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Original Article

Roles of miR-200a in renal fibrosis through regulating ZEB1 and ZEB2

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Abstract: Objective: This study aims to investigate the role and mechanism of miR-200a in the epithelial-mesenchymal transition (EMT) of kidney epithelial cells. Methods: Through treatment with transforming growth factor-β1 (TGFβ1), renal epithelial cells HKC were induced to EMT. MiR-200a expression was detected by qRT-PCR and EMT related proteins E-Cadeherin and Vimentin were detected by Western blot. Bioinformatics methods were used to predict the target genes of miR-200a. After transfection with miR-200a mimics or siRNA of ZEB1 and ZEB2, the expressions of ZEB1, ZEB2 and other EMT related proteins were detected by Western blot. The HKC cells with stable expression of ZEB1 and ZEB2 were selected by G418 pressure screening. EMT changes were evaluated for HKC cells. Dual luciferase assay was applied to identify the target of miR-200a. Results: MiR-200a expression was significantly decreased in EMT processes in HKC cells induced by TGFβ1, and E-Cadherin expression was down-regulated while Vimentin expression was up-regulated after TGFβ1 treatment. The results indicated that TGFβ1 can induce the occurrence of EMT. In vitro results showed that miR-200a induced EMT in HKC cells. Over-expressed miR-200a down-regulated the expressions of ZEB1 and ZEB2, and EMT of HKC cells were significantly repressed. After RNAi interference for ZEB1 and ZEB2, the EMT process in HKC cells was inhibited. HKC cells with stable expression of ZEB1 and ZEB2 were selected by G418 pressure screening, and the selected cells stably expressed green fluorescence. Increased expression of ZEB1 and ZEB2 significantly promoted EMT process in HKC cells. These results of dual luciferase assay indicated that miR-200a regulated the expression of ZEB1 and ZEB2 through complementary binding. Conclusion: MiR-200a expression was significantly down-regulated in EMT cell model induced by TGFβ1. MiR-200a can inhibit EMT process in HKC cells through directly target ZEB1 and ZEB2, which indicates that miR-200a may be a potential therapeutic target for renal fibrosis.

Keywords: MiR-200a, ZEB1, ZEB2, renal fibrosis

Introduction

Chronic kidney disease (CKD) is a common kidney disease in clinic, and the incidence rate increased significantly worldwide as the aging population, diabetes, obesity and hypertension patients increased in recent years [1, 2]. Renal Interstitial Fibrosis (RIF), tubular atrophy and peritubular capillaries is the final pathological changes of CKD, which caused irreversible damage to kidneys [3, 4]. The associated molecular mechanisms were still unclear. It was studied that under the stimulation of the continuous injury, chronic inflammation and other factors, epithelial-mesenchymal transition (EMT) can occur in tubular epithelial cells [5, 6]. The epithelial cells can be transmitted into mesenchymal cells, which damages tubulointerstitium and eventually develop to tubulointerstitial fibrosis [7, 8]. So EMT process is the key step in RIF progression. It is important and valuable to explore the molecular mechanisms of EMT.

MicroRNA (miRNA) is one class of non-coding RNA with length about 18-22 nucleotides. MiRNA can inhibit translation of target genes through complementary pairing with the 3'UTR of target mRNA, which is an important post-transcription regulator [9, 10]. It was demonstrated that miRNA participated in different physiological and pathological processes, such as tumor, cardiovascular diseases, and diabetes [11, 12]. MiR-200a is widely concerned as its tumor suppressor effects [13]. It was shown that miR-200a can weaken the invasion and metastasis of tumor cells through inhibiting the

EMT, while there is no report about roles of miR-200a in RIF. In this study, we investigated the roles of miR-200a in RIF through qRT-PCR and Western blot methods. We try to deeply discuss. The biological functions of miRNA in kidney fibrosis were discussed.

Materials and methods

HKC cell culture

The normal HKC cells were purchased from cell bank of Shanghai Institute for Biological Science, CAS. The DMEM/F12 medium with 10% FBS was used to culture the cells in 37°C within 5% CO_2 incubator. When spread to 70%-80% area in flasks, the cells were used to for passage based on routine procedure.

EMT induction by TGFβ1

The recombinant TGFβ1 protein (BD, New Jersey, USA) was added to culture medium of HKC cells at 10 ng/ml concentration and incubated for 72 h to induce EMT. At 24 h, 48 h, and 72 h of incubation, the HKC cells were collected to extract total RNA and proteins.

RNA extraction

For 24-well plate, 0.5 ml Trizol reagent (Invitrogen, California, USA) was added for each well. Phenol-chloroform method was used to extract total RNA. The cDNA was synthesized from total RNA by the reverse transcription with miRNA cDNA Kit (Takara Company, Dalian, China) and stored in -20°C. The reverse system of miRNA included: 5 μl miRNA template, 2× miRNA Reaction Buffer Mix 10 μl, 0.1% BSA 2 μl, miRNA PrimeScript RT Enzyme Mix 2 μl, totally 20 μl. The reaction was performed at 37°C for 60 min with PolyA primer.

Real-time PCR to detect the expression of miR-200a

The SYBR Green qPCR was used to detect miR-200a expression in HKC cells after treatment with TGFβ1, and U6 was internal reference. The primers of miR-200a was 5' CACTGUCTGGTAACGT 3' and Uni-miR qPCR Primer provided by kit. The 30 μl miRNA reaction system included 10 μl qRT-PCR-Mix, 1 μl forward primer and 1 μl reverse primer, 5 μl cDNA and 13 μl ddH_2O, and the cycle conditions were the following: 95°C for 10 min, and followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. Each sample had 3 replicates and average was used. The relative expression of miR-200a was calculated by the $2^{-\Delta\Delta T}$ method.

MiR-200a mimics transfection

HKC cells were divided into Control group, Negative control group, and miR-200a mimics group. All cells were cultured in antibiotics free DMEM F12 medium with 10% FBS. When cell density reached about 70%~90%, 1.25 μl of miR-200a mimics (20 pmol/μl) and 1 ul of lipo2000 was added into EP tubes containing 50 μl Opti Memi medium respectively. The 2 tubes were mixed together after 5 min standing, then followed by another 20 min incubation at room temperature. The mixture was added to each well and DMEM F12 medium containing 10% FBS was replaced after 6 h. The cells were harvested after transfection 48 h and Western blot was used to detect the expression of ZEB1, ZEB2 and EMT related proteins.

Western blot analysis

The collected cells were washed twice by precooled PBS, and then lysed with RIPA containing PMSF to lysis for 5 min on ice. The supernatant was centrifuged at 4°C 12000 rpm/min for 10 min. Then the supernatant was mixed with 2× loading buffer and boiled at 100°C for 10 min, and 5 μl mixture was separated by SDS-PAGE (100 V constant voltage) and then transferred to PVDF membrane (300 mA constant current 2 h). After blocking by 50 g/l skim milk for 1 h at room temperature, primary antibodies (ZEB1 1:1000, ZEB2 1:1000, Vimentin 1:800, E-Cadherin 1:1000, and GAPDH 1:5000) were added to incubate overnight at 4°C. The second antibody was HRP-conjugated goat anti-mouse (1:5000) and goat anti-rabbit IgG (1:2000). All antibodies were bought from Abcam Company (Boston, USA). After incubation for 1 h for secondary antibodies at room temperature, TBST was used to wash the membrane for 5 times (each time for 5 min). Finally, the membrane was developed by enhanced ECL (Beyotime Biotechnology, Beijing, China).

RNA interference by siRNA

HKC cells were divided into 4 groups: Control, Negative control, siR-ZEB1, and siR-ZEB2, and liposome method was used to transfect siR-

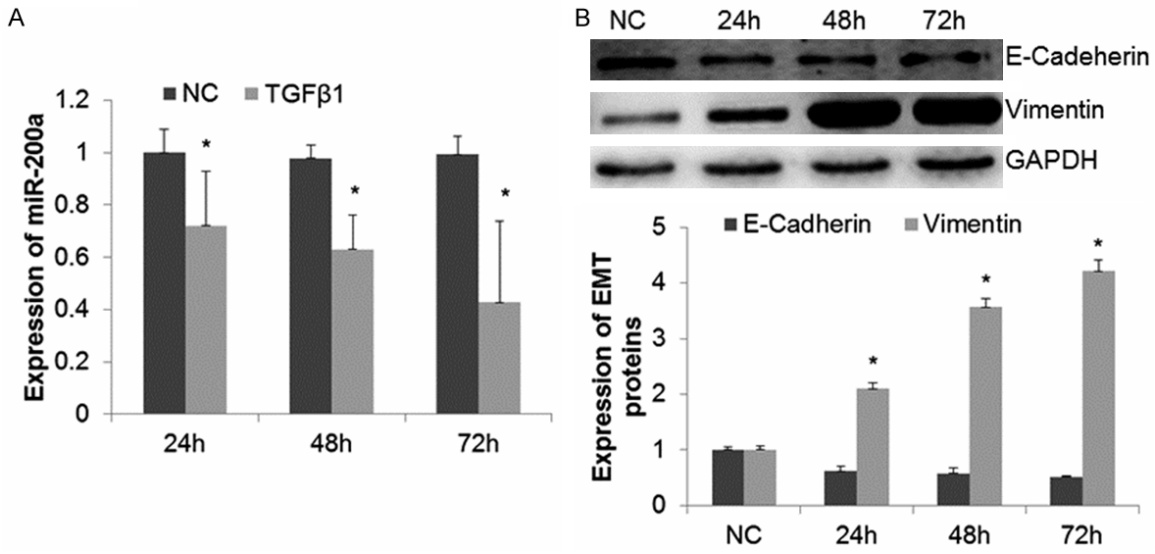

Figure 1. The expression of miR-200a in HKC cells and EMT changes induced by TGFβ1. A. MiR-200a was decreased in HKC cells treated with TGFβ1 compared with negative control cells by qRT-PCR (*P < 0.05), and miR-200a expression was time dependent. B. Expressions of E-Cadherin protein and Vimentin protein in HKC cells treated with TGFβ1 by Western blot. Compared with Negative control, E-Cadeherin expression intensity was decreased while Vimentin intensity was increased after TGFβ1 treatment (*P < 0.05).

NAs. After siRNA transfection 24 h, FAM fluorescence was observed under microscope to estimate the transfection efficiency. After transfection 48 h, Western blot was used to detect the expression of ZEB1, ZEB2 and EMT related proteins.

ZEB1 and ZEB2 over expression and miR-200a co-transfection

The over-expression plasmids for ZEB1 and ZEB2 (CMV-MCS-EGFP-SV40-Neomycin-ZEB1) were constructed. Positive HKC cells were enriched by G418 pressure screening method, and the transfection efficiency was evaluated by observing EGFP green fluorescence. Western blot was used to detect the expression change of ZEB1 and ZEB2. After co-transfection with miR-200a, the expressions of EMT related proteins were detected.

Dual luciferase assay

Based on the bioinformatics prediction, the wild-type 3'UTR and the mutant 3'UTR of ZEB1 and ZEB2 were synthesized in vitro and were cloned into the downstream of pMIR-REPORT luciferase vector by Spe-1 and HindIII enzyme. HEK293T cells were co-transfected with miR-200a mimics and wild-type c-Met 3'UTR or the mutant 3'UTR. After transfection for 24 h, cells were lysed and luciferase intensity was measured by GloMax 20/20 luminometer (Promega, Wisconsin, USA) based on the standard protocol of the luciferase kit (Promega, Wisconsin, USA). The intensity of *Renilla* was used as control, and the fluorescence intensity in different groups was analyzed.

Statistical analysis

The SPSS 16.0 software was used to do statistical analysis. All the data were shown as the mean ± SD, and difference were determined by two-tailed Student's t-test. P < 0.05 was considered as statistically significant.

Results

MiR-200a expression in HKC cells induced by TGFβ1

To check the association of miR-200a expression with EMT process in HKC cells, we used qRT-PCR to detect miR-200a expression in HKC cells after treatment with TGFβ1. As shown in **Figure 1A**, compared with negative control, miR-200a expression was decreased in HKC cells treated with TGFβ1. And miR-200a expression decreased significantly depending on the treatment time of TGFβ1 (P < 0.05). We also detected the expression of EMT related pro-

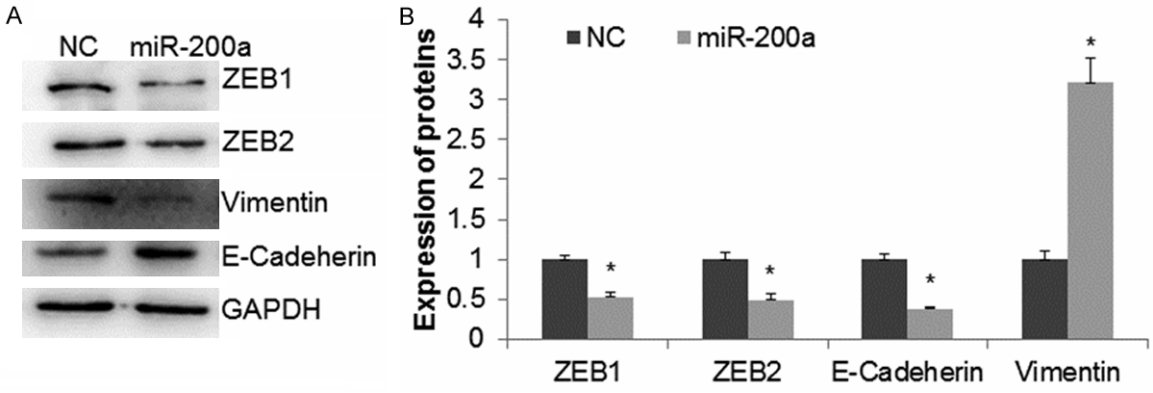

Figure 2. The expression of ZEB1, ZEB2 and EMT related proteins in HKC cells after miR-200a over-expression. A. Expressions of ZEB1, ZEB2, E-Cadherin, and Vimentin in HKC cells after miR-200a over-expression by Western blot. B. Compared with Negative control, the expression intensities of ZEB1, ZEB2, and E-Cadeherin were down-regulated in HKC cells over-expressed miR-200a, while intensity of Vimentin was up-regulated (*P < 0.05).

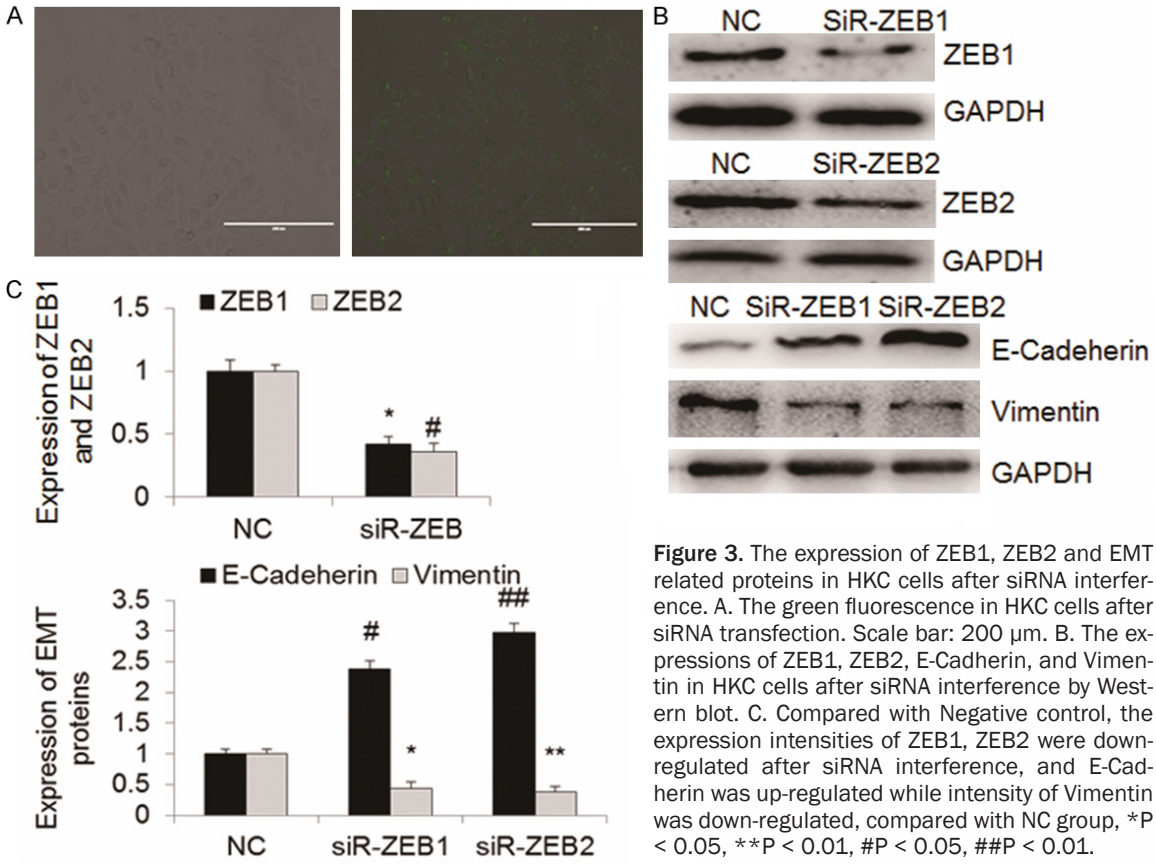

Figure 3. The expression of ZEB1, ZEB2 and EMT related proteins in HKC cells after siRNA interference. A. The green fluorescence in HKC cells after siRNA transfection. Scale bar: 200 μm. B. The expressions of ZEB1, ZEB2, E-Cadherin, and Vimentin in HKC cells after siRNA interference by Western blot. C. Compared with Negative control, the expression intensities of ZEB1, ZEB2 were down-regulated after siRNA interference, and E-Cadherin was up-regulated while intensity of Vimentin was down-regulated, compared with NC group, *P < 0.05, **P < 0.01, #P < 0.05, ##P < 0.01.

teins in HKC cells. As shown in **Figure 1B**, Vimentin protein was up-regulated while E-Cadeherin protein was down-regulated (P < 0.05), which indicated that TGFβ1 can succeed to induce EMT of HKC cells. MiR-200a may be associated with the EMT process induced by TGFβ1.

Regulation on ZEB1, ZEB2, and EMT related proteins by over-expressed miR-200a

To study how miR-200a participated in the EMT process, we applied Western blot to detect the expression of EMT related genes that were predicted by bioinformatics methods as potential

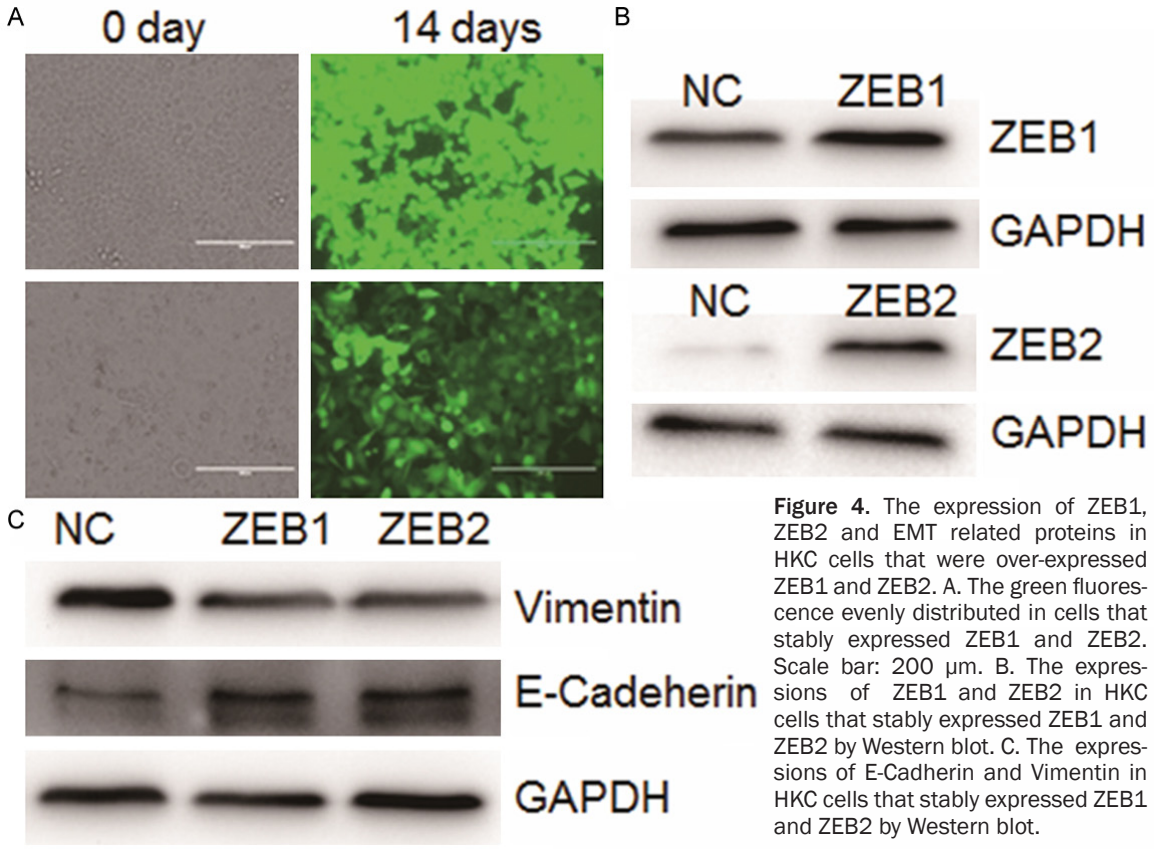

Figure 4. The expression of ZEB1, ZEB2 and EMT related proteins in HKC cells that were over-expressed ZEB1 and ZEB2. A. The green fluorescence evenly distributed in cells that stably expressed ZEB1 and ZEB2. Scale bar: 200 μm. B. The expressions of ZEB1 and ZEB2 in HKC cells that stably expressed ZEB1 and ZEB2 by Western blot. C. The expressions of E-Cadherin and Vimentin in HKC cells that stably expressed ZEB1 and ZEB2 by Western blot.

target genes of miR-200a. ZEB1 and ZEB2 were the predicted target genes of miR-200a, and the 2 genes were also EMT related genes that play important roles in EMT process. We used liposome method to over-express miR-200a in HKC cells, and then detected the expressions of ZEB1, ZEB2, and EMT related proteins. As shown in **Figure 2**, the expressions of ZEB1, ZEB2, and E-Cadherin were decreased while Vimentin expression was up-regulated. The results indicated that miR-200a may participate in EMT through regulating ZEB1 and ZEB2 expression.

Effects of ZEB1 and ZEB2 siRNA on EMT in HKC

To validate the roles of ZEB1 and ZEB2 in EMT process of HKC cells, we used RNAi interference to down-regulate the expressions of ZEB1 and ZEB2, and then detected the expressions of EMT related proteins. As shown in **Figure 3A**, after siRNA transfection 24 h, the green fluorescence was evenly distributed in HKC cells, and the transfection efficiency reached more than 90%, which was satisfied to following experiments. After interference for 48 h, the expression of ZEB1 and ZEB2 proteins were significantly down-regulated, which indicated that the effects of RNAi was efficient. And E-Cadherin protein was up-regulated while Vimentin protein was down-regulated, which indicated that EMT process in HKC was inhibited (**Figure 3B** and **3C**). The results indicated that down-regulated ZEB1 and ZEB2 inhibited EMT process in HKC cells, which was consistent with the over-expressed miR-200a results.

Over-expressed ZEB1 and ZEB2 inhibited the regulation of miR-200a

To investigate whether the over-expressed ZEB1 and ZEB2 can relieve the regulation by miR-200a, we transfected miR-200a in the HKC cells that can stably express high level of ZEB1 and ZEB2 to detect the changes of EMT. Through G418 pressure screening and Western blot, we selected HKC cells that stably expressed ZEB1 and ZEB2. As shown in **Figure 4A** and **4B**, green fluorescence stably expressed in HKC cells, and the expressions of ZEB1 and ZEB2 were significantly up-regulated ($P < 0.05$).

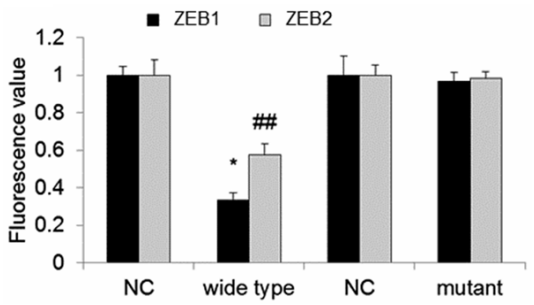

Figure 5. ZEB1 and ZEB2 are directly targeted by miR-200a. Dual luciferase assay showed that miR-200a can direct regulate ZEB1 and ZEB2 by binding to 3'UTR. *P < 0.05, ##P < 0.01.

After over-expressing miR-200a by liposome method, the expression of EMT related proteins were detected. As shown in **Figure 4C**, after miR-200a over-expression, Vimentin expression was decreased in HKC cells that stably expressed ZEB1 and ZEB2 than control, while E-Cadherin expression was increased. The results showed that over-expressed ZEB1 and ZEB2 may inhibit the roles of miR-200a on EMT in HKC cells, which indicated that miR-200a may play regulatory roles in EMT through regulating ZEB1 and ZEB2 expression.

ZEB1 and ZEB2 are directly targeted by miR-200a

To determine whether ZEB1 and ZEB2 were targeted directly by miR-200a, we detected the GFP intensity by dual luciferase assay. As shown in **Figure 5**, we found that the GFP intensity was significantly decreased when co-transfected with miR-200a mimics and pMIR-REPORT-ZEB1/ZEB2 wild type vector compared with NC ($P < 0.05$), while no significant difference between NC and mutant pMIR-REPORT-ZEB1/ZEB2 mutant vector ($P > 0.05$). The results validated the prediction that miR-200a can directly bind to the "seed region" in 3'UTR of ZEB1 and ZEB2 mRNA.

Discussion

It was studied that the etiology of renal fibrosis was related to the imbalance of extracellular matrix (ECM) degradation and production, and also closely associated with the proliferation of fibroblasts [14]. The excessive accumulation and deposition of EMC is an important pathological feature in renal fibrosis [15], and myofibroblasts (MFB) is the primary effector cells which play key roles in this process [16]. In recent years, several studies show that epithelial-mesenchymal transition (EMT) is one of the major ways leading to abnormal proliferation of fibroblasts [17]. EMT is referred to the phenomenon that epithelial cells were transmitted and differentiated to mesenchymal cells under external stimuli, and the molecular biomarkers are reducing expression of epithelial cell marker protein (E-Cadherin) and increasing expression of mesenchymal cell markers (Vimentin). EMT plays an important role in kidney disease, and reverse EMT process may improve early renal fibrosis. Although it has been confirmed that EMT plays important roles in renal fibrosis, it is still unclear about the exact molecular mechanism.

MiR-200a is a tumor suppressor factor, which can inhibit epithelial-mesenchymal transition of variety of tumor cells in vitro, then inhibits the invasion and metastasis. We hypothesize that miR-200a plays roles in renal fibrosis. So we used TGFβ1 to treat kidney epithelial cells to construct EMT model. It was shown that E-Cadherin (kidney epithelial cells marker) was down-regulated and Vimentin (mesenchymal markers) was up-regulated significantly after TGFβ1 treatment 72 h, which indicated that EMT cell model was successfully constructed. With EMT of HKC cells, miR-200a expression was significantly reduced, which indicated that miR-200a was associated with EMT. ZEB1 and ZEB2 were predicted to be target genes of miR-200a, and ZEB1 and ZEB2 played important roles in EMT of tumor cells. Therefore, we over-expressed miR-200a in HKC cells in vitro, then the expressions of ZEB1 and ZEB2 were significantly decreased ($P < 0.05$). Meanwhile, E-Cadherin was upregulated while Vimentin was significantly reduced, which indicated that EMT process was reversed. This result suggests that overexpressed miR-200a inhibits EMT in HKC cells, which may play an important role in the development of renal fibrosis. To determine the roles of ZEB1 and ZEB2 in EMT process, we applied siRNA and overexpression technology to study the function. It was shown that the EMT degree was slowed after down-regulating ZEB1 and ZEB2 by RNAi, while the EMT degree was speeded up after over-expressed ZEB1 and ZEB2. The results indicated that ZEB1 and ZEB2 promoted EMT process of HKC cells. The results of dual luciferase assay showed that ZEB1 and ZEB2 were directly targeted by miR-200a.

In summary, miR-200a inhibits the transmission from epithelial cells to mesenchymal cells through directly regulating the expression of ZEB1 and ZEB2. MiR-200a is an inhibition factor for EMT, which has clinical value as it is also a potential drug target in kidney fibrosis.

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Disclosure of conflict of interest

None.

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