Original Article
EPA attenuates epithelial-mesenchymal transition and fibrosis through the TGF-β1/Smad3/ILK pathway in renal tubular epithelial HK-2 cells by up-regulating miR-541

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Abstract: Background: It was reported that eicosapentaenoic acid (EPA) could prevent tubulointerstitial injury in kidney. EPA could also inhibit the epithelial-mesenchymal transition (EMT) of HK-2 cells stimulated by albumin (Alb) in vitro. However, the regulating molecular mechanism of EPA remains to be elucidated. Methods: An immortalized human proximal tubular cell line (human kidney-2 (HK-2) cells) was used in all experiments. MTT assay was employed to determine the effect of Alb or EPA on the cell viability of HK-2 cells. The miR-541 expression, the mRNA levels of EMT markers E-cadherin, α-smooth muscle actin (α-SMA), and fibrogenesis markers Collagen I and fibronectin (FN) were examined by RT-qPCR assay. The protein levels of E-cadherin, α-SMA and Collagen I, transforming growth factor β1 (TGF-β1)/Smad3/integrin-linked kinase (ILK) pathway-related protein TGF-β1, pSmad2/3, Smad7 and ILK were measured by western blot. Enzyme-linked immunosorbent assay (ELISA) was performed to detect FN expression. The target relationship between miR-541 and TGF-β1 was confirmed by bioinformatics, luciferase reporter assay and western blot. Results: Low doses of Alb had no effect on the cell viability of HK-2 cells, while EPA repressed the cell viability of HK-2 cells in a concentration-dependent manner. EPA could inhibit EMT and fibrosis and increase the miR-541 expression of HK-2 cells exposed to Alb. Interestingly, introduction of miR-541 effectively abolished the EMT and fibrosis of HK-2 cells stimulated by Alb. Bioinformatics analysis predicted TGF-β1 as a target gene of miR-541, and subsequent luciferase reporter assay and western blot further supported the prediction. miR-541 counter-regulated TGF-β1 expression, and inhibited the TGF-β1/Smad3/ILK pathway. Alb treatment activated the TGF-β1/Smad3/ILK pathway, while EPA inhibited the activation of the pathway. miR-541 inhibitors reversed the effects of EPA on EMT, fibrosis, and TGF-β1/Smad3/ILK pathway-related protein expression induced by Alb. Conclusion: EPA attenuates EMT and renal fibrosis through the TGF-β1/Smad3/ILK pathway in renal epithelial cells by targeting miR-541.

Keywords: EPA, Alb, miR-541, EMT, renal fibrosis, TGF-β1/Smad3/ILK signaling pathway

Introduction
Chronic kidney disease (CKD) is a common disease worldwide. A large portion of cases eventually develop end-stage kidney failure, a damaging condition that demands lifelong dialysis or kidney transplantation [1]. A better understanding of the cellular and molecular mechanisms of renal EMT and fibrosis is helpful for developing effective strategies for CKD treatment.

Eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid, derived from fish oil, was beneficial for inflammatory and oxidative mechanisms involved in atherosclerotic plaque formation and progression [2]. A previous study revealed that the efficacy of polyunsaturated fatty acids, such as EPA and docosahexaenoic acid (DHA), on IgA nephropathy patients with proteinuria depended on the dose and size [3]. EPA inhibited the early progression of tubulointerstitial injury in Thy-1 nephritis models, and the inhibitory effect was related to its regulation of IκBα [4]. MicroRNAs (miRNAs) comprise a large group of regulatory RNAs with length ~22 nucleotides.
that repress the expression of their target messenger RNAs (mRNAs) by binding to 3'-untranslated regions (3'-UTR) of target genes [5]. Over the past decade, miRNA has been proved to exert vital functions in various cellular processes, including development, differentiation, proliferation, apoptosis, and cell cycle [6]. It is also reported to be a reliable biomarker for certain cancers [7]. Several miRNAs have been shown to be involved in the development of HK-2 cells, such as miR-4756 [8], miR-34a [9] and miR-210 [10]. Zhang et al. reported that miR-541 plays a significant role in suppressing proliferation and neurite outgrowth of PC12 neurons in vitro by targeting Synapsin I [11]. MiR-541 is a target of microphthalmia-associated transcription factor (MITF), and the MITF/miR-541 axis is a novel regulating pathway of cardiac hypertrophy [12]. However, the role of miR-541 on progress of HK-2 cells remains unclear.

Conversions of epithelial cells to mesenchymal cells, epithelial-mesenchymal transition (EMT) is an important process that involves in kidney ontogeny [13]. miRNAs, vital regulators of cancer metastasis and EMT, participate in progression of various tumors through regulating EMT-related genes [14]. Endothelial cadherin (E-cadherin) is main adhesion receptor of endothelial adherent junctions, and the loss of E-cadherin is a key event during EMT. EMT induced by high glucose leads to increased expression of mesenchymal markers α-SMA, FSP-1, and type I collagen [15].

Transforming growth factor β1 (TGF-β1) is a common profibrotic cytokine that plays an important role in chronic inflammatory changes of the interstitium and accumulation of extracellular matrix during renal fibrosis [16]. A prior study indicated that TGF-β1 induced EMT in renal fibrosis primarily by the Smad signaling pathway [17]. Integrin linked kinase (ILK) is an intracellular serine/threonine kinase participating in cell-matrix interactions and mediating integrin signaling in diverse types of cells [18, 19].

In this study, the effect of EPA on viability of HK-2 cells was detected; the effect of Alb, EPA, and miR-541 on the expression levels of EMT, fibrosis, and TGF-β1/Smad3/ILK pathway-related proteins were analyzed, and the underlying mechanism was also explored.

Materials and methods

Cell culture and treatment

The human proximal tubule cell line (HK-2) was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute 1640 (RP-MI1640) Medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) in a 37°C, 5% CO₂ humidified atmosphere.

Alb and EPA were purchased from Sigma Chemical Co. Ltd (Dorset, UK). 5 mg/mL Alb or (and) 30 μmol/L EPA were applied to treat HK-2 cells for 24 h, which was utilized to validate the effects of Alb or EPA on the EMT and fibrosis, and miR-541 expression of HK-2 cells.

Cell transfection and Alb or EPA treatments

miR-541 mimics and its negative control miR-NC mimics, miR-541 inhibitors and its negative control miR-NC inhibitors, were purchased from RiboBio Co. Ltd. (Guangzhou, China).

As a pretreatment, HK-2 cells were treated with miR-541 mimics or miR-541 inhibitors for 24 h using Lipofectamine™ 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) following the protocol of manufacturer strictly. Then cells were washed and treated with or without 5 mg/mL Alb, with or without 30 μmol/L EPA for another 24 h.

Cell viability assay

HK-2 cells were seeded into 96-well plates (3 × 10³ cells), then treatment with 5 mg/mL Alb or different concentrations of EPA (0 μmol/L, 10 μmol/L, 30 μmol/L and 50 μmol/L) was carried out. After 12 h, 24 h, 48 h and 72 h of treatment, 10 μL MTT (5 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added into each well and incubated for another 4 h at 37°C, respectively. Then supernatant was discarded, and then 150 μL dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added in each well. The absorbance at 490 nm was measured with a microplate read-
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**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted from HK-2 cells utilizing the RNA Isolation Kit (Sigma-Aldrich) following the instruction of manufacturer. 500 ng total RNA was used as template for reverse transcription reaction with a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). For mRNA detection, RT-qPCR was performed with a SYBR Green mix (Takara). For miR detection, an all-in-one miRNA RT-qPCR Detection Kit (GeneCopoeia Inc., Rockville, MD, USA) was utilized. GAPDH was used as an internal control for E-cadherin, α-SMA, Collagen I and FN, and U6 was used as an internal control for miR-541. Above primers were listed as below: E-cadherin, 5'-CCACATACACTCTCTTCTCAAC-3' (sense) and 5'-TGACCACACTGATGACTCCTG-3' (antisense); α-SMA, 5'-TCCGTAATGGGTCTGC-3' (sense) and 5'-ATGCTTGCTAGTCCAT-3' (antisense); Collagen I, 5'-CCGTCACCTCAAGATGCA-3' (sense) and 5'-CTTGAAGTTGCCAGTCTGC-3' (antisense); FN, 5'-CATGGCTGATTGCAATTG-3' (sense) and 5'-GGTACCTGAATTGGCTAT-3' (antisense); GAPDH, 5'-AGTCCACTGGCGTCTTCAC-3' (sense) and 5'-GCTTGACAAAGTGGTCGTTGA-3' (antisense). Primers for miR-541 and U6 were purchased from TIANGEN (Shanghai, China). The qPCR was repeated for three times. The expression of E-cadherin, α-SMA, Collagen I, FN, and miR-541 were analyzed using the classic $2^{-\Delta\Delta C_{t}}$ method.

**Western blot assay**

Total cell protein was extracted from HK-2 cells using a Protein Extraction Kit (Bio-Rad) referring to the manufacturer's instructions. Protein concentrations were detected by bicinchoninic acid assay (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). 30 μg protein samples were separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were first blocked with 5% skim milk for 1 h at room temperature, then incubated with primary antibody against TGF-β1 (1:1000 dilution), pSmad2/3 (1:1000 dilution), Smad7 (1:1000 dilution), ILK (1:1000 dilution) and GAPDH (1:2000 dilution) overnight at 4°C, then incubated with corresponding secondary antibodies (1:1000 dilution) for 2 h at room temperature. The bands were visualized using a chemiluminescence kit (Merck Millipore, Darmstadt, Germany), and the images were analyzed using Quantity One software (Bio-Rad). All antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

**ELISA**

After different treatments, the supernatant was collected from each well, and the level of FN was monitored by a Human FN Quantikine ELISA Kit (R&D Systems, Abingdon, UK) according to the protocols supplied by the manufacturer.

**Luciferase reporter assay**

Using the TargetScan database (http://www.targetscan.org), the binding site in miR-541 that could directly bind with TGF-β1 3'-UTR was predicted. The fragment from TGF-β1 containing the predicted binding site (GGCCCA) was amplified by and cloned into pGL3 luciferase promoter vector (pGL3-empty, Promega, Madison, WI, USA) to generate TGF-β1-WT. A fragment from TGF-β1 containing the mutant of predicted binding site (UAAGCAU) was applied to generate TGF-β1-MUT. HK-2 cells were co-transfected with miR-541 and TGF-β1-WT or TGF-β1-MUT. Then luciferase activity was quantified using the Dual Luciferase Reporter Detection System (Promega).

**Statistical analysis**

Data were analyzed with SPSS 22.0 software (SPSS, Chicago, IL, USA). All data were shown as mean ± S.D. (standard deviation). Student’s t-test was utilized to analyze differences between two groups (Figures 1A, 3B and 4B-F), and LSD-t was utilized to analyze differences among three or more groups (the rest of figures). P value < 0.05 was considered significant.

**Results**

**EPA inhibited the cell viability of HK-2 cells in a concentration-dependent manner**

To clarify the effects of Alb and EPA on cell viability of HK-2 cells, we treated HK-2 cells with 5 mg/mL Alb and different concentrations of EPA, MTT assay was conducted to detect the cell viability of HK-2 cells with different treatments.
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The cell viability of cells treated with 5 mg/mL Alb showed no obvious change, compared to cells treated with 0 mg/mL Alb (Figure 1A). As shown in Figure 1B, the cell viability of HK-2 cells treated with EPA significantly decreased, and EPA inhibited the cell viability of HK-2 cells in a concentration-dependent manner.

EPA could inhibit EMT and fibrosis of HK-2 cells induced by Alb to some extent

To determine the effects of Alb and EPA on EMT and fibrosis of HK-2 cells, we detected whether EMT or fibrosis in HK-2 cells were activated after treatment with Alb or (and) EPA. The mRNA levels of EMT markers E-cadherin and α-SMA, and fibrosis markers Collagen I and FN were evaluated by RT-qPCR assay. Alb treatment apparently inhibited E-cadherin expression, while up-regulated expression levels of α-SMA, Collagen I and FN. No obvious change was observed in levels of EMT markers and fibrosis markers of HK-2 cells treated with 30 μmol/L EPA alone. But co-treatment of Alb with EPA partially reversed the modulation of Alb on these markers (Figure 2A-D). Subsequent western blot assay was applied to clarify the modulation of Alb and EPA on EMT and fibrosis of HK-2 cells, we transfected HK-2 cells with miR-541 mimics. RT-qPCR assay was utilized to verify the transfection efficiency (Figure 3B). RT-qPCR assay was also performed to examine the mRNA levels of E-cadherin, α-SMA, Collagen I, and FN. Distinctly, introduction of miR-541 attenuated the down-regulation of E-cadherin and up-regulation of α-SMA, Collagen I and FN triggered by Alb (Figure 3C-F). Western blot indicated that up-regulation of miR-541 also weakened the modulation of Alb on protein levels of E-cadherin, α-SMA, and Collagen I (Figure 3G, 3H). We can conclude from ELISA that gain of miR-541 effectively undermined the up-regulation of FN induced by Alb (Figure 3I).

miR-541 targeted TGF-β1 and regulates the TGF-β1/Smad3/ILK signaling pathway

Online software Targetscan was utilized to predict the potential target gene of miR-541 and identified that miR-541 could bind with the 3’-UTR of TGF-β1. The binding site was highlighted in Figure 4A. To confirm the target relationship between miR-541 and TGF-β1, luciferase activity assay was conducted according to manufacturer’s instruction. The luciferase activity of HK-2 cells co-transfected with TGF-β1-WT and miR-541 mimics was notably decreased, while in cells co-transfected with TGF-β1-WT and miR-541 inhibitors, luciferase activity was strikingly elevated. However, luciferase activity of cells co-transfected with TGF-β1-MUT and miR-541 mimics or miR-541 inhibitors was essentially unchanged (Figure 4B, 4C). Subsequent western blot assay was applied to clarify the modulation of miR-541 on TGF-β1 expres-
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The protein level of TGF-β1 was down-regulated by miR-541 mimics (Figure 4D), while up-regulated by miR-541 inhibitors (Figure 4E). Western blot was also conducted to detect the impact of miR-541 on TGF-β1/Smad3/ILK signaling pathway. We found that levels of both pSmad2/3 and ILK were decreased, while Smad7 expression was elevated in HK-2 cells transfected with miR-541 mimics (Figure 4F).

EPA regulates the TGF-β1/Smad3/ILK pathway, EMT and fibrosis of HK-2 cells by targeting miR-541

To investigate the mechanism by which EPA regulates the TGF-β1/Smad3/ILK pathway, EMT and fibrosis of HK-2 cells, we treated HK-2 cells with Alb, EPA, and miR-541 inhibitors. Western blot for TGF-β1/Smad3/ILK pathway-related proteins was conducted. Results indicated that EPA inhibited the activation of the TGF-β1/Smad3/ILK pathway caused by Alb, and miR-541 inhibitors reversed the effects of EPA on pathway-related proteins expression induced by Alb (Figure 5A). RT-qPCR assay, western blot, and ELISA were used to detect EMT and fibrosis-related proteins expression after treatment with Alb, EPA, and miR-541 inhibitors, and suggested that miR-541 inhibitors also abolished the impact of EPA on EMT and fibrosis-related protein expression induced by Alb at both the mRNA and protein level (Figure 5B, 5C).

Discussion

EPA has been reported to prevent the progression of IgA nephropathy and diabetic nephropathy. Nevertheless, the mechanism by which EPA exerts its inhibitory effect on the progression of kidney injury needs to be further elucidated.

Recently, EPA was used as a medicine for hyperlipidemia and arteriosclerotic obstructions [4]. EPA was a potent inhibitor of inflammatory responses in HK-2 cells; this effect was mediated by the activation of PPAR-γ [20]. EPA treatment could reduce cardiac hypertrophy, albuminuria, renal fibronectin expression, and infiltration of monocytes/macrophages, and protect against Ang II-induced end-organ damage.
In colon cancer cells, EPA as Free Fatty Acid (EPA-FFA) treatment counteracted NOTCH1 activation stimulated by inflammation [22]. EPA had a protective effect against the palmitate-induced renal tubular cell damage by repressing activation of PKCθ [23]. Yang et al. proposed that the EPA level in plasma and cells of hemodialysis patients was lower than that in people without kidney disease [24]. EPA inhibited the cell activation and proliferation of mesangial cells in anti-Thy 1.1 (ATS) glomerulonephritis, reduced proteinuria, and decreased histologic...
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In our study, EPA inhibited the cell viability of HK-2 cells in a concentration-dependent manner.

**miR-541** has been found to be down-regulated in the hippocampus after traumatic brain injury (TBI), and participates in regulation of TBI pathophysiology [26]. Yang et al. demonstrated that overexpression of miR-541 largely contributes to the proliferation and invasion of vascular smooth muscle cells (VSMCs) through a direct interaction with the 3'-UTR of Interferon regulatory factor 7 (IRF7). Therefore, miR-541 may be a potential therapy target for VSMC growth-related diseases [27]. miR-541 suppressed proliferation and invasion of squamous cell lung carcinoma cell lines by down-regulating high-mobility group AT-hook2 [28]. Furthermore, it is reported that miR-541 is related to myocardial hypertrophy [12] and SiO₂ induced pulmonary fibrosis [29].

There are four key cellular events during EMT: (1) loss of epithelial adhesion properties; (2) de novo expression of α-SMA and actin reorganization; (3) damage of tubular basement membrane; and (4) elevated cell migration and invasion. EMT typically occurs at the late stage during the pathogenesis of renal fibrosis in various animal models, and is essential for renal fibrosis [30]. EPA promotes glucose intolerance, mesangial matrix accumulation, and tubulointerstitial fibrosis in KKAy/Ta mice. The mechanism may be associated with its down-regulation on MCP-1 [31]. Emerging evidence suggests that EMT is regulated by certain miRNAs, for example, overexpression of miR-4756 accelerated the EMT and endoplasmic reticulum (ER) stress stimulated by Alb through targeting Sestrin2 [8]. Loss of miR-23a suppressed the HG-induced EMT and renal fibrosis in HK-2 cells by up-regulation of SnoN [32]. miR-300 counter-regulated EMT by direct targeting Twist and therefore repressed human epithelial cancer cell invasion and metastasis [33]. Down-regulation of miR-18a-5p led to an apparent induction of EMT, and inhibited breast cancer progression and metastasis [34]. In the current study, EPA could inhibit EMT and fibrosis of HK-2 cells induced by Alb partially, which validated by RT-qPCR assay, western blot assay and ELISA. Also, EPA regulated miR-541 expres-
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In Alb-stimulated HK-2 cells. Interestingly, the gain of miR-541 also inhibited EMT and fibrosis of HK-2 cells induced by Alb in part; then we tried to investigate the underlying mechanism. By utilizing bioinformatics analysis, we identified TGF-β1 as a downstream target of miR-541. TGF-β1 could activate and complete the entire EMT course that consists of four key steps [30]. TGF-β1-Smad3-ILK is an important pathway in regulation of renal tubular EMT [18]. P311 participates in the pathogenesis of renal fibrosis through suppressing the EMT process induced by TGF-β1 by TGF-β1/Smad3/ILK pathway, indicating a significant role of TGF-β1 in promoting tubular EMT by the TGF-β1/Smad3/ILK pathway [19]. In our study, up-regulation of miR-541 inhibits the TGF-β1/Smad3/ILK pathway. EPA also inhibited the activation of the TGF-β1/Smad3/ILK pathway by targeting miR-541. Therefore, we concluded that EPA attenuates EMT and fibrosis through the TGF-β1/Smad3/ILK pathway in renal epithelial cells by up-regulating miR-541.

In summary, EPA represses the cell viability of HK-2 cells. EPA could partially inhibit the EMT and fibrosis of HK-2 cells and miR-541 expression suppression induced by Alb. Up-regulation of miR-541 effectively abolished the EMT and fibrosis of HK-2 cells stimulated by Alb. TGF-β1 is a downstream gene of miR-541, and is counter-regulated by miR-541. miR-541 inhibited the TGF-β1/Smad3/ILK pathway. EPA inhibited the activation of the TGF-β1/Smad3/ILK pathway caused by Alb, and miR-541 inhibitors reversed the effects of EPA on the activation of TGF-β1/Smad3/ILK induced by Alb. Therefore, EPA attenuates EMT and fibrosis through the

Figure 5. EPA regulated the TGF-β1/Smad3/ILK pathway, EMT, and fibrosis of HK-2 cells by targeting miR-541. HK-2 cells were treated with Alb, EPA, and miR-541 inhibitors meanwhile. A. The protein levels of TGF-β1, pSmad2/3, Smad7 and ILK were analyzed through western blot. B. The evaluation of mRNA levels of E-cadherin, α-SMA, Collagen I and FN in HK-2 cells post-treatment. C. The E-cadherin, α-SMA, Collagen I and FN expression were determined by western blot or ELISA. ***P < 0.001 compared to cells without treatment. ***P < 0.001 compared to cells treated with 5 mg/mL Alb, EPA, and miR-NC inhibitors.
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Disclosure of conflict of interest

None.

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References


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