Oxidored-nitro domain-containing protein 1 promotes liver fibrosis by activating the Wnt/β-catenin signaling pathway *in vitro*

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Abstract. Hepatic fibrosis is a characteristic of various types of chronic liver diseases, and may further develop into liver cirrhosis and liver cancer. Oxidored-nitro domain-containing protein 1 (NOR1) expression levels are greater in hepatitis, cirrhosis and hepatocellular carcinoma samples compared with from normal liver samples. However, the importance of NOR1 in liver fibrosis remains to be elucidated. The present study aimed to investigate the effect of NOR1 on the proliferation and matrix expression of human hepatic stellate cells (HSCs) *in vitro*. Additionally, the molecular mechanisms underlying the role of NOR1 in the activation of HSCs was investigated. The present study determined that transforming growth factor β1 (TGF-β1) may induce NOR1 expression in HSCs in a dose-dependent manner, as determined by reverse transcription-quantitative polymerase chain reaction and western blot analysis. NOR1-small hairpin (sh)RNA was transfected into TGF-β1-treated HSCs to knock down NOR1. The MTT assay revealed that TGF-β1-induced cell proliferation was significantly inhibited in the NOR1-shRNA group. In addition, NOR1 knockdown significantly inhibited TGF-β1-induced protein expression of fibrosis indexes, including collagen 1, 3 and α-smooth muscle actin (α-SMA). Subsequently, NOR1-pcDNA3.1 was transfected into HSCs to overexpress NOR1. It was revealed that NOR1 overexpression may activate the Wnt/β-catenin pathway in HSCs. The gain-of function experiments demonstrated that NOR1 overexpression promoted cell proliferation and the expression of fibrosis indexes; however, these effects may be attenuated by dickkopf-1, an inhibitor of the Wnt/β-catenin signaling pathway. In conclusion, the present study demonstrated that NOR1 activates HSCs and contributes to liver fibrosis *in vitro* and this effect was achieved through the activation of the Wnt/β-catenin pathway. Therefore, the current study may provide a novel target for the treatment of chronic liver diseases.

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Introduction

Liver fibrosis results from the imbalance between production and dissolution of the extracellular matrix, which is characteristic got the majority of chronic liver diseases (1). Advanced liver fibrosis leads to cirrhosis, liver failure and portal hypertension (2). The majority of patients with hepatocellular carcinoma (HCC) have a history of liver fibrosis and cirrhosis (3-6). The pathogenesis of liver remains to be fully elucidated and the recovery remains unsatisfactory. Therefore, the present study aimed to investigate the cellular and molecular mechanisms of the development of liver fibrosis, in order to improve the therapeutic options for the management of patients with chronic liver diseases.

Oxidored-nitro domain-containing protein 1 (NOR1) is a novel member of nitroreductases that was initially isolated from nasopharyngeal carcinoma (NPC) (7). The NOR1 gene shares 40% homology with nitroreductase from *Escherichia coli* and has the similar function of reducing nitrogen (7).

The NOR1 gene was associated with liver cancer (8-11). Transfection of the NOR1 gene in HepG2 cells leads to an increased expression of zinc finger protein, tumor necrosis factor receptor, and protein tyrosine phosphatase receptor (9), which are important proteins that are involved in gene transcription and signal transduction associated with cancer. Recently, Li *et al* (11) determined that there is a trend for increased positive rate of NOR1 expression from normal liver samples to hepatitis, cirrhosis and HCC samples, indicating that NOR1 may be a predictive biomarker in HCC development (11). To the best of our knowledge, the role of the NOR1 gene in liver fibrosis has not been previously reported.

The present study performed *in vitro* studies to investigate the effect of NOR1 on the proliferation and matrix expression of human hepatic stellate cells (HSCs). Additionally, the current study examined whether NOR1 exerts its effect on HSCs activation through the Wnt/β-catenin signaling pathway. This may elucidate the role of NOR1 in liver fibrosis.

Materials and methods

Cell culture and treatment. Human hepatic stellate cells (HSCs) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in Stellate Cell Medium (ScienCell Research Laboratories) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified atmosphere at 37˚C of 5% CO_2. Dickkopf-1 (DKK-1) and transforming growth factor β1 (TGF-β1) were obtained from Peprotech, Inc. (Rocky Hill, NJ, USA) and were dissolved in phosphate buffered saline (PBS). TGF-β1 was diluted into concentrations of 1, 2, 5, 10 and 20 ng/ml. HSCs were incubated with 200 ng/ml DKK-1 or different concentrations of TGF-β1 at 37˚C for indicated times.

Cell transfection. NOR1-small hairpin (sh)RNA and NOR1-pcDNA3.1 plasmid were synthesized by Shenzheng Zhonghong Boyuan Biological Technology Co., Ltd. (Shenzheng, China). They were transfected into HSCs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. shRNAs were transfected at a final concentration of 50 nM; the plasmids were transfected at a final concentration of 1 μg.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the cells using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). Primers used were: NOR1, 5'-tcaagggattcatccgagac-3' (forward) and 5'-ggatactcggatgtgcaggt-3' (reverse); and GAPDH, 5'-cgaccactttgtcaagctca-3' (forward) and 5'-aggggtctacatggcaactg-3' (reverse). A total of 1 μl cDNA was used for qPCR using the SYBR-Green PCR kit (Thermo Fisher Scientific, Inc.) on a 7,900 real-time PCR system (Thermo Fisher Scientific, Inc.). PCR was performed with 40 cycles at 95˚C for 30 sec, 59˚C for 30 sec and 72˚C for 30 sec. Gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (12), normalizing Cq values to the internal control GAPDH. This experiment was replicated three times.

Western blot analysis. The cells were washed twice with PBS and lysed with cell lysis buffer (Biovison, Inc., Milpitas, CA, USA). Total protein concentration was determined using a BCA Protein Assay kit (Pierce, Thermo Fisher Scientific, Inc.). Total protein (20 μg/lane) were resolved by SDS-PAGE on a 10% gel and were then transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). After washing with PBS, the membranes were incubated at 4˚C overnight with 5% non-fat milk, and then at 37˚C for 2 h with the following primary antibodies: Rabbit polyclonal to NOR1 (cat. no. ab155535; dilution, 1:400), mouse monoclonal to wnt3a (cat. no. ab81614; dilution, 1:800), rabbit polyclonal to β-catenin (cat. no. ab16051; dilution, 1:500), rabbit polyclonal to Axin 2 (cat. no. ab32197; dilution, 1:400), mouse monoclonal to GAPDH (cat.no. ab8245; dilution, 1:1,000), mouse monoclonal to a-smooth muscle actin (α-SMA; cat. no. ab7817; dilution, 1:800; all from Abcam, Cambridge, MA, USA), rabbit polyclonal to collagen 1 (cat. no. BA0325; dilution, 1:500), mouse monoclonal to collagen 3 (cat. no. BM1625; dilution, 1:500; all from Boster Biological Technology, Ltd., Wuhan, China). After washing with TBST twice, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies [goat anti-rabbit IgG-HRP (cat. no. BA1054; dilution, 1:1,000) and goat anti-mouse IgG-HRP (cat. no. BA1050; dilution, 1:1,000); Boster Biological Technology, Ltd.] at 37˚C for 1 h. Immunoreactive bands were visualized with the enhanced chemiluminescence western blotting kit (Pierce; Thermo Fisher Scientific, Inc.).

MTT assay. The cells were seeded into the 96-well plates and maintained at 37˚C in a humidified atmosphere of CO_2 in air. After a 24 h incubation, the cells were treated with TGF-β1 or DKK-1 for further incubation of 12, 24, 48 and 72 h. At the end of cell incubation, 10 μl MTT (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was added into each well and incubated for 4 h at 37˚C. After that, 100 μl dimethyl sulfoxide (Sigma-Aldrich; Merck Millipore) was added into each well for 30 min. The absorbance at 490 nm was determined (Multiskan Ascent 354 microplate reader; Thermo Labsystems; Thermo Fisher Scientific, Inc.) for quantitative measurement of cell growth.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed with SPSS version 19.0 (IBM SPSS, Armonk, NY, USA) using Student's t-test or one-way analysis of variance followed by a least significant difference test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of TGF-β1 on NOR1 expression in HSCs. The HSCs were treated with 1, 2, 5, 10 and 20 ng/ml TGF-β1 for 48 h and then RT-qPCR and western blotting were performed to detect NOR1 expression. As presented in Fig. 1A, the mRNA expression level of NOR1 was increased with the increasing of TGF-β1 concentration, the maximum effect was observed at 20 ng/ml. NOR1 protein was absent in HSCs; however, an increase of NOR1 protein expression was observed in HSCs following treatment with 5, 10 and 20 ng/ml TGF-β1 (Fig. 1B).

Effect of NOR1 on HSCs activation. NOR1-shRNA was transfected into HSCs to knock down NOR1 expression and the cells were treated with 20 ng/ml TGF-β1 for 48 h. Subsequently the cell proliferation and the expression of fibrosis indexes in HSCs was examined. As demonstrated in Fig. 2A, TGF-β1-induced NOR1 protein expression was significantly suppressed following NOR1-shRNA transfection. The effect of NOR1 on HSCs proliferation was investigated using an MTT assay. HSCs proliferation increased under TGF-β1 treatment; however, NOR1 knockdown significantly inhibited TGF-β1-induced HSCs proliferation (Fig. 2B). The expression of fibrosis indexes in HSCs, such as collagen 1, 3 and α-SMA was examined using western blot analysis. As presented in Fig. 2C, the protein expression levels of collagen 1, 3 and α-SMA were significantly increased in HSCs group treated with TGF-β1; however, NOR1-knockdown cells exhibited significantly reduced protein expression levels of collagen 1, 3 and α-SMA.

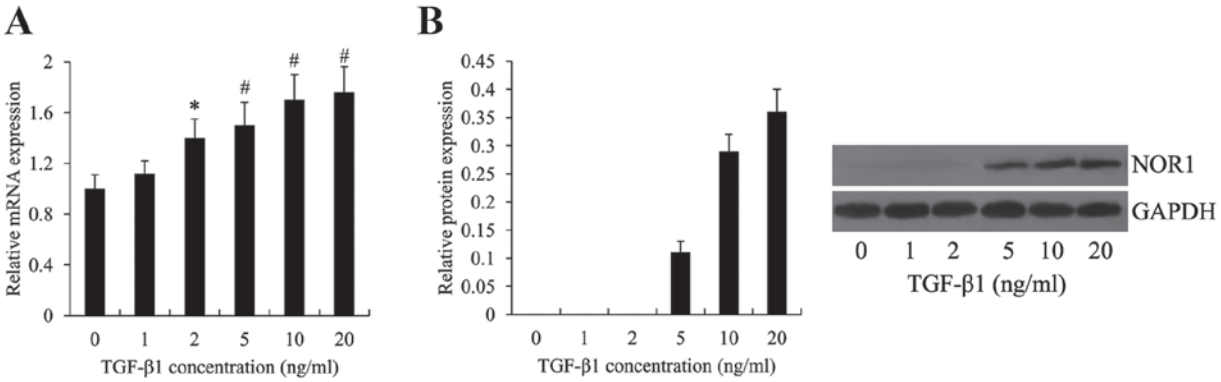

Figure 1. NOR1 expression was induced in HSCs by TGF-β1 treatment. Relative NOR1 (A) mRNA and (B) protein expression levels in HSCs following treatment with 1, 2, 5, 10 and 20 ng/ml TGF-β1. *P<0.05 and #P<0.01 vs. 0 ng/ml. NOR1, oxidored-nitro domain-containing protein 1; TGF-β1, transforming growth factor β1; HSCs, hepatic stellate cells.

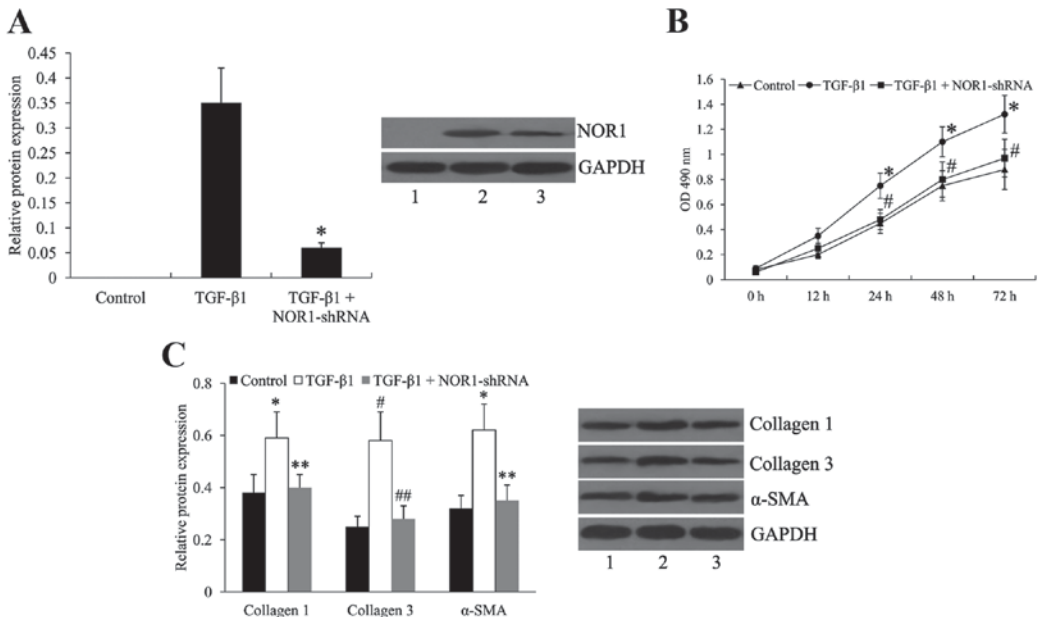

Figure 2. Effect of NOR1 on HSC activation. (A) Relative protein expression of NOR1 in TGF-β1-treated HSCs following transfection with NOR1-shRNA. *P<0.01 vs. TGF-β1 group. (B) Cell proliferation of TGF-β1-treated HSCs following transfection with NOR1-shRNA. *P<0.05 vs. control group; #P<0.05 vs. TGF-β1 group. (C) Relative protein expression of collagen 1, collagen 3 and α-SMA in TGF-β1-treated HSCs following transfection with NOR1-shRNA. *P<0.05 and #P<0.01 vs. control group; **P<0.05 and ##P<0.01 vs. TGF-β1 group. Lane 1, control; lane 2, TGF-β1; lane 3, TGF-β1 + NOR1-shRNA. NOR1, oxidored-nitro domain-containing protein 1; TGF-β1, transforming growth factor β1; HSCs, hepatic stellate cells; α-SMA, α-smooth muscle actin; shRNA, small hairpin RNA.

Effect of NOR1 on Wnt/β-catenin pathway in HSCs. NOR1-pcDNA3.1 was transfected into HSCs to overexpress NOR1 and the protein expression levels of wnt3a, β-catenin and Axin2 were quantified. NOR1 protein was highly expressed in HSCs transfected with NOR1-pcDNA3.1 (Fig. 3A). The protein expression levels of wnt3a, β-catenin and Axin2 were significantly increased in NOR1-overexpressed cells compared with the control cells (Fig. 3B).

Wnt/β-catenin pathway mediates the effect of NOR1 on HSCs activation. To investigate whether Wnt/β-catenin pathway mediates the effect of NOR1 on HSCs activation, DKK1 was used to suppress Wnt/β-catenin pathway and then cell proliferation and the expression of fibrosis indexes in HSCs were examined. As demonstrated in Fig. 4A, DKK1 treatment significantly inhibited the protein expression levels of wnt3a, β-catenin and Axin2 in HSCs. The MTT assay revealed that NOR1 overexpression promoted HSCs cell proliferation; however, this effect was reversed by DKK1 treatment (Fig. 4B). Additionally, the protein expression levels of collagen 1, 3 and α-SMA were significantly increased in HSCs transfected with NOR1-pcDNA3.1. DKK1 significantly inhibited the increased protein expression levels of collagen 1, 3 and α-SMA in HSCs (Fig. 4C).

Discussion

The NOR1 gene is expressed ubiquitously in human tissues (7). NOR1 is suggested to be a candidate tumor repressor gene in the development and progression of NPC (7), cervical (13) and prostate cancer (14). Previous studies reported the association between NOR1 and HCC (8-11). NOR1 is important in the chemical carcinogenesis of hepatic cancer (10). Overexpression

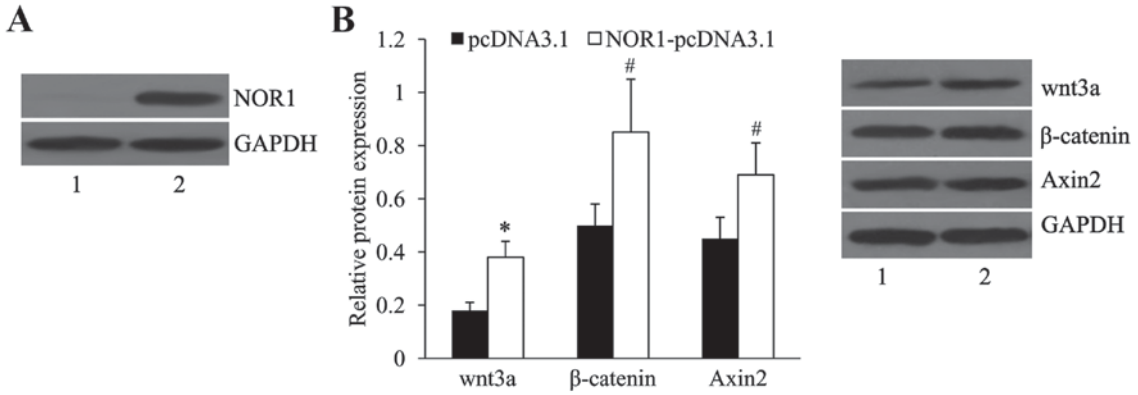

Figure 3. Wnt/β-catenin signaling pathway was induced by NOR1 in HSCs. (A) Western blot images for NOR1 expression in HSCs following transfection with NOR1-pcDNA3.1 plasmid. (B) Relative protein expression of wnt3a, β-catenin and Axin2 in HSCs following transfection with NOR1-pcDNA3.1 plasmid. *P<0.05 and $^{\#}$P<0.01 vs. pcDNA3.1 group. Lane 1, pcDNA3.1; lane 2, NOR1-pcDNA3.1. NOR1, oxidored-nitro domain-containing protein 1; HSCs, hepatic stellate cells.

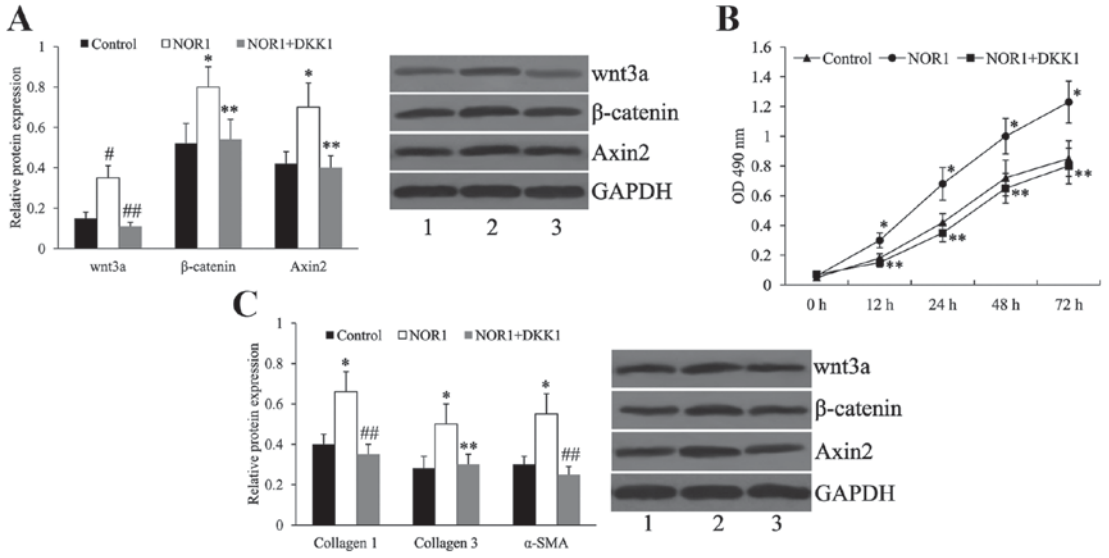

Figure 4. Wnt/β-catenin signaling pathway mediates the effect of NOR1 on HSCs activation. (A) Relative protein expression of wnt3a, β-catenin and Axin2 in NOR1-overexpressed HSCs following DKK1 treatment. (B) DKK1 suppressed NOR1-induced HSCs proliferation. (C) DKK1 inhibited NOR1-induced collagen 1, collagen 3 and α-SMA expression. *P<0.05 and $^{\#}$P<0.01 vs. control group; **P<0.05 and $^{\#\#}$P<0.01 vs. NOR1 group. Lane 1, control; lane 2, NOR1; lane 3, NOR1 + DKK1. NOR1, oxidored-nitro domain-containing protein 1; HSCs, hepatic stellate cells; α-SMA, α smooth muscle actin; DKK1, dickkopf-1.

of NOR1 may enhance CB1954-induced cell death in HepG2 cells (10). Li *et al* (11) reported that NOR1 expression was increased in patients with HCC and its expression was correlated with clinicopathological parameters of HCC (11). It has been previously established that ~90% of HCC cases are associated with fibrotic or cirrhotic livers (5,6). Hepatic fibrosis may further develop into liver cirrhosis and liver cancer. The present study, revealed the role of NOR1 in liver fibrosis *in vitro*. Hepatic stellate cells are the major cell types involved in liver fibrosis (15,16). When the liver is damaged, hepatic stellate cells are activated. The activated hepatic stellate cells are characterized by proliferation, contractility and collagen secretion, leading to cirrhosis (17-19). The present study performed *in vitro* experiments with HSCs to investigate the role of NOR1 in liver fibrosis. TGF-β1 is an important profibrotic protein, which induces fibrosis in a variety of organs (20-23). The current study revealed that TGF-β1 may induce NOR1 expression in HSCs in a dose-dependent manner. Furthermore, it was revealed that cell proliferation and the expression of fibrosis indexes induced by TGF-β1 were significantly inhibited in NOR1-shRNA-transfected HSCs. These findings confirmed that NOR1 was involved in liver fibrosis and NOR1 knockdown attenuated TGF-β1-induced HSCs activation.

Gui *et al* (10) reported that the expression of growth factor receptor-bound protein 2 and activated mitogen-activated protein kinase signal transduction were regulated by NOR1 in HepG2 cells (10). Shen *et al* (24) screened the genes differentially expressed in HepG2 cells transfected with NOR1 gene and the DNA microarray data revealed that 59 genes were upregulated and 103 genes were downregulated in NOR1-overexpressing HepG2 cells compared with the control (24). Subsequently, the present study investigated the molecular mechanisms underlying the role of NOR1 in liver fibrosis. Previous studies revealed that Wnt signaling has an essential role in the pathogenesis of liver fibrosis (25-27). Expression of Wnt genes was induced in activated HSC and Wnt signaling contributed to HSC activation and liver

fibrogenesis (28). Blockage of the Wnt/β-catenin signaling pathway inhibited HSC activation (29). In the present study, DKK-1 was used to block the Wnt signaling pathway to confirm whether it was involved in mediating NOR1-induced HSCs activation. The findings revealed that NOR1 overexpression may activate the Wnt/β-catenin pathway in HSCs. The gain-of function experiments performed by the current study demonstrated that NOR1 activates HSCs and contributes to liver fibrosis; however, these effects may be attenuated by DKK1. These findings suggested that the Wnt/β-catenin pathway mediates the effect of NOR1 on liver fibrosis.

In conclusion, the present study demonstrated that NOR1 promoted liver fibrosis *in vitro* and this effect was achieved through the activation of the Wnt/β-catenin pathway. To the best of our knowledge, the present study was the first to reveal the role of NOR1 in liver fibrosis and suggested a novel target for the treatment of chronic liver diseases.

Acknowledgements

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