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Original Article

MiR-21 overexpression enhances TGF-β1-induced epithelial-to-mesenchymal transition by target RECK in hepatic oval cells

Shengqiang Gao, Dongdong Chen, Liangwang Zou, Lidong Huang, Ruijie Dai, Jie Zheng, Zhichao Mo, Weijian Hu, Yunfeng Shan

Department of Hepatobiliary Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, P. R. China

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Abstract: MiRNAs have been shown to play fundamental role in diverse biological and pathological processes, including fibrotic diseases. In the present study, we investigated whether miR-21 regulated the fibrogenic epithelial-mesenchymal transition (EMT) in rat hepatic oval cells WB-F344 and explored underlying mechanisms. The results showed that treatment of WB-F344 cells with pro-fibrogenic factor TGF-β1resulted in increased expression of miR-21 and promoted fibrogenic EMT in hepatic oval cells. Downregulation of miR-21 expression by transfection of miR-21 inhibitor lentivirus into WB-F344 cells inhibited fibrogenic EMT induced by TGF-β1. Furthermore, overexpression of miR-21 alone also resulted in EMT-like transformation in WB-F344 cells. TGF-β1 treatment resulted in decreased RECK and anti-miR-21 canceled this effect. Overexpression of miR-21 in WB-F344 cells also downregulated RECK. Inhibition of RECK by RECK siRNA enhanced TGF-β1 and miR-21-induced fibrogenic EMT. In summary, our results identify miR-21 as a key regulator of fibrogenic EMT in hepatic oval cells via RECK. Targeting miR-21 may provide a new therapeutic strategy against hepatic fibrosis.

Keywords: miR-21, TGF-β1, EMT, RECK

Introduction

In almost all patients with chronic liver injury, we find a reversible scarring response liver fibrosis. Accmulation of extracellular matrix (ECM) is characteristic of liver fibrosis, finally inducing to liver cirrhosis. Hepatic oval cells have emerged as a frequent part of normal repair, and a crucial element in the pathobiology of fibrotic liver diseases. Apart of that, another related mesenchymal cell types may also play partly contributions to total ECM accumulation [1], including classical portal fibroblasts [1], bone marrow derived cells [2], fibroblasts derived from epithelial-mesenchymal transition (EMT) as well [3].

EMT is a process in which epithelial cells lose basal-apical polarity, become more spindle-shaped, and acquire invasion like cancer stem cell (CSC) phenotypes with a heightened propensity which can enhance migration [4]. And EMT can greatly accelerate deposition of ECM components [5]. It has been reported that epithelial cells are potential sources of fibroblasts via EMT in hepatic fibrosis, lung [6] and renal [7].

MicroRNAs (miRNAs), small, regulatory RNA molecules of 21-23 nucleotides, are involved in the regulation of biological processes, including cell proliferation, differentiation, apoptosis, homeostasis, and stress responses [8]. They act as negative regulators of gene expression by inhibiting mRNA translation or promoting mRNA degradation [9]. We all know that miRNAs participate in activation of HSC and the liver fibrotic process [10]. During recent years, additional roles of miR-21 in renal, pulmonary and cardiac fibrosis have been described. A growing body of evidence suggests that miR-21 enhances the malignant behavior of cells, including invasion and migration [11]. Additionally, miR-21 is a component of the cellular signaling circuitry

that regulates the EMT program [12], indicating that it is involved in the process of EMT. However, the biological functions of miR-21 in the regulation of EMT remain to be investigation.

Materials and methods

Cell culture and treatments

Rat hepatic oval cells WB-F344 was obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Life Technologies/Gibco, Grand Island, NY), 100 IU/ml penicillin and 100 mg/ml streptomycin (Life Technologies/Gibco, Gaithersburg, MD) and incubated at 37°C in a humidified chamber supplemented with 5% CO2. Cells grown to subconfluence were washed three times with serum-free RPMI-1640 and serum starved for 24 h at 37°C. The cells were washed once again with serum-free RPMI-1640 and incubated with either RPMI-1640 alone or RPMI-1640 supplemented with 10 ng/ml TGF-β1 for indicated time at 37°C. RECK siRNA and Control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz). RECK siRNA was added 30 min before TGF-β1 treatment or 12 h after transfection of pre-miR-21.

Transient transfection analysis

Cells (5×10^5) were seeded in six-well plates and grown to 80% confluence. Transfection with miR-21 enhanced lentivirus (pre-miR-21), miR-21 inhibitor lentivirus (anti-miR-21) and scramble (Genechem, Shanghai, China) into HOC was performed at a concentration of 100multiplicity of infection(MOI) according to the manufacturer's protocol. We put it incubated for 48 hours at room temperature.

Real-time PCR

Total cellular RNA was isolated by use of Trizol (Invitrogen) according to the manufacturer's protocol. The RT primers for miR-21 and U6 small nuclear RNA (snRNA) were as follows: miR-21, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3', and U6 snRNA, 5'-CGCTTCACGAATTTGCGTGTCA-3'. The sequences of mature miRNAs were from Sanger miRBase (http://microrna. sanger.ac.uk/sequences/). Forward (F) and reverse (R) primers were as follows: miR-21-F, 5'-GCGGCGGTAGCTTATCAGACTG-3'; miR-21-R, 5'-ATCCAGTGCAGGGTCCGAGG-3'; U6-F, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; U6-R, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. All of the primers were synthesized by Invitrogen. Quantitative real-time PCR was performed with an Applied Biosystems 7500 machine and $SYBR^{Green}$ Real-time PCR Master Mix-Plus-(Toyobo, Japan). U6 snRNA was used as internal controls to determine relative expressions of miRNAs. All Real-time RT-PCRs were performed at least 3 separate times in triplicate and the data are presented as mean ± SD. Gene expression profiles were normalized to U6 snRNA and calculated using the ΔΔCt ($2^{-\Delta\Delta Ct}$) levels.

Western blot

Protein concentrations were measured with the BCA protein assay according to the manufacturer's manual (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts (80 mg) of protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The PVDF membranes were blocked with 50% skimmed milk, treated with primary antibody at 4°C overnight, washed and then incubated with the secondary horseradish (1:5000) for 2 h. Bands were detected with Enhanced Chemiluminescence (ECL). Immunoblotting was performed with E-Cadherin, vimentin, N-cadherin and RECK antibody (Cell Signaling Technology, 1:1000). Densitometry was detected by Imagine J. Western blot analyses were performed at least in triplicate.

Statistical analyses

Derived values are presented as the means ± SD. Comparison of mean data among multiple groups was analyzed by one-way analysis of variance (ANOVA), and a multiple range least significant difference (LSD) was used for intergroup comparisons. P values < 0.05 were considered statistically significant. All statistical analyses were performed with SPSS 16.0.

Results

TGF-β1 upregulated miR-21 and induced EMT in WB-F344 cells

TGF-β1 is one of the most important factor promoting liver fibrosis [13]. TGF-β1 can promote the occurrence of epithelial mesenchymal transition [14]. EMT was induced in WB-F344 cells

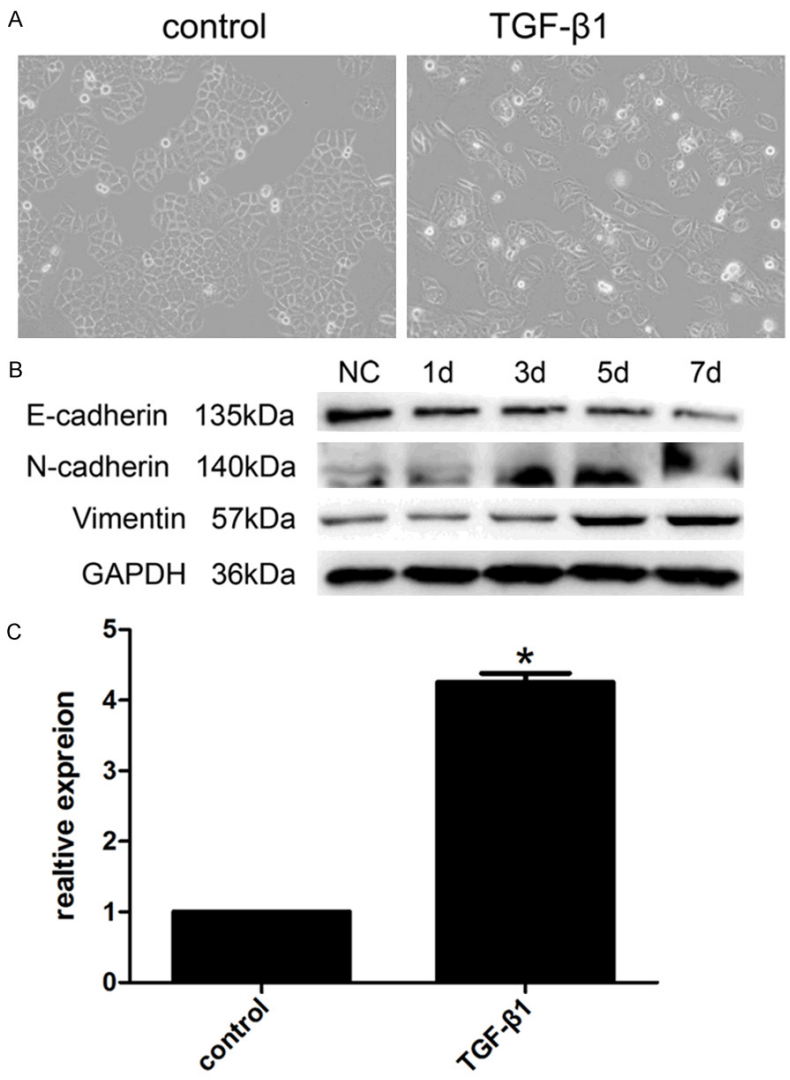

Figure 1. TGF-β1 upregulated miR-21 and induced EMT in WB-F344 cells. A. WB-F344 cells were treated with 10 ng/mL TGF-β1 for 5 days. The changes in cell morphology were observed under microscope (× 200). B. WB-F344 cells were treated with 10 ng/ml TGF-β1 for different times. The protein expression of E-cadherin and vimentin was examined by Western blot. C. WB-F344 cells were treated with 10 ng/mL TGF-β1 for 5 days. miR-21 expression was measured by real-time PCR. (*P < 0.05).

by exposure to 10 ng/ml TGF-β1. TGF-β1 treatment could lead WB-F344 cells to undergo EMT-like transformation evidenced by loss of cell-cell adhesion and alterations of morphology from a round compact shape to a spindle-like shape with irregular processes (**Figure 1A**). Western-blots demonstrated a phenotypic transition from epithelial properties to mesenchymal-like properties evidenced by loss of the epithelial marker, E-cadherin and in turn increased expression of the mesenchymal marker, N-cadherin and vimentin (**Figure 1B**). We then examined the expression changes of miR-21 in WB-F344 cells by TGF-β1 treatment. Expression of miR-21 was upregulated time-dependently after 10 ng/ml TGF-β1 treatment, suggesting that it may play an important role in TGF-β1 induced EMT (**Figure 1C**).

miR-21 mediated TGF-β1-induced EMT in WB-F344 cells

In order to confirm the role of miR-21 in EMT, we examined whether inhibition of miR-21 could attenuate EMT induced by TGF-β1 in WB-F344 cells. After down-regulation of miR-21 expression in WB-F344 cells by transfection of miR-21 inhibitor lentivirus, the EMT was inhibited significantly. Western blot showed that TGF-β1- induced downregulation of E-cadherin and upregulation of N-cadherin and vimentin were significantly restored by downregulation of miR-21 expression. (**Figure 2A**). Next, we found that overexpression of miR-21 alone also resulted in EMT-like transformation in WB-F344 cells. Transfection of miR-21 enhanced lentivirus into WB-F344 cells lead to reduced E-cadherin and increased N-cadherin and vimentin expression (**Figure 2B**). Were transfected with miR-21 inhibition and enhanced lentivirus lentivirus to WB-F344 cells were detected by PCR the expression of miR-21, the result is significantly suppressed and enhanced miR-21 (**Figure 2C**).

RECK mediated TGF-β1-and miR-21-induced EMT in WB-F344 cells

According microRNA target gene databases we search to miR-21 target genes is RECK, RECK is a tumor suppressor. Zhang [15] et al study

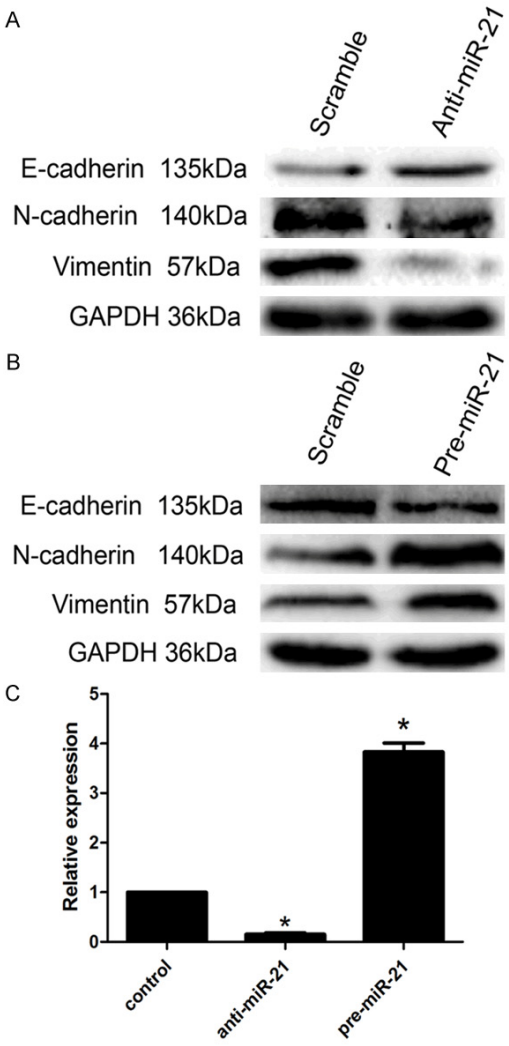

Figure 2. A. Cells were treated with 10 ng/ml TGF-β1 for 3 days after anti-miR-21 was transfected. The protein expression of E-cadherin, N-cadherin and vimentin was examined by western blot. B. Cells were transfected with pre-miR-21. The protein expression of E-cadherin, N-cadherin and vimentin was examined by Western blot. C. Cells were transfected with anti-miR-21 and pre-miR-21, miR-21 expression was measured by real-time PCR. (*P < 0.05).

found that gastric RECK expression and miR-21 was negatively correlated. So we asked whether RECK mediates the process of EMT induced by TGF-β1- and miR-21 in WB-F344 cells. The results showed that TGF-β1 treatment resulted in decreased RECK. However, downregulation of miR-21 by transfection of miR-21 inhibitor lentivirus into WB-F344 cells abolished this effect, indicating that miR-21 regulates TGF-β1-induced RECK in WB-F344 cells (**Figure 3A**). Furthermore, overexpression of miR-21 alone in WB-F344 cells downregulated RECK (**Figure 3B**), suggesting that RECK is a miR-21 target in WB-F344 cells.

We next examined whether modulation of RECK affected miR-21- induced EMT in WB-F344 cells. The results showed RECK siRNA, significantly enhanced TGF-β1- and miR-21-induced EMT by decreasing E-cadherin and increasing N-cadherin and vimentin (**Figure 3C** and **3D**), suggesting RECK mediates the EMT induced by TGF-β1 and miR-21 in WB-F344 cells.

Discussion

miR-21 in a number of fibrosis of organs universal expression. Inhibition of miR-21 expression can reduce organ fibrosis, such as, lung [16], heart [17], kidney diseases [18] and myofibroblasts fibrosis [19]. It suggests that the role for miR-21 in the EMT of fibrotic diseases is important. Wang et al [20] demonstrated that miR-21 overexpression can contribute to TGF-β1-induced EMT by inhibiting target smad7, and that targeting miR-21 may be a better alternative to directly suppress TGF-β1-mediated fibrosis in diabetic nephropathy. Liu et al [21] reported that up-regulation of miR-21 in the lungs of mice with bleomycin-induced fibrosis and also in the lungs of patients with idiopathic pulmonary fibrosis. Kumarswamy et al [22] reported that TGF-β1 treatment of endothelial cells significantly increased miR-21 expression and induced endothelial-to-mesenchymal transition characterized by suppression of endothelial in cardiac fibrosis. Liu et al [23] showed that miR-21 over-expression enhanced TGF-β1-induced EMT characterized by upregulating of vimentin and downregulating of E-cadherin in human hepatocytes. Targeting miR-21 may provide a new therapeutic strategy against hepatic fibrosis. However, expression changes in miR-21 and the role of miR-21 in EMT during liver fibrosis in hepatic oval cell have not yet been defined. In the present study, we showed that downregulation of miR-21 prevented TGF-β1-induced fibrogenic EMT in hepatic oval cells, suggesting that miR-21 is required for TGF-β1-induced fibrogenic EMT in hepatic oval cells. More importantly, miR-21 manipulations showed substantial impact on EMC phenotype, since overexpression of miR-21 in hepatic oval cells markedly promoted biomarkers of fibrogenic EMT. Previous study has demonstrated

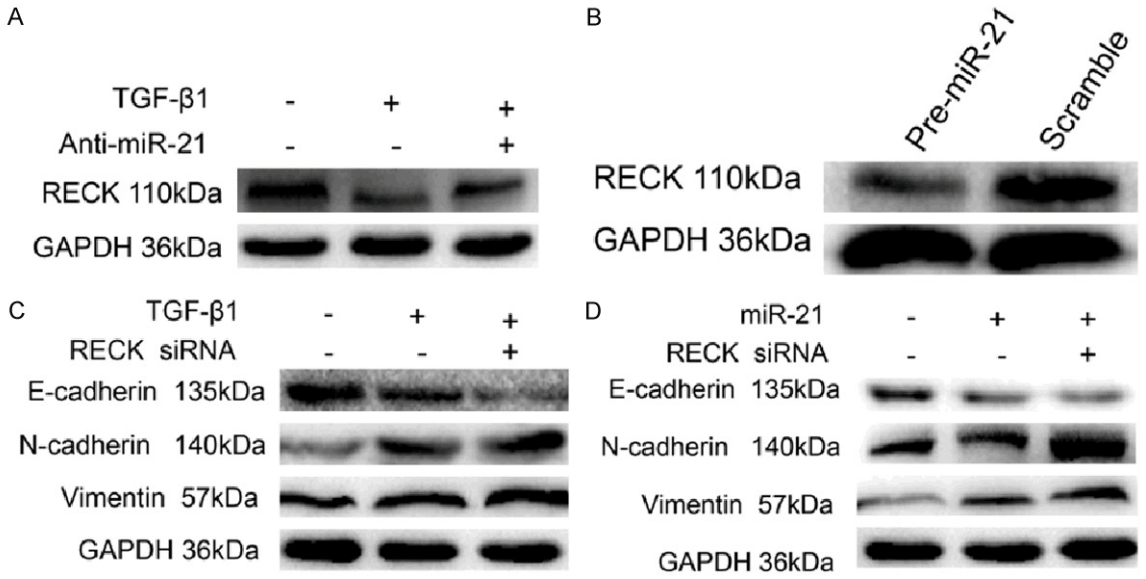

Figure 3. RECK mediated TGF-β1- and miR-21-induced EMT in WB-F344 cells. A. Cells were treated with 10 ng/mL TGF-β1 12 h after anti-miR-21 was transfected. The protein expression of RECK was examined by western blot. B. Cells were transfected with pre-miR-21. The protein expression of RECK was examined by Western blot. C. Cells were treated with 10 ng/mL TGF-β1 30 min after 20 μmol/L RECK siRNA treatment. The protein expression of E-cadherin, N-cadherin and vimentin was examined by western blot. D. Cells were transfected with pre-microR-21 or in combination treatment with 20 μmol/L RECK siRNA. The protein expression of E-cadherin, N-cadherin and vimentin was examined by western blot.

that miR-21 stimulates the activation of hepatic stellate cells [24] and hepatocytes [23]. Taken together, our data suggest that miR-21 may be important target for treatment of liver fibrosis.

TGF-β is master player of fibrosis and EMT [25]. TGF-β initiates signaling by binding to type I and type II receptor serine/threonine kinase on the cell surface [26], regulating gene expression by receptor-mediated activation of Smad (R-Smad) transcription factors [27].

MiR-21 target genes includes PDCD4 [28], TPM1 [29], PTEN [30], Sprouty protein [31], RECK [15]. RECK (reversion inducing-cysteine-rich protein with kazal motifs), a unique membrane-anchored MMP regulator, is expressed in various organs, including developing vasculature, skeletal muscles, neuromuscular junctions, cartilage, fibroblasts, and neural precursor cells [32-35]. The RECK gene encodes a membrane-anchored glycoprotein capable of regulating several members of the MMP family (MMP2, MMP7, MMP9, and MT1-MMP) [32, 36] and some other extracellular metalloproteinases. RECK expression is regulated at multiple levels; while Sp1 activation represses RECK transcription [37], post-transcriptional modifications such as acetylation, methylation and modulation by microRNAs have been shown to target RECK. Multiple microRNAs, including miR-21, target RECK 3'UTR, and repress its expression in various cancer cells. Tumor suppressor reck is a target gene of miR-21 [38, 39]. Expression of RECK inhibit tumor cell invasion and metastasis [40]. Tumor suppressor RECK is a target gene of miR-21. RECK expression is usually reduced or absent in human malignancies, including renal-, lung-, and glia-derived tumors. That RECK directly interacts with transcription factors. According to my experiments RECK directly inhibit EMT transcription unit, for example Twist1 and zeb1, eventually lead to the weakening of the EMT, over-expression of Twist1 represses Ecadherin transcription activity.

Hepatic oval cells are considered primitive and pluripotent stem cells, and in vivo experiments confirmed that it can differentiate into hepatocytes, bile duct cells, pancreatic cells, intestinal epithelial cells et al [41]. Yovchev et al [42] reported freshly purified from rat hepatic oval cells can co-expression of epithelial and mesenchymal markers. EMT also demonstrated in

vivo in rats oval cells in the induction of certain conditions, eventually differentiate into mesenchymal tumor tissue.

In the present study, as expected, TGF-β1 inhibited the expression of RECK protein and upregulated miR-21 expression in hepatic oval cells. These results indicate that miR-21 mediates TGF-β1-induced RECK in hepatic oval cells. Our results demonstrated that RECK siRNA enhanced TGF-β1 or miR-21 induced fibrotic EMT in hepatic oval cells, suggesting that RECK mediated TGF-β1 or miR-21 induced fibrogenic EMT in hepatic oval cells. In conclusion, our results identify miR-21 as a key regulator of fibrogenic EMT in hepatic oval cells via RECK. Targeting miR-21 may provide a new therapeutic strategy against hepatic fibrosis.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yunfeng Shan, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, Zhejiang, P. R. China. Tel: +86-13857763998; Fax: +86-577-86689900; E-mail: shanyfeng@126.com

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