Bone morphogenetic protein-7 regulates Snail signaling in carbon tetrachloride-induced fibrosis in the rat liver

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Abstract. The aim of this study was to explore the molecular mechanism of the bone morphogenetic protein-7 (BMP-7) downregulation of Snail-mediated E-cadherin repression and mesenchymal-epithelial transition (MET) induction, since little is presently known about this issue. In this study, our aim was to elucidate the underlying mechanism by which cells acquire liver fibrosis characteristics after epithelial-mesenchymal transition (EMT). Cell cultures were exposed to Snail alone or in the presence of BMP-7; control cultures were exposed to medium only. The expression of the mRNA encoding α-smooth muscle actin (α-SMA), Snail, and E-cadherin in rat liver epithelial cells was determined by real-time quantitative PCR (RT-PCR) and the main results were confirmed by ELISA. Cell differentiation was determined by analysis of the expression of α-SMA, Snail and E-cadherin by western blotting and co-immunoprecipitation. We demonstrated Snail-induced upregulation of mRNAs encoding α-SMA, Snail, and E-cadherin by western blotting and co-immunoprecipitation. We demonstrated Snail-induced upregulation of mRNAs encoding α-SMA and downregulation of mRNAs encoding E-cadherin in rat liver epithelial cells when compared with unstimulated cells, and confirmed these results at the protein level. BMP-7 downregulated Snail-induced α-SMA and upregulated E-cadherin release compared with untreated and Snail-treated cells. In summary, we demonstrated that BMP-7 induces MET through decreased downregulation of Snail. In addition, Snail1 directly regulates Nanog promoter activity. Notch signaling is also involved in this process.

Introduction

Epithelial-mesenchymal transition (EMT) is a critical developmental process that plays a central role in the formation and differentiation of multiple tissues and organs. During EMT, epithelial cells lose cell-cell adhesion and apical polarity and acquire mesenchymal features, including motility, invasiveness and resistance to apoptosis (1). One of the key hallmarks of EMT is loss of E-cadherin, a cell-adhesion protein that is regulated by multiple transcription factors, including Snail, Slug and Twist. These transcription factors act as E-box repressors and block E-cadherin transcription (2). Transforming growth factor (TGF)-β1 induces EMT in epithelial cells through the upregulation of Snail1 in Smad-dependent signaling (3). The inhibition of Snail1 in mesenchymal cells results in decreased Nanog promoter luciferase activity and loss of self-renewal characteristics in vitro. BMP-7 induces mesenchymal-epithelial transition (MET) through Snail1 and Nanog downregulation. In mesenchymal cells post-EMT, Snail1 directly regulates Nanog expression and loss of Snail1 causes liver fibrosis.

Snail1 and Snail2 belong to the Snail superfamily of zinc finger (ZF) transcription factors (3) and have emerged as important repressors of E-cadherin and inducers of EMT (4). Vertebrate Snail1 and Snail2 factors share a high degree of homology at the DNA-binding C-terminal region, containing four and five C2H2 ZFs, respectively, and at the N-terminal region that contains the SNAG transactivation domain (5). Snail1 and Snail2 present a similar modular organization of nuclear import sequences, distributed among several ZFs (6). Snail factors have emerged as essential regulators of physiological and pathological EMT processes (7). Post-translational modifications of mammalian Snail have been shown to modulate Snail1 stability and functional repressor activity. In particular, phosphorylation by GSK3β or PKD1 plays a negative role (8), while phosphorylation by PAK1, CK2, PKC or Lats2 or interaction with lysyl oxidase-like 2/3 (LOXL2/3), exerts a positive effect on Snail1 functionality (9).

Our hypothesis is that mesenchymal cells acquire liver fibrosis traits after EMT through Snail1-dependent mechanisms. In this study, we demonstrate that BMP-7 induces MET through Snail1 in rat liver fibrosis cells (post-EMT).

Materials and methods

Animals. Adult gender-matched (n=20 each) C57BL rats weighing 200±10.2 g were purchased from Tongji University...
Laboratories (Shanghai, China) and fed with a commercial diet and water. All animal experiments were performed according to the National Institutes of Health (NIH) guidelines for the ethical care and use of laboratory animals, and the experimental protocol was approved by the Tongji Animal Care and Use Committee of China.

Rat liver fibrosis models. A total of 40 adult numbered rats were sorted into liver fibrosis model and normal control groups. A total of 20 rats in the liver fibrosis group received intraperitoneal injections of 40% CCl₄ and olive oil admixture (0.5 ml/100 mg, Sigma-Aldrich, St. Louis, MO, USA) tert as previously described (4). Rats were sacrificed after 8 weeks of treatment.

Chemicals and materials. Glass slides (75x25 mm²) were obtained from Gibco (Carlsbad, CA, USA). (3-Acryloxypropyl) trichlorosilane was purchased from Gelest, Inc. (Morrisville, PA, USA). Streptavidin-conjugated Alexa 546, AlexaFluor 488 anti-mouse IgG, BMP-7 and Snail were obtained from Sigma-Aldrich. Mouse anti-E-cadherin antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Concentrated phosphate-buffered saline (10X PBS) was purchased from Lonza (China). Minimal essential medium (MEM), sodium pyruvate, non-essential amino acids, fetal bovine serum (FBS), Superscript III, RNaseOut (RNase inhibitor) and dNTPs were purchased from Genetix (China). Goat anti-rat cross-adsorbed albumin antibody was obtained from Sigma-Aldrich. Formalin was purchased from Fisher Scientific (China). ApopTag Red in situ Apoptosis Detection kit was obtained from Chemicon (China). DAPI stain mounting media were purchased from VECTaShield (China).

Cell culture and transfections. Established LEPC cells were obtained from the ATCC collection (LGC Standards-SLU, Barcelona, Spain). Cell lines were maintained in DMEM supplemented with 10% FBS and antibiotics (100 µg/ml ampicillin, 32 µg/ml gentamicin; Sigma-Aldrich). Stable and transient transfections were performed using Lipofectamine reagent following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA) or rat anti-HA (Pierce Biotechnology, Inc., Rockford, IL, USA; 110:000) or sheep anti-mouse (Pierce Biotechnology, Inc.; 1:1000). For detection of E-cadherin, α-smooth muscle actin (α-SMA) and Snail expression, western blotting was performed on whole-cell lysates using rat anti-E-cadherin ECCD2 mAb (1:200), produced in our laboratory from the ECCD2 hybridoma, a gift of M. Takeichi, Ricken Center, Japan), mouse anti-α-SMA (1:500, Dako, Carpinteria, CA, USA) or rat anti-Snail (Roche Diagnostics), followed by HRP-coupled secondary antibodies.

Real-time quantitative PCR (RT-PCR) analysis. RT-PCR analysis of cDNA samples was performed with specific primers designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primers used for Snail were 5’-AAAGATCTCAGCTGCTGGAAG-3’ (forward) and 5’-GCTTCGGATGTGCATCTTGA-3’ (reverse) and those used for β-actin were 5’-GCAAGAACCTGTACGGCAACA-3’ (forward) and 5’-TGCATCCGTGCAATG-3’ (reverse). Total RNA was extracted from cultured cells using an RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized using 1 μg of RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT) primers. Transcript levels were assessed by RT-PCR (ABI 7300; Applied Biosystems) and all experiments were normalized to β-actin.

In vivo ubiquitination assay. The cells were treated with 10 µM MG132 for 6 h, 24 h after transfection. The treated cells were then harvested with PBS containing 10 mM N-ethylmaleimide (NEM) and 1 mM dithiothreitol (DTT). The cells were washed with PBS, centrifuged and subjected to one freeze-thaw cycle. Cell pellets were then resuspended in 200 µl buffer 1 [10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM NEM, 1 mM DT T, 5 mM NaF, 1 mM Na₃VO₄ and protease inhibitor BI et al: BMP-7 REGULATES SNAIL SIGNALING IN THE RAT LIVER 1023

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cocktail] and sonicated in a water bath (Bioruptor; Diagenode, Denville, NJ, USA). Next, 500 µl buffer 2 (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.5% NP-40, 5 mM EDTA, 1 mM EGTA, 10 mM NEM, 1 mM DTT, 5 mM NaF, 1 mM Na3VO4, and protease inhibitor cocktail) was added and the extracts were subjected to a 30-min rotation at 4°C. The extracts were then centrifuged. We added 2 µg of anti-Flag M2 antibody and protein A/G beads to the supernatant, which was then incubated for 2 h. The beads were then washed three times, resuspended in loading buffer and boiled. Immunoblotting was performed as described above.

Groups for a role for Snail in rat liver fibrosis. A total of 15 adult numbered rats were randomly sorted into 3 groups: i) normal control group: 5 rats received intraperitoneal injections of olive oil (0.5 ml/100 mg) twice per week; ii) liver fibrosis model group: 5 rats received intraperitoneal injections of 40% CCl4 and olive oil admixture (0.5 ml/100 mg); iii) BMP-7-treated group: 5 rats received intraperitoneal injections of 40% CCl4 and olive oil admixture (0.5 ml/100 mg) twice per week and BMP-7 (300 µg/kg) at the same time.

Statistical analysis. All results shown in the bar graphs are expressed as the fold ratio relative to untreated or control cells. Statistical analysis was performed using SPSS version 17 statistical software (SPSS Inc., Chicago, IL, USA). Student's t-test was used when comparing two groups. One-way ANOVA was used when comparing multiple groups, followed by Tukey's post-hoc test. P<0.001 was considered to indicate a statistically significant result.

Results

General remarks and groups. None of the animals died during the study period. Body weight gain was lower in the Snail-treated compared to the control rats (data not shown). A total of 40 adult numbered rats were randomly sorted into 4 groups: i) normal control group: 5 rats received intraperitoneal injections of olive oil (0.5 ml/100 mg) twice per week; ii) liver fibrosis model group: 10 rats received intraperitoneal injections of 40% CCl4 and olive oil admixture (0.5 ml/100 mg) tert as previously described (4); iii) Snail-treated group: 10 rats received intraperitoneal injections of olive oil (0.5 ml/100 mg) twice per week and Snail (500 µg/kg) at the same time; iv) BMP-7-treated group, 10 rats received intraperitoneal injections of 40% CCl4 and olive oil admixture (0.5 ml/100 mg) twice per week and BMP-7 (300 µg/kg) at the same time. The rats were sacrificed after 8 weeks of treatment.

A role for Snail in rat liver fibrosis. The present study demonstrated that treatment with Snail induced EMT and increased liver injury during CCl4-induced liver fibrosis in rats.
was accompanied by increased expression of liver fibrosis mesenchymal markers, including α-SMA, but inhibition of E-cadherin (P<0.001; Figs. 1-5). The ratio of α-SMA/GAPDH in liver fibrosis model group was higher than that in control group, with 200 mg/µl insulin containing Snail-treated for 96 h, ratio of α-SMA increased significantly in liver fibrosis model group than control group. But this was reverse in ratio of E-cadherin/GAPDH (P<0.001; Fig. 5).

A role for Snail in rat liver fibrosis. The present study demonstrated that rats treated with Snail had increased hepatic fibrosis in CCl₄-induced rat liver injury. This was accompanied by increased expression of hepatic fibrosis mesenchymal markers, including α-SMA, but repression of E-cadherin (Figs. 1-4).

A role for BMP-7 in rat liver fibrosis. We demonstrated that CCl₄-induced fibrosis is reversed in rats treated with BMP-7. Significantly more BMP-7 and less Snail mRNA were expressed in the hepatic fibrosis model group than in the controls (P<0.001; Figs. 1-4). This was accompanied by reduced expression of hepatic fibrosis mesenchymal markers, including α-SMA, but increased expression of E-cadherin (Fig. 1). Thus, a strategy that specifically increased BMP-7 in myofibroblasts from cirrhotic livers tended to reverse the myofibroblastic phenotype and caused the cells to acquire a more quiescent and epithelial phenotype.

Discussion

BMP-7 induces MET through Snail1, which represses α-SMA by binding to E-box promoter elements (11). In the present study, Snail stimulation of epithelial liver fibrosis cells resulted in a mesenchymal phenotype with fibroblastoid appearance and loss of E-cadherin. However, the underlying mechanism has not yet been elucidated. Based on our results, we hypothesize that these liver fibrosis characteristics are Snail-dependent. Inhibition of Snail1 causes the downregulation of Nanog and CD₄₄ and loss of self-renewal, as evidenced by decreased liver fibrosis formation. Liver fibrosis cells are more mesenchymal in character, with increased Snail1, Zeb1 and Zeb2 mRNA expression and decreased E-cadherin expression. Notably, although Smads and Snail proteins are known to play a central role in liver fibrosis cell growth, Notch signaling is also capable of inhibiting liver fibrosis growth through the induction of EMT (12). Notch1 is known to regulate Snail and Slug mRNA levels, but efforts have not been made to examine alternative functions of NICD and Snail expression in liver fibrosis (13). In addition, Notch1 is involved in the mesenchymal program by activating Snail expression in liver fibrosis development (10). However, since Notch signals and cellular functions vary according to cell type and cellular environments, the role of Notch in liver fibrosis needs further investigation.
environment, these inconsistencies may be caused by the different cell types and conditions. We hypothesized that ROS stress upregulated Snail mRNA and protein expression (14). E-cadherin expression decreased in Snail-overexpressed cells compared with control cells (P<0.001). Our study provides one clue for understanding the complex regulation mechanism of p53, MDM2, Notch1 and Snail in the EMT process. The regulation of these proteins and their physiological contribution to EMT require further investigation. However, the mechanism that we have described presents substantial evidence of cross-interference between the Notch and Snail signaling pathways, which may be mediated by BMP-7. In addition, Snail1 is one of a number of regulators of EMT, and thus manipulation of multiple factors may be required to fully inhibit liver fibrosis initiation.

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