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

Bin Du, Li-Ming Ma, Mian-Bo Huang, Hui Zhou, Hui-Lin Huang, Peng Shao, Yue-Qin Chen, Liang-Hu Qu *

Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory for Biocontrol, Sun Yat-sen University, Guangzhou 510275, PR China

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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

* Corresponding author. Address: Biotechnology Research Center, Sun Yat-sen University, Guangzhou 510275, PR China. Fax: +86 20 84036551.
E-mail address: lssqlh@mail.sysu.edu.cn (L.-H. Qu).

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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