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MicroRNA-145 inhibits hepatic stellate cell activation and proliferation by targeting ZEB2 through Wnt/β-catenin pathway

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ABSTRACT

The activation of hepatic stellates cells (HSCs) is well believed to play a pivotal role in the development of liver fibrosis. MicroRNA-145 (miR-145) is known to suppress the progression of hepatocellular carcinoma, and is previously reported to be associated with Wnt/β-catenin pathway, but its role in the progression of hepatic fibrosis and activation of HSCs remains unknown and is warranted for investigation. In the present study, we found that the expression of miR-145 is significantly down-regulated *in vivo* in CCl_4-induced mice liver fibrosis as well as in transforming growth factor-β1 (TGF-β1) induced HSC-T6 cell lines and human hepatic stellate cell line LX-2 *in vitro*. Furthermore, over-expression of miR-145 inhibited TGF-β1-induced the activation and proliferation of HSC-T6 cells *in vitro*. Mechanistically, we identified that zinc finger E-box-binding homeobox 2 (ZEB2), a key mediator of epithelial-to-mesenchymal transition, acted as a functional downstream target for miR-145. Interestingly, ZEB2 was shown to be involved in the TGF-β1-induced HSCs activation by regulating Wnt/β-catenin signaling pathway. Taken together, our results revealed the critical regulatory role of miR-145 in HSCs activation and implied miR-145 as a potential candidate for therapy of hepatic fibrosis by regulation of Wnt/β-catenin through targeting ZEB2.

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1. Introduction

Hepatic fibrosis is a common wound-healing response to chronic liver injuries, including continuous alcoholic toxicity, viral infection and non-alcoholic steatohepatitis (Fagone et al., 2015; Schuppan and Kim, 2013). Hepatic stellate cells (HSCs) play an important role in the pathogenesis of liver fibrosis (Puche et al., 2013). Activated HSCs stimulate the collagen production and excessive accumulation of extracellular matrix (ECM), which gives rise to the occurrence of liver fibrosis (Peverill et al., 2014). Inhibiting activation and proliferation of HSCs or accelerating apoptosis of activated HSCs would reverse the process of liver fibrosis (Lee and Friedman, 2011).

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs, which exert a post-transcriptional and negative regulation on protein expression through binding to the 3′-untranlated regions (3′-UTR) in target mRNAs (Ambros, 2004; Ul Hussain, 2012). It has been demonstrated that several miRNAs could regulate the activation and proliferation of HSCs through regulating anti- and pro-fibrotic gene expression (He et al., 2012; Noetel et al., 2012; Vettori et al., 2012). MiR-145 is known to be a tumor-suppressor gene, which can inhibit the development of tumor by regulating cell growth, invasion and metastasis (Sachdeva and Mo, 2010). Recently, miR-145 is considered to be a regulator in some fibrosis diseases (Wang et al., 2014b; Zhu et al., 2015). In a previous study, miR-145 was reported to play a specific role in the regulation of matrix gene expression in smooth muscle cells, suggesting that miR-145 acts to suppress TGF-β dependent ECM accumulation and fibrosis (Zhao et al., 2015). However, the functional significance of miR-145 in the hepatics fibrosis and activation of HSCs remains unclear.

Abbreviations: HSCs, hepatic stellates cells; miR-145, mircoRNA-145; TGF-β1, transforming growth factor-β1; ZEB2, zinc finger E-box-binding homeobox 2; ECM, extracellular matrix; miRNAs, microRNAs; UTR, untranlated regions; siRNA, small interfering RNA; HE, hematoxylin and eosin; Col-I, type I collagen; α-SMA, α-smooth muscle actin.

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Recently, Wnt/β-catenin signaling was shown to be involved in pathogenesis of different kinds of diseases. Wnt/β-catenin signaling has been also shown to be activated in HSCs during liver fibrosis, and some molecules of the Wnt/β-catenin signaling pathway are up-regulated and implicated in the process (Zhang et al., 2013). Recently, there are conflicting studies on the regulation of miR-145 in Wnt/β-catenin signaling pathway. Inhibition of miR-145 blocks the up-regulation of Wnt/β-catenin pathway in acute myocardial infarction (Mayorga and Penn, 2012), while miR-145 deregulates nuclear transcription of β-catenin and consequent down-regulation of its transcriptional targets c-Myc and CyclinD1 in human colon cancer (Yamada et al., 2013), and whether miR-145 can modulate Wnt/β-catenin pathway in liver fibrosis need to do further research.

ZEB2 (also known as SIP1) is one of the family of zinc-finger E-box binding proteins (Verschueren et al., 1999), and ZEB2 expression has been shown to be positively regulated by TGF-β signaling (Comijn et al., 2001). A previous study indicated that repression of ZEB2 expression could decrease the expression of α-smooth muscle actin (α-SMA) in cardiac myofibroblast (Cunnington et al., 2014). One study has shown that miR-200a suppresses Wnt/β-catenin pathway through regulating the activity of β-catenin partly by targeting the E-cadherin repressors ZEB1/ZEB2 in gastric adenocarcinoma (Cong et al., 2013), but there is no direct experiment verifying the regulating effect of ZEB2 on Wnt/β-catenin pathway.

In this study, we demonstrated that miR-145 was down-regulated in mice fibrotic tissues, activated HSC-T6 cells and LX-2 cells after TGF-β1 treatment, which is inversely correlated with ZEB2 expression. In addition, over-expression of miR-145 not only decreased the expression of ZEB2 but also inhibited the activation and proliferation of HSC-T6 cells and inactivated the Wnt/β-catenin signal pathway. Furthermore, we found that down-regulating ZEB2 with small interfering RNA (siRNA) can decrease the expression of β-catenin, c-Myc and cyclinD1 as well as suppress the activation and proliferation of TGF-β1-induced HSC-T6 cells. Our results suggest anti-fibrosis effect of miR-145, which may provide a promoting and novel therapeutic strategy for hepatic fibrosis.

2. Materials and methods

2.1. Animal models of liver fibrosis

Adult male (16–20 g, C57BL/6) mice were all provided by Experimental Animal Center of Anhui Medical University (Hefei, Anhui, China). The animal experimental procedures were approved by the University Animal Care and Use Committee. All mice were randomly dived into normal group and model group. The model group mice were hypodermic injected with 20% carbon tetracholoride (CCl_4; Shantou Xilong Chemistry Plant, China) diluted with olive twice a week (1 ml/kg). Normal control animals were treated with the same volume of olive oil at the same time intervals. At week 4, all mice were sacrificed and livers were harvested for the future experiment.

2.2. Primary HSCs isolation

Primary HSCs were isolated using collagenase-pronase perfusion followed by Nycodenz two-layer discontinuous density gradient centrifugation. The mice were anesthetized with chloral hydrate. The portal vein was severed and inserted by special needle. Inferior vena cava was cut after the perfusion setup was stabilized. The blood was flushed out by perfusion buffer and liver then turned to white, perfusion with enzymes fix consisted of protease, collagenase IV and DNAase (sigma, GER) until liver was digested completely. The digested liver was carefully excised and minced under sterile conditions. Then the cell suspension was filtered to remove undigested debris. Cell suspension was centrifuged by several times, and adjusted the density to 1.040–1.060 g/ml with Nycodenz mixture. HSCs were collected by removing the cell layer from the gradient interface, and centrifuged finally.

2.3. Cell culture

Rat HSC cell line HSC-T6 was acquired from the institute for Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM high glucose medium (Hyclone), respectively, supplemented with 10% fetal bovine serum (sijiqing, Hangzhou, Zhejiang, China). LX-2 cells, immortalized human HSCs with a myofibroblast-like phenotype, were obtained from Dr. Scott Friedman (Mount Sinai School of Medicine). LX-2 cells were maintained in Dulbecco's modified Eagle's medium (DMED, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). All cells were incubated at 37 °C in a humidified incubator containing 5% $CO_{2.}$ When cells grown to 70%-80% confluence, we cultured the cells in a 6-well plate and incubated for 12 h before transfection.

2.4. MiRNA mimics, miRNA inhibitor and siRNA transfection

The cells were transfected with miR-145 mimics, inhibitor, ZEB2 small interfering(si) RNA or corresponding control RNA using lipofectamine 2000 (Invitrogen, USA) according to the manufacture's instructions, changing the culture medium 6 h after transfection and TGF-β1 was added. Cells were maintained at 37 °C in a CO_2 incubator for 24 h and collected for qPCR or Western blot analysis. MiR-145 mimics, miR-145 inhibitor and ZEB2 siRNA were synthesized by Gene Pharma (Shanghai, China). The oligonucleotides sequences were as follows: ZEB2-siRNA (rat): 5′-CCAUCUCUGCUCAGAGUCCAATT-3′, 5′-UUGGACUCUGAGCAGCA GAGCAGAUGGTT-3′; and Negative control: 5′-UUCUCCGAACGUGUCACGUT T-3′, 5′-ACGUGACA CGUUCGGAGAATT-3.′

2.5. Quantitative real-time PCR analysis

Total RNA, including miRNA, was extracted from the cells or liver tissues, using Trizol Reagent (Invitrogen) according to the manufacture's protocol. cDNA was generated from total RNA from each sample, using a transcriptor first-strand cDNA synthesis kit (TaKaRa, Shiga, Japan). Real time PCR was performed in a detection system with SYBR-Green Master Mix (TaKaRa). qRT-PCR primers were purchased from Invitrogen. For miR-145, the procedure was performed according to the manuscript of the one-step miRNA qRT-PCR Detection Kit (biomics, Nantong, JiangSu, China). Each measurement was repeated at least in triplicate and the ratio for the mRNA of interest was normalized to β-actin and expressed as the mean ± standard errors of the mean(SEM). The primers used were listed as following: ZEB2 (forward: 5′-GCAATGTAGGTCTCTGCTGC-3′; reverse: 5′-CTCCCCTTTGCTCCTTCTCA), β-actin (rat) (forward: 5′-CCCATCTATGAGGGTTACGC-3′; reverse: 5′-TTTAATG TCACGCACGATTTC-3′), β-actin (mouse) (forward: 5′-GGGAAATCGTGCGTGAC-3′; reverse: 5′-AGGCTGGAAAAGAGCCT-3′), ZEB2 (human) (forward: 5′-CCCTTCTGCGACATAAATACGA-3′; reverse: 5′-TGTGATTCATGTGCTGCGAGT-3′), GAPDH (human) (forward: 5′-ACCACAGTCCATGCCATCAC-3′; reverse: 5′-TCCACCACCCTGTTGCTGTA-3′).

2.6. Protein isolation and western blot analysis

Protein was extracted from cells or liver tissues using RIPA lysis buffer (Beyotime, China). After centrifugation for 30 min at

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