Cellular Signalling 58 (2019) 1–8

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

MiR-146a attenuates liver fibrosis by inhibiting transforming growth factor-β1 mediated epithelial-mesenchymal transition in hepatocytes

Yanting Zou[a,b], Shuyu Li[a,b], Zhengliang Li[a,b], Dongqiang Song[c], Shuncai Zhang[a,b,*], Qunyan Yao[a,b,*]

[a] Department of Gastroenterology and Hepatology, Zhongshan Hospital, Fudan University, Shanghai, PR China
[b] Shanghai Institute of Liver disease, Shanghai, PR China
[c] Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, PR China

ARTICLE INFO

Keywords:
Liver fibrosis
EMT
miR-146a
TGF-β1
SMAD4

ABSTRACT

Epithelial-mesenchymal transition (EMT) has emerged as a vital process in embryogenesis, carcinogenesis, and tissue fibrosis. Transforming growth factor-beta 1 (TGF-β1)-mediated signaling pathways play important roles in the EMT process. MicroRNA-146a (miR-146a) has been suggested as a significant regulatory molecule in fibrogenesis. Therefore, the present study aimed to evaluate the effect of miR-146a on the EMT of hepatocytes and to investigate the role of overexpressing miR-146a on rat hepatic fibrosis. The results showed that the miR-146a level decreased during the EMT process of L02 hepatocytes induced by TGF-β1 *in vitro*. Moreover, miR-146a overexpression led to significant reduction of EMT-related markers expression in hepatocytes. Subsequent experiments revealed that miR-146a attenuated the EMT process in hepatocytes by targeting small mothers against decapentaplegic (SMAD) 4. Meanwhile, restoration of SMAD4 expression rescued the inhibitory effect of miRNA-146a on EMT. Further *in vivo* studies revealed that intravenous injection of miR-146a-expressing adenovirus (Ad-miR-146a) successfully restored the miR-146a levels and mitigated fibrogenesis in the livers of CCl4-treated rats. More importantly, after Ad-miR-146a treatment, inhibition of both EMT traits and SMAD4 expression was observed. The results of the present study showed that miR-146a/SMAD4 is a key signaling cascade that inhibits hepatocyte EMT, and the introduction of miR-146a might present a promising therapeutic option for liver fibrosis.

1. Introduction

Hepatic fibrogenesis is the common stage of various chronic liver diseases. Overproduction and deposition of the extracellular matrix (ECM) is a key event in hepatic fibrosis. Myofibroblasts, the primary ECM-producing cells, are the main effector cells of liver fibrosis. Hepatic stellate cells (HSCs) are the major source of myofibroblasts in fibrotic livers [1]. Myofibroblasts are also derived from epithelial cells through epithelial mesenchymal transition (EMT) [2–5]. EMT is the process by which fully differentiated epithelial cells transform into fully differentiated mesenchymal cells [6]. A growing body of evidence indicates that EMT promotes the development of hepatic fibrosis [7–11]. In chronic liver diseases, hepatocytes undergo EMT induced by adverse stimuli. These cells lose their epithelial markers, such as epithelial (E)-cadherin and zonula occludens-1 (ZO-1), which are replaced by the mesenchymal markers, α-smooth muscle actin (α-SMA), vimentin, matrix metalloproteinase (MMP)-2, MMP-9, and collagens [12]. This transformation leads to increased deposition of the ECM [13].

Emerging evidence demonstrates that microRNAs (miRNAs) play important roles in regulating EMT [14]. MiRNAs are small noncoding RNAs that play negative regulatory roles in gene expression at the post-transcription and/or transcription levels. They have been reported as significant mediators in the development of hepatic fibrogenesis, including the EMT of hepatocytes [15]. In view of the indispensable role of EMT in hepatic fibrogenesis, the identification of significant candidate miRNAs that regulate the EMT of hepatocytes might lead to improved treatment of hepatic fibrosis. MicroRNA-146a (miR-146a) was identified as a multi-functional miRNA that can regulate multiple signaling pathways involved in the fibrosis of different organs, including C-X-C motif chemokine ligand (CXCL)8/CXCL1, nuclear factor kappa B (NF-κB), transforming growth factor beta (TGF-β)/small mothers against decapentaplegic (SMAD), and wnt signaling pathways [16–19].

* Corresponding authors at: Department of Gastroenterology and Hepatology, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Xuhui District, Shanghai, PR China.
E-mail addresses: zhang.shuncai@zs-hospital.sh.cn (S. Zhang), yao.qunyan@zs-hospital.sh.cn (Q. Yao).

https://doi.org/10.1016/j.cellsig.2019.01.012
Received 5 October 2018; Received in revised form 5 January 2019; Accepted 31 January 2019
Available online 31 January 2019
0898-6568/

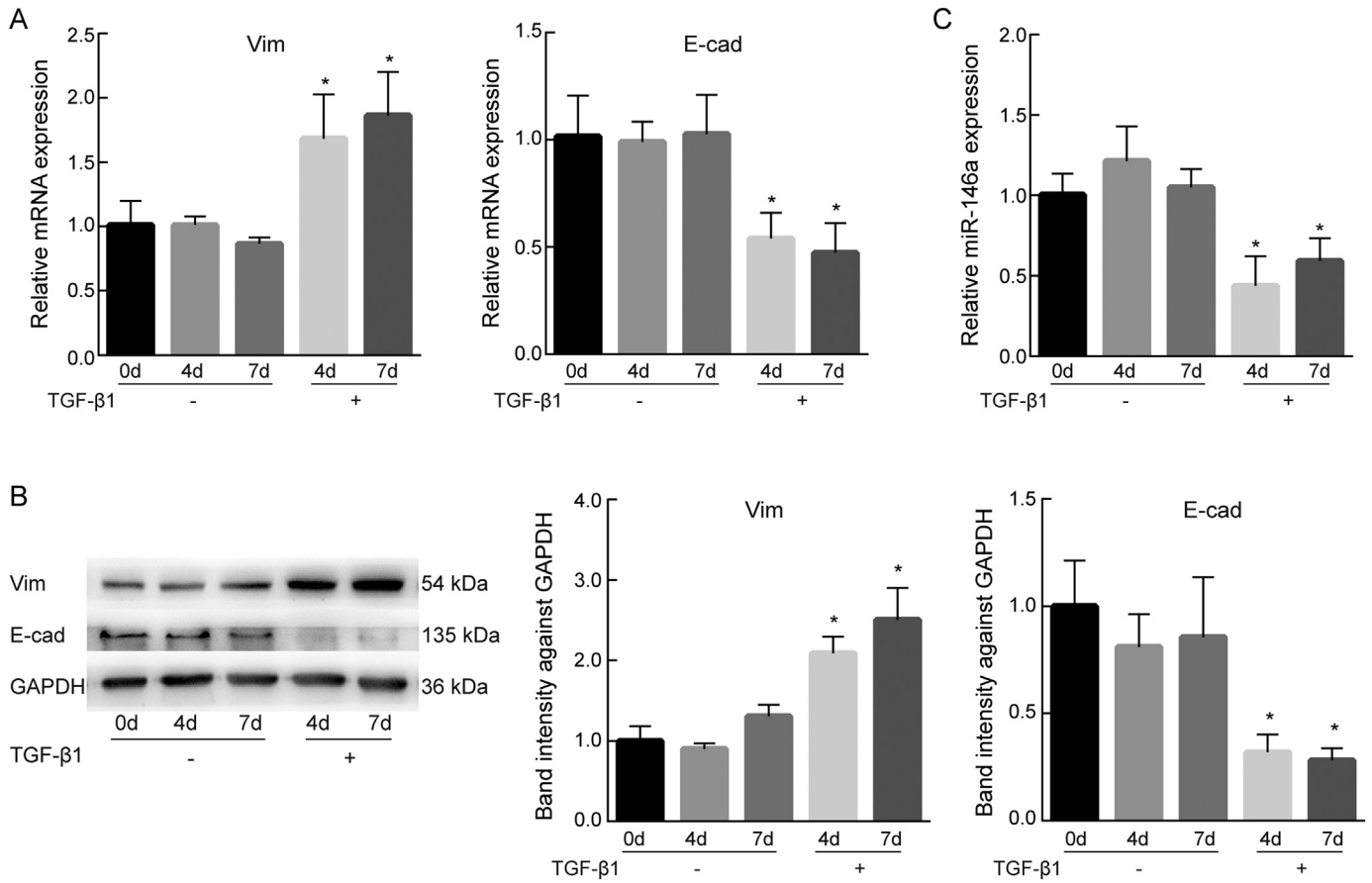

Fig. 1. The expression of vimentin (vim), E-cadherin (E-cad), and miR-146a in L02, with or without TGF-β1 treatment, for 4 or 7 days. (A) The levels of vimentin and E-cadherin mRNA expression were determined using qRT-PCR. (B) Representative images of western blotting for vimentin and E-cadherin protein levels. (C) MiR-146a expression was determined using qRT-PCR. Data are expressed as the mean ± SD of three independent experiments. *$P < .05$ compared with the groups without TGF-β1 treatment.

Our previous study showed that the hepatic contents of miR-146a were lower in rats with carbon tetrachloride (CCl4)-induced hepatic fibrosis. Furthermore, we demonstrated that miR-146a suppressed the effects of TGF-β1 and lipopolysaccharide (LPS) during HSC activation by directly targeting interleukin 1 receptor associated kinase 1 (IRAK1), TNF receptor associated factor 6 (TRAF6), and SMAD4 [20].

However, little attention has been paid to the effect of miR-146a on the EMT process in hepatocytes and its therapeutic potential in hepatic fibrosis *in vivo*. Therefore, in the present study, we aimed to evaluate the effect of miR-146a on the EMT of hepatocytes and to investigate the role of overexpressing miR-146a on rat hepatic fibrosis. The results indicated that miR-146a inhibited the EMT of hepatocytes by targeting SMAD4. Overexpression of miR-146a in hepatocytes attenuated EMT associated traits. Furthermore, delivery and overexpression of miR-146a alleviated liver fibrosis and attenuated EMT marker expression in a rat hepatic fibrosis model, implying that miR-146a could represent a target in liver fibrosis treatment.

2. Materials and methods

2.1. Cell culture and treatments

The immortalized human hepatocyte cell line L02 was cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 1% penicillin and streptomycin. The EMT of hepatocytes was induced by stimulation with TGF-β1 (5 ng/ml, Peprotech, Rocky Hill, NJ, USA) for 24 h to 7 days.

2.2. Cell transfections

The lentivirus vectors for miR-146a, miR-146a inhibitor, and scrambled miR-control carrying a green fluorescent protein sequence were obtained from Hanyinbt (Shanghai, China). For infection of cells, cells were plated in 6-well plates at a density of 2×10^5 cells per well and cultured until they reached 30%–50% confluence. The above cells were infected with lentivirus in the presence of 5 mg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's protocol. Eight hours after viral infection, the culture medium was replaced by fresh medium. Then the infected cells were continuous exposured to 2 μg/ml puromycin (InvivoGen, San Diego, CA, USA). Green fluorescence under a fluorescence microscope was utilized for transduction efficiency measurement (Supplementary Fig. 1). For transient transfections, 200 nM of vector DNA was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, US). The SMAD4 plasmid was purchased from Genepharma (Shanghai, China). Transfection efficiency was monitored by western blotting at 48 h after transfection.

2.3. Animal model

Male Sprague-Dawley rats weighing 180–220 g were purchased from the Experimental Animal Center of Fudan University. The animal maintenance and experimental procedures were approved by the Institutional Animal Care and Use Committee of Fudan University. Twenty-four rats were divided into four groups of six rats each. For the CCl4 group, 1 ml/kg of CCl4 (Sigma-Aldrich), diluted 1:1 in olive oil,

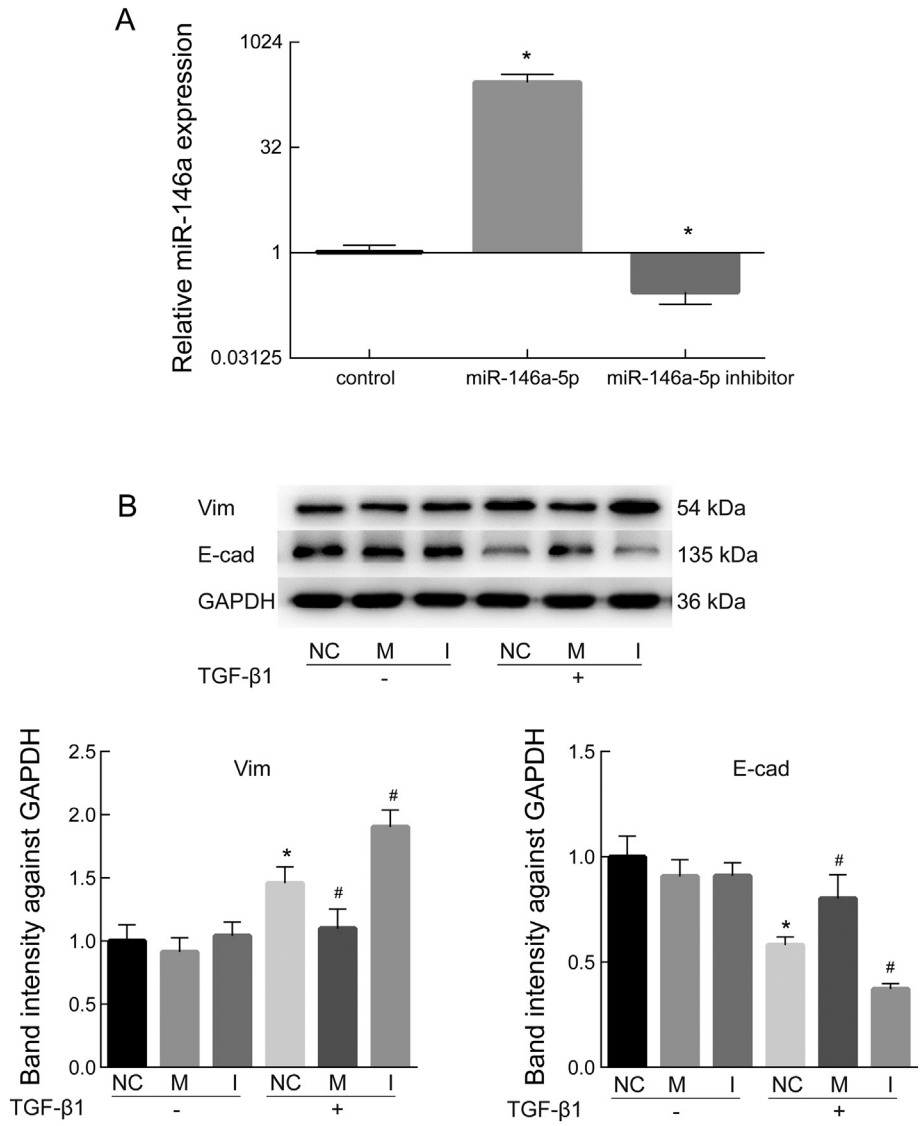

Fig. 2. MiR-146a attenuates the EMT progress of L02 cells. (A) Changes in miR-146a expression in L02 cells transfected with lentivirus vectors expressing miR-146a, miR-146a inhibitor, and the control. (B) Representative images of the protein levels of vimentin and E-cadherin in miR-146a (M), miR-146a inhibitor (I), and negative control (NC) lentivirus vector-transfected cells. Data are expressed as the mean ± SD of three independent experiments. *$P < .05$ compared with groups without TGF-β1 treatment; #P < .05 compared with the corresponding negative control group.

was injected intraperitoneally twice a week. The Ad-control and Ad-miR-146a groups were administered with 5×10^9 infective units of adenovirus (Obio Technology, Shanghai, China) by intravenous injection into the tail vein every two and a half weeks along with the CCl4 treatment described above. The control group received intraperitoneally injections of olive oil vehicle and intravenous injection of PBS for comparison. Five weeks after treatment with CCl4, the rats were sacrificed under anesthesia. A portion of their livers were excised for histological examination and fixed in 4% paraformaldehyde. The remnant liver tissues were frozen immediately in liquid nitrogen and stored at −80 °C for western blotting analysis and RNA isolation.

2.4. RNA isolation and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells or liver tissue using Trizol (Invitrogen) according to the manufacturers' protocols. The expression of miR-146a was assessed using a Bulge-Loop™ miRNA qRT-PCR Starter Kit (RiboBio, Guangzhou, China). A PrimeScript RT reagent Kit and SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) were used to assess the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), vimentin, and E-cadherin. The fold-change for the miRNA and mRNA relative to U6 and GAPDH, respectively, was determined using the formula $2^{-\Delta\Delta}$Ct [21]. Supplementary Table 1 shows the primer sequences. These experiments were repeated at least three times independently.

2.5. Western blotting

Total protein was extracted from cultured cells and liver tissues using cold Radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). Nuclear and cytoplasmic proteins were isolated from cells using Nuclear and Cytoplasmic Protein Extraction Kits (Beyotime). The protein contents were analyzed using a BCA protein assay kit (Vazyme, Nanjing, China). Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% nonfat milk in Tris-buffered saline-Tween-20 for 1 h, the membranes were probed with primary antibodies recognizing vimentin (Cell Signaling Technology, Danvers, MA, USA), E-cadherin, SMAD4, SMAD2/3, phosphorylated (p)SMAD2/3 (Abcam, Cambridge, MA,

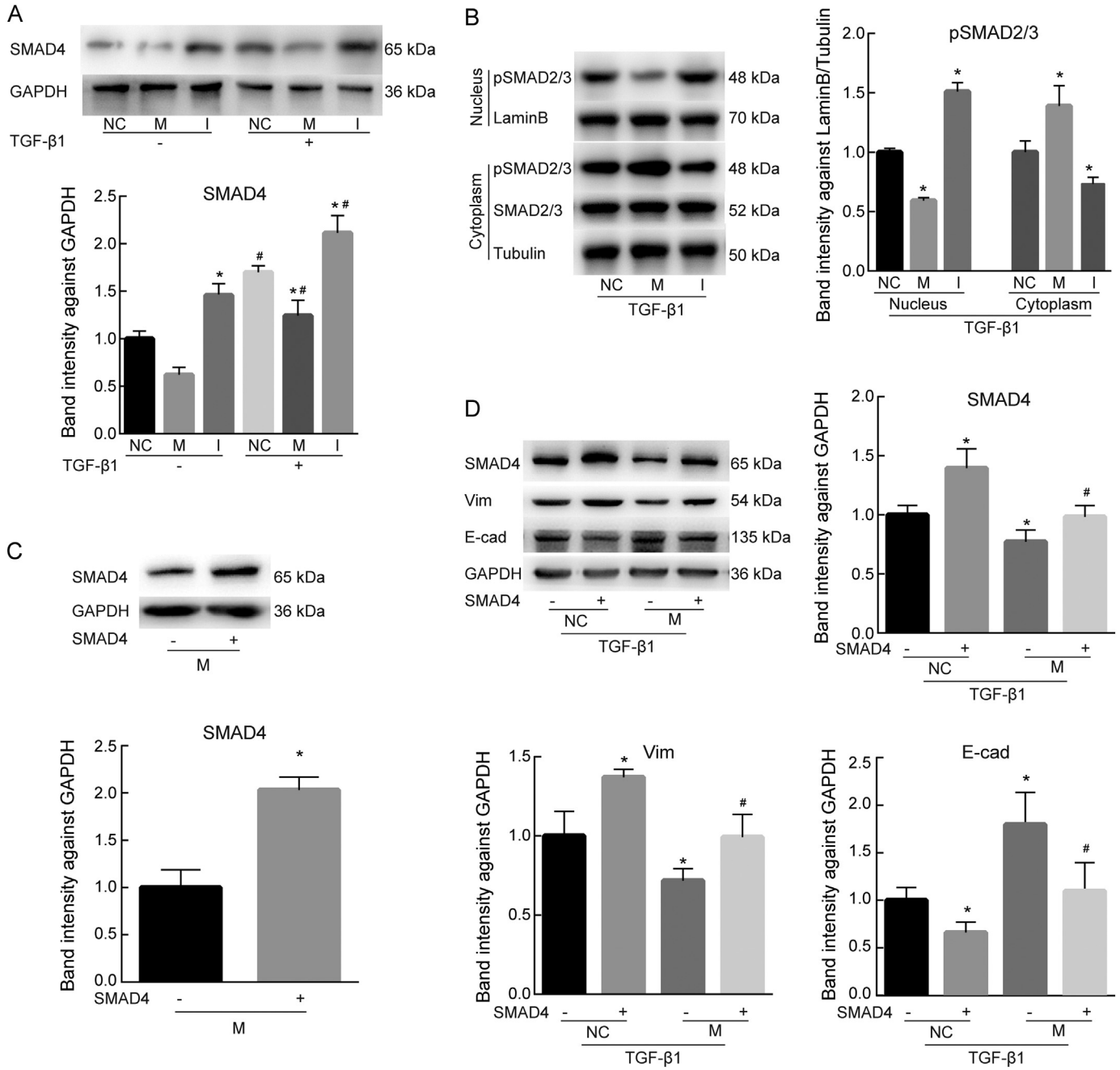

Fig. 3. MiR-146a attenuates the EMT process in L02 cells by targeting SMAD4. (A) The expression of SMAD4 in miR-146a (M), miR-146a inhibitor (I), and negative control (NC) lentivirus vector-transfected cells were measured using western blotting. *$P < .05$ compared with the corresponding negative control group; #P < .05 compared with the corresponding groups without TGF-β1 treatment. (B) MiR-146a decreased the level of pSMAD2/3 in the nuclei of TGF-β1-treated L02 cells, while it increased that in the cytoplasm. *P < .05 compared with the control group. (C) Transfection of a SMAD4 overexpressing plasmid increased the expression of SMAD4 in miR-146a overexpressing L02 cells. *P < .05 compared with the control group. (D) Overexpression of SMAD4 rescued the attenuation of EMT traits caused by miR-146a overexpression in TGF-β1-treated hepatocytes. Data are presented as the mean ± SD from three independent experiments. *$P < .05$ compared with the first group; #P < .05 compared with the third group; there is no difference between the first and the last group.

USA), or GAPDH (Vazyme) overnight at 4 °C. Then, horseradish peroxidase (HRP)-conjugated secondary antibody (Vazyme) was used in a standard enhanced chemiluminescence reaction (Thermo Fisher Scientific, Waltham, MA, USA) to visualize the immunoreactive proteins.

2.6. Histological and immunohistochemical (IHC) analyses

Formalin fixed, paraffin embedded liver tissues were cut into 4 μm sections. The sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome. A trained pathologist, who was blinded to the treatment details, evaluated the fibrosis of each liver on a scale of 0–4. The positively stained area was measured using NIH ImageJ (NIH, Bethesda, MD, USA).

For immunohistochemistry, sections were dewaxed using xylene and rehydrated with a series of decreasing concentrations of ethanol. The treated sections were incubated with antibodies recognizing vimentin, E-cadherin, α-SMA, SMAD4, or pSMAD2/3 (Abcam). A SignalStain Boost hRP-Polymer solution (Cell Signaling Technology) and 3,3′-diaminobenzidine tetrachloride (Sigma-Aldrich) were used to

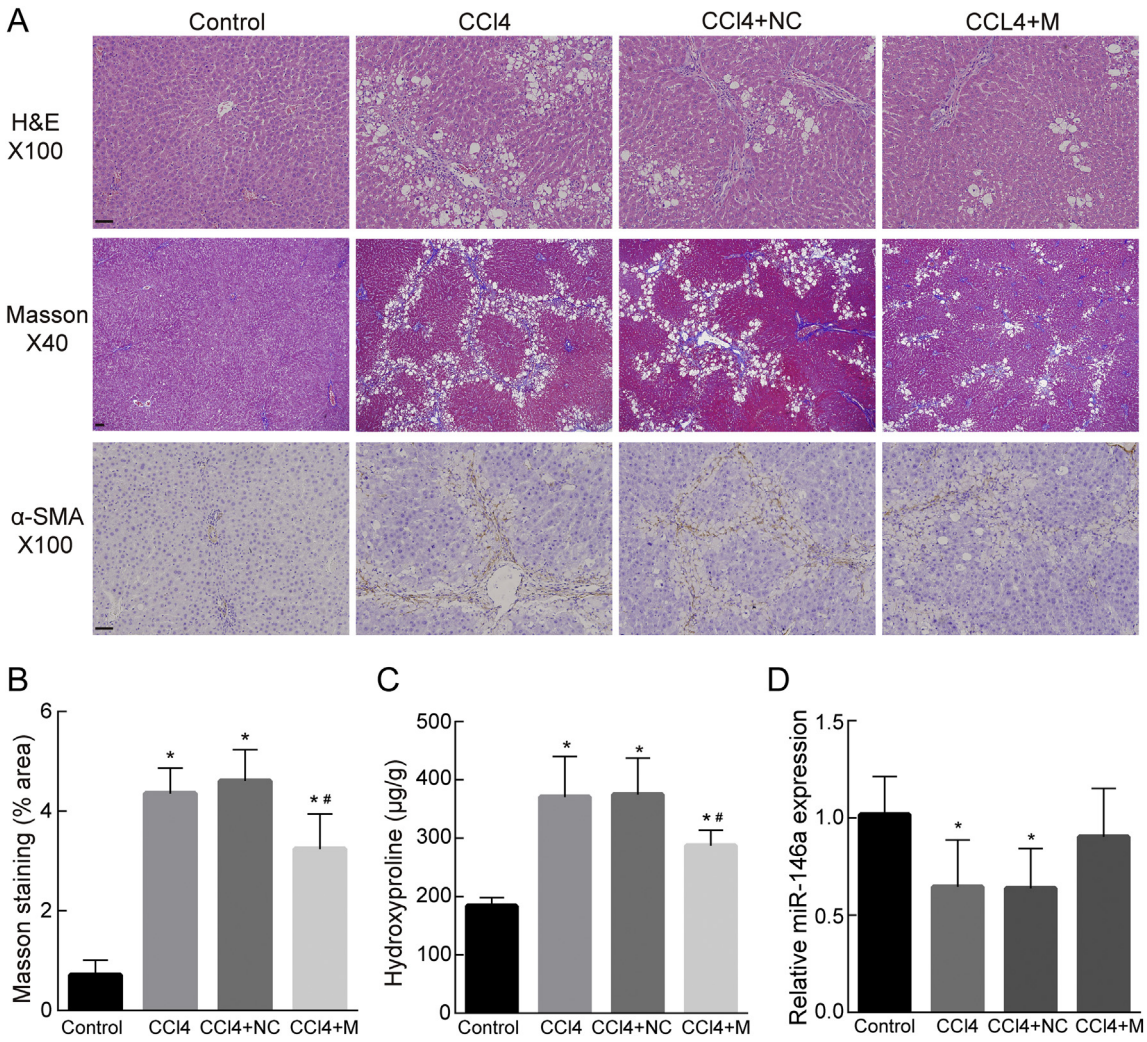

Fig. 4. MiR-146a suppresses CCl4-induced liver fibrosis in rats. (A) Hematoxylin and eosin staining (× 100), Masson trichrome staining (× 40) and immunohistochemistry for α-SMA of liver sections in rats from the control group, CCl4 group, CCl4 + Ad-negative control group (CCl4 + NC), and CCl4 + Ad-miR-146a group (CCl4 + M). (B) Quantification of the Masson-positive area. (C) Quantification of hepatic hydroxyproline content. (D) The expression of miR-146a in hepatic tissues. $N = 6$ for each group. Scale bar: 200 μm. *$P < .05$ compared with the control group, #P < .05 compared with the CCl4 + N group.

detect the bound antibodies.

2.7. Measurement of the hydroxyproline content

The hepatic hydroxyproline content was detected using a Hydroxyproline Colorimetric Assay Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

All results are expressed as mean ± SD. Data with a normal distribution were compared using one-way analysis of variance and Student's *t*-test using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). $P < .05$ was considered statistically significant.

3. Results

3.1. MiR-146a decreases in the EMT process of L02 hepatocytes induced by TGF-β1

TGF-β1 is a key cytokine in the initiation and progression of hepatic fibrogenesis. For various epithelial cells, culturing with TGF-β1 is a convenient way to induce EMT [22]. When cultured and stimulated with TGF-β1 *in vitro*, L02 hepatocytes convert to a fibroblast-like phenotype, with changes in the expression levels of specific proteins. QRT-PCR and western blotting analysis showed an increase in vimentin and a decrease in E-cadherin levels in L02 hepatocytes stimulated with TGF-β1 (Fig. 1A & 1B). During the EMT process of L02 hepatocytes, miR-146a levels decreased in TGF-β1-treated hepatocytes compared with that in the untreated group (Fig. 1C).

3.2. MiR-146a attenuates the progression of EMT in hepatocyte in vitro

To study the role of miR-146a in EMT, we transfected L02 hepatocytes with lentivirus vectors expressing miR-146a, miR-146a inhibitor, or miR-control before TGF-β1 treatment. In the miR-146a group, miR-146a was significantly overexpressed. By contrast, transfection with the miR-146a inhibitor decreased miR-146a expression (Fig. 2A). Western blotting analysis showed that overexpression of miR-146a inhibited the TGF-β1-induced increased vimentin levels, whereas it increased the expression of E-cadherin. Conversely, knockdown of miR-146a promoted EMT progression of hepatocytes (Fig. 2B).

3.3. MiR-146a attenuates EMT process in L02 by targeting SMAD4

The TGF-β/SMAD pathway functions as a potent inducer of EMT

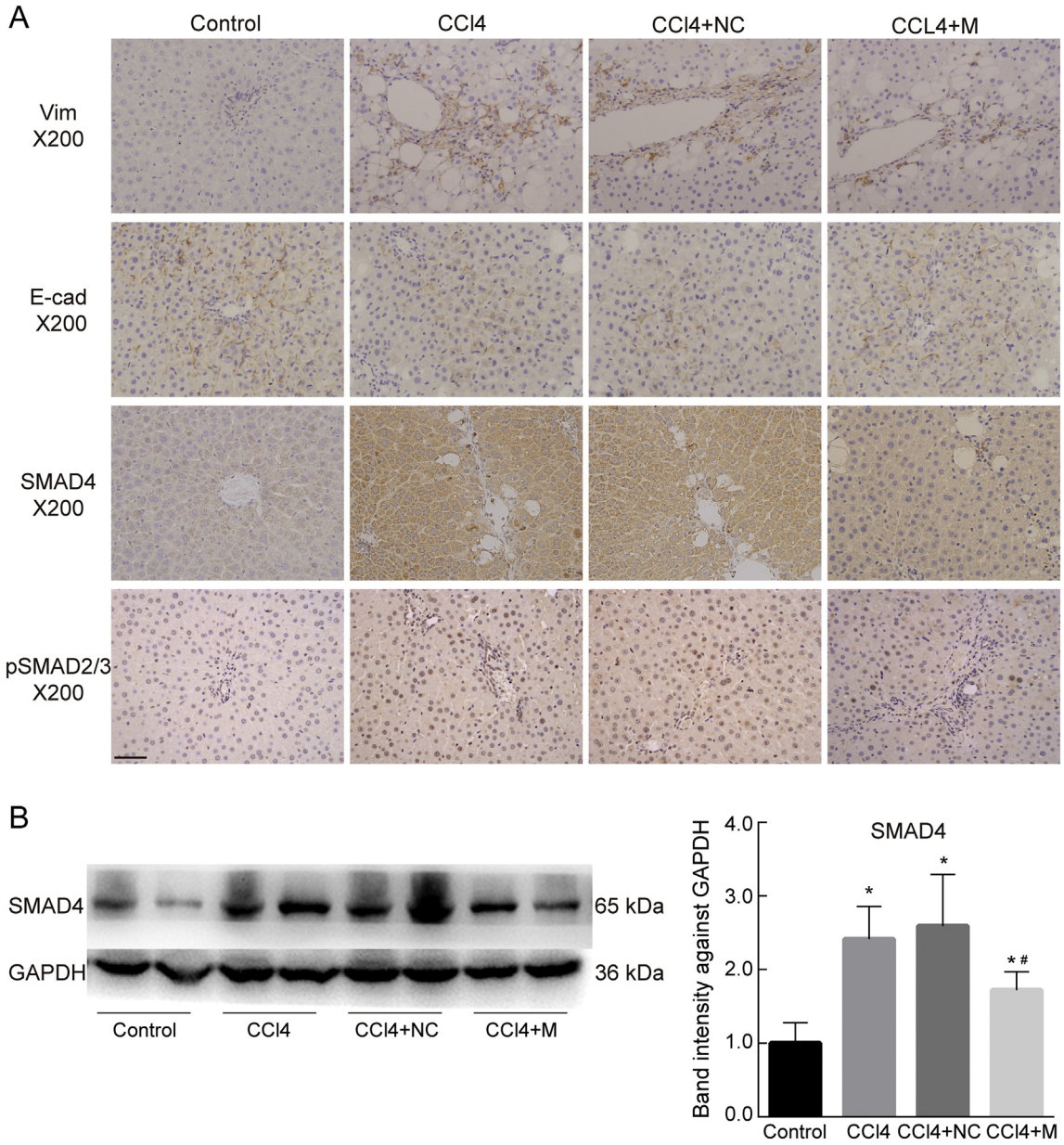

Fig. 5. MiR-146a suppresses EMT and TGF-β/SMAD signaling pathways in rats. (A) Typical patterns of vimentin, E-cadherin, SMAD4, and pSMAD2/3 staining in liver tissues. (B) Representative images of western blotting for SMAD4 protein levels in liver tissues. N = 6 for each group. Scale bar: 200 μm. *P < .05 compared with the control group, #P < .05 compared with the CCl4 and CCl4 + NC group.

[23]. Acting as the unique co-SMAD of this signaling pathway, SMAD4 plays an important part in EMT. Several studies have disclosed that miR-146a could regulate the TGF-β signaling pathway by targeting SMAD4 [24–28]. Therefore, we attempted to demonstrate whether miR-146a attenuated EMT in L02 cells by targeting SMAD4. First, we found that the expression level of SMAD4 was reduced after transfection with the lentivirus expressing miR-146a, which attenuates EMT progress of L02 hepatocytes stimulated by TGF-β1, and the level of SMAD4 increased after transfection with the miR-146a inhibitor (Fig. 3A). MiR-146a did not alter the levels of SMAD2/3. Nevertheless, miR-146a reduced the level of pSMAD2/3 in the nucleus, while it increased that in the cytoplasm, which suggested that miR-146a inhibits the nuclear translocation of pSMAD2/3 induced by TGF-β1 (Fig. 3B). Next, we performed rescue experiments. Fig. 3C shows that transfection with the SMAD4 plasmid increased the expression level of SMAD4 significantly. Overexpression of SMAD4 rescued the attenuation of EMT caused by miR-146a overexpression in TGF-β1-treated hepatocytes (Fig. 3D). These results suggested that miR-146a attenuates EMT of hepatocytes by targeting SMAD4.

3.4. MiR-146a suppresses liver fibrosis and EMT of hepatocytes via targeting SMAD4 in vivo

In order to confirm the effects of miR-146a on hepatic fibrosis *in vivo*, we injected Ad-miR-146a through the tail vein in CCl4-treated rats. Ad-miR-146a injection attenuated the decrease of miR-146a in CCl4-treated rats (Fig. 4D). H&E staining, Masson staining, and IHC for α-SMA demonstrated fewer hepatic injuries in the CCl4 + Ad-miR-146a group than in the CCl4 and CCl4 + Ad-control groups (Fig. 4A). Compared with that in the Ad-control-infected rats, the Masson-positive area (Fig. 4B) and hydroxyproline content (Fig. 4C) in Ad-miR-146a-infected rats were reduced. Moreover, adenovirus-mediated miR-146a expression alleviated the EMT traits in the fibrotic liver, manifesting as downregulated vimentin levels and upregulated E-cadherin levels

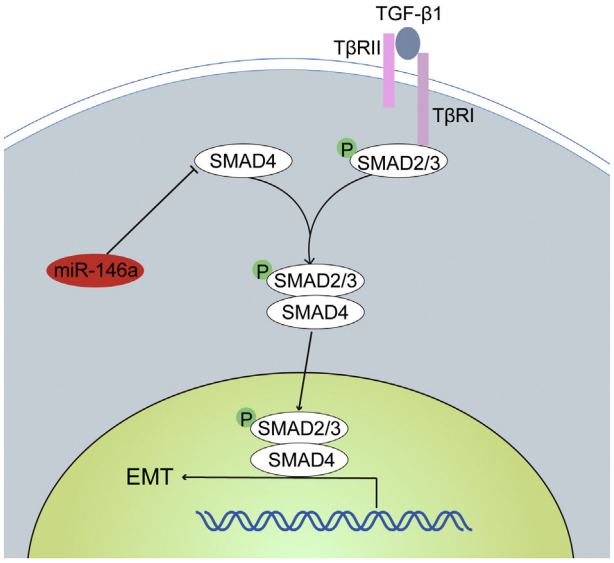

Fig. 6. Schematic diagram of the anti-EMT effect of miR-146a in hepatocytes. MiR-146a targets SMAD4 and decreases the nuclear translocation of pSMAD2/3, leading to the inhibition of the canonical TGF-β signaling pathway, which result in attenuation of EMT in hepatocytes. TβR: TGF-β receptor.

compared with those in CCl4-treated rats (Fig. 5A). We then examined SMAD4 levels in liver tissues. IHC and western blotting showed that intensity of SMAD4 staining was weaker in the CCl4 + Ad-miR-146a group than in the CCl4 + control group (Fig. 5A & B). Moreover, the difference of pSMAD2/3 between these groups is similar (Fig. 5A). Taken together, these findings suggested that miR-146a attenuates EMT of hepatocytes by targeting SMAD4 to alleviate liver fibrosis *in vivo*.

4. Discussion

Continuous injury to the liver generates fibrogenesis, which is accompanied by EMT of liver epithelial cells. There are three EMT subtypes. Type 1 is limited to embryogenesis. Type 2 concerns the conversion of epithelial cells to fibroblast-type cells and appears as fibrogenesis during chronic tissue damage. Type 3 EMT plays an important role in tumor metastasis and invasion [29]. Hepatic fibrosis is a potential outcome of type 2 EMT. Several groups have shown that hepatocytes, HSCs, and cholangiocytes can be induced to undergo EMT [30]. When undergoing EMT, these epithelial cells acquire the characteristics of mesenchymal cells, showing downregulation of epithelial gene expression, such as E-cadherin. A previous study demonstrated that loss of E-cadherin promoted the expression of TGF-β1 and its target genes in HSCs, thus facilitating hepatic fibrosis [31]. Typically, transdifferentiation of quiescent HSCs into myofibroblast-like cells is regarded as the pivotal event in liver fibrosis [32]. Additionally, growing evidence shows that myofibroblasts are also derived from hepatocytes and cholangiocytes through EMT during hepatic fibrogenesis [33]. Hepatocyte EMT might contribute to the accumulation of ECM and promote the progression of hepatic fibrosis [9].

Although substantial progress has been made in determining the mechanism and treatment of liver fibrosis, there are concerns regarding the treatment course in patients [34]. Therefore, it is imperative to develop more effective therapies to treat hepatic fibrosis. Recently, miRNAs have been documented as significant regulators of EMT, in which they target elements of different signaling pathways in hepatic fibrogenesis [35–39]. For instance, miR-706 is downregulated in fibrotic hepatocytes and was proved to prevent EMT by directly binding to the 3′-untranslated region (UTR) of protein kinase C alpha and TAO kinase 1 [35]. Overexpression of miR-181a could induce EMT-like changes to hepatocytes *in vitro*, and this phenotypic change was reversed using a miR-181a inhibitor [36]. MiR-21 also promotes the EMT process of hepatocytes by targeting and regulating the expression of hepatocyte nuclear factor 4 alpha, which is an important transcription factor that is essential for epithelial phenotype maintenance of hepatocyte [37]. MiR-101 not only prevented hepatocyte EMT *in vitro* but also inhibited mesenchymal markers upregulation in the hepatocytes of CCl4 treated mice [38].

As a multifunctional miRNA, miR-146a has been shown to attenuate the HSC proliferation induced by TGF-β1 [40]. Our previous study also reported a protective role of miR-146 in activated HSCs [20]. In the present study, we further verified that miR-146a was associated with the EMT process in hepatocytes. The expression of miR-146a decreased after TGF-β1 treatment of hepatocytes. Overexpression of miR-146a attenuated the downregulation of E-cadherin and upregulation of vimentin induced by TGF-β1 in hepatocytes. Taken together, these results support the view that miR-146a alleviates TGF-β1 induced EMT-associated traits. We then determined the functional target gene of miR-146a in modulating the EMT process. Given its antagonistic effect on TGF-β1, we hypothesized that miR-146a might target downstream molecules of the TGF-β1 signaling pathway, among which SMADs were probable candidates. SMADs have central roles in most actions of the TGF-β family [41]. SMAD4 is an indispensable element of the SMAD pathway. Upon TGF-β receptor activation, SMAD4 combines with R-SMADs to form a complex, which transduces signals from the extracellular environment to the nucleus [41]. SMAD4 was proven to be a direct target of miR-146a in certain other types of cells [24–28]. Our study suggested that SMAD4 is a direct target of miR-146a, and this targeting alleviated TGF-β/SMAD signaling pathway and EMT-associated traits in hepatocytes *in vitro*. Overexpression of SMAD4 partially rescued the effect of miR-146a overexpression in hepatocytes. Our data demonstrated that miR-146a reduces the EMT traits of TGF-β1 treated hepatocytes, at least partially, by targeting SMAD4 (Fig. 6).

In this study, we evaluated the effects of miR-146a in CCl4-induced liver fibrosis by intravenous injection of Ad-miR-146a. The results demonstrated the effect of miR-146a for alleviating hepatic fibrosis *in vivo*. Currently, traditional therapy is not sufficiently effective to cure hepatic fibrosis. MiRNAs might represent promising alternative targets for liver disease treatment. Several recent studies have reported therapeutic benefits of modulating miRNAs to treat liver fibrosis in different animal models. According to one study, lentivirus-mediated overexpression of miR-101 could attenuate hepatic fibrosis induced by CCl4 in mice and regulated profibrotic signaling pathways in both hepatocytes and HSCs [42]. MiR-29 is another antifibrotic miRNA. Overexpression miR-29 *via* intravenous injection of Ad-miR-29 or using an estradiol-inducible system exhibited prominent antifibrotic effects in CCl4-induced liver fibrosis in male mice [43]. Inhibition of pro-fibrotic miRNAs is another therapeutically effective measure to treat hepatic fibrosis. Okada et al. repressed miR-214 using a locked nucleic acid (LNA)-anti-miR-214 in platelet-derived growth factor-c transgenic mice. Their data showed attenuation of hepatic fibrosis in the LNA-anti-miR-214 group compared with that of the control [44]. Expression of miRNAs can be modulated specifically in the liver using a liver-specific delivery system, for example, the combination of rAAV8 and miRNA-Tough Decoy RNAs. Using this delivery system, He et al. showed efficient inhibition of miR-21 and attenuation of liver fibrosis in mice with hepatic schistosomiasis [45].

In conclusion, the results of the present study indicated that miR-146a alleviates liver fibrosis as a suppressor of EMT traits of hepatocytes by regulating the TGF-β signaling pathway. The identification of miR-146a's function and its target gene (SMAD4) may lead to a deeper understanding of the mechanisms of hepatic fibrosis and to the development of novel treatments.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.01.012.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant numbers: 81500460 and 81400628), Zhongshan Hospital (grant number: 2017ZSYQ11) and the Science and Technology Commission of Shanghai Municipality (grant number: 16411952300).

References

[1] M.E. Zoubek, C. Trautwein, P. Strnad, Reversal of liver fibrosis: from fiction to reality, Best Pract. Res. Clin. Gastroenterol. 31 (2017) 129–141.
[2] K.A. Rygiel, H. Robertson, H.L. Marshall, M. Pekalski, L. Zhao, T.A. Booth, D.E. Jones, et al., Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease, Lab. Investig. 88 (2008) 112–123.
[3] S.J. Lee, K.H. Kim, K.K. Park, Mechanisms of fibrogenesis in liver cirrhosis: the molecular aspects of epithelial-mesenchymal transition, World J. Hepatol. 6 (2014) 207–216.
[4] M. Pinzani, Epithelial-mesenchymal transition in chronic liver disease: fibrogenesis or escape from death? J. Hepatol. 55 (2011) 459–465.
[5] M. Zeisberg, C. Yang, M. Martino, M.B. Duncan, F. Rieder, H. Tanjore, R. Kalluri, Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition, J. Biol. Chem. 282 (2007) 23337–23347.
[6] E.W. Thompson, I. Haviv, The social aspects of EMT-MET plasticity, Nat. Med. 17 (2011) 1048–1049.
[7] J.P. Thiery, Epithelial-mesenchymal transitions in development and pathologies, Curr. Opin. Cell Biol. 15 (2003) 740–746.
[8] M. Iwano, D. Plieth, T.M. Danoff, C. Xue, H. Okada, E.G. Neilson, Evidence that fibroblasts derive from epithelium during tissue fibrosis, J. Clin. Invest. 110 (2002) 341–350.
[9] M. Zeisberg, C. Yang, M. Martino, M.B. Duncan, F. Rieder, H. Tanjore, R. Kalluri, Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition, J. Biol. Chem. 282 (2007) 23337–23347.
[10] W.K. Syn, Y. Jung, A. Omenetti, M. Abdelmalek, C.D. Guy, L. Yang, J. Wang, et al., Hedgehog-mediated epithelial-to-mesenchymal transition and fibrogenic repair in nonalcoholic fatty liver disease, Gastroenterology 137 (2009) 1478–1488.
[11] A. Kaimori, J. Potter, J.Y. Kaimori, C. Wang, E. Mezey, A. Koteish, Transforming growth factor-beta1 induces an epithelial-to-mesenchymal transition state in mouse hepatocytes in vitro, J. Biol. Chem. 282 (2007) 22089–22101.
[12] W.R. Bi, G.T. Xu, L.X. Lv, C.Q. Yang, The ratio of transforming growth factor-beta1/bone morphogenetic protein-7 in the progression of the epithelial-mesenchymal transition contributes to rat liver fibrosis, Genet. Mol. Res. 13 (2014) 1005–1014.
[13] E.A. Barnes, H.L. Kenerson, X. Jiang, R.S. Yeung, Tuberin regulates E-cadherin localization: implications in epithelial-mesenchymal transition, Am. J. Pathol. 177 (2010) 1765–1778.
[14] X. Zhao, Y. Lu, Y. Nie, D. Fan, MicroRNAs as critical regulators involved in regulating epithelial- mesenchymal transition, Curr. Cancer Drug Targets 13 (2013) 935–944.
[15] X. Xin, Y. Zhang, X. Liu, H. Xin, Y. Cao, M. Geng, MicroRNA in hepatic fibrosis and cirrhosis, Front. Biosci. (Landmark Ed) 19 (2014) 1418–1424.
[16] L. Amrouche, G. Desbuissons, M. Rabant, V. Sauvaget, C. Nguyen, A. Benon, P. Barre, et al., MicroRNA-146a in Human and Experimental Ischemic AKI: CXCL8-Dependent Mechanism of Action, J. Am. Soc. Nephrol. 28 (2017) 479–493.
[17] B. Feng, S. Chen, A.D. Gordon, S. Chakrabarti, miR-146a mediates inflammatory changes and fibrosis in the heart in diabetes, J. Mol. Cell. Cardiol. 105 (2017) 70–76.
[18] Y. Morishita, T. Imai, H. Yoshizawa, M. Watanabe, K. Ishibashi, S. Muto, D. Nagata, Delivery of microRNA-146a with polyethylenimine nanoparticles inhibits renal fibrosis in vivo, Int. J. Nanomedicine 10 (2015) 3475–3488.
[19] J. Du, X. Niu, Y. Wang, L. Kong, R. Wang, Y. Zhang, S. Zhao, et al., MiR-146a-5p suppresses activation and proliferation of hepatic stellate cells in nonalcoholic fibrosing steatohepatitis through directly targeting Wnt1 and Wnt5a, Sci. Rep. 5 (2015) 16163.
[20] Y. Zou, Y. Cai, D. Lu, Y. Zhou, Q. Yao, S. Zhang, MicroRNA-146a-5p attenuates liver fibrosis by suppressing profibrogenic effects of TGFbeta1 and lipopolysaccharide, Cell. Signal. 39 (2017) 1–8.
[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[22] J. Xu, S. Lamouille, R. Derynck, TGF-beta-induced epithelial to mesenchymal transition, Cell Res. 19 (2009) 156–172.
[23] J. Zavadil, E.P. Bottinger, TGF-beta and epithelial-to-mesenchymal transitions, Oncogene 24 (2005) 5764–5774.
[24] D.H. Kim, M.S. Chang, C.J. Yoon, J.M. Middeldorp, O.M. Martinez, S.J. Byeon, S.Y. Rha, et al., Epstein-Barr virus BARF1-induced NFkappaB/miR-146a/SMAD4 alterations in stomach cancer cells, Oncotarget 7 (2016) 82213–82227.
[25] B. Xiao, E.D. Zhu, N. Li, D.S. Lu, W. Li, B.S. Li, Y.L. Zhao, et al., Increased miR-146a in gastric cancer directly targets SMAD4 and is involved in modulating cell proliferation and apoptosis, Oncol. Rep. 27 (2012) 559–566.
[26] Z. Liu, C.L. Lu, L.P. Cui, Y.L. Hu, Q. Yu, Y. Jiang, T. Ma, et al., MicroRNA-146a modulates TGF-beta1-induced phenotypic differentiation in human dermal fibroblasts by targeting SMAD4, Arch. Dermatol. Res. 304 (2012) 195–202.
[27] J. Li, J. Huang, L. Dai, D. Yu, Q. Chen, X. Zhang, K. Dai, miR-146a, an IL-1beta responsive miRNA, induces vascular endothelial growth factor and chondrocyte apoptosis by targeting Smad4, Arthritis Res Ther 14 (2012) R75.
[28] H. Zhong, H.R. Wang, S. Yang, J.H. Zhong, T. Wang, C. Wang, F.Y. Chen, Targeting Smad4 links microRNA-146a to the TGF-beta pathway during retinoid acid induction in acute promyelocytic leukemia cell line, Int. J. Hematol. 92 (2010) 129–135.
[29] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, J. Clin. Invest. 119 (2009) 1420–1428.
[30] S.S. Choi, A.M. Diehl, Epithelial-to-mesenchymal transitions in the liver, Hepatology 50 (2009) 2007–2013.
[31] I.J. Cho, Y.W. Kim, C.Y. Han, E.H. Kim, R.A. Anderson, Y.S. Lee, C.H. Lee, et al., E-cadherin antagonizes transforming growth factor beta1 gene induction in hepatic stellate cells by inhibiting RhoA-dependent Smad3 phosphorylation, Hepatology 52 (2010) 2053–2064.
[32] F. Tacke, R. Weiskirchen, Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques, Expert Rev. Gastroenterol. Hepatol. 6 (2012) 67–80.
[33] S.J. Lee, K.H. Kim, K.K. Park, Mechanisms of fibrogenesis in liver cirrhosis: the molecular aspects of epithelial-mesenchymal transition, World J. Hepatol. 6 (2014) 207–216.
[34] V. Hernandez-Gea, S.L. Friedman, Pathogenesis of liver fibrosis, Annu. Rev. Pathol. 6 (2011) 425–456.
[35] R. Yin, D. Guo, S. Zhang, X. Zhang, miR-706 inhibits the oxidative stress-induced activation of PKCalpha/TAOK1 in liver fibrogenesis, Sci. Rep. 6 (2016) 37509.
[36] J. Brockhausen, S.S. Tay, C.A. Grzelak, P. Bertolino, D.G. Bowen, W.M. D'Avigdor, N. Teoh, et al., miR-181a mediates TGF-beta-induced hepatocyte EMT and is dysregulated in cirrhosis and hepatocellular cancer, Liver Int. 35 (2015) 240–253.
[37] K. Wu, C. Ye, L. Lin, Y. Chu, M. Ji, W. Dai, X. Zeng, et al., Inhibiting miR-21 attenuates experimental hepatic fibrosis by suppressing both the ERK1 pathway in HSC and hepatocyte EMT, Clin. Sci. (Lond.) 130 (2016) 1469–1480.
[38] S. Zhao, Y. Zhang, X. Zheng, X. Tu, H. Li, J. Chen, Y. Zang, et al., Loss of MicroRNA-101 Promotes Epithelial to Mesenchymal transition in Hepatocytes, J. Cell. Physiol. 230 (2015) 2706–2717.
[39] W. Dai, J. Zhao, N. Tang, X. Zeng, K. Wu, C. Ye, J. Shi, et al., MicroRNA-155 attenuates activation of hepatic stellate cell by simultaneously preventing EMT process and ERK1 signalling pathway, Liver Int. 35 (2015) 1234–1243.
[40] Y. He, C. Huang, X. Sun, X.R. Long, X.W. Lv, J. Li, MicroRNA-146a modulates TGF-beta1-induced hepatic stellate cell proliferation by targeting SMAD4, Cell. Signal. 24 (2012) 1923–1930.
[41] J. Massague, How cells read TGF-beta signals, Nat. Rev. Mol. Cell Biol. 1 (2000) 169–178.
[42] X. Tu, H. Zhang, J. Zhang, S. Zhao, X. Zheng, Z. Zhang, J. Zhu, et al., MicroRNA-101 suppresses liver fibrosis by targeting the TGFbeta signalling pathway, J. Pathol. 234 (2014) 46–59.
[43] Y. Zhang, L. Wu, Y. Wang, M. Zhang, L. Li, D. Zhu, X. Li, et al., Protective role of estrogen-induced miRNA-29 expression in carbon tetrachloride-induced mouse liver injury, J. Biol. Chem. 287 (2012) 14851–14862.
[44] H. Okada, M. Honda, J.S. Campbell, K. Takegoshi, Y. Sakai, T. Yamashita, T. Shirasaki, et al., Inhibition of microRNA-214 ameliorates hepatic fibrosis and tumor incidence in platelet-derived growth factor C transgenic mice, Cancer Sci. 106 (2015) 1143–1152.
[45] X. He, J. Xie, D. Zhang, Q. Su, X. Sai, R. Bai, C. Chen, et al., Recombinant adeno-associated virus-mediated inhibition of microRNA-21 protects mice against the lethal schistosome infection by repressing both IL-13 and transforming growth factor beta 1 pathways, Hepatology 61 (2015) 2008–2017.