

miR-192 prevents renal tubulointerstitial fibrosis in diabetic nephropathy by targeting Egr1

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN), as the most common and serious diabetic microvascular complication, has become the first cause of end-stage renal disease (ESRD) in many countries and regions. However, the pathogenesis of renal fibrosis during the development of DN remains unknown.

MATERIALS AND METHODS: The expression levels of miR-192 and early growth response factor 1 (Egr1) were determined by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) and Western blotting in the renal tissues of Otsuka-Long-Evans-Tokushima-Fatty (OLETF) and Long-Evans-Tokushima-Otsuka (LETO) rats. The diabetic kidney environment was simulated by a high-sugar medium. The expression levels of miR-192 and Egr1 were further measured in the HK-2 cell line. Egr1 was verified as a potential target of miR-192 by using bioinformatics analysis and luciferase activity assay. The expression level of Egr1 was determined by overexpressing and knocking down the expression of miR-192. In addition, Western blotting was used to determine changes in Transforming growth factor-beta 1 (TGF- β 1) and fibronectin (FN).

RESULTS: Compared with the kidney tissue of LETO rats, the expression of miR-192 was decreased in OLETF rats, whereas the expression of Egr1 was increased. We found the same phenomenon in the HK-2 cell line cultured in the high-glucose medium. Next, miR-192 can act on Egr1 through 3'-UTR to reduce the expression of Egr1 verified by luciferase assay. In addition, the expression levels of TGF- β 1 and FN changed significantly, as the expression level of Egr1 increased or decreased.

CONCLUSIONS: MiR-192 causes degradation of TGF- β 1 and FN through targeting Egr1 and affects the progression of TIF and even DN.

Key Words:

MiR-192, Egr1, Renal tubulointerstitial fibrosis, Diabetic nephropathy.

Introduction

In 2015, a total of 124,111 new cases of end-stage renal disease (ESRD) were reported. Diabetic nephropathy (DN), the most common and severe diabetic microvascular complication, has become the leading cause of ESRD in many countries and regions^{1,2}. Using renin-angiotensin system inhibitors to control blood glucose and blood pressure fluctuations is widely accepted as the standard treatment for DN. However, the mechanism of how DN causes ESRD is not yet clear. Oxidative stress and cytokines (transforming growth factors, vascular endothelial growth factors) are considered as important factors in the pathogenesis^{3,4}. During the development of DN, under the action of inflammatory reaction, high glucose environment, and immune factors, the renal tubules are easily damaged, which accelerates the progress of ESRD⁵. Tubular interstitial fibrosis (TIF) acts an important role in the progression of DN.

MicroRNAs (miRNAs) are small RNAs of 21-25 nucleotides that bind to partially complementary sequences in the 3'-untranslated region of mRNA (3'-UTR) and negatively affect post-transcriptional regulation⁶. Current studies^{7,8} have found that many miRNAs are involved in the pathogenesis of DN, such as miR-135a and miR-18a-5p. Previous studies^{9,10} have revealed that miR-192 is enriched in extracellular vesicles in urine and plays the role of an early diagnostic marker of DN, and can also be used as a potential therapeutic target of DN. MiR-192 can modulate the process of DN by regulating TGF- β (Transforming growth factor beta) and FN (fibronectin)^{11,12}. However, whether miR-192 has other regulatory mechanisms in TIF is still unknown.

Early growth response factor 1 (Egr1) is a transcription factor that binds to DNA. Egr1 is located on human chromosome 5q31 and encodes a protein of 80 kDa¹³. Previous studies have suggested that Egr1 shows differential expression in diabetic mice and provides a new approach for the targeted treatment of DN¹⁴. Egr1 is involved in renal fibrosis through multiple pathways, such as promoting the proliferation of mesangial cells, facilitating the expression of TGF- β , and accelerating the transdifferentiation of renal tubular epithelial cells to the mesenchymal cell^{15,16}. The impact of Egr1 on TIF in DN process has not been clarified.

In this study, we found that miR-192 is low in the kidneys of OLETF; in contrast, Egr1 is highly expressed. *In vitro* experiments, we found that decreased miR-192 expression and increased Egr1 expression were shown in high-glucose cultured HK-2 cell lines. By bioinformatics prediction, luciferase activity assay, quantitative real-time polymerase chain reaction (qRT-PCR), and Western blotting, it was revealed that Egr1 is a downstream target for miR-192. We also verified the relationship between Egr1 and TGF- β 1 in the progression of FN. Finally, it was demonstrated that miR-192 exerts its TIF role in DN through Egr1.

Materials and Methods

Animals

The study was approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University Animal Center. Male Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats were used as a model for spontaneous type 2 diabetes, and Male Long-Evans-Tokushima-Otsuka (LETO) rats served as non-diabetic controls. All experimental rats were cage-fed in a specific-pathogen-free (SPF) grade environment and fed with standard rat feed and clean drinking water. LETO rats developed proteinuria from the 30th week. Each strain was randomized and we took three rats each for sacrifice. Rat kidney tissue was removed and placed in liquid nitrogen for subsequent analysis.

Immunohistochemical (IHC) Analysis

Rat kidney tissue was fixed with 4% paraformaldehyde for one day, and then, the fixative was washed away. The specimens were washed, and the washed specimens were embedded in paraffin and then cut into thin tissue sections with a microtome. The water bath temperature was main-

tained at about 45°C, and the slices were placed in warm water to allow them to expand. Finally, the slides were placed horizontally on a 60°C baking machine for 2 hours. The samples were deparaffinized, washed, and then placed in normal goat serum at a concentration of 10% and blocked for 30 minutes at room temperature. Incubate the specimen overnight at 4°C. The sections were then washed 3 times with phosphate buffered saline (PBS) solution. Samples were incubated with the secondary antibody for 30 minutes at room temperature. The specimens were again washed 3 times for 5 minutes each with PBS solution. Diaminobenzidine (DAB) was added thereto for staining, the specimen was washed with water and, then, hematoxylin was added for staining. Finally, we washed the slides and observed the results under a microscope.

Cell Culture

HK-2 cells (human renal tubular epithelial cell line) were provided by the Shanghai Academy of Sciences. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco, Rockville, MD, USA) at 37°C in a humidified cell incubator in a 5% CO₂ atmosphere. Cells were passaged 2-4 days depending on the cell growth. High-sugar medium: First, 0.45 g of D-Glucose was added to 500 mL of a DMEM medium with a glucose concentration of 25 mM (the medium glucose was adjusted to 30 mM). Then, they were mixed and sterilized by filtration. Finally, 36 mL of high-glucose DMEM medium and 4 mL of FBS were added to a sterile 50 mL centrifuge tube, thoroughly mixed and stored at 4°C.

Cell Transfection of Mimics, Inhibitor, Plasmid And Small Interfering RNA

The hsa-miR-192-mimics, hsa-miR-192-inhibitor, NC, plasmid Egr1, empty vector, siRNA-Egr1, and siRNA-NC were purchased from Gene Pharma (Shanghai, China). The lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transient transfection following the manufacturer's instructions. The cells were collected 48 hours after transfection.

Total RNA Extraction

The mortar is sterilized by high-temperature sterilization. Each time about 20 mg of kid-

ney tissue was cut into a mortar with a suitable amount of liquid nitrogen. The tissue was quickly crushed into fine particles and then 1 mL TRIzol solution (Invitrogen, Carlsbad, CA, USA) was added. In the case of cell samples, when the cells covered the bottom of the dish, the cells were washed twice with PBS, added with TRIzol solution (1 mL), and collected in an Eppendorf tube (EP) tube with a scraper. The next step of extraction follows the instructions. Finally, the RNA sample was stored in a -80°C freezer.

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

Samples of total RNA were taken from the -80°C freezer and thawed on ice. Reverse transcription was performed according to the instructions of the PrimeScript RT reagent (TaKaRa, Otsu, Shiga, Japan). PCR was performed using an SYBR Green Master Mix II (TaKaRa, Otsu, Shiga, Japan) on an ABI 7900 fast real-time PCR system machine (ABI, Foster City, CA, USA). All miRNA samples were calibrated with U6, while all mRNA samples were calibrated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The reaction conditions were set as follows: pre-denaturation was performed at 95°C for 5 minutes; PCR was then performed for 40 cycles of 95°C for 15 seconds per cycle and 60°C for 1 minute per cycle. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method. Using Primer 5.0 to design gene primers, specific gene primer sequences are as follows: Egr1: F: 5'-TCAGCACCTTCCAGTCTGC-3', R: 5'-TGCTGTCATGTCTGAAAGACCC-3'; GAPDH: F: 5'-AAGGTGAAGGTCGGAGTCA-3', R: 5'-GGAAGATGGTGTATGGGATTT-3'; miR-192 F: 5'-CTGACCTATGAATTGACAGCC-3', R: 5'-GAGAAGAT TAGCATGGCCC-3'; U6: F: 5'-CTCGCTTCGGCAGCACATATACT-3', R: 5'-ACGCTTCACGAATTTGCGTGTC-3'. Each sample was repeated three times.

Western Blotting

The mortar is autoclaved and sterilized in advance. 0.5 M Ethylene Diamine Tetraacetic Acid (EDTA), protease inhibitors and phosphatase inhibitors were added to radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) at a ratio of 100:1. The kidney tissue stored in liquid nitrogen was then removed, quickly placed in a mortar and a lysed solution was added in advance, ground on ice until the tissue

was finely granular, and then all the components were transferred to a 1.5 mL centrifuge tube. Meanwhile, for the cell sample, after the cells are covered with the bottom of the dish, the dish is washed twice with PBS, and the RIPA lysate is added to continue the above steps. The EP tube was placed on a cryogenic centrifuge and centrifuged for 15 minutes at a temperature of 4°C at a speed of 15000 g. The supernatant was then transferred using a pipette tip. A quarter volume of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) Protein Loading Buffer to the protein sample was added and mixed thoroughly. Then, the sample was put in boiling water for 5 minutes and taken. Proteins extracted from NSCLC cells and tissues were separated by 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk powder for 2 hours, the membranes were incubated overnight at 4°C with the following specific primary antibodies: Egr1 (CST, 4154, Danvers, MA, USA), TGFβ1 (CST, 3712, Danvers, MA, USA), FN (Sigma, St. Louis, MO, USA), GAPDH (CST, 2118, Danvers, MA, USA). The membrane was then incubated with HRP-conjugated anti-rabbit IgG (1: 2,000) for 2 hours at room temperature and then three times with Tris-buffered saline and Tween-20 (TBS-T) buffer. Bound secondary antibodies were detected by an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL, USA). GAPDH was used as an internal control.

Luciferase Reporter Assay

The 3'-UTR sequence or mutated sequence of Egr1 and the predicted target site were inserted into the pLG3 promoter vector (Promega, Madison, WI, USA), respectively. They were named pLG3-Egr1-wt and pLG3-Egr1-mut. HK-2 cells were seeded in 24-well plates and transfected with 100 ng of pLG3-Egr1-wt or pLG3-Egr1-mut, miR-192 mimic and NC with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, transfected cells were obtained. Relative luciferase activity was measured using the Luciferase Assay Kit (Promega, Madison, WI, USA).

Statistical Analysis

All experimental data were analyzed using GraphPad software 5.0 (La Jolla, CA, USA)

and Statistical Product and Service Solutions (SPSS) 18.0 (Chicago, IL, USA) for statistical analysis. The p -values were analyzed using Student's t -test, and Spearman's test. Comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ for the difference was considered statistically significant.

Results

Low Expression Level of miR-192 Was Inversely Correlated With Egr1 Expression in OLETF Rats

To determine whether the expression of miR-192 is different in kidneys of type 2 diabetic OLETF rats with DN, we performed qRT-PCR. As shown in Figure 1A, miR-192 expression was

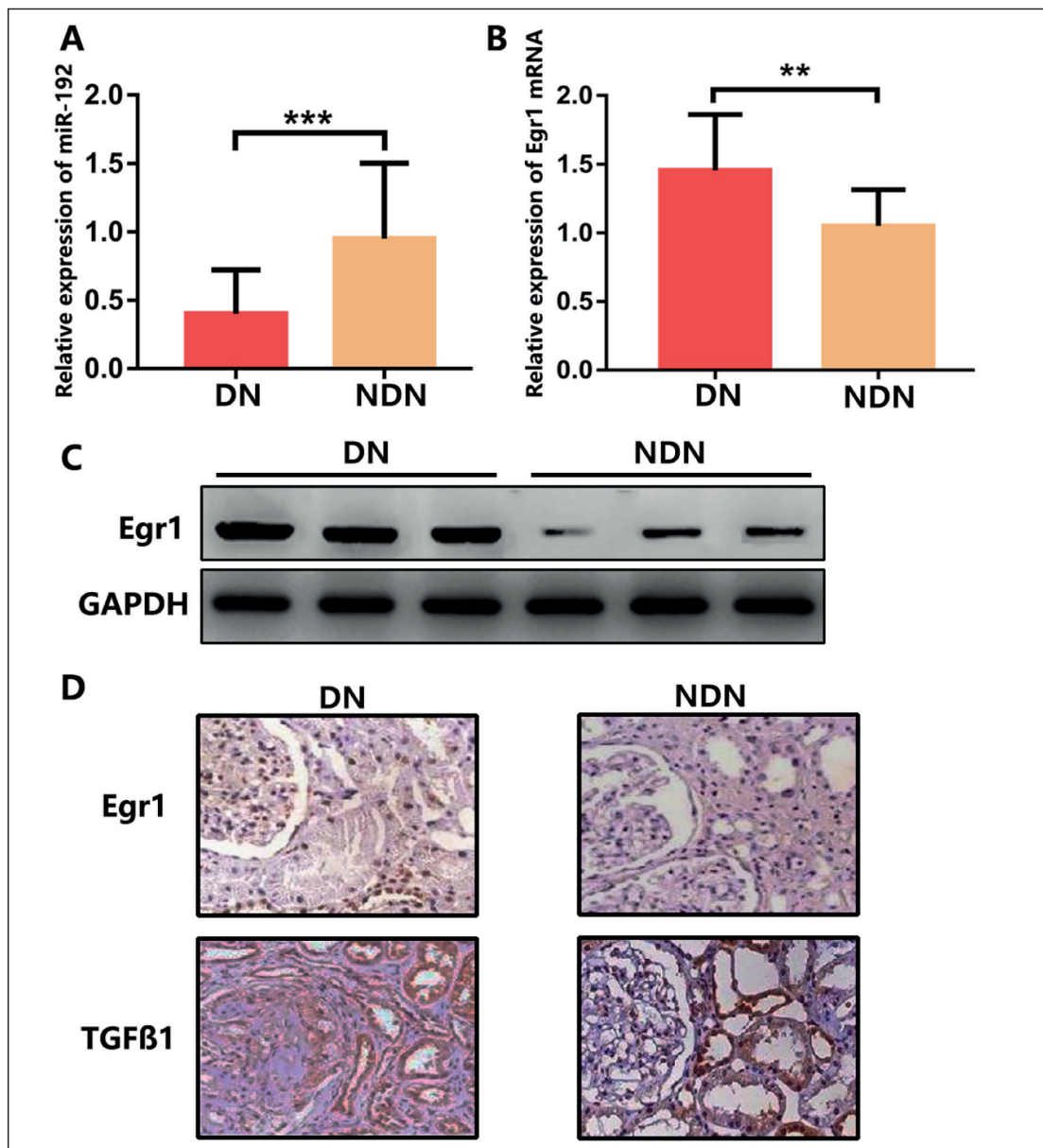


Figure 1. Ectopic expression of miR-192 and Egr1 in rat kidney tissue. **A**, Analysis of miR-192 expression level in DN and NDN rats. **B**, Analysis of Egr1 expression level in DN and NDN rats. **C**, In DN, the expression of Egr1 protein was significantly higher than that of NDN. **D**, The IHC of Egr1 and TGF-β1 in DN and NDN. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

reduced in the kidney tissue of DN compared with NDN. At the same time, we also measured the expression level of Egr1 mRNA in tissue samples and found that Egr1 was increased in the kidney tissue of DN (Figure 1B). To further determine the difference in expression of Egr1, we performed Western blotting. As shown in Figure 1C, the expression of Egr1 in DN rats was significantly increased. Meanwhile, we also performed immunohistochemistry (IHC). As shown in Figure 1D, in addition to the abnormal expression level of gr1 in DN rats, there was also an increase in the expression of TGF- β 1. The above experiments revealed that, *in vitro*, the expression of miR-192

decreased with the progression of DN, while the expression of Egr1 and TGF- β 1 increased, and there may be a link between them.

Low Expression Level of miR-192 and High Expression Level of Egr1 in HK-2 Cells

We further validated changes in miR-192 and Egr1 expression during DN progression *in vivo* experiments. We placed the HK-2 cell line in a pre-configured high glucose DMEM medium in the logarithmic growth phase and collected cells at 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h. As shown in Figure 2A and 2B, by qRT-PCR, we found that

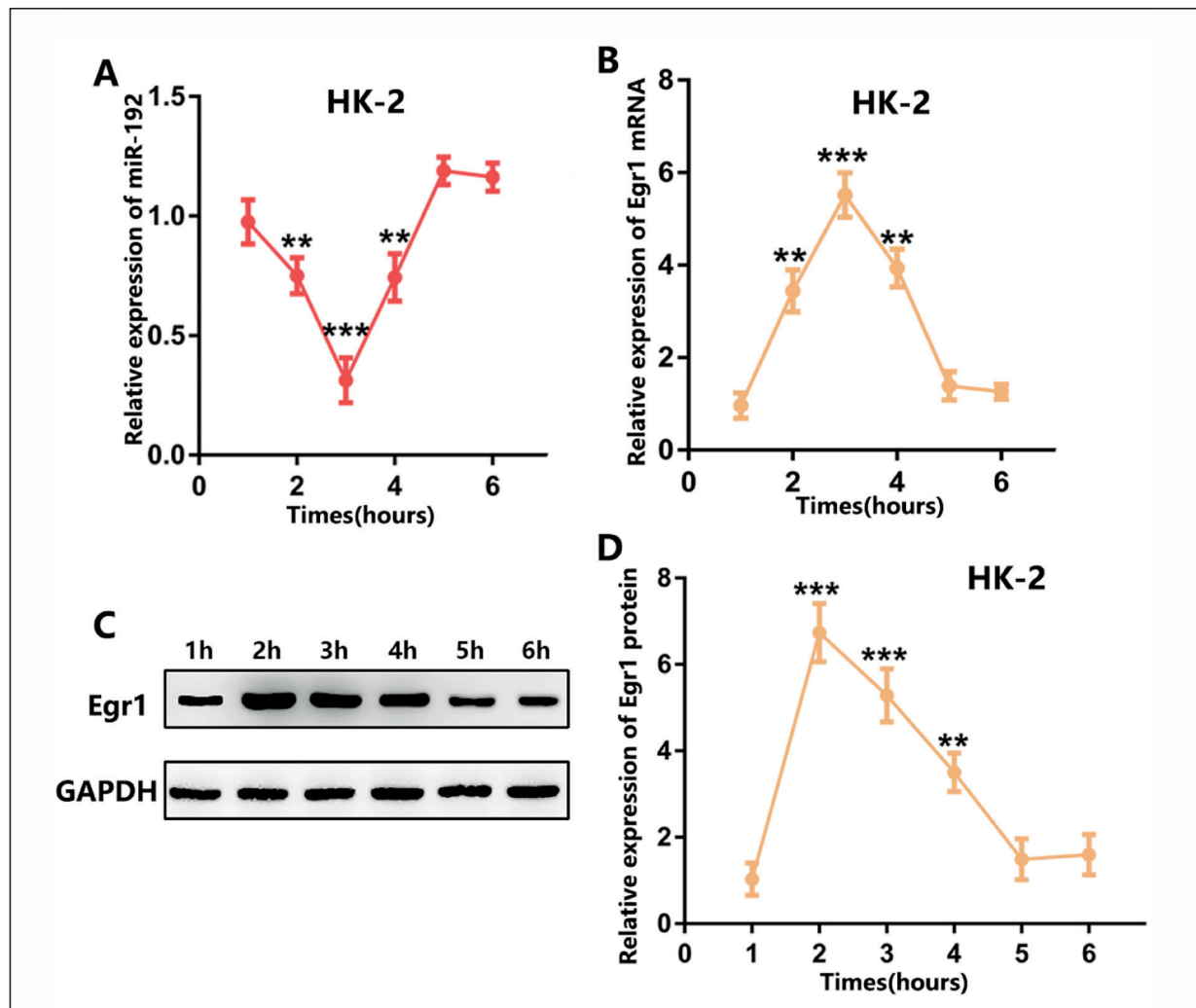


Figure 2. High glucose inhibits miR-192 expression and promotes Egr1 expression *in vitro* in a time-dependent manner. *A*, The expressions of miR-192 were measured at the indicated time points by qRT-PCR in HK-2 cells treated with high glucose (HG). *B-D*, The expressions of Egr1 were measured at the indicated time points by qRT-PCR (*B*) and Western blotting (*C* and *D*) in HK-2 cells treated with high glucose (HG). Data are presented as the mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001.

miR-192 expression decreased in high glucose cultures, and reached a minimum at 3 h, then recovered, and there was no difference in expression after 5 h. On the other hand, Egr1 mRNA reached the highest level at 3 h and showed no difference after 5 h. Protein levels were determined by Western blotting and we found that the expression of Egr1 peaked at 2 h and then declined to the 5 h level (Figure 2C, 2D). From this, we speculate that there is a highly possible link between miR-192 and Egr1.

MiR-192 Suppress Egr1 Expression Via Interacting Directly With a Hypothetic Binding Site of Egr1-3'-UTR

To explore the potential target of miR-192, MicroRNA.org, MiRWalk, and TargetScan were adopted. The predicted binding sites of miR-192 and Egr1 are shown in Figure 3A. To further verify the binding of miR-192 and Egr1, we performed luciferase activity assay *via* inserting the wild-type and mutant sites of Egr1 into the pGL3 luciferase reporter vector. Then, we co-transfected pGL3 luciferase reporter vector and miR-192 mimics or NC into HK-2 cells. After 48 hours, we found that the fluorescence intensity of the wild-type group was significantly reduced compared to the fluorescence of the mutant-type group (Figure 3B). The above results demonstrated that Egr1 is a potential target of miR-192.

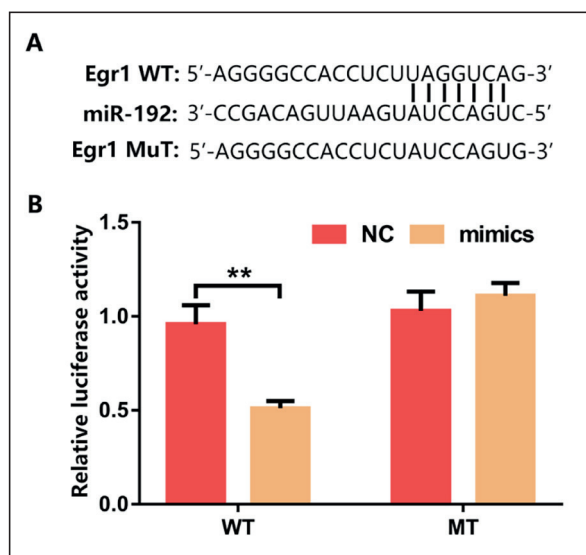


Figure 3. MiR-192 inhibited Egr1 expression. **A**, Double fluorescence experiment mutation sites. **B**, The fluorescence intensity of the wild-type group was significantly weaker than that of the mutant group. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MiR-192 Inhibits Renal Interstitial Fibrosis By Targeting Egr1

To verify the targeting of miR-192, we transfected miR-192 mimics, mimics NC, inhibitor, and inhibitor NC into HK-2 cells. After 24 hours, the expression level of miR-192 was determined by qRT-PCR (Figure 4A). At the same time, we also found that when miR-192 was over-expressed, the expression level of Egr1 mRNA decreased; when miR-192 was inhibited, the expression of Egr1 mRNA increased (Figure 4B). By Western blotting, we further validated the inhibitory effect of over-expressed miR-192 on Egr1. Furthermore, some of the proteins associated with renal interstitial fibrosis, such as TGF- β 1 and FN, also decreased in expression as the expression of miR-192 increased (Figure 4C). To further validate our suggestion, we transfected plasmid Egr1 into cells treated with mimics while si-Egr1 into cells treated with inhibitor. By using qRT-PCR to measure changes in Egr1 mRNA after transfection, we found that plasmid Egr1 counteracted the inhibitory effect of over-expression of miR-192 on Egr1. Meanwhile, si-Egr1 reversed the inhibitory effect of miR-192 on Egr1 (Figure 5A). By Western blot, we further confirmed the targeting effect of miR-192 on Egr1 and also demonstrated that miR-192 inhibits the expression of TGF- β 1 and FN to inhibit renal interstitial fibrosis (Figure 5B, 5C).

Discussion

The main treatment of diabetic nephropathy is still multi-factor intervention targeting glucose, blood lipids and blood pressure, including drugs that block renin angiotensin^{17,18}. At present, new biomarker research and progress in therapeutic drugs play a pivotal role. Here, we provide evidence that miR-192 inhibits the progression of TIF by regulating Egr1. All experimental data indicated that miR-192 may function as a promoter in DN progression.

Abnormal miRNA expression is revealed to involve in various mechanisms of DN progression¹⁹⁻²¹. miR-192 can be used as an early diagnostic marker and therapeutic target in the progression of DN^{11,12}. However, it remains unknown that whether miR-192 could inhibit TIF. Egr1 is also reported to participate in all aspects of the DN process^{8,22,23}. In this experiment, we constructed a diabetic rat model (OLETF) and a control group (LETO). By qRT-PCR and Western blot-

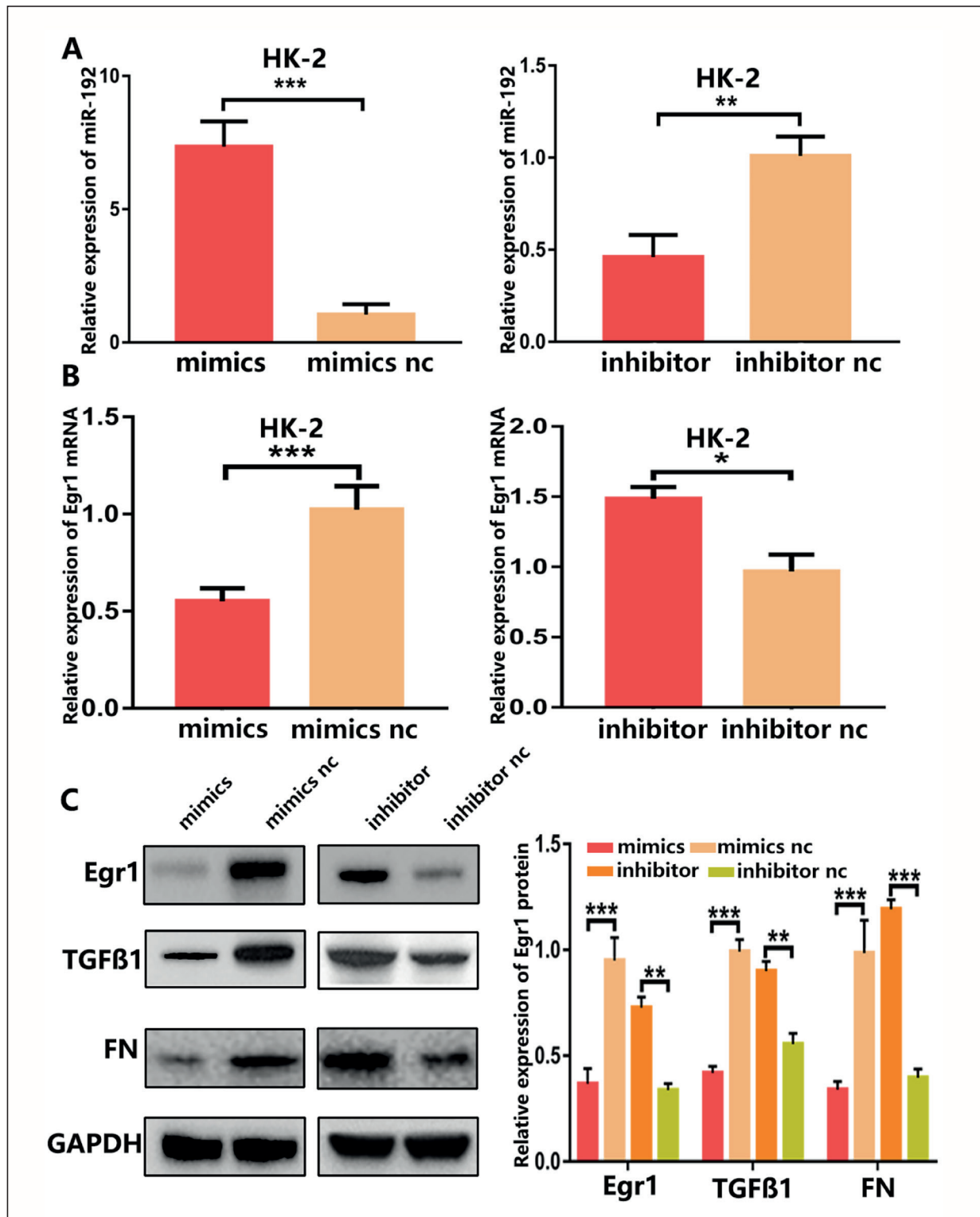


Figure 4. Overexpression of miR-192 inhibits the expression of Egr1. *A-B*, The expressions of miR-192 were measured by qRT-PCR in HK-2 cell after transfected. *C-D*, The expressions of Egr1 were measured by qRT-PCR after transfected. *E*, After overexpression of miR-192, the expression levels of Egr1, TGF-β1, and FN protein decreased significantly, whereas the expression of Egr1 protein increased. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

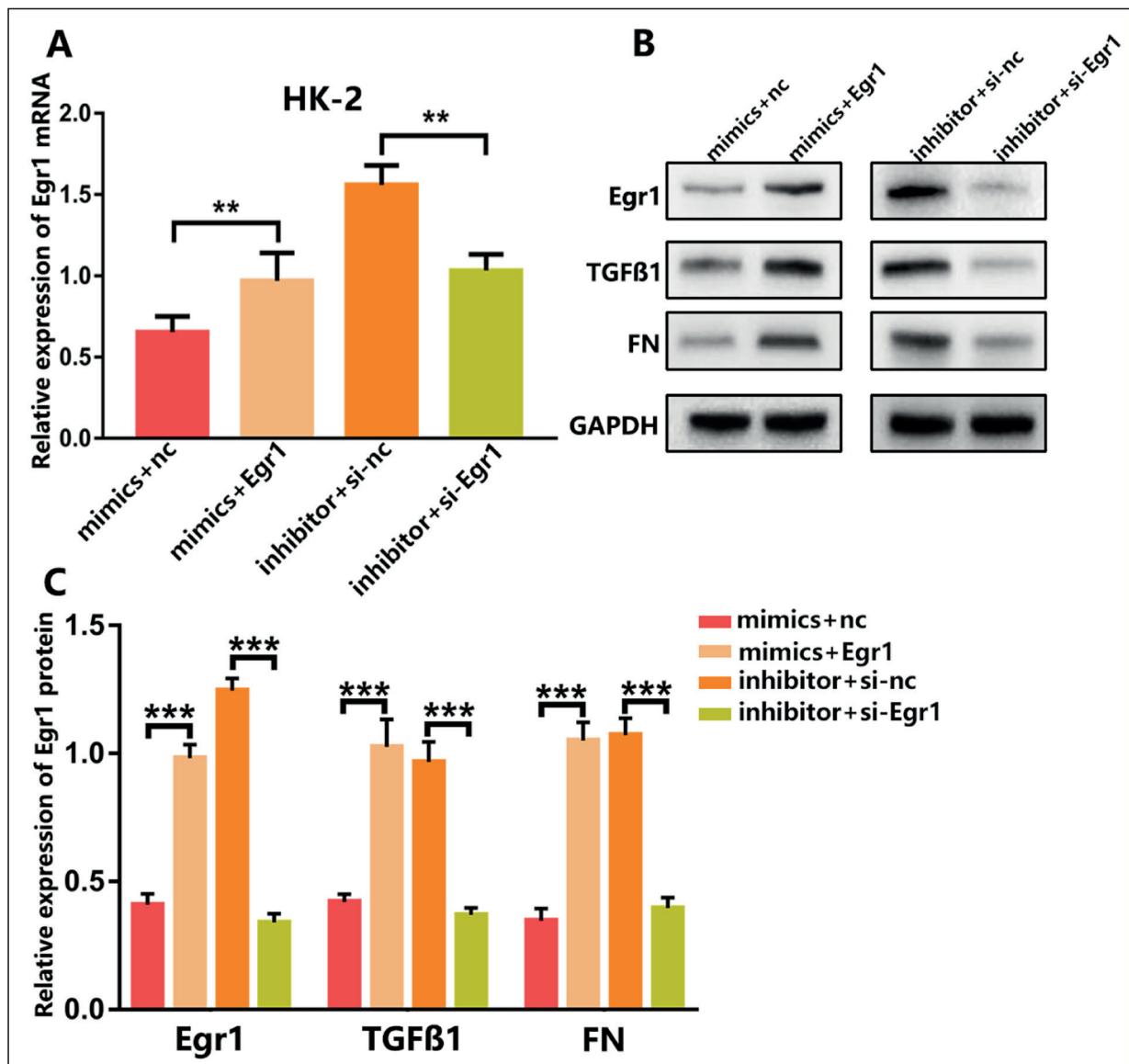


Figure 5. MiR-192 delays the TIF process by targeting Egr1. **A**, The expression level of Egr1 mRNA was measured in rescue experiments. **B**, The expression levels of Egr1, TGF- β 1, and FN protein was measured in rescue experiments. Data are presented as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ting, we found that the expression of miR-192 decreased in DN rats, while the expression of Egr1 increased. And through *in vitro* experiments to create a high-sugar environment, we verified this phenomenon again in the HK-2 cell line. By overexpression of miR-192, we found that the expression level of Egr1 decreased; at the same time, knocking down the expression of miR-192 increased the expression of Egr1. Besides, we also demonstrated that overexpressed miR-192 inhibited the effect of TGF- β 1 and FN proteins. Finally, we again showed the regulation of miR-

192 on Egr1, TGF- β 1, and FN through rescue experiments. miR-192 could target Egr1 to delay the progression of TIF.

Conclusions

Our current study showed that miR-192 has an inhibitory effect on the progression of TIF and DN *in vitro* and *in vivo*. Besides, miR-192 inhibits the progression of TIF by inhibiting Egr1. Our results suggested that miR-192 can serve as an innovative and prospective therapeutic target for DN.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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