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High glucose down-regulates miR-29a to increase collagen IV production in HK-2 cells

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ABSTRACT

Deposition of collagen IV in proximal tubule cells (PTCs) plays an important role during diabetic nephropathy, but the mechanism underlying excessive production of collagen IV remains poorly understood. In this study, we examined the miRNA profile of HK-2 cells and found that high glucose/TGF-β1 induced significant down-regulation of miR-29a. We then showed that miR-29a negatively regulated collagen IV by directly targeting the 3′UTRs of *col4a1* and *col4a2*. These results suggest that miR-29a acts as a repressor to fine-tune collagen expression and that the reduction of miR-29a caused by high glucose may increase the risk of excess collagen deposition in PTCs.

1. Introduction

Diabetic nephropathy (DN) is a serious complication of diabetes and is the most common cause of kidney failure in diabetic patients. Studies have shown that the pathogenesis of DN is attributable to hyperglycemia-activated downstream pathways, among which transforming growth factor β1 (TGF-β1) plays the most important role [1,2]. Both in vitro and in vivo studies have demonstrated that elevated glucose levels activate TGF-β1 and subsequently increase the production of collagen IV in proximal tubule cells (PTCs) [3–5]. Excessive collagen IV-induced basement membrane thickening underlies the critical morphological characteristics of tubulointerstitial injury in DN [3]. However, the molecular mechanism by which collagen IV is induced by high glucose remains poorly understood.

MicroRNAs (miRNAs), which are short (~22 nt) non-coding RNAs, have been shown to play key roles in diverse biological and pathological processes [6]. Recently, several studies have demonstrated that dysregulation of miRNAs is linked to hyperglycemia-induced DN. For example, miR-192 is involved in TGF-β1-mediated collagen I and III synthesis [7], and up-regulated miR-377 increases fibronectin production in DN [8]. However, these studies focused on glomerular mesangial cells (GMCs), not PTCs. In fact, PTCs constitute the bulk of the renal cortical cells, and their central role in tubulointerstitial injury in DN has been extensively studied [9,10]. So far, however, the expression and function of miRNAs in PTCs during DN have not been reported. Thus, further investigation of miRNA regulation in PTCs is essential to unravel the molecular mechanisms underpinning tubulointerstitial changes in DN.

In this study, we determined the miRNA expression profile of a cell line derived from human PTCs (HK-2 cells), and we found that high glucose/TGF-β1 induced significant down-regulation of miR-29a. We further validated that miR-29a negatively regulated collagen IV protein by directly targeting the 3′UTRs of collagen IV transcripts. The results suggest that miR-29a acts as a repressor to fine-tune collagen expression and accumulation in HK-2 cells.

2. Materials and methods

2.1. Cell culture

The proximal tubule cell line HK-2 was purchased from the cell culture center of the Chinese Academy of Medical Sciences. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 mM glucose, 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin (Gibco/Life Technologies, Paisley, UK) and incubated at 37 °C in a humidified incubator with 5% CO_2.

Subconfluent HK-2 cells were seeded at a 10% density on 10-cm Petri dishes and maintained in medium containing 5 mM D-glucose

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(NG) or 30 mM D-glucose (HG) for seven days. Medium was changed every two days to maintain glucose levels in the desired range. For the TGF-β1 assay, 3×10^5 cells were planted in each well of a 6-well plate. Experiments were performed after cells were cultured in medium containing low serum (2% FBS) and 10 ng/ml TGF-β1 (PeproTech, Rocky Hill, NJ, USA) for 72 h.

2.2. MiRNA microarray

RNA from HK-2 cells treated with NG or HG was used for miRNA microarray analysis. Microarray procedures and data analysis were performed at Beijing CapitalBio Corporation as described previously [11]. Briefly, total RNA extracted from HK-2 cells treated with NG or HG was covalently labeled with Cy3 (green channel) or Cy5 (red channel), respectively. Dye switching was done to eliminate dye bias. Pairs of labeled samples were hybridized to dual-channel microarrays. Fluorescence scanning used a double-channel laser scanner. The analog signal was transformed to a digital signal using image analysis software. Signal intensities for each spot were calculated by subtracting local background from total intensity. Raw data were normalized and analyzed using the Significance Analysis of Microarrays (SAM, Stanford University, CA, USA) software.

2.3. Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells and samples were reverse-transcribed to cDNA using the primescript™ RT reagent kit (Takara, Tokyo, Japan). Real-time PCR was carried out using SYBR Premix ExTaq™ (Takara) according to manufacturer's instructions. The input was normalized by U6 snRNA. Hsa-miR-29a and U6 primer sets were purchased from RiBiBio Corporation (Guangzhou, China). Experiments were performed in triplicate, and the results are represented as mean ± S.E.M.

2.4. Northern blot analysis

Total RNA was extracted from HK-2 cells at indicated days using TRIzol (Invitrogen, Carlsbad, CA, USA). Low molecular weight RNA was subsequently isolated by precipitation in PEG8000/NaCl as previously described [12]. Forty micrograms of low molecular weight RNA was run on a denaturing 10% polyacrylamide gel, transferred to PVDF membrane (Amersham/GE Healthcare, Piscataway, NJ, USA), subjected to UV light irradiation for 4 min and baked at 80 °C for 50 min. The hsa-miR29a oligonucleotide probe (5′-TAACCGATTTCAGATGGTGCTA-3′) was 5′ end-labeled with [γ-^{32}P] ATP. The membranes were pre-hybridized for at least 1 h, then hybridized overnight at 42 °C. After three washes ($2 \times$ SSC, 0.5% SDS), membranes were exposed to a phosphor screen and visualized by Typhoon 8600 imager (Amersham). $tRNA^{Thr}$ was used as a loading control.

2.5. Luciferase reporter assays

For construction of reporter plasmids, synthesized 54-bp DNA oligonucleotides containing the putative binding sites for miR-29a in the 3′UTR of *col4a1* or *col4a2* were annealed and cloned into EcoRI/BglII-digested phRL-null (Promega, Madison, WI). The *col4a1* and *col4a2* 3′UTR target sites were cloned using the following oligonucleotides: *col4a1*-site1: sense, AATTCTGAAGCCTGACTCAGC TAATGTCACAACATGGTGCTACTTCTTCTA and antisense, GATCTAGA AGAAGTAGCACCATGTTGTGACATTAGCTGAGTCAGGCTTCAG; *col4a1*-site2: sense, AATTCTGTGAAGTGAGAACTCCATCAGAAAACCAAAGG GTGCTAGGAGGTGTA and antisense, GATCTACACCTCCTAGCACCC TTTGGTTTTCTGATGGAGTTCTCACTTCACAG; *col4a2*: sense, AATT CGCCGGCGCGTGCCAGGAAGGGCCATTTTGGTGCTTATTCTTAA and antisense, GATCTTAAGAATAAGCACCAAAATGGCCCTTCCTGGCACG CGCCGGCG. As a negative control response element, we used a mutated sequence (underline) by inserting the following oligonucleotides: *col4a1*-site1 MUT: sense, AATTCTGAAGCCTGACTCAGCTA ATGTCACAACATAATTATACTTCTTCTA and antisense, GATCTAGAA GAAGTATAATTATGTTGTGACATTAGCTGAGTCAGGCTTCAG; *col4a1*-site2 MUT: sense, AATTCTGTGAAGTGAGAACTCCATCAGAAAACC AAAGAATTATAGGAGGTGTA and antisense, GATCTACACCTCCTA TAATTCTTTGGTTTTCTGATGGAGTTCTCACTTCACAG; *col4a2* MUT: sense, AATTCGCCGGCGCGTGCCAGGAAGGGCCATTTTAATTATTATT CTTAA and antisense, GATCTTAAGAATAATAATTAAAATGGCCCTT CCTGGCACGCGCCGGCG. HK-2 cells were plated in 48-well plates and co-transfected with 0.1 μg of the phRL-null reporter plasmid and 30 nM miR-29a or miR-neg mimic (Genepharma, Shanghai, China) in each well using Lipofectamine 2000 (Invitrogen). Luciferase assays were performed 48 h after transfection using the Dual-Luciferase® Reporter Assay System (Promega) on a GloMax™ 96 Microplate Luminometer (Promega). Renilla luciferase activity was normalized to firefly luciferase expression for each sample.

2.6. Transient transfection of miRNA mimic or anti-miR miRNA inhibitors

HK-2 cells were seeded in 24-well plates at 10^5 cells/well. After 24 h, 50 nM hsa-miR-29a mimic or negative control (miR-neg) and the indicated amounts of anti-miR-29a or control anti-miR (Genepharma) were transiently transfected into HK-2 cells by Lipofectamine 2000 (Invitrogen). Seventy-two hours after transfection, cell extracts were used for real-time PCR or western blot.

2.7. Western blot analysis

Collagen IV protein was analyzed by western blotting from total cell lysates as described previously [13]. Proteins were extracted with RIPA lysis buffer. Collagen IV protein was revealed with a collagen IV polyclonal antibody (Abcam, Cambridge, MA, USA). Signals were detected by Super signal Western Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Western blotting of tubulin on the same membrane was used as a loading control.

2.8. Statistics assay

Results are expressed as arbitrary units and are presented as the mean ± S.E.M. In each experiment, all determinations were performed at least in triplicate. Statistical significance was assessed using Student's *t* test for experiments with multiple groups. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Expression profiling of miRNAs in PTCs

To investigate whether miRNAs are involved in the pathogenesis of DN, it is essential to first establish the miRNA expression profile in PTCs. Therefore, we determined the miRNA expression profile of a common proximal tubule cell line, HK-2 cells, using an established microarray platform. The microarray data revealed that there were about 100 miRNAs for which the signal intensity was more than 1500. Among these miRNAs, the miR-30 family members, miR-565 and miR-21 were the most highly expressed in HK-2 cells. According to the expression level, the 40 most highly expressed miRNAs in HK-2 cells were arranged and shown in Fig. 1. Generally, the miRNA expression level correlates with functional importance, so dysregulation of some of these 40 miRNAs is perhaps involved in PTC dysfunction during DN. Intriguingly, several

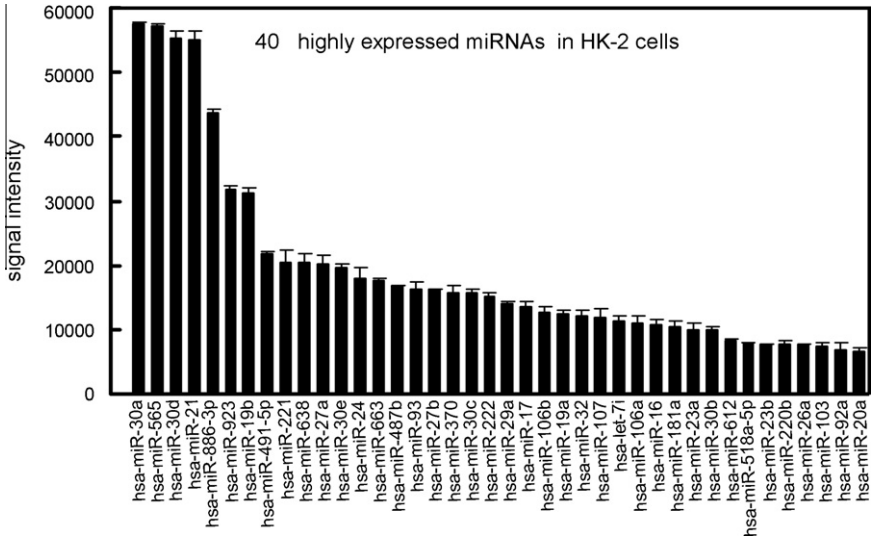

Fig. 1. MiRNA expression profile of HK-2 cells. MiRNAs are ranked from left to right on the basis of their expression level.

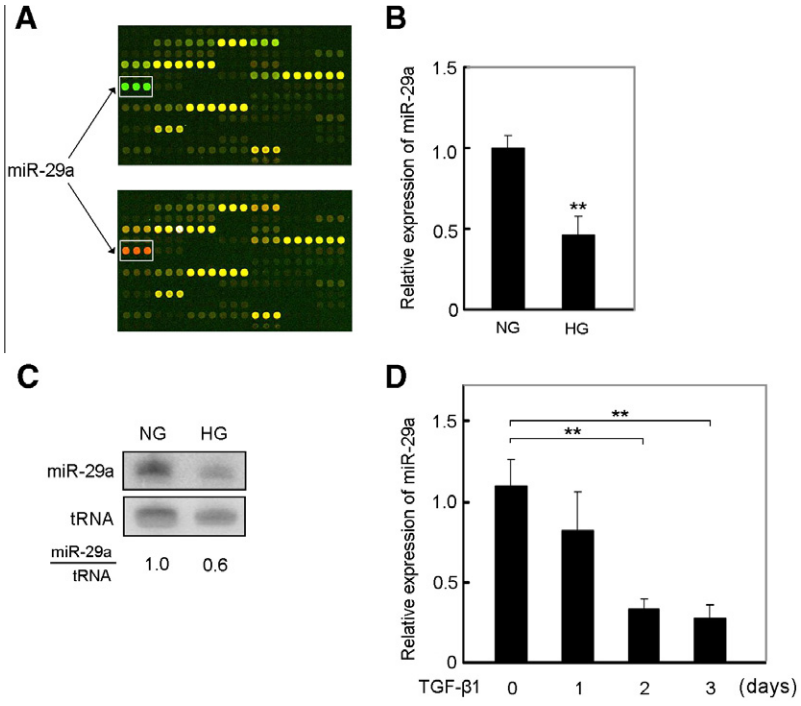

Fig. 2. Down-regulation of miR-29a by HG or TGF-β1 in HK-2 cells. (A) Detection of miRNAs by dual-channel microarray. Part of the microarray image showed that there were significant differences in miR-29a levels between NG- and HG- treated HK-2 cells. The upper panel, total RNA extracted from NG- and HG- treated HK-2 cells was covalently labeled with Cy3 (green channel) and Cy5 (red channel), respectively, and hybridized to the array. The lower panel, the RNA of NG- and HG- treated HK-2 cells labeled with Cy5 and Cy3 dye, reversely. (B and C) MiR-29a was down-regulated by HG. Real-time PCR (B) and northern blotting (C) were performed to detect miR-29a in HK-2 cells treated with NG or HG for seven days and normalized to U6 snRNA or $tRNA^{Thr}$, respectively. (D) MiR-29a was down-regulated by TGF-β1 treatment. Real-time PCR analysis of miR-29a in TGF-β1-stimulated HK-2 cells at different days and normalized to U6 snRNA. All data represent the means of three independent experiments ± S.E.M. with $n = 3$. $^{**}P < 0.01$.

miRNAs highly expressed in GMCs, such as miR-192 and miR-377, did not appear in this list, implying different miRNA profiles for PTCs and GMCs.

3.2. MiR-29a is down-regulated in response to high glucose or TGF-β1 stimulation in HK-2 cells

To determine whether miRNAs are involved in PTC regulation during DN, we compared the HK-2 miRNA profile after treatment with high glucose (HG) for seven days with that of cells exposed to normal glucose (NG) by dual-channel miRNA microarrays. The SAM (Significance Analysis of Microarrays) results showed that there were 14 miRNAs down or up-regulated more than 30% upon HG treatment (Table 1). Among these miRNAs, we focused on miR-29a, which showed the greatest fold change and was one of the 40 highly expressed miRNAs in HK-2 cells. As shown in Fig. 2A, the dual-channel miRNA microarray images reveal the significant difference in miR-29a expression between NG and HG treatments.

Table 1
Differentially expressed miRNAs in HK-2 cells treated with NG or HG.

Gene name	Fold change	Score	Regulation
Hsa-miR-29a	0.113	−12.663	Down
Hsa-miR-29c	0.445	−18.826	Down
Hsa-miR-195	0.556	−3.843	Down
Hsa-miR-532-5p	0.599	−3.109	Down
Hsa-miR-923	0.623	−2.305	Down
Hsa-miR-29b	0.637	−12.233	Down
Hsa-miR-16	0.668	−4.685	Down
Hsa-miR-362-3p	0.692	−2.172	Down
Hsa-miR-374b	0.694	−8.845	Down
Hsa-miR-596	1.304	3.165	Up
Hsa-miR-296-5p	1.339	6.601	Up
Hsa-miR-523	1.342	4.969	Up
Hsa-miR-760	1.390	3.154	Up
Hsa-miR-298	1.458	3.880	Up

The expressions of miRNAs were detected by miRNA microarray and SAM was used for data analysis.

To validate the microarray data, we further examined the expression of miR-29a in NG and HG-induced HK-2 cells. Consistent with the microarray analysis, real-time PCR showed that the miR-29a expression level fell by 40–50% ($P < 0.01$) when cells were stimulated with HG for seven days (Fig. 2B). Northern blot analysis revealed a similar pattern (Fig. 2C). Taken together, these results confirm that miR-29a is significantly down-regulated by HG.

It was previously shown that long-term exposure of PTCs to high glucose induced TGF-β1 synthesis, whereas TGF-β1 knockout attenuated collagen IV biosynthesis in PTCs [2]. Thus, we presumed that TGF-β1 was involved in the down-regulation of miR-29a in HG-treated HK-2 cells. To confirm this, miR-29a levels were examined by real-time PCR after HK-2 cells were treated with TGF-β1 for 1, 2 or 3 days. Our data showed that miR-29a expression was also reduced in TGF-β1-treated HK-2 cells (Fig. 2D), confirming that miR29a is down-regulated by HG/TGF-β1 pathway in HK-2 cells.

3.3. MiR-29a down-regulates collagen IV protein in HK-2 cells

The striking down-regulation of miR-29a in HK-2 cells after elevation of the glucose concentration suggested that miR-29a might participate in the dysfunction of PTCs treated with HG. Previous reports indicated that the miR-29 family regulated the expression of collagen I or III in metastasis, myocardial fibrosis, and osteoblast differentiation [14–16]. As increased production of collagen IV in PTCs is an important pathological change during DN progression, we presumed that the down-regulation of miR-29a might promote the production of collagen IV in these cells. A series of functional studies were performed to determine the role of miR-29a in the regulation of collagen IV. First, we confirmed by western blot that high glucose induced the expression of collagen IV in HK-2 cells. As shown in Fig. 3A, collagen IV protein in HK-2 cells gradually in-

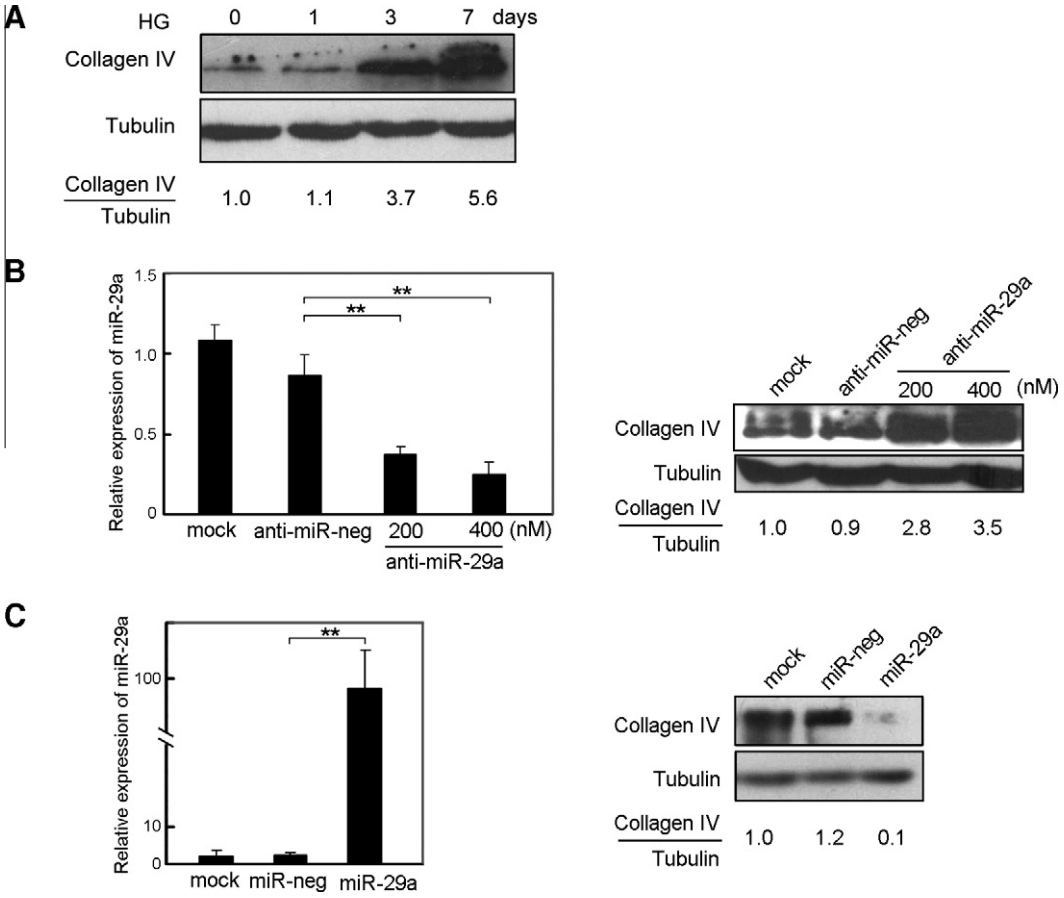

Fig. 3. MiR-29a regulates collagen IV in HK-2 cells. (A) HG induced collagen IV expression. Western blot of protein extracts from HK-2 cells treated with NG or HG for different durations using antibodies against collagen IV or tubulin. (B) Anti-miR-29a increased collagen IV protein expression. Real-time PCR for mature miR-29a in HK-2 cells transfected with 200 or 400 nM anti-miR-29a, anti-miR-neg or mock to determine the knockdown efficiency of anti-miR-29a (left panel). Western blots for collagen IV or tubulin using protein extracts from HK-2 cells transfected with 200 or 400 nM anti-miR-29a, anti-miR-neg or mock (right panel). (C) MiR-29a mimic decreased collagen IV protein expression. Real-time PCR for mature miR-29a in HK-2 cells transfected with miR-29a mimic, miR-neg or mock to determine the efficiency of the miR-29a mimic (left panel). Western blots for collagen IV or tubulin using protein extracts from HK-2 cells transfected with miR-29a mimic, miR-neg or mock in HK-2 cells treated with HG (right panel). Data represent three independent experiments ± S.E.M. with $n = 3$. $^{**}P < 0.01$.

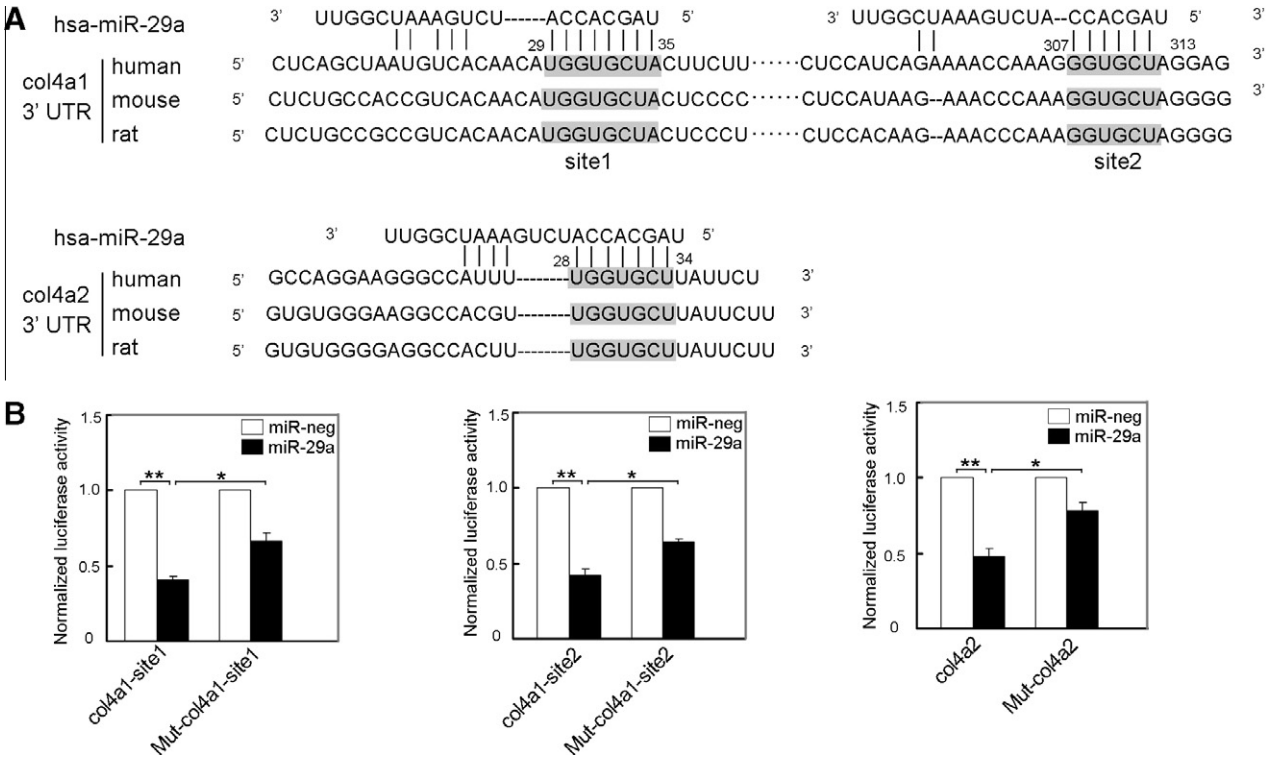

Fig. 4. MiR-29a directly targets *col4a1* and *col4a2*. (A) Sequence alignment between miR-29a and predicted binding sites in the 3'UTR of *col4a1* and *col4a2* for human, mouse and rat. (B) HK-2 cells were transfected with luciferase expression plasmids containing *col4a1* or *col4a2* 3'UTRs with the predicted miR-29a target site or plasmids containing a mutated sequence. The cells were also treated with either miR-29a mimic or negative mimic. Forty-eight hours after transfection, cells were assayed for firefly luciferase and Renilla luciferase. Data represent three independent experiments ± S.E.M. with $n = 3$. $^{*}P < 0.05$; $^{**}p < 0.01$.

creased with longer exposure to HG, supporting the negative correlation between the expression of miR-29a and collagen IV in HK-2 cells after seven days of HG induction.

Next, we determined whether transfection of miR-29a or anti-miR-29a affected collagen IV protein expression. A scrambled sequence (anti-miR-neg) was transfected as a negative control, and the knockdown efficiency of anti-miR-29a was confirmed by real-time PCR (Fig. 3B, left panel). Down-regulation of endogenous miR-29a with anti-miR-29a significantly induced collagen IV protein (Fig. 3B, right panel). In addition, we compared results of two different dose levels and observed that anti-miR-29a may increased collagen IV protein in a dose-dependent manner.

To determine whether overexpression of miR-29a has the opposite effect on collagen IV protein, HK-2 cells were transfected with double-stranded RNA oligonucleotides containing the mature miR-29a sequence (miR-29a mimic) or control miRNAs (miR-neg). Real-time PCR results showed that, compared with miR-neg, miR-29a mimic increased mature miR-29a levels by ~100-fold, confirming the efficiency of the mimic (Fig. 3C, left panel). We then treated HK-2 cells with this miR-29 mimic and found that it significantly decreased the collagen IV protein level (Fig. 3C, right panel). These data suggest that miR-29a acts as a repressor to regulate collagen IV protein expression level in HK-2 cells.

3.4. Col4a1 and col4a2 are directly targeted by miR-29a

The above results suggest that collagen IV is regulated by miR-29a in PTCs. However, this regulation could be indirect. To investigate whether miR-29a directly targets *col4a1* and *col4a2* (the two main subunits of collagen IV in PTCs), we screened the 3′UTR of *col4a1* and *col4a2* for complementarity to seed sequences of miR-29a via TargetScan software (www.targetscan.org) and found that miR-29a has two putative target sites in the 3′UTR of *col4a1* (position 29–35 nt and 307–313 nt) and one site in the 3′UTR of *col4a2* (position 28–34 nt, Fig. 4A). Moreover, these target site sequences are highly conserved among human, mouse and rat, suggesting that miR-29a may act as a direct regulator of collagen IV.

To test whether the putative miR-29a target sequences in the *col4a1* and *col4a2* 3′UTRs directly regulate gene expression, we constructed six luciferase expression plasmids containing the predicted miR-29a target sites in the *col4a1* or *col4a2* 3′UTRs or mutated seed region sequences, named *col4a1*-site1/Mut-*col4al*-site1, *col4a1*-site2/Mut-*col4al*-site2 and *col4a2*/Mut-*col4a2*, respectively. MiR-29a mimic was co-transfected with these reporters into HK-2 cells. As shown in Fig. 4B, compared with the miRNA negative mimic, transfection of the miR-29a mimic decreased the luciferase activity of *col4a1*-site1, *col4a1*-site2 and *col4a2* by 40.5%, 41.9% and 47.4%, whereas the mutant sequence restored levels to 66.4%, 64.0% and 77.8%, respectively. Taken together, our data demonstrate that miR-29a can directly regulate *col4a1* and *col4a2* through their 3′UTRs.

4. Discussion

The two main cell types in the renal cortex, GMCs and PTCs, are both linked to hyperglycemia-induced DN [1,3]. Previous studies found that up-regulation of miR-192 [7] and miR-377 [8] in GMCs contributed to the accumulation of extracellular matrix (ECM) proteins. Recently, a new study reported that miR-216a and miR-217 suppressed PTEN to activate Akt, which led to glomerular mesangial expansion and hypertrophy [17]. However, it was not clear whether these miRNAs were involved in PTC dysfunction during DN. Through a miRNA array approach, we determined the PTC miRNA expression profile and found that it was quite different from that of GMCs. For example, several miRNAs, including

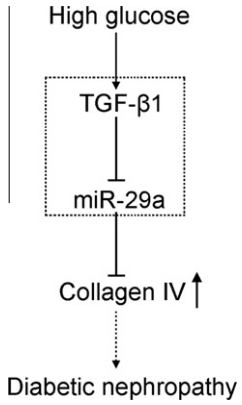

Fig. 5. A model for the role of miR-29a in the collagen IV increase induced by high glucose. In response to high glucose, TGF-β1 is activated [2] and triggers the down-regulation of miR-29a in PTCs. Consequently, collagen IV is up-regulated and contributes to tubulointerstitial fibrosis and DN.

miR-192 and miR-215, that were highly expressed in GMCs were almost undetectable in PTCs. Our data also revealed that miR-29a negatively regulates the expression level of collagen IV in PTCs, although it was not reported to be expressed in GMCs. These differences imply that miRNAs modulate the pathogenesis of DN in a cell type-specific manner.

Our array showed that more than 100 miRNAs can be detected in HK-2 cells, with the majority in very low abundance. Among the 40 most highly expressed miRNAs in HK-2, the expression level of miR-29a was present at medium, compared with more highly expressed miRNAs such as miR-30a and miR-21 (Fig. 1). Recent studies revealed that miRNAs regulated their targets in different patterns. In some cases, they seem to act as rheostats or "dimmer switches" to fine-tune the expression of target mRNAs [18], whereas in other cases they function as "on–off" switches [19]. Medium-level miRNAs like miR-29a in HK-2 cells often fall into the former category and co-exist with their targets (such as collagen IV) in the same cells. Under normal serum glucose, miR-29a expression maintains the physiological expression level of collagen IV in PTCs. After high glucose induction, miR-29a is down-regulated, permitting significant changes in collagen IV deposition in the long-term, and eventually leading to DN. In fact, many studies have revealed that miRNAs fine-tune key proteins to affect physiological or pathological processes [20] such as cardiogenesis [21], innate immune response [22] and hematopoietic differentiation [23]. Thus, fine-scale adjustments to target output by medium-expressed miRNAs may be a universal mechanism for regulating gene expression, especially in long-term processes of biochemical metabolism.

In summary, our studies revealed a new pathway for spatiotemporal control of collagen IV in PTCs during DN progression: high levels of glucose stimulate TGF-β1 synthesis and subsequently decrease the expression of miR-29a, and this elevates the level of collagen IV, which in turn mediates the pathogenesis of DN. A model depicting this new pathway is shown in Fig. 5.

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