Int J Clin Exp Med 2015;8(11):20610-20616
www.ijcem.com /ISSN:1940-5901/IJCEM0011591

Original Article

MicroRNA profiling identifies miR-129-5p as a regulator of EMT in tubular epithelial cells

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Received June 18, 2015; Accepted October 25, 2015; Epub November 15, 2015; Published November 30, 2015

Abstract: The importance of microRNAs in various diseases has been demonstrated, but their potential role in the pathogenesis of renal fibrosis needs to further research. We have profiled changes in microRNA levels in human kidney proximal tubular cell line HK-2 with TGF-β treatment and identified significantly altered miRNAs. miR-129-5p, was one of the significant down-regulated miRNAs in experimental models. PDPK1 was a potential target gene of miR-129-5p and luciferase assay analysis identified PDPK1 as a new direct target gene of miR-129-5p. Further research indicated that miR-129-5p suppressed PDPK1 mRNA and protein levels in HK-2 cells. miR-129-5p inhibited EMT via PDPK1 in HK-2 cells. In a conclusion, our findings suggested that miR-129-5p may function as a suppressor in renal fibrosis by targeting PDPK1.

Keywords: miR-129-5p, PDPK1, tubular epithelial cells

Introduction

Renal fibrosis is a common phenomenon of chronic kidney disease progressing to end-stage renal failure. Renal fibrosis is generally characterized either by interstitial extracellular matrix (ECM), or myofibroblast accumulation, and destruction of renal tubules [1, 2]. Although there are great advancement in diagnostic techniques and improvement in treatment modalities, the cure effect of renal fibrosis is still not good. There are many reports show that numerous pathways are activated during renal fibrosis. However, the mechanism is still far from clear, there needs to identify new molecular targets for renal fibrosis.

MicroRNAs (miRNAs) are small RNA and negative regulate gene expression in various cells [3]. The target genes of miRNAs can be down-regulated on post-transcriptional levels. Their function in the cells depends on their direct target genes [4, 5]. Emerging evidence showed that a set of miRNAs involves in the regulation of basic cellular processes such as cell proliferation, differentiation, apoptosis and metastasis in renal fibrosis. Previous reports showed that miR-146a, miR-145, miR-217 and other miRNAs play roles in renal fibrosis [6-8]. However, the molecular mechanism of renal fibrosis regulated by miRNAs needs to further research for finding new diagnostic marker or therapeutic methods.

Transforming growth factor-beta (TGF-β), a critical molecule, plays important roles in fibrosis [1, 2]. The study will focus on investigating the miRNAs profile of renal fibrosis in the cell model with TGF-β treatment to find different miRNAs and selecting significant miRNAs to explore their underlying roles in renal fibrosis.

Materials and methods

Tubular epithelial cell lines

Human kidney proximal tubular cell line HK-2 was purchased from the Institute of Basic Medical Science, Beijing, China. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Germany)/F-12 supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and maintained at 37°C in humidified air with 5% CO_2 [9].

miRNA array

HK-2 cells were exposed to TGF-β (10 ng/mL) for 24 h, and miRNAs were extracted using mirVana miRNA Isolation kit (Ambion) according to manufacturer's instructions. miRNA array was assayed by Kangcheng Company (Shanghai, China).

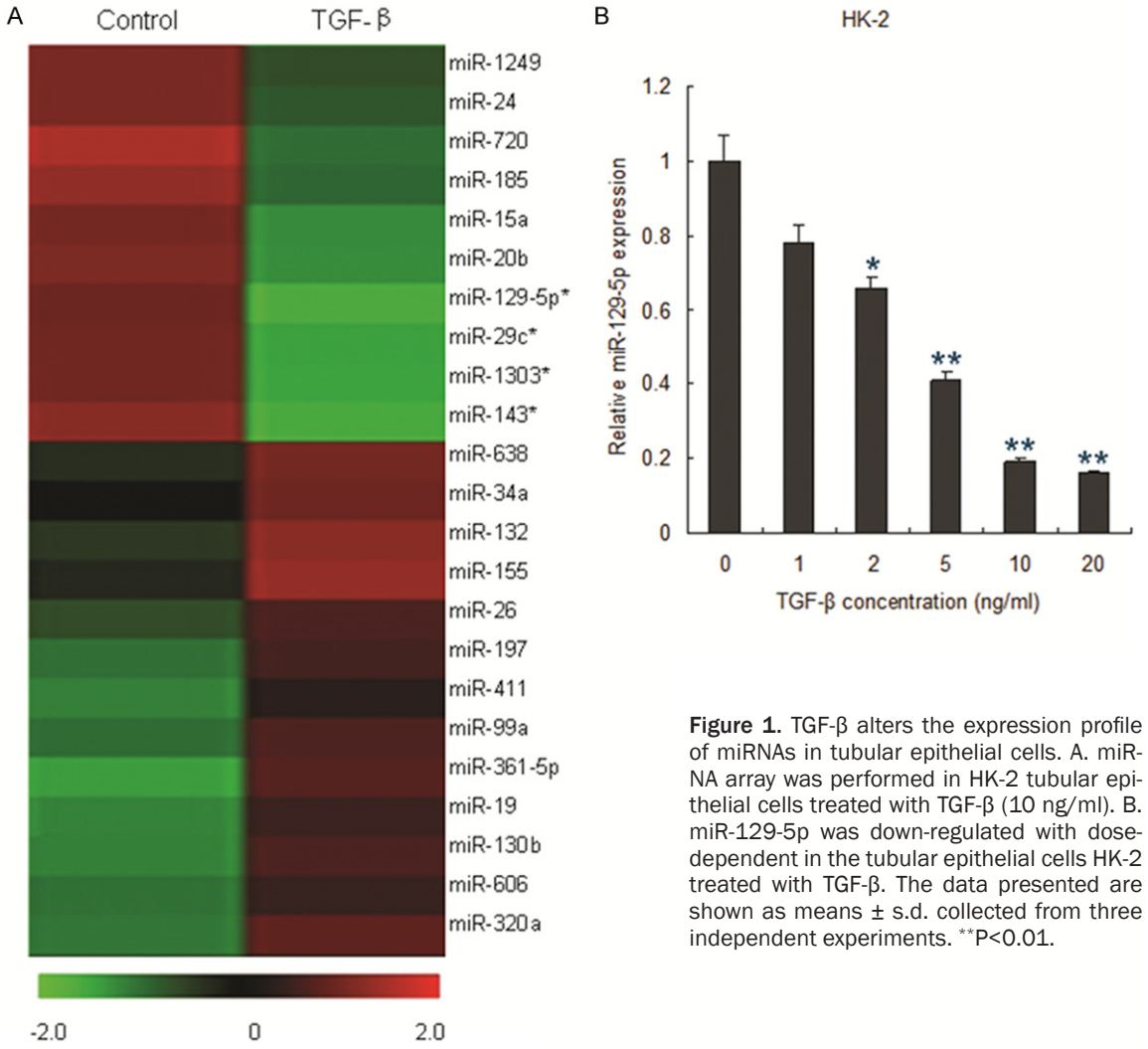

Figure 1. TGF-β alters the expression profile of miRNAs in tubular epithelial cells. A. miRNA array was performed in HK-2 tubular epithelial cells treated with TGF-β (10 ng/ml). B. miR-129-5p was down-regulated with dose-dependent in the tubular epithelial cells HK-2 treated with TGF-β. The data presented are shown as means ± s.d. collected from three independent experiments. $^{**}P<0.01$.

Vector construction and transfection

To generate the PDPK1 expression vector, the open reading frame of human PDPK1 cDNA was cloned into the pcDNA 3.1. The primers used for the amplification of the open-reading frame of the PDPK1 cDNA were 5'ATGGCCAGGACCACCAGCCAGCTGTATGACG3' (forward primer) and 5'AGTGACGTGTCGCCGCAGGCCCACCGA3' (reverse primer). For transient transfection, the cells were transfected with pcDNA 3.1 vector or pcDNA 3.1-PDPK1 at about 80% confluence using the Lipofectamine™2000 (Invitrogen, San Diego, CA, USA) reagent according to the manufacturer's instructions.

Luciferase assays

About 10^5 HK-2 cells were seeded in 12-well plates for 24 h before transfection. The cells were cotransfected with miR-129-5p or control combining with 100 ng of wild-type or mutant PDPK1 3'-UTR psiCHECK-2 plasmid (Promega) using Lipofectamine reagent (Invitrogen). Cell lysates were harvested 48 h after transfection and then firefly and Renilla luciferase activities were measured by the Dual- Luciferase Reporter Assay System (Promega) on a Berthold AutoLumat LB9507 rack luminometer. Renilla luciferase activities were normalized to firefly luciferase activities to control for transfection efficiency.

Western blotting

Total protein was extracted from the cells with miR-129-5p or PDPK1 transfection. Protein was separated in 10% SDS-PAGE and then transferred to PVDF membranes (Millipore). The membranes were incubated with primary

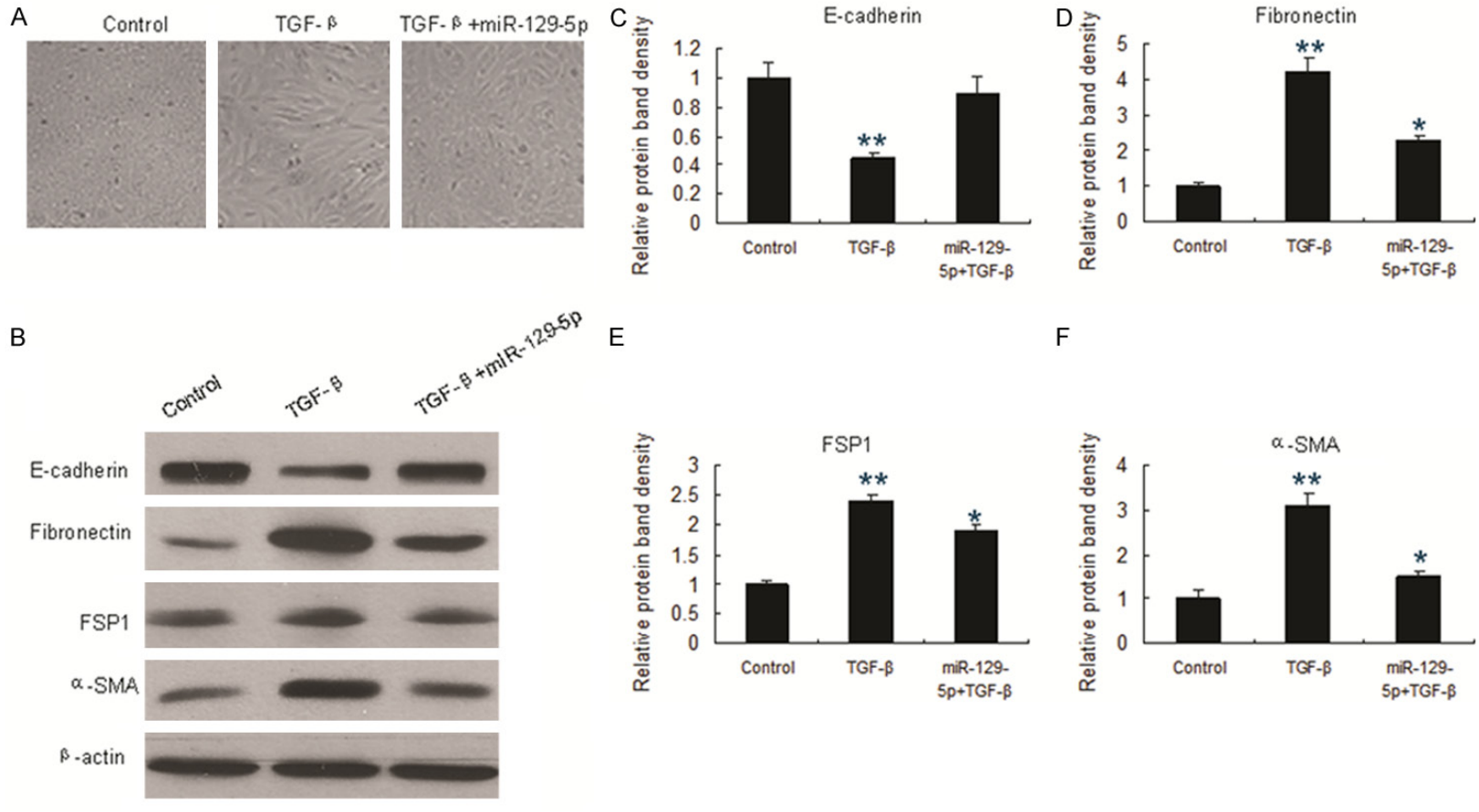

Figure 2. miR-129-5p inhibits EMT of tubular epithelial cells induced by TGF-β. A. miR-129-5p inhibited EMT of HK-2 cells stimulated by TGF-β. HK-2 cells were transfected with miR-129-5p mimics (miR-129-5p) and miRNA control (Control) for 24 h and then exposed to TGF-β (10 ng/ml) for 24 h. E-cadherin and Fibronection were strained by immunofluresence. B. miR-129-5p changed EMT associated protein levels in HK-2 cells. HK-2 cells were transfected with miR-129-5p mimics (miR-129-5p) and miRNA control (Control) for 24 h and then exposed to TGF-β (10 ng/ml) for 24 h. Total protein was extracted for western blotting. C-F. Qualification of protein bands from B. The data presented are shown as means ± s.d. collected from three independent experiments. $^{**}P<0.01$; $^{*}P<0.05$.

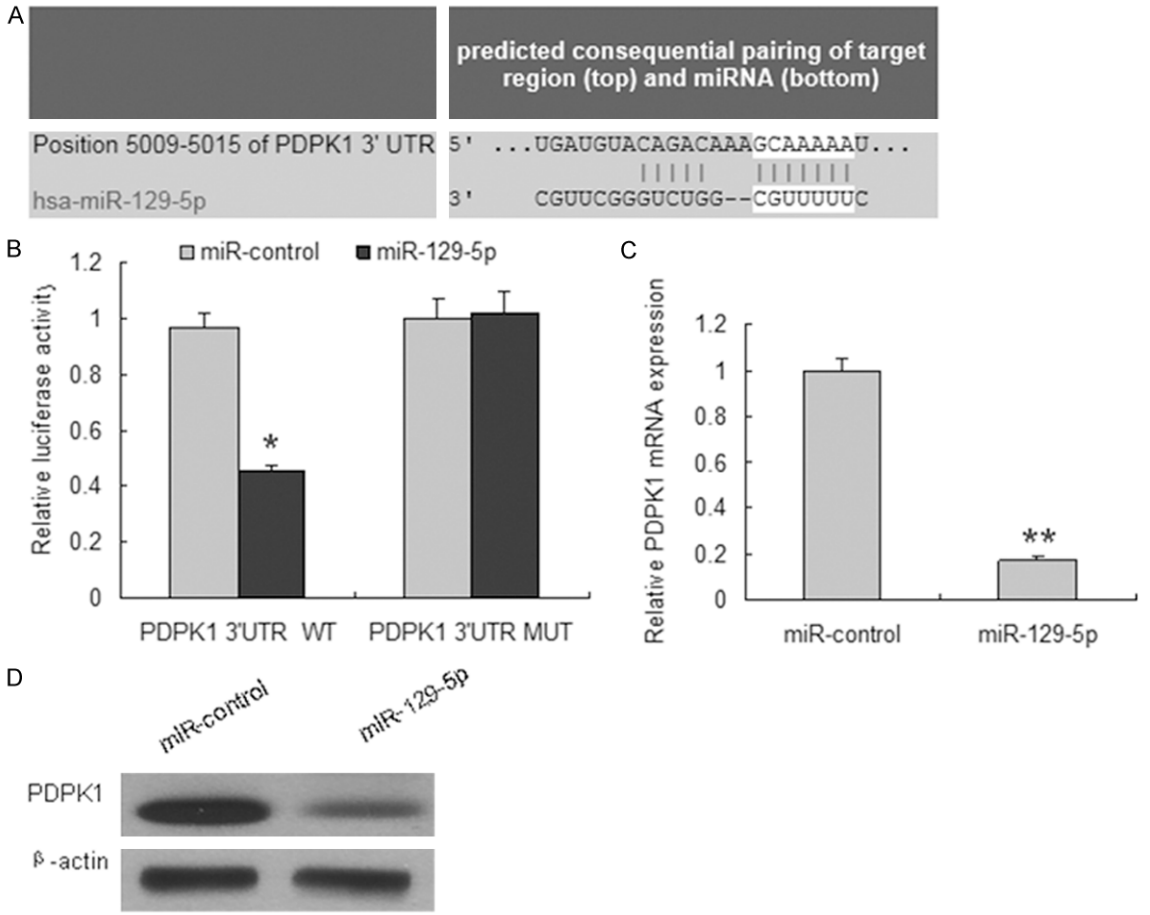

Figure 3. miR-129-5p down-regulates PDPK1 expression. A. The 3'-UTR of the PDPK1 gene contains binding sites for miR-129-5p according to bioinformatic analysis. B. miR-129-5p suppressed the expression of a luciferase reporter gene harboring the 3'-UTR of PDPK1. C. miR-129-5p down-regulated PDGFRA mRNA in HK-2 cells. Cells were transfected with miR-129-5p or control for 48 hours, then collected for real-time PCR. D. anti-miR-129-5p up-regulated PDPK1 mRNA in HK-2 cells. Cells were transfected with anti-miR-129-5p or control for 48 hours, then collected for real-time PCR. E. miR-129-5p down-regulated PDPK1 protein in HK-2 cells. Cells were transfected with anti-miR-129-5p or control for 48 hours, then collected for western blotting. The data presented are shown as means ± s.d. collected from three independent experiments. $^{**}P<0.01$.

antibodies overnight at 4°C and then with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature. The protein bands were visualized using the enhanced chemiluminescence system and exposed to Kodak XOMAT film (Rochester, New York, USA). The primary antibodies PDPK1, E-cadherin, fibronection, FSP1 and α-SMA were purchased from Cell Signaling (Danvers, MA, USA). The β-actin antibody was purchased from Sigma.

qRT-PCR

Total RNA from the cells were isolated and performed for qRT-PCR. The primers based on the microRNA sequences DNA were amplified with specific primers and Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen) on an ABI PRISM 7900 real-time PCR system (Applied Biosystems). The relative expression levels were calculated by comparing Ct values of the samples with those of the reference, all data normalized to GAPDH or U6 control.

Statistical analysis

Statistical analysis was carried out using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Student's t test was used to analyze the results expressed as mean ± SD. A *P* value of <0.05 was considered to be statistically significant. All experiments were performed in triplicate.

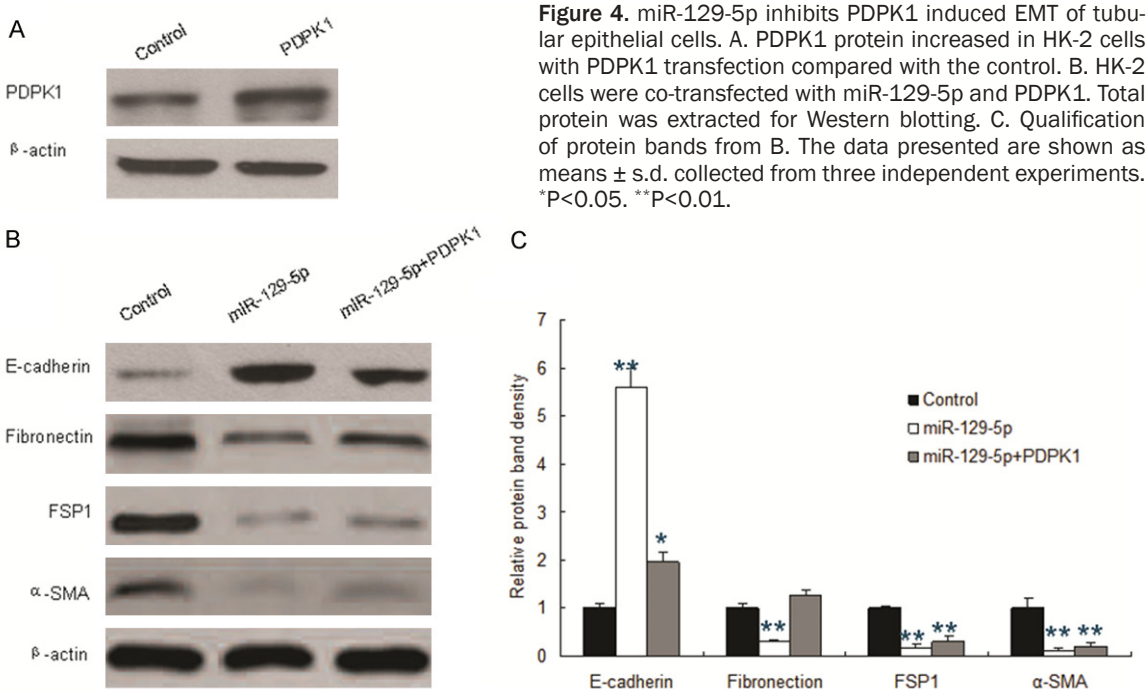

Figure 4. miR-129-5p inhibits PDPK1 induced EMT of tubular epithelial cells. A. PDPK1 protein increased in HK-2 cells with PDPK1 transfection compared with the control. B. HK-2 cells were co-transfected with miR-129-5p and PDPK1. Total protein was extracted for Western blotting. C. Qualification of protein bands from B. The data presented are shown as means ± s.d. collected from three independent experiments. $^{*}P<0.05$. $^{**}P<0.01$.

Results

TGF-β alters the expression profile of miRNAs in tubular epithelial cell

To find out the different miRNAs that involves in renal fibrosis, tubular epithelial cells were treated with TGF-β and performed for miRNA array analysis. The results showed that there were many different miRNAs altered in a tubular epithelial cell line HK-2 with TGF-β treatment (**Figure 1A**). miR-129-5p is the one of most significant one. The result confirmed miR-129-5p was inhibited greatly in HK-2 cells treated with different concentration of TGF-β (**Figure 1B**).

miR-129-5p inhibits EMT of tubular epithelial cells induced by TGF-β

To further investigate the effect of miR-129-5p on EMT, tubular epithelial cells HK-2 were transfected with miR-129-5p mimics and miRNA control, the result showed that EMT of HK-2 induced by TGF-β (10 ng/ml) was inhibited (**Figure 2A**). The EMT associated proteins were also examined by western blotting and it was found that E-cadherin protein levels increased and Fibronection, FSP1, α-SMA decreased in HK-2 cells with miR-129-5p transfection (**Figure 2B** and **2C**). The data indicated that miR-129-5p could inhibit EMT of tubular epithelial cells induced by TGF-β.

miR-129-5p down-regulates PDPK1 expression in HK-2 cells

miR-129-5p plays suppressing roles in EMT of HK-2 cells, however, its target gene is not fully clear. Bioinformatic analysis showed that 3-phosphoinositide dependent protein kinase-1 (PDPK1) (NM_002613) was directly suppressed by miR-129-5p (**Figure 3A**). As shown in **Figure 3B**, the luciferase activity of PDPK1 in HK-2 cells was much lower than in control cells. The luciferase activity of mutation was rescued in the cells. We next examined whether miR-129-5p could regulate endogenous PDPK1 expression in the cells. In HK-2 cells, compared with control, endogenous PDPK1 mRNA (**Figure 3C**) and protein levels (**Figure 3D**) were down-regulated in the cells with miR-129-5p transfection.

miR-129-5p inhibits PDPK1 induced EMT of tubular epithelial cells

Above data showed that PDPK1 was the target gene of miR-129-5p, we want to know whether miR-129-5p inhibits EMT by targeting PDPK1. HK-2 cells were transfected with PDPK1 plasmids and PDPK1 protein increased (**Figure 4A**).

Next, HK-2 cells were co-transfected with miR-129-5p mimics and PDPK1 and the data showed that miR-129-5p inhibited EMT of HK-2 cells induced by PDPK1 overexpression (**Figure 4B** and **4C**).

Discussion

In the present study, our findings showed that miR-129-5p is usually downregulated in renal fibrosis and plays an important role in metastasis by targeting PDPK1. Data of miRNA profile indicated that there are many changes of miRNAs in the established renal fibrosis models. The up-regulated miRNA included miR-633, miR-34a, miR-132, miR-155; the down-regulated miRNs included miR-15a, miR-20b, miR-29c, miR-1303, miR-143. In this study, we chosen miR-129-5p because of its significant different from other down-regulated miRNAs and its previous reported roles in diseases, which show that miR-129-5p could inhibit cancer cell proliferation, migration and invasion [10-17]. With bioinformatics prediction and it was identified that PDPK1 is the direct target of miR-129-5p. Overexpression of miR-129-5p decreased the levels of PDPK1 protein and mRNA, suggesting that PDPK1 is a downstream gene of miR-129-5p in cells. But miR-129-5p in HK-2 cells may be targets other genes in different biological systems. Absolutely, there are molecules influencing miR-129-5p which involves in rental fibrosis development, growth and other functions.

3-Phosphoinositide-dependent kinase 1 (PDK1), encoded by the gene *PDPK1*, is a molecular kinase belonging to the phosphoinositide-3-kinase (PI3K) signaling pathway [18, 19]. PDPK1 is usually activated in many types of cancer and its role in renal disease including fibrosis is lack of evidence from the reports. Our study showed that PDPK1 is overexpressed in tubular epithelial cells with TGF-β treatment and miR-129-5p could inhibit its expression on post-transcriptional levels.

To draw a conclusion, our present study verified that the deregulated expression of miR-129-5p was associated with poor prognosis and aggressive phenotype of renal fibrosis. This study also implied that miR-129-5p played an important role in the regulation of EMT of tubular epithelial cells by directly targeting PDPK1. Generally, the study suggested that miR-129-5p might be a potential prognostic biomarker and a therapeutic molecule in renal fibrosis.

Disclosure of conflict of interest

None.

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