Inhibitory effects of long noncoding RNA MEG3 on hepatic stellate cells activation and liver fibrogenesis

Yong He, Yu-ting Wu, Cheng Huang, Xiao-Ming Meng, Tao-tao Ma, Bao-Ming Wu, Feng-yun Xu, Lei Zhang, Xiong-Wen Lv, Jun Li

School of Pharmacy, Anhui Key Laboratory of Bioactivity of Natural Products, Anhui Medical University, Hefei 230032, China
The Key Laboratory of Anti-Inflammatory and Immune Medicine, Anhui Medical University, Ministry of Education, Hefei 230032, China
Institute for Liver Diseases of Anhui Medical University, ILD-AMU, Anhui Medical University, Hefei 230032, China

ABSTRACT

Long noncoding RNAs (IncRNAs) are increasingly recognized as major players in governing fundamental biological processes through diverse mechanisms. Maternally expressed gene 3 (MEG3) is an imprinted gene located at 14q32 that encodes a IncRNA correlated with several human cancers. Recently, the methylation-dependent downregulation of MEG3 has been described in liver cancers. However, its biological functional role in liver fibrosis remains unknown. In our study, MEG3 levels were remarkably decreased in CCl4-induced mouse liver fibrosis models and human fibrotic livers as demonstrated by real-time quantitative PCR. Moreover, the expression of MEG3 was downregulated in human hepatic stellate cell lines LX-2 cells in response to transforming growth factor-β1 (TGF-β1) stimulation in dose and time-dependent manner. Enforced expression of MEG3 in LX-2 cells inhibited TGF-β1-induced cell proliferation, while promoting cell apoptosis. In addition, hypermethylation of MEG3 promoter was identified by methylation-specific PCR and MEG3 expression was robustly increased by the inhibition of methylation with either 5-aza-2-deoxycytidine (5-azadC), or siRNA to DNA methyltransferase 1 (DNMT1) in TGF-β1-induced LX-2 cells. More importantly, overexpression of MEG3 could activate p53 and mediate cytochrome c release, subsequently leading to caspase-3-dependent apoptosis in TGF-β1-treated LX-2 cells. These findings suggested that MEG3 may play an important role in stellate cell activation and liver fibrosis progression and act as a novel potential therapeutic target for liver fibrosis.

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1. Introduction

Hepatic fibrosis, characterized by the excessive deposition of extracellular matrix (ECM) in response to chronic hepatic injury, is the major determinant of morbidity and mortality in patients with liver disease [1,2]. Following chronic liver injury of any cause, hepatic stellate cells (HSCs) become activated and transdifferentiate to myofibroblast-like cells by the secretion of excessive ECM proteins including α-smooth muscle actin (α-SMA) and alpha1(I) collagen (Col1A1), by the release of pro-inflammatory and pro-fibrogenic factors including transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) [3]. It is well accepted that activation of HSCs is a pivotal event during liver fibrogenesis [4–6]. However, better figuring out the mechanisms of HSC activation is still beyond our reach because of its complexity.

Regulatory noncoding RNAs (ncRNAs), such as microRNAs (miRNAs), a great variety of long noncoding RNAs (IncRNAs), play increasingly key roles in the development of human diseases [7–10]. lncRNA is commonly defined as a RNA molecular which is larger than 200 nucleotides (nt) with limited or no protein-coding capacity [11,12]. A large and growing body of literature shows that involvement of IncRNAs in liver diseases, and in liver cancers in particular [10,13]. For example, an IncRNA, down-regulated expression by HBx (termed IncRNA-Dreh), which could inhibit hepatocellular carcinoma (HCC) growth and metastasis in vitro and in vivo, act as a tumor suppressor in the development of HBV–HCC [14]. Deregulation of IncRNA MVIH (IncRNA associated with microvascular invasion in HCC) was a predictor for poor recurrence-free survival (RFS) of HCC patients after hepatectomy and the inhibition of phosphorylglucerase kinase 1 (PGK1) secretion by its association with MVIH contributed to active angiogenesis both in vitro and in vivo [15]. Furthermore, IncRNA highly up-regulated in liver cancer (HULC) was dramatically up-regulated in HCC and the up-regulated HULC by HBx promoted...
proliferation of hepatoma cells through suppressing p18 [16]. However, whether lncRNA is involved in the development of nonneoplastic liver diseases has not been elucidated so far.

Maternally expressed gene 3 (MEG3), a lncRNA, is expressed in many normal tissues and functions as a lncRNA tumor suppressor [17, 18]. Recently, the loss of MEG3 expression has been gradually proved to be a primary feature of human cancers, such as liver cancer [17,19], gastric cancer [20,21], lung cancer [22], glioma [23], cervical cancer [24], bladder cancer [25] and so on. Furthermore, promoter hypermethylation or hypermethylation of the differentially methylated regions (DMRs) has been found to contribute to the loss of MEG3 expression in cancers [26–28]. Moreover, MEG3 could induce the accumulation of p53 (TP53) protein, selectively regulate p53 target gene expression leading to cell growth inhibition [29]. However, very little is known about MEG3 expression level and its biological role in the progression of liver fibrosis.

In this study, we firstly demonstrated that MEG3 expression was significantly decreased in both CCl4-induced mouse liver fibrosis models and human fibrotic livers. Furthermore, our results showed the antifibrotic effect of MEG3 on TGF-β1-induced LX-2 cells activation. The effect of DNA methylation on MEG3 expression was also investigated. We further verified that overexpression of MEG3 could induce the activation of p53 and mediate cytochrome c release subsequently leading to caspase-3-dependent apoptosis in TGF-β1-treated LX-2 cells. Taken together, our findings indicated that lncRNA MEG3 may represent a novel regulator to modulate stellate cells activation and could serve as a potential therapeutic target against liver fibrosis.

2. Materials and methods

2.1. Animals, mouse models of liver fibrosis, patients

Each 6 adult (8-week-old) male C57BL/6J mice from the Experimental Animal Center of Anhui Medical University were used for CCl4 liver injury model. The animal experimental procedures were reviewed and approved by the University Animal Care and Use Committee. Liver fibrosis was generated by biweekly intraperitoneal dose of a 10% solution of CCl4 in olive oil (0.02 ml/g/mouse) for the first 4 weeks and then once a week as previously described [30]. At weeks 4, 6 or 8, the mice were sacrificed. Control mice were treated intraperitoneally with the same volume of olive oil at the same time intervals. 24 h after the last CCl4 injection mice were sacrificed and liver tissues were harvested for the further analysis. The liver tissues were used for hematoxylin and eosin (H&E) staining and Masson staining by fixation with 10% formalin.

Human liver fibrotic tissue samples were obtained from patients undergoing partial liver resection at the Department of Surgery, the First Affiliated Hospital of Anhui Medical University. As a control group, matched healthy volunteers received normal aminotransferase activities, no history of liver disease or alcohol abuse and tested negative for HBV, HCV and HIV infections in this study. All of the patients or their guardians provided written informed consent, and Medicine’s Ethics Committee of Anhui Medical University approved all aspects of this study in accordance with the Helsinki Declaration. The liver tissues were also used for hematoxylin and eosin (H&E) staining and Masson staining by fixation with 10% formalin.

2.2. Cell culture

An immortalized human HSC line, LX-2 cell line, was donated by Dr. Lieming Xu from Shuguang Hospital, Shanghai University of Traditional Chinese Medicine. The LX-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine and incubated at 37 °C at an atmosphere of 5% CO2. LX-2 cells were plated in a 6-well plate and cultured for 24 h before transfection.

2.3. Total RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from liver tissues and LX-2 cells using TRIzol reagents (Invitrogen, USA), and the first-strand cDNA was synthesized using Thermoscript RT-PCR synthesis kit (Fermentas, USA) according to the manufacturer’s instructions. Real-time quantitative PCR analyses for mRNA of α-SMA, Col1A1, DNM1, TIMP-1, MMP-2, MMP-9 and GAPDH were performed by using Thermoscript RT-PCR kits (Fermentas,USA) in an ABI Prizm step-one plus real-time PCR System (Applied Biosystems, USA). The mRNA level of GAPDH was used as an internal control. Primer sequences were listed in Table 1. We used specific primers for human MEG3 (forward, 5′-CTGCCCCATTCACACTCAG-3′; and reverse, 5′-CTCT CCGGCTTGGCGGTAGGGGC-3′) from previous publication [23] and for mouse MEG3 (forward, 5′-GGACCCTCAGC ACAACAGGT-3′; and reverse, 5′-GTCCCCACGAGTTCA-3′) from previous publication [31]. GAPDH was used as references for MEG3. Each test was done in triple replication and the 2−ΔΔCT method was used to calculate the expression of mRNA and MEG3 in tissue samples and cells.

2.4. Plasmid construction

pCI-MEG3 was obtained from Addgene bank (Addgene plasmid 44727). The plasmid pCI-MEG3 was first constructed by Yunli Zhou and colleagues [29]. We acknowledge the principal investigators. Ectopic expression of MEG3 was achieved by using the pCI-MEG3 transfection and empty pCI vector (pCI-control) was used as a control.

2.5. Transfection of LX-2 cells

On the day of transfection, the cells were plated in DMEM supplemented with 10% FBS at a density of 2–3 × 104 cells/ml and were transfected with pCI-MEG3 and pCI-control using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. The culture medium was changed 6 h after transfection, and TGF-β1 (Peprotech, USA) was added at a concentration of 10 ng/ml or pitfibrin-μ (Selleckchem, USA) was incubated with concentration of 5 nM and compared to transfected cells without pitfibrin-μ. Cells were harvested after 48 h for RT-qPCR, western blot, cell proliferation and apoptosis analyses.

2.6. Treatment of LX-2 cells with 5-azaC

The cells were seeded overnight in culture dishes, 1 μM 5-aza-2′-deoxycytidine (5-azaC) (Sigma-Aldrich, St. Louis, MO) was added and was refreshed every 48 h until the 48 h treatment finished.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Number of gene primer (5′ → 3′)</th>
</tr>
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<tbody>
<tr>
<td>α-SMA</td>
<td>F: 5′-GGCTCTGGGCCTCTGTAAGG-3′</td>
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<tr>
<td></td>
<td>R: 5′-CTCTGCTGTCGGTCTCATC-3′</td>
</tr>
<tr>
<td>Col1A1</td>
<td>F: 5′-CCCGGGGTGTCAGCAACTCCT-3′</td>
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<tr>
<td></td>
<td>R: 5′-TCCACATGTTATCCAGCAG-3′</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>F: 5′-AATTCGACTCTGCATCATAC-3′</td>
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<tr>
<td></td>
<td>R: 5′-TCCGTTTTCCAGCAATGAG-3′</td>
</tr>
<tr>
<td>MMP2</td>
<td>F: 5′-AATCAGTATGATCGCCAG-3′</td>
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<tr>
<td></td>
<td>R: 5′-GACACAGGAAAGTTCGTCG-3′</td>
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<tr>
<td>GAPDH</td>
<td>F: 5′-ACCACGCTCATGGCCTAG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TCCACACCTGTTGCTGTA-3′</td>
</tr>
</tbody>
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F, forward primer; R, reverse primer.

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2.7. Small interfering RNAs and transfection

The cells were cultured in serum-free DMEM for 12 h. On the day of transfection, the cells were plated in DMEM supplemented with 10% FBS at a density of 2–3 × 10⁵ cells/ml and were transfected with siRNA-DNMT1 or negative control siRNA (GenaPharma, China) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s protocol. The culture medium was changed 6 h after transfection, and TGF-β₁ (Peprotech, USA) was added at a concentration of 10 ng/ml. The sequences of oligonucleotides used are as follows: siRNA-DNMT1: forward: 5′-GAGGCCUAUAAUGCAAAGATT-3′, reverse: 5′-UCUUUGCAUUAVAGCCUC TT-3′, and negative control siRNA: 5′-UUCUCCGAACTT-3′; 5′-ACGUGACACGUUCGGAGATT-3′;

2.8. Cell proliferation assays

A Cell Counting Kit-8 (CCK-8) assay was used to measure cell proliferation. The cells were resuspended in 200 μl of cell culture medium and seeded at a density of 5 × 10⁴ cells per well in 96-well culture plates, incubated overnight to allow for cell attachment, after which they were transfected with pCI-MEG3, siRNA-DNMT1 or negative control. The culture medium was changed 6 h after transfection, and TGF-β₁ (Peprotech, USA) was added at a concentration of 10 ng/ml. 10 μl of CCK-8 reagent (obio, Shanghai, China) were added to each well 24, 48, 72 h before the end of incubation. The optical density value (OD) of each well was measured at a wavelength of 450 nm on Thermomax microplate reader (Bio-Tek EL, USA). The result of cell proliferation measurement is expressed as the absorbance at OD450. All experiments were performed in triplicate and repeated at least three times.

2.9. Cell cycle and apoptosis analysis

For cell cycle analysis, we performed Cell Cycle and Apoptosis Analysis Kit (Beyotime, China). The cells were washed for three times by cold PBS, and then cells were fixed in 70% ethanol in PBS at −20 °C for 24 h. After fixation, cells were washed with cold PBS and stained with 0.5 ml of propidium iodide (PI) staining buffer, which contains 200 mg/ml RNase A, 50 μg/ml PI, at 37 °C for 30 min in the dark. Analyses were performed on BD FACSVerse (BD Biosciences). The cell debris and fixation artifacts (aggregates of cells) were gated out and cell populations that were at the G0/G1, S, and G2/M phases were quantified by the FlowJo data analysis software package (TreeStar, USA). The experiments were repeated for three times.

For apoptosis analysis, quantification of apoptotic cells was performed with Annexin-V-FITC Apoptosis Detection Kit (Bestbio, China) according to the manufacturer’s instruction. Cells were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. Analyses were performed on BD FACSVerse (BD Biosciences). Apoptosis was analyzed with FACS using FlowJo data analysis software package (TreeStar, USA). The percentage of early apoptotic cells were compared to control groups from each experiment. All of the samples assayed were in triplicates.
2.10. Methylation-specific polymerase chain reaction

The methylation status of the MEG3 promoter region was determined by methylation-specific PCR (MSP) using bisulfite-modified DNA. Genomic DNA was extracted using the QIAamp DNA mini kit (Axygen). Two primer sets were used to amplify the promoter region of the MEG3 that incorporated a number of CpG sites, one specific for the methylated sequence (MEG3-M, forward: 5’-TATGAGTTGTAAGCGGTAAGTTGTAACG-3’; reverse: 5’-TAGAAGCTTAGGCTAGGCATCT-3’) and the other for the unmethylated sequence (MEG3-U, forward: 5’-
GAATATGAGTGTAGTG GTAGAGTTT-3'; reverse: 5'-TACAACTTAAAC AAAAAAATCATACT-3'). The primers used in the present study detect specifically the promoter sequence of the MEG3 rather than that of the MEG3 pseudogene. M and U are the PCR products of methylated and unmethylated alleles, respectively. The polymerase chain reactions for MEG3-M and MEG3-U were carried out in a 50 μl volume containing 1× polymerase chain reaction buffer (15 mmol/l MgCl2), 2.5 mmol/l mixture of dNTPs, 10 pM of each primer, 4 U HotStart Taq DNA polymerase (Qiagen, Frankfurt, Germany), and 25 ng to 50 ng of bisulfite-modified DNA. Amplification was performed in a thermocycler with the following conditions: 94 °C for 3 min, cycled at 94 °C for 15 s, 60 °C or 57 °C for 15 s, and 72 °C for 15 s (35 cycles), followed by an extension at 72 °C for 7 min. Methylation-specific PCR experiments were performed at least in duplicate.

2.11. Western blot analysis

Liver tissues and cells were lysed with RIPA lysis buffer (Beyotime, China), cytosolic protein was isolated by Cell Mitochondria Isolation Kit (Beyotime, China), and 25 ng to 50 ng of bisulfite-modified DNA. Amplification was performed in a thermocycler with the following conditions: 94 °C for 3 min, cycled at 94 °C for 15 s, 60 °C or 57 °C for 15 s, and 72 °C for 15 s (35 cycles), followed by an extension at 72 °C for 7 min. Methylation-specific PCR experiments were performed at least in duplicate.

2.12. Immunohistochemistry

Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. Slides were deparaffinized and treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were further blocked by 2% bovine serum albumin followed by incubation with primary antibody against α-SMA (1:200) and DNMT1 (1:50) for 16 h at 4 °C. After rinsing, the sections were incubated with biotinylated secondary antibody for 60 min at room temperature. α-SMA and DNMT1 expression was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. The sections were counter stained with Mayer’s hematoxylin for 30 s, dehydrated, and mounted by dehydrated and DAB stained, and DNMT1 and α-SMA positive areas within the fibrotic region were then observed.

2.13. Double immunofluorescence staining

Tissues from mice livers were fixed with 4% paraformaldehyde. The tissues were permeabilized with 0.2% Triton X-100 in 1% bovine serum albumin (BSA) for 10 min, blocked with 5% BSA for one hour at room temperature. To determine the colocalization of MEG3 and α-SMA, FITC-conjugated MEG3 probes (Exiqon) in combination with Cy3-conjugated anti-α-SMA antibody (1:50 dilution; Boster, China) were used in the hybridization assays. The cells were mounted with SlowFade Gold antifade reagent with DAPI (Sigma, MO, USA) and images were taken using fluorescence microscopy. MEG3 was shown as green fluorescence and α-SMA as red fluorescence.

2.14. Statistical analysis

Data are represented as mean ± SD. The data were analyzed by One-Way ANOVA (LSD) using the SPSS 15.0 software to determine their significant differences. For changes in mRNA or protein levels, mRNA (relative expression) and protein (densitometric values) to respective house-keeping controls were compared. P < 0.05 was considered statistically significant.
3. Results

3.1. Downregulation of long noncoding RNA MEG3 in murine and human fibrotic livers

To identify the changes in the MEG3 expression profile between advanced liver fibrosis and non-fibrotic liver, the degree of liver fibrosis was first determined by hematoxylin eosin (H&E) staining and Masson staining. Histopathological analysis showed that the degree of liver fibrosis progressed in mice that received CCl4 relative to mice receiving olive oil alone (Fig. 1a). Immunohistochemical and western blot results demonstrated that the myofibroblast markers α-SMA was stained to investigate the cellular events in mice and the levels of α-SMA and Col1A1 were consistently increased in CCl4 mice compared with that in control mice (Fig. 1b, c). Real-time qPCR result showed that MEG3 was progressively downregulated as liver fibrosis progressed (Fig. 1d).

Next, we correlated these findings with the data from human patients and analyzed liver fibrosis samples from patients undergoing partial liver resection as well as liver samples from non-fibrotic livers. Histologically, the liver fibrosis tissues exhibited connective tissue fibers extending from the central vein, early septal formation and collagen deposition were also established by H&E staining and Masson staining (Fig. 2a). Similarly, the expression of α-SMA and Col1A1 protein in livers of patients with liver fibrosis was greater than that with healthy livers as shown by Immunohistochemical and western blot (Fig. 2b, c). Interestingly, MEG3 was also downregulated in human liver fibrotic tissues compared with the control liver tissues (Fig. 2d). To determine if down-regulation of MEG3 in fibrotic mice was due to reduced expression in HSCs, we used co-labeling MEG3 (ISH with anti-MEG3 probe) and α-SMA (IHC) to identify whether MEG3 is expressed in HSCs. Immunofluorescence revealed abundant MEG3 labeling in the liver of control mice. More importantly, the double immunofluorescence staining showed that MEG3 expression was primarily colocalized with that for α-SMA, suggesting that HSCs might be one of the main sources of the MEG3 levels present in CCl4-treated livers (Fig. 2e). Thus, it was concluded that downregulation of MEG3 might have important roles in liver fibrosis development and progression.

3.2. MEG3 is downregulated in TGF-β1-induced LX-2 cells

Stimulation of HSCs by TGF-β1 is believed to be the key fibrogenic response in liver fibrosis because the expression of α-SMA can be upregulated in HSCs treated with TGF-β1 [32]. Here, we showed that treatment of LX-2 cells with TGF-β1 at the concentration of 0, 2, 10 and 30 ng/ml for 48 h could cause the increased expression of α-SMA protein (Fig. 3a). The expression of α-SMA protein was also increased in LX-2 cells stimulated with TGF-β1 (10 ng/ml) for 0, 24, 48 and 72 h (Fig. 3b). These results suggested that α-SMA protein expression could be increased time- and dose-dependently by TGF-β1. Meanwhile, MEG3 was downregulated in the time- and dose- dependent manner when the cells were treated with TGF-β1 at the concentration of 0, 2, 10 and 30 ng/ml for 48 h, or when the cells were treated with TGF-β1.
3.3. Effect of MEG3 on TGF-β1-induced LX-2 cells in vitro

The significant decrease of MEG3 expression in liver fibrosis and activated HSCs prompted us to explore the possible functional role of MEG3 in stellate cell activation. MEG3 was overexpressed in LX-2 cells by transfecting them with pCI-MEG3. Real time qPCR analysis of MEG3 levels was performed 48 h posttransfection and revealed that MEG3 expression was significantly increased in LX-2 cells compared with respective control (Fig. 4a). Firstly, we investigated the effects of overexpression of MEG3 on cell proliferation. The CCK-8 assay found that cell proliferation was significantly suppressed in TGF-β1-induced LX-2 cells transfected with pCI-MEG3 compared with respective controls (Fig. 4b). Furthermore, the flow cytometric analyses showed that the proportion of apoptotic cells induced by transfection of pCI-MEG3 was greater than that induced by transfection of pCI-control (Fig. 4c). Besides, overexpression of MEG3 could increase the percentage of the activated HSCs in G0/G1 phase and decrease the population of cells in S phases (Fig. 4d). Specifically, transfection with pCI-MEG3 in TGF-β1-treated LX-2 cells significantly downregulated α-SMA and Col1A1 mRNA and protein expression and affected the mRNA expression of fibrosis-related genes, such as tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase 2 (MMP-2), MMP-9 via unknown mechanisms (Fig. 4e).

3.4. Effect of DNA methylation on MEG3 expression

Then, to determine whether DNA methylation contributes to the loss of MEG3 expression, we performed Methylation-Specific PCR to examine the role of methylation in deregulation of MEG3 in liver fibrotic tissues. We found that the promoter regions of MEG3 gene from the liver tissues of CCl4-treated mice and human liver fibrotic tissues in which MEG3 expression were downregulated were strongly methylated, whereas the control liver tissues in which MEG3 expression were present had unmethylated MEG3 promoter region (Fig. 5a). Importantly, treatment of LX-2 cells with TGF-β1 (10 ng/ml) significantly induced hypermethylation of the MEG3 gene (Fig. 5b). These results indicated aberrant methylation of MEG3 gene promoter, which likely contributed to the loss of MEG3 expression in CCl4–treated mice, human liver fibrotic tissues and activated LX-2 cells.

To further confirm that the loss of MEG3 expression was caused by promoter methylation in vitro, we evaluated the effect of the methylation inhibitor 5-azadC on MEG3 expression. We found that stimulation with 5-azadC (1 μM) significantly abolished TGF-β1-induced aberrant hypermethylation of the MEG3 and MEG3 expression was robustly increased in TGF-β1-induced LX-2 cells. The CCK-8 was performed to examine the effect of 5-azadC (1 μM) on TGF-β1-induced HSC proliferation. The results are expressed as relative expression against control expression without treatment. *P < 0.05 vs control, #P < 0.05 vs model.

**Fig. 5.** Effects of 5-azadC on MEG3 promoter methylation and MEG3 expression in TGF-β1-treated LX-2 cells. a. Results of MSP analysis of MEG3 gene in CCl4-induced mouse liver fibrotic tissues, liver tissues of patients with liver fibrosis, and control liver tissues. M and U, PCR products of methylated and unmethylated alleles, respectively. b. MSP analysis of MEG3 promoter from untreated LX-2 cells and TGF-β1-treated LX-2 cells challenged with or without 5-azadC (1 μM) for 48 h. c. LX-2 cells were treated with or without TGF-β1 (10 ng/ml) and 5-azadC (1 μM) for 48 h. Real time qPCR analyses of MEG3 were performed. The results are expressed as relative expression against control expression without treatment. *P < 0.05 vs control, #P < 0.05 vs model. d. LX-2 cells were treated with or without TGF-β1 (10 ng/ml) and 5-azadC (1 μM) for 48 h. The mRNA and protein expression of α-SMA and Col1A1 was analyzed by real time qPCR and western blot. The results are expressed as relative expression against control expression without treatment. *P < 0.05 vs control, #P < 0.05 vs model. e. The CCK-8 was performed to examine the effect of 5-azadC (1 μM) on TGF-β1-induced HSC proliferation. The results are expressed as relative expression against control expression without treatment. *P < 0.05 vs control, #P < 0.05 vs model.
To better investigate the methylation-dependent mechanism of MEG3 alteration, we examined the expression of the main DNA methyltransferases (DNMT) 1 in liver fibrotic tissues and TGF-β1-induced LX-2 cells. Immunohistochemical analysis showed that DNMT1 expression was significantly increased in the liver tissues from CCl4-treated mice and human liver fibrotic tissues compared with the control liver tissues (Fig. 6a), which was further confirmed by western blot (Fig. 6b). Moreover, treatment with TGF-β1 (10 ng/ml) markedly induced upregulated expression of DNMT1 mRNA and protein in LX-2 cells (Fig. 6c). Next, to determine whether DNMT1 was involved in the loss of MEG3 expression in TGF-β1-induced LX-2 cells, the expression of DNMT1 gene was inhibited by RNA interference. Results of real time PCR and western blot indicated that cells transfection with DNMT1 siRNA significantly suppressed DNMT1 mRNA and protein expression (Fig. 6d). Importantly, the demethylation of MEG3 gene and an increase in MEG3 expression were observed in cells incubated with siRNA to DNMT1 (Fig. 6f). More interestingly, DNMT1 knockdown with siRNA could markedly reduce α-SMA and CollA1 mRNA and protein expression in TGF-β1-induced LX-2 cells compared with control siRNA-transfected cells (Fig. 6g). These data indicated that MEG3 expression could be modulated by alterations in methylation and downregulation of MEG3 observed in liver fibrotic tissues and TGF-β1-induced LX-2 cells might have been partly due to hypermethylation of MEG3 gene promoter.

3.5. MEG3 increases activation of p53 protein

Recently, accumulating studies reported that re-expression of MEG3 could lead to activation of p53 protein and its downstream targets that contributed to cell apoptosis and cell growth inhibition [18,20,24,29]. To further explore the potential mechanisms involved in MEG3-induced cell apoptosis and growth arrest, we examined the expression of p53 protein after transfection with pcI-MEG3. The results of western blot analysis showed that the level of p53 protein was markedly increased in TGF-β1-treated LX-2 cells transfected with pcI-MEG3 compared with the control (Fig. 7a). Next, we measured the signaling marker proteins, caspase 3 and caspase 9 by Western blot. Interestingly, active forms of caspase 3 and caspase 9 were significantly upregulated in TGF-β1-treated LX-2 cells transfected with pcI-MEG3 (Fig. 7b). There is evidence that p53 participates in the mitochondrial apoptosis pathway and leads to caspase activation through induction of the release of cytochrome c [33–35]. Therefore, we evaluated the extent of cytochrome c translocation into cytoplasm in TGF-β1-treated LX-2 cells after transfection of pcI-MEG3 for 48 h. Herein, a dramatic increase in cytoplasmic cytochrome c was observed in pcI-MEG3-transfected TGF-β1-treated LX-2 cells (Fig. 7c). To further confirm whether MEG3 induced apoptosis by the mitochondrial apoptosis pathway, we investigated mitochondrial-dependent apoptosis-related proteins, Bax and Bcl-2. More interestingly, we found that overexpression of MEG3 significantly induced the Bax/Bcl-2 ratio in TGF-β1-treated LX-2 cells, which was accompanied by cytochrome c release into the cytoplasm (Fig. 7d). Moreover, pifithrin-μ, the inhibitor of mitochondrial translocation of p53 [36,37], did suppress MEG3-induced apoptosis associated with a cleavage of caspase 3, strongly suggesting that MEG3-induced apoptosis in TGF-β1-induced LX-2 cells was partly mediated by p53-dependent mitochondrial apoptosis pathway (Fig. 7e, f).

4. Discussion

Recent improvements in genome-wide surveys have revealed that only ~1% of the human genome encodes proteins and the majority of the human genome transcripts are non-coding RNAs, in particular, long non-coding RNAs (lncRNAs) without evident protein coding function [38,39]. There are over 3000 huma lncRNAs greater than 200 nt in length, but less than 1% of them have been manifested in human diseases [40,41]. Although only a minority has been characterized in detail and they were previously disregarded as transcriptional “noise”, they are becoming increasingly appreciated in diverse biological processes for their potential diagnostic, prognostic or therapeutic importance [10,42].

Liver fibrosis, an excessive wound healing response that occurs in most forms of chronic liver disease, is characterized by a preponderance of fibrogenesis (the excess synthesis and deposition of ECM). Activation of HSCs is widely accepted to play a critically important role in excessive deposition of ECM [2,43]. Recently, accumulating evidence demonstrated that non-coding RNAs, microRNAs (miRNAs) in particular, may play key roles in regulating HSC functions such as cell proliferation, differentiation, and apoptosis [44]. However, the overall pathophysiological contributions of lncRNAs to the development of liver fibrosis and activation of HSCs remain blank.

Maternally expressed gene 3 (MEG3, also known as gene trap locus 2 (GTL2)) is reciprocally imprinted with the paternally expressed gene DLK1 constituting an imprinting domain on human chromosome 14q32 and on mouse chromosome 12 [45]. As a lncRNA, MEG3 does not encode any proteins and has functions at the RNA level [18,45]. Specifically, the methylation-dependent dysregulation and function of MEG3 have been profoundly described in development and progression of liver cancer. For instance, the expression of the DLK1/MEG3 locus was deregulated in human HCC accompanied by extensive aberrations in DNA methylation leading to aberrant expression of MEG3 RNA [19]. Enforced expression of MEG3 in HCC cells significantly decreased both anchorage-dependent and -independent cell growth, and induced apoptosis [46]. However, whether MEG3 is involved in the liver fibrogenesis is poorly understood. Therefore, we focused on investigating the role of MEG3 in the development of liver fibrosis and TGF-β1-induced HSC activation.

In this study, we found that the expression of MEG3 was consistently decreased in CCl4-induced mouse progressed liver fibrosis model when compared to normal tissues. Meanwhile, MEG3 was also found to be downregulated in human liver fibrotic tissues compared with the control liver tissues. More importantly, double immunofluorescence staining confirmed that HSCs may be one of the main sources of the MEG3 levels present in CCl4-treated mice [47]. We therefore tested whether TGF-β1 might regulate the expression of MEG3 in HSCs. In line with the downregulation of MEG3 in liver fibrogenesis, stimulation of human hepatic stellate cell lines LX-2 cells with recombinant TGF-β1 at the concentration of 0, 2, 10 and 30 ng/ml for 48 h or with TGF-β1 (10 ng/ml) for 0, 24, 48 and 72 h resulted in a significant decrease of MEG3 levels, correlating with an increase in α-SMA expression in these cells. These data strongly suggested that the decreased expression of MEG3 may occur in the development of liver fibrosis and activation of HSCs. It is well accepted that chronic hepatitis can develop into liver cirrhosis and HCC, liver fibrosis and
HCC development are also strongly correlated. Thus, more effects are needed to better elucidate the critical function and mechanisms of lncRNA MEG3 in the progression of liver disease.

The methylation of the C5 position of the cytosine base with S-adenosyl methionine as the methyl donor was reported in approximately 70–80% of CpG dinucleotides in somatic mammalian cells and to some extent in non-CpG sequences in embryonic stem cells [48,49]. DNA methylation is currently the most widely studied epigenetic modification and its correlation to pathogenesis of liver fibrosis has been well established [49,50]. Furthermore, previous studies demonstrated that DNA methylation contributed to the loss of MEG3 expression in tumors [26,46,50,51]. Therefore, it is reasonable to hypothesize that the decreased expression of MEG3 in liver fibrosis and activation of HSCs may be due to its promoter hypermethylation. In our study, we showed that MEG3 promoter hypermethylation was observed in liver tissues of CCl4-treated mice, human liver fibrotic tissues, and TGF-β1-treated LX-2 cells by MSP. Importantly, the methylation inhibitor 5-azadC significantly abolished TGF-β1-induced aberrant hypermethylation of MEG3 and restored MEG3 expression in TGF-β1-treated LX-2 cells, which suggested that 5-azadC prevents MEG3 methylation and subsequently increases MEG3 expression, followed by inhibiting HSC activation and proliferation.

DNA methyltransferase 1 (DNMT1) is mainly responsible for the maintenance of methylation pattern on the daughter strand after DNA methylation [52]. In recent study, we found

![Fig. 7. Effect of MEG3 on p53 activation. a. Activation of p53 by MEG3 in TGF-β1-treated LX-2 cells. b. Proteolytic cleavage of caspase-3 and caspase-9 was analyzed by western blot in MEG3-transfected TGF-β1-treated LX-2 cells. c. Effect of MEG3 on the release of cytochrome c into the cytosol in TGF-β1-treated LX-2 cells. d. The protein levels of Bax and Bcl-2 were determined by Western blot in MEG3-transfected TGF-β1-treated LX-2 cells. e and f. Effect of pifithrin-μ (the inhibitor of mitochondrial translocation of p53) on MEG3-induced apoptosis in TGF-β1-treated LX-2 cells. The inhibitor pifithrin-μ attenuated cleavage of caspase 3 which blocked apoptosis. The results are expressed as relative expression against control expression without treatment. *P < 0.05 vs pCI-control.](image-url)
that the level of DNMT1 expression was analyzed via immunofluorescence staining in control liver tissues and liver fibrotic tissues. The result suggested that more nuclear DNMT1 labeling in livers of rats that had received CCl_{4} as compared to control livers [53]. More importantly, our previous study also revealed that TGF-β1 treatment markedly induced nuclear translocation of DNMT1 and upregulated expression of DNMT1 in hepatic stellate cells [54,55]. In particular, it has been postulated that DNMT1 was upregulated and knockdown of DNMT1 may lead to loss of promoter hypermethylation resulting in reactivation of the corresponding genes in activated HSCs [54–56]. Therefore, DNMT1 may play an important role in the development of liver fibrosis and activation of HSC. In this study, we also found that DNMT1 was upregulated in the liver tissues from CCl_{4}-treated mice, human liver fibrotic tissues and cultured HSCs after activation. Depletion of DNMT1 by RNAi suppressed LX-2 cell activation and proliferation, and reversed the methylation of the MEG3 promoter and subsequently restored MEG3 expression. Intriguingly, treatment with 5-azaC or knockdown of DNMT1 could downregulate the mRNA and protein level of α-SMA and Col1A1 in TGF-β1-treated LX-2 cells. Thus, it is possible that overexpression of DNMT1 contributes to hypermethylation of the MEG3 promoter with the loss of In order to emphasize the impact of dysregulated expression and function of MEG3, we investigated the biological role of MEG3 in TGF-β1-induced LX-2 cells by applying gain-of-function approach. Results indicated that ectopic expression of MEG3 in TGF-β1-induced LX-2 cells inhibited cell proliferation, led to the promotion of cell apoptosis, and the decreased expression of fibrosis-related genes expression.

The known functions of IncRNAs were summarized as signals, decoys, guides, and scaffolds resulting in modulating activity or localization of proteins, inducing chromatin remodeling, generating alternative splicing and so on [9,57]. MEG3 was believed to function to suppress tumor cell related genes expression. Additionally, overexpression of MEG3 was reported to significantly activate the level of p53 pro-expression, and functions, Endocrinol. 151 (2010) 939–947.


