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Original article

MicroRNA-21 activates hepatic stellate cells via PTEN/Akt signaling

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ABSTRACT

Activation of hepatic stellate cells is the key event in the liver fibrosis. miRs have been shown to play fundamental role in diverse biological and pathological processes. In the present study, we investigated the fibrogenic role of miR-21 in human hepatic stellate LX-2 cells and explored underlying mechanisms. The results showed that treatment of LX-2 cells with platelet-derived growth factor (PDGF)-BB significantly stimulated α1(I) collagen mRNA synthesis and the protein expression of α-SMA, which are characteristics of activation of hepatic stellate cells and simultaneously increased miR-21 expression. Downregulation of miR-21 expression by transfection of anti-miR-21 into LX-2 cells prevented PDGF-BB-induced LX-2 cell activation. Overexpression of miR-21 expression alone also stimulated LX-2 cell activation, while downregulation of miR-21 expression suppressed LX-2 cell activation. miR-21 also played a role in mRNA expression and activity of matrix metalloproteinase 2 (MMP2) in LX-2 cells. Moreover, overexpression of miR-21 decreased protein expression of PTEN in LX-2 cells, resulting in activation of the Akt. Inhibition of Akt signaling by specific inhibitor LY 294002 blocked miR-21-induced fibrogenic effects in LX-2 cells. In summary, miR-21 is an important mediator in LX-2 cell activation. The fibrogenic effects of miR-21 on LX-2 cell activation are mediated through PTEN/Akt pathway. miR-21 may be a potential novel molecular target for the liver fibrosis.

1. Introduction

Progressive liver fibrosis is the late stage of various chronic liver diseases. Fibrosis is characterized by an excessive deposition of several extracellular matrix (ECM) proteins in which type I collagen predominates. The excess deposition of ECM disrupts the normal architecture of the liver resulting in pathophysiological damage to the organ, which eventually undergoes the liver fibrosis-cirrhosis. It has been known that the activated hepatic stellate cell (HSC) is responsible for the excess synthesis and deposition of ECM following a fibrotic stimulus [1,2]. In addition, activated HSCs proliferate thereby effectively increasing the population of fibrogenic cells and amplifying the fibrotic response [3]. Despite fundamental advances in understanding the pathophysiology of liver fibrosis, detail mechanism of the liver fibrosis remain elusive [3]. Therefore, a comprehensive understanding of the molecular mechanisms involved in HSC activation will provide new therapeutic targets to inhibit the progression of this devastating disease.

MicroRNAs (miRs) are a newly discovered class of posttranscriptional regulators. A miR is 22 ribonucleotides long, and genetically encoded, with a potential to recognize multiple mRNA targets guided by sequence complementarity and RNA-binding proteins. This class of molecules has the capacity to specifically inhibit translation or induce mRNA degradation, through predominantly targeting the 3′ untranslated regions (UTRs) of mRNA [4]. miRs are differentially expressed during development and various diseases, thus implicating them in normal and pathological cellular mechanisms [5]. miR-21 is one of the most commonly and highly upregulated miRNA in many forms of cancer, suggesting that it is oncomir [6]. Like carcinogenesis, fibrogenesis is associated with relentless proliferation of myofibroblastic cells. The role of miR-21 in the fibrogenesis of heart, lung and kidney recently has been investigated [7–9]. However, the effect of miR-21 on the liver fibrosis remains unclear.

In this study, we investigated the fibrogenic role of miR-21 in human hepatic stellate LX-2 cells and mechanisms involved. The results showed that platelet-derived growth factor (PDGF)-BB, a potent mitogen of HSCs, upregulated miR-21 expression and stimulated LX-2 cell activation. Downregulation of miR-21 expression abrogated PDGF-BB-induced LX-2 cell activation. Overexpression of miR-21 alone mimicked PDGF-BB mediated

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effects, whereas inhibition of miR-21 expression reversed these effects. In addition, miR-21 played a role in matrix metalloproteinase (MMP) 2 mRNA expression and activity. miR-21 regulated the protein expression of PTEN and Akt in LX-2 cells. Inhibition of Akt activation by specific inhibitor LY 294002 suppressed the fibrogenic effects of miR-21 in LX-2 cells. Fifty μM LY294002 were added 6 h after transfection of pre-miR-21.

2. Materials and methods

2.1. Cell culture and treatments

The human immortalized HSC line, LX-2, was purchased from American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C in a humidified chamber supplemented with 5% CO_2.

Cells grown to subconfluence were washed three times with serum-free DMEM and serum starved for 24 h at 37 °C. The cells were washed again once with serum-free DMEM and incubated with either DMEM alone or DMEM supplemented with 5 ng/mL PDGF-BB (Prospec) for indicated time at 37 °C. LY 294002 (EMD Biosciences), a specific PI3 K/Akt inhibitor, was used to evaluate the role of Akt pathways in the fibrogenic effects of miR-21 in LX-2 cells. Then, 50 μM LY294002 were added 6 h after transfection of pre-miR-21.

2.2. Transient transfection analysis

LX-2 cells were re-seeded at a density of 2×10^5 cells per well in 6-well Plates 24 h prior to the transfection. Pre-miR-21, anti-miR-21 and scramble were transfected into the cells at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. Pre-miR-21, anti-miR-21 and scramble (Ambion) were mixed with lipofectamine 2000 respectively in DMEM and incubated for 25 min at room temperature. The complexes were then added to culture medium. After 6 h, the culture medium was changed. Samples were collected for measurement of mRNA and miR-21 after 48 h and for protein expression after 72 h.

2.3. Real-time PCR

Total RNA including the miRNA fraction was isolated using mirVana™ miRNA isolation kit according to the manufacturer's protocol (Ambion). miR-21 expression was determined by TaqMan MicroRNA Assay (Applied Biosystems), with small nuclear RNA U6 as an endogenous control for normalization according to manufacturer's instructions. Gene expression profiles were normalized to U6 snRNA and calculated using the $\triangle\triangle Ct(2^{-\triangle\triangle Ct})$ levels. mRNA expression of precollagen type I and MMP2 was analyzed by Syber Green method using the primers as follows: The sequences of primers were used as follows: β-actin: sense, 5′-TGAAGTACCCCATCGAGCACG-3′, antisense, 5′-CAAACATGATCTGGGTCATCTTCTC-3′; α1(I) collagen: sense, 5′-AAGGTGTTGTGCGATGACG-3′, antisense, 5′-GGAGACCACGAGGACCAGAG-3′; MMP2: sense, 5′-AACTACGATGATGACCGCAAG-3′, antisense, 5′-GACAGACGGAAGTTCTTGGTG-3′.

2.4. Western blot

Parental and transfected cells were washed with pre-chilled PBS and solubilized in 1% Nonidet P-40 lysis buffer. Homogenates were clarified by centrifugation at 20,000 × g for 15 min at 4 °C and the protein concentration was measured by bicinchoninic acid protein assay kit (Pierce Biotechnology). Then, 50 μg of protein from each sample was subjected to SDS-PAGE on SDS-acrylamide gel. Separated proteins were transferred to PVDF membranes (Millipore) and incubated with primary antibody at 1:1000 dilution (Santa Cruz); followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody as the secondary antibody (1:3000) for 2 h at room temperature. The specific protein was detected using enhanced chemiluminescence system (Amersham).

2.5. Zymography

Activities of MMP2 were detected by zymography following the manufacturer's instructions (Millipore). Briefly, cell culture supernants were separated in a 10% polyacrylamide gel containing 1 mg/mL gelatin. Gels were washed with 2.5% Triton X-100 for 30 min 2 or 3 times, followed by washing once with reaction buffe containing 50 mM/L Tris-HCl (pH 7.5), 200 mM, NaCl, 5 mM $CaCl_2$, and 0.02% Brij-35 to remove the SDS and then incubated overnight at 37 °C. Afterwards, the gels were stained with 0.5% Coomassie Blue R-250 (Sigma) for 1 hour and de-stained with an appropriate Coomassie R-250 de-staining solution (methanol: acetic acid: water = 50:10:40).

2.6. Statistical analysis

The data were analyzed using the SPSS11.5 software program. Results are expressed as mean ± SEM. Analysis of variance (ANOVA), followed by Bonferroni's *t* test, was used to assess differences among groups. The *P* value < 0.05 was considered to be statistically significant.

3. Results

3.1. MicroR-21 mediated PDGF-BB induced LX-2 cell activation

PDGF-BB is one of the most potent mitogens for HSC [10]. In our study, treatment of LX-2 cells with 5 ng/mL PDGF-BB significantly stimulated α1(I) collagen mRNA synthesis and the protein expression of α-SMA which are characteristics of HSC activation (Figs. 1A and B). Meanwhile, PDGF-BB also significantly increased miR-21 expression measured by real-time PCR (Fig. 1C). These results suggest that miR-21 may be involved in LX-2 cell activation.

To determine if miR-21 mediated the role of PDGF-BB in LX-2 cell activation, we transiently transfected anti-miR-21 into LX-2 cells to downregulate miR-21 expression and then treated LX-2 cells with PDGF-BB. The results showed that expression of miR-21 was significantly decreased after transfection of anti-miR-21 into LX-2 cells (data not shown). At the same time, PDGF-BB-induced expression of α-SMA and α1(I) collagen was also inhibited compared to control group (Fig. 1D), suggesting that miR-21 mediates the fibrogenic role of PDGF-BB in LX-2 cells.

In order to determine if miR-21 would mimic PDGF-BB-induced fibrogenic effects, pre-miR-21 and anti-miR-21were transfected into LX-2 cells. miR-21 overexpression in LX-2 cells produced significantly higher level of α-SMA and α1(I) collagen than the control group (Fig. 2A). Conversely, downregulation of miR-21 expression by transfection of anti-miR-21 decreased the expression of α-SMA and α1(I) collagen (Fig. 2B). These results suggest that miR-21 also has a direct fibrogenic role in LX-2 cells.

3.2. MicroR-21 stimulated MMP2 expression and activity in LX-2 cells

MMP2 mRNA expression was upregulated after treatment LX-2 cells with PDGF-BB and significantly attenuated when miR-21 was inhibited (Fig. 3A). Similarly, miR-21 overexpression alone also upregulated MMP2 mRNA expression in LX-2 cells (Fig. 3B). In

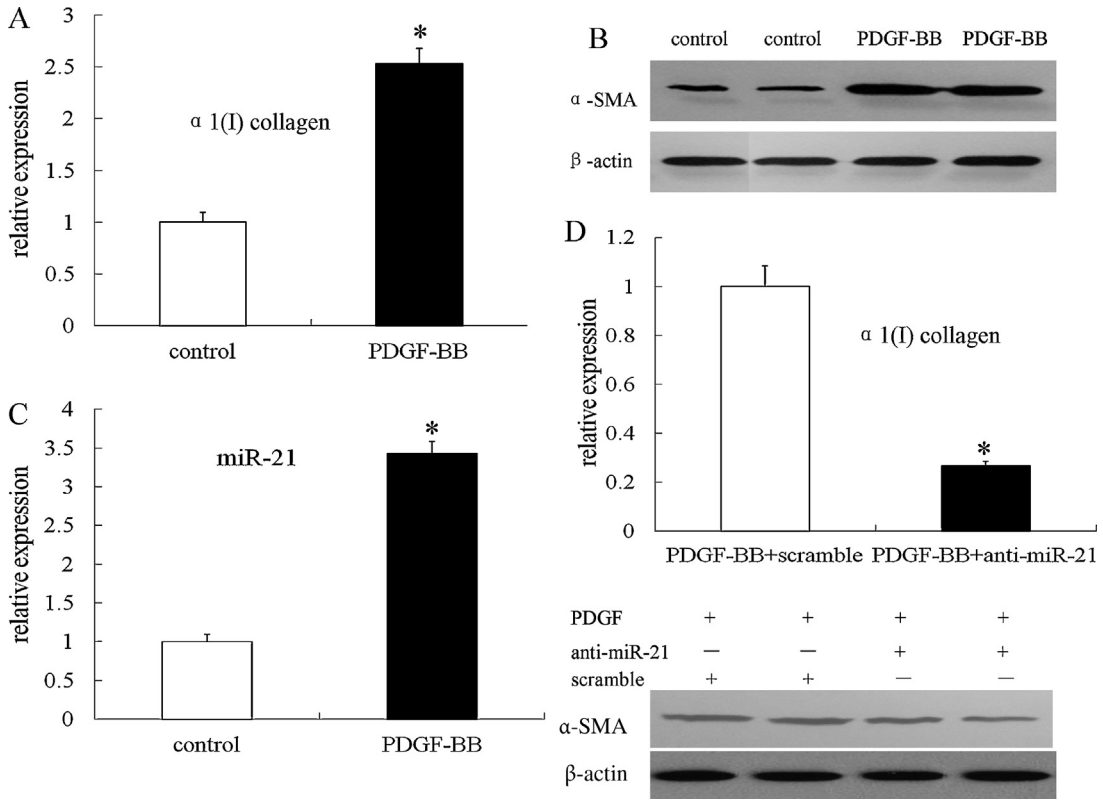

Fig. 1. MicroR-21 mediated PDGF-BB induced LX-2 cell activation. A. LX-2 cells were treated with 5 ng/mL PDGF-BB for 48 h. mRNA expression of α1(I) collagen was measured by real-time PCR. B. LX-2 cells were treated with 5 ng/mL PDGF-BB for 72 h. The protein expression of α-SMA was examined by Western blot. C. LX-2 cells were treated with 5 ng/mL PDGF-BB for 48 h. MicroR-21 expression was measured by real-time PCR. D. Downregulation of microR-21 expression blocked PDGF-BB induced mRNA expression of α1(I) collagen (upper) and protein expression of α-SMA (lower) (* $P < 0.05$).

addition, PDGF-BB treatment and overexpression of miR-21 resulted in enhanced MMP2 activity in LX-2 cell culture supernatants based on zymography assays, which also could be attenuated by miR-21 inhibition (Fig. 3C). These results suggest that miR-21 plays a role in MMP2 expression and activation.

3.3. PTEN/Akt pathway mediated the fibrogenic effects of microR-21 in LX-2 cells

One of the targets of miR-21 is PTEN that is the negative regulator of PI3K/Akt pathway [11]. PI3K/Akt pathway is believed

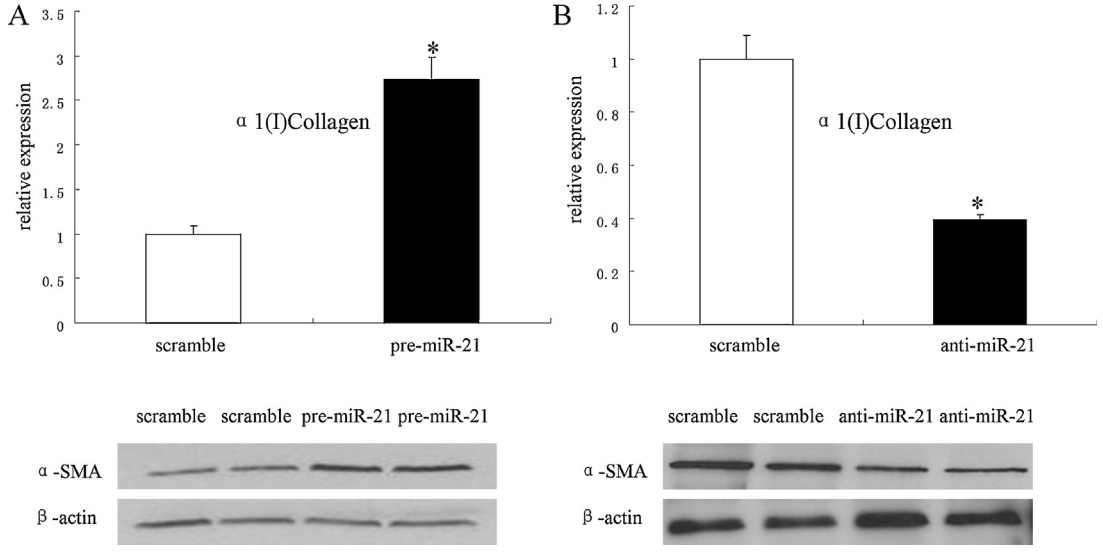

Fig. 2. The direct role of microR-21 in LX-2 cell activation. A. Pre-microR-21 was transfected into LX-2 cells. mRNA expression of α1(I) collagen (upper) and protein expression of α-SMA (lower) was examined by real-time PCR and Western blot respectively. B. Anti-miR-21 was transfected into LX-2 cells. mRNA expression of precollagen I (upper) and protein expression of α-SMA (lower) was examined by real-time PCR and Western blot respectively (* $P < 0.05$).

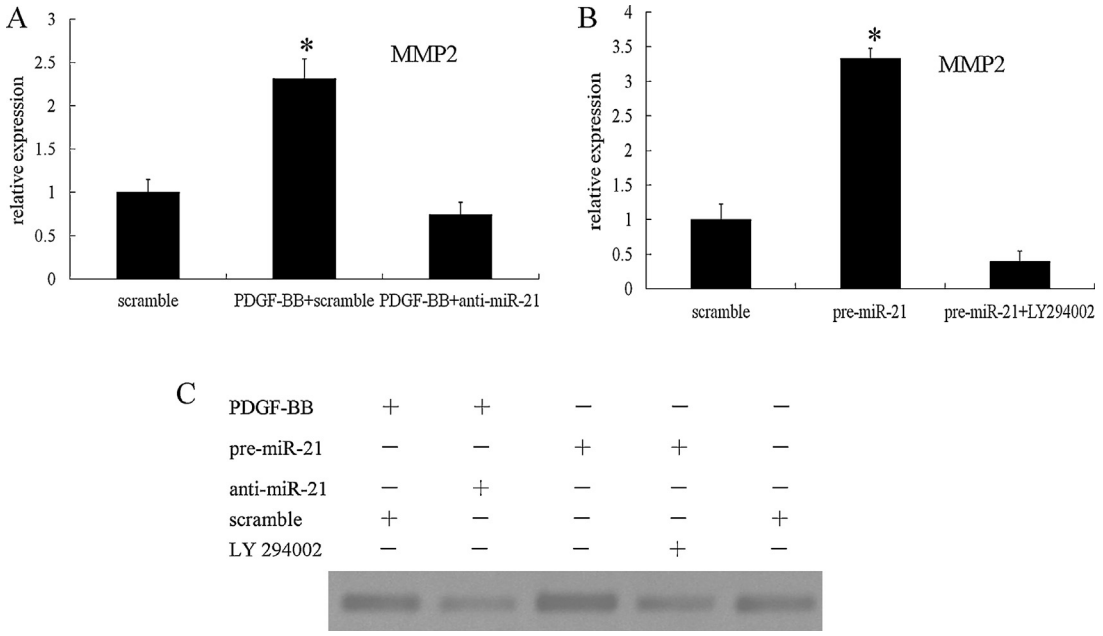

Fig. 3. MicroR-21 stimulated MMP2 expression and activity in LX-2 cells. A. LX-2 cells were treated with 5 ng/mL PDGF-BB alone or in combination with transfection of anti-miR-21. mRNA expression of MMP2 was measured by real-time PCR. B. LX-2 cells were transfected with pre-microR-21 alone or in combination with LY 294002 treatment. mRNA expression of MMP2 was measured by real-time PCR. C. LX-2 cells were treated with 5 ng/mL PDGF-BB alone or in combination with transfection of anti-miR-21 and LX-2 cells were transfected with pre-microR-21 alone or in combination treatment with LY 294002. MMP2 activity in LX-2 cell culture supernatants was measured by zymography assays (* $P < 0.05$).

to regulate the liver fibrosis [12]. To examine whether PTEN/Akt pathway is regulated by miR-21 in LX-2 cells, we transiently transfected pre-miR-21 or anti-miR-21 into LX-2 cells to overexpress or downregulate miR-21 expression. As shown on Fig. 4A, the protein expression of PTEN was decreased and meanwhile Akt phosphoralation was increased in pre-miR-21-transfected cells. In contrast, inhibition of miR-21 expression by transfection of anti-miR-21 into LX-2 cells increased protein expression of PTEN and decreased Akt phosphoralation. These results confirmed that miR-21 regulated the PTEN/Akt pathway in LX-2 cells.

The above results indicate that PTEN/Akt signaling may play a role in miR-21-induced fibrogenic effects in LX-2 cells. Subsequently, we used LY 294002, a specific inhibitor for Akt activation, to determine the role of Akt signaling in miR-21-induced fibrogenic effects in LX-2 cells. The results showed that 50 μM LY 294002 significantly abolished miR-21 mediated increase in

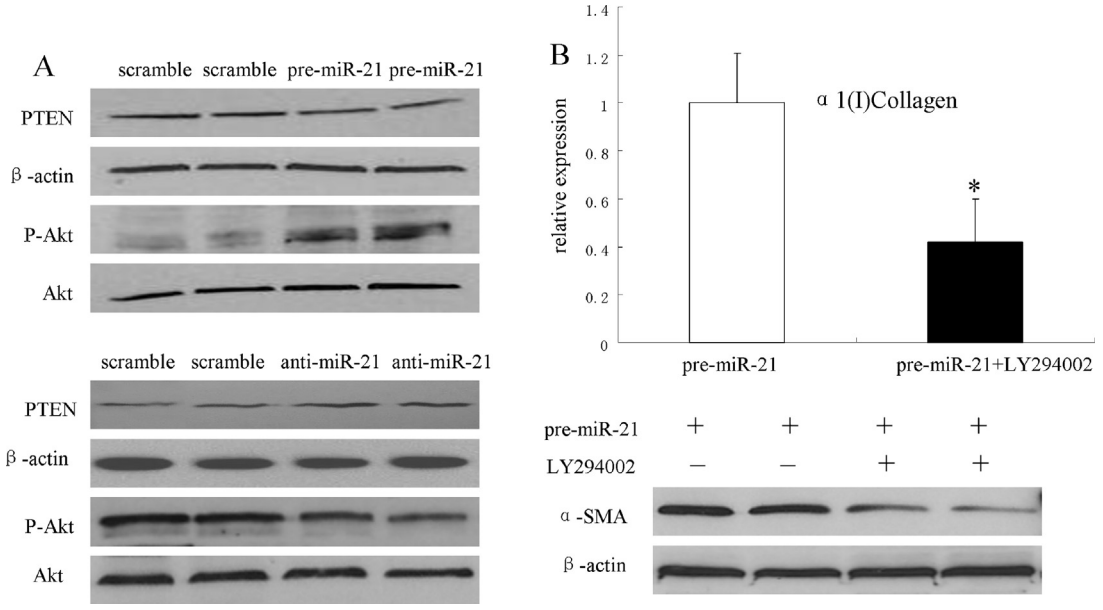

Fig. 4. PTEN/Akt pathway mediated the fibrogenic effects of microR-21 in LX-2 cells. A. LX-2 cells were transiently transfected with pre-microR-21 or microR-21. The protein expression of PTEN and Akt was examined by Western blot. B. LX-2 cells were transiently transfected with pre-microR-21 or in combination treatment with LY 294002. mRNA expression of α1(I) collagen (upper) and protein expression of α-SMA (lower) were examined by real-time PCR and Western blot respectively (* $P < 0.05$).

α1(I) collagen production and a-SMA expression in LX-2 cells (Fig. 4B). Inhibition of Akt activation also suppressed MMP2 mRNA expression and activity induced by miR-21 (Figs. 3B and C). Therefore, these results suggest that the PTEN/Akt pathway mediates the fibrogenic effects of miR-21 in LX-2 cells.

4. Discussion

The effects of different miRs on the liver fibrosis have been studied. Murakami et al. [13] showed that the progression of liver fibrosis is related to overexpression of the miR-199 and 200 families. Roderburg et al. [14] showed that miR-29 mediates the regulation of liver fibrosis. miR-21 is involved in a variety of physiological and pathological events [15,16]. The fibrotic effect of miR-21 has been confirmed in the lung, heart, and kindey [7–9]. Thum et al. have shown that miR-21 is upregulated selectively in fibroblasts of the pressure-overloaded heart, but not in cardiomyocytes. Upregulation of miR-21 in cardiac fibroblasts promotes fibroblast survival and growth factor secretion. Most importantly, injection of chemically modified antisense oligonucleotides specific for miR-21 (antagomiR-21) into mice subjected to pressure overload of the left ventricle by transverse aortic constriction (TAC) reduces myocardial fibrosis [7]. The study from Liu et al. indicates that miR-21 is highly upregulated in the lungs of mice with bleomycin induced lung fibrosis and in the lungs of patients with idiopathic pulmonary fibrosis. miR-21 is primarily enriched in myofibroblasts in the fibrotic lungs. Sequestration of miR-21 in mouse lungs effectively attenuated bleomycin induced fibrosis suggesting a central role for miR-21 in the pathogenesis of lung fibrosis [8]. miR-21 is also upregulated associated with unilateral ureteral obstruction (UUO)-induced renal fibrosis. Blocking miR-21 *in vivo* attenuated UUO-induced renal fibrosis [9]. However, the effect of miR-21 on the liver fibrosis remains unclear. Our study showed that miR-21 was upregulated after treatment of LX-2 cells with PDGF-BB accompanying LX-2 cell activation, suggesting that it may play an important role in PDGF-BB induced fibrogenic effects. Downregulation of miR-21 expression blocked PDGF-BB-induced LX-2 cell activation. These results suggest that miR-21 may play an important role in PDGF-BB induced liver fibrogenesis. More direct evidence for the role of miR-21 in the LX-2 cell activation was further confirmed. Overexpression of miR-21 promoted and downregulation of miR-21 blocked LX-2 cell activation respectively, implying that miR-21 also has direct fibrogenic effect in the liver.

Fibrosis reflects a balance between matrix production and degradation. Thus, like matrix production, the degradation of extracellular matrix is a key event in hepatic fibrosis that is both regulable and amenable to therapies. Early disruption of the normal hepatic matrix by matrix-degrading proteases hastens its replacement by scar matrix, which has deleterious effects on cell function. Activation of matrix metalloproteinases (MMPs) allows degradation of fibrillar collagen and cleavage of the basement membrane surrounding necrotic myocytes — a prerequisite for the invasion and proliferation of cells, e.g. fibroblasts, inflammatory, and vascular cells — ensuring tissue repair and scar formation [3]. Stellate cells are a key source of the basement membrane proteases MMP-2 of liver [17]. The effects of PDGF-BB and miR-21 on MMP2 expression and activity have been extensively studied. The study from Kenagy et al. shows that PDGF-BB-induced migration of arterial smooth muscle cells is dependent on MMP2 [18]. miR-21 also plays an important role in TGF-β-induced MMP2 expression and activity in human umbilical vein endothelial cells [19]. In addition, inhibition of miR-21 has been shown that suppressed MMP2 activity *in vitro* and *in vivo* [19]. Roy et al. show that miR-21 regulates MMP2 expression in cardiac fibroblasts of the infarct zone via a PTEN pathway [20]. Our study showed that miR-21 mediated PDGF-BB-induced MMP2 mRNA expression and activity in LX-2 cells, further suggesting that miR-21 gets involved in LX-2 activation.

There are many signaling pathways involved in liver fibrosis [12]. One of the pathways is PI3K/Akt signaling. The PI3K/Akt signaling pathway is activated in HSC by PDGF-BB and promotes cellular proliferation and collagen expression [10,21]. It is well established that PTEN is negative regulator of PI3K/Akt pathway [22]. Overexpression of PTEN inhibits HSC activation and proliferation through negative regulation of PI3K/Akt. [23]. It is also known that PTEN is one of targets of miR-21 [11]. The studies show that signaling of miR-21 on fibrosis is different based on the cell types studied. Upregulation of miR-21 in cardiac fibroblasts targeted the sprouty homologue 1 (Spry1), thereby indirectly enhancing the activity of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, suggesting that miR-21 is a critical regulator of ERK-MAP kinase activity in cardiac fibroblasts [7]. Liu et al. [8] found that a potential mechanism for the role of miR-21 in lung fibrosis is through regulating the expression of Smad7. However, TGF-β-mediated endothelial-to-mesenchymal transition is regulated at least in part by miR-21 via the PTEN/Akt pathway [19]. In our study, miR-21 exhibited inhibition of PTEN and activation of Akt in HSC. Importantly, Akt inhibition blocked the fibrogenic effect of miR-21 in LX-2 cells. These findings demonstrate that PTEN/Akt pathway mediates the fibrogenic effects of miR-21 in LX-2 cells.

In conclusions, miR-21 stimulated the fibrogenic effects in LX-2 cells through PTEN/Akt pathway. Combining our study with other studies on the profibrotic role of miR-21 in different organs such as heart, lung, and kidney, we suggest that miR-21 may also be a fibromir. Further in vivo study on the role of miR-21 in liver fibrosis is needed.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

Acknowledgments

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