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MeCP2 controls the expression of RASAL1 in the hepatic fibrosis in rats

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ABSTRACT

Hepatic stellate cells (HSCs) activation is an essential event during liver fibrogenesis. A major pathway is the transition of HSCs into hepatic myofibroblasts. The methyl-CpG-binding protein MeCP2 which promotes repressed chromatin structure is selectively detected in myofibroblasts of diseased liver. Overexpression of this protein results in an increase of global methylation levels. Treatment of HSCs with DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-azadC) blocks the cell proliferation. 5-azadC also prevents loss of Ras GTPase activating-like protein 1 (RASAL1) expression that occurs during HSCs proliferation. To further explore the underlying molecular mechanisms, we hypothesized that this perpetuation of fibrogenesis was caused by DNA methylation. Results demonstrated that hypermethylation of RASAL1 is associated with the perpetuation of fibroblast activation and fibrogenesis in the liver. knockdown of MeCP2 using siRNA technique increased RASAL1 in both mRNA and protein level in myofibroblasts. These studies demonstrated that MeCP2 and DNA methylation may provide molecular mechanisms for perpetuated fibroblast activation and fibrogenesis in the liver.

1. Introduction

Hepatic fibrosis is commonly observed after chronic liver injury and is regarded as a risk factor for liver cirrhosis and hepatocellular carcinomas (Ogawa et al., 2010). Severe or chronic injury is associated with persistence and proliferation of wound healing myofibroblasts and formation of crosslinked scars known as fibrotic tissue (Desmouliere et al., 2005). What perpetuates scarring in the setting of fibrosis is not yet known (Strutz and Muller, 2006). Activated fibroblasts are commonly considered as main mediators of liver fibrosis. A major pathway is the transition of HSCs into hepatic myofibroblasts. Cell transdifferentiation is a generic process by which myofibroblasts are generated in chronic tissue injury such as the liver, kidney, pancreas and lung (Apte and Wilson, 2004; Desmouliere et al., 2004; Mann and Smart, 2002; Thannickal et al., 2004; Wheeler and McNally, 2005). It has been hypothesized that such maintenance of the excited stage reflects the failure of the fibroblasts in the fibrotic liver to return to their resting state as they do in physiological wound healing (Burke and Tosh, 2005). In response to liver injury, quiescent HSCs are activated and transdifferentiate into myofibroblast-like cells characterized by increased cell proliferation (Mann and Smart, 2002; Benyon and Arthur, 2001). This phenomenon is observed not only in fibrotic liver fibroblasts but also in activated fibroblasts isolated from some given fibrotic organ (Kalluri and Zeisberg, 2006; Lafyatis, 2006; Weber and Brilla, 1992).

Myofibroblast transdifferentiation is therefore a highly conserved physiological process that must be tightly regulated to ensure limited and controlled scar formation (Mann et al., 2010). The MTD or so-called "activated" HSCs undergoes increased proliferation and expressions of α-smooth muscle actin (α-SMA) contributing to the progression of fibrosis (Olsen et al., 2011). It acquires a plethora of biochemical and functional phenotypic characteristics that was not observed with the quiescent HSCs (Kristensen et al., 2000; Mann and Smart, 2002; Sancho-Bru et al., 2005). The MTD is highly profibrogenic and produces large quantities of collagen such as type I collagen (Benyon and Arthur, 2001; Bataller and Brenner, 2005). These dramatic functional changes are associated with a global reprogramming of the HSCs transcriptome, including the up and down regulations of several hundred different genes (Sancho-Bru et al., 2005). RASAL1 is a member of the RAS-GAP family which play an important role in the constitutive activity of Ras signal (Calvisi et al., 2010; Walker et al., 2004). It has been previously reported that the decreased expressions of RASAL1 contributed to renal fibrosis progression (Bechtel et al., 2010). Therefore, in our study, we explored the involvements of decreased RASAL1 expressions in hepatic fibrosis.

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Improved understanding of the relationship between DNA methylation and gene transcription provides new epigenetic paradigms with which to explain how the differentiated state of a mammalian cell can be regulated at a global level (Jaenisch and Bird, 2003; Margueron et al., 2005). For example, methyl-CpG-binding proteins such as MeCP2, which is most abundantly expressed as a chromosomal protein and requires a single methylated CpG site for preferential binding to DNA, once bound to methylated DNA, is thought to silence transcription of down stream genes by virtue of its interaction with a histone deacetylase/Sin3complex (Margueron et al., 2005; Nan et al., 1997). In the present study, it has been demonstrated a role for MeCP2 as an orchestrator of MTD (Mann et al., 2007). Treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-azadC) on HSC blocks MTD and prevents the down-regulation of the HSC proliferation related genes such as RASAL1. Here we hypothesized that the modification of MeCP2 on RASAL1 expression may be one of the possible mechanisms by which the fibrotic fibroblasts were activated.

2. Materials and methods

2.1. Materials and reagents

CCl_4 was obtained from Shantou Xilong Chemistry Plant (Shantou, China). 5-azadC (5-aza-2′-deoxycytidine), dimethyl sulfoxide (DMSO) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) were purchased from Sigma Inc.(St. Louis, MO, USA). Mouse anti-α-SMA monoclonal antibodies, goat anti- RASAL1 polyclonal antibody and rabbit anti-MeCP2 polyclonal antibody were purchased from Boster (Wuhan, China), Santa Cruz (California, USA) and Abcam (Cambridge, UK), respectively. ERK1/2, phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA). PDGF-BB (Peprotech, USA). MeCP2, RASAL1, α-SMA, Collagen I and β-actin primers were produced by Shanghai Sangon Biological and Technological Company (Shanghai, China). Streptavidin peroxidase (SP) immunohistochemical kit was acquired from Zhongshan Biotechnology Corporation (Beijing, China). Secondary antibodies for goat anti-rabbit immunoglobulin (Ig)G horse radish peroxidase (HRP), rabbit anti-goat IgG HRP, goat anti-mouse IgG HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). β-actin antibody was obtained from Boster (Wuhan, China).

2.2. CCl_4 liver injury model

Liver fibrosis was generated by 12-weeks treatment of adult male Sprague–Dawley (200–220 g) rats with CCl_4 (CCl_4/olive oil, 1:1(vol/vol) per kg body weight by intraperitoneal injection twice weekly) as previously described (Lafyatis, 2006). Vehicle control animals were treated intraperitoneally with 1 ml of olive oil/kg body weight at same time intervals. At 24 h after the final CCl_4 administration, tissures were harvested for the further analysis. Animals were provided by the Experimental Animal Center of Anhui Medical University. The animal experimental protocol was approved by the University Animal Care and Use Committee.

2.3. Cell culture and cell treatment with PDGF-BB

The HSC-T6 cell line was obtained from shanghai fumeng gene Biological Corporation (Shanghai, China). HSC-T6 cells was passaged for 48 h and serum - starved with 0.5%FCS for 24 h before adding 10 ng/ml recombinant murine PDGF-BB (Peprotech, USA). Cultured HSC loose their vitamin A, enter the cell cycle and display de novo expression of a wide variety of profibrogenic and proinflammatory molecules that are characteristic of HM and which are described in detail elsewhere (Benyon and Arthur, 2001; Desmouliere et al., 2004; Oakley et al., 2005). In addition, the HSC-T6 cells were cultured on plastic in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA), supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum, respectively. Cell cultures were maintained at 37 °C at an atmosphere of 5% CO_2.

2.4. MTT cell viability assay

Cells (5×10^3/ml) were cultured with various concentrations of 5-azadC and PDGF- BB for 2 days in 96-well plates. After culture, 5 mg/ml MTT (Sigma) reagent was added and incubated for 4 h at 37 °C before adding DMSO to dissolve formazan crystals and measuring in triplicate at 490 nm wavelength using a Thermomax microplate reader (bio-tek EL, USA). All experiments were performed in triplicate and repeated at least three times.

2.5. Immunohistochemistry

The sections were dewaxed in xylene and dehydrated in alcohol, antigen retrieval was achieved by microwaving in citric saline for 15 min. Thin sections were deparaffinized and treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were further blocked by 1% bovine serum albumin and were then incubated with primary antibody against α-SMA (1:100) and MeCP2(1:500), then incubated overnight at 4 °C. After rinsing, the sections were incubated with biotinylated secondary antibody for 20 min at room temperature. MeCP2 and α-SMA expression was visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. The sections were counter stained with Mayers Haematoxylin for 30 s, dehydrated, MeCP2 and α-SMA positive areas with in the fibrotic region were then observed. Quantitative analysis was calculated from five fields for each liver slice.

2.6. 5-Aza-2′-deoxycytidine treatment

HSC-T6 cells were seeded overnight in 24-well plates, 5-azadC (Sigma–Aldrich, St. Louis, MO) was added and was refreshed every 24 h until 48 h treatment finished. The medium containing PBS only was regarded as a control.

2.7. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from rat liver tissues and HSC-T6 cells using TRIzol reagents (Invitrogen). The first-strand cDNA was synthesized from total RNA using Thermoscript RT-PCR synthesis kit (Fermentas) according to the manufacturer's instructions. RT-PCR was carried out under standard protocol using the following primers: β-actin (forward: 5′-TGAGCTGCGTGTGGCCCCTGAG-3′; reverse: 5′-GGG GCATCGGAACCGCTCATTG-3′), RASAL1 (forward: 5′-CCCACAGACCCTCCAG C AG-3′; reverse: 5′-GTTCGTGCCACCTCACGCC-3′), MeCP2: (forward: 5′-CAGCT CCAACAGGATTCCATGGT-3′; reverse: 5′-AGGCAGGCAAAGCAGAGACATCA-3′), α-SMA: (forward: 5′-TGGCCACTGCTGCTTCCTCTTCTT-3′; reverse: 5′-GGGG CCAGCTTCGTCATACTCCT-3′), collagen I: (forward: 5′-TACAGCACGCTTGT GGATG-3′; reverse: 5′-TTGAGTTTGGGTTGTTGGTC-3′). PCR was performed at 94 °C for 2 min, followed by 30–33 cycles of amplification at 94 °C for 36 s, 52 °C for 36 s and 72 °C for 1 min by using ABI9700. The band intensities were measured by adensitometer and the results were normalized with β-actin. The results were repeated by at least three times independently from three different pools of templates, while each pool of template was extracted from at least eight ventricles.

2.8. RNA interference (RNAi) analysis

RNAi experiments in HSC-T6 cells were performed by forward transfection in day 2 cultured HSCs (2×10^5 cells per 200 mm^2 dish) using Lipofectamine™2000 (Invitrogen) according to the manufacturer's protocol. For MeCP2 and RASAL1 immunoblotting, HSC-T6 cells were cultured in serum-free DMEM for 12 h and then subjected to reverse transfection with RNAiMax in Opti-MEM. Small interfering RNA (siRNA) oligonucleotides against MeCP2 genes or scrambled sequences were synthesized by the Shanghai GenaPharma Corporation. The following siRNA sequences were used: si-MeCP2 (rat), 5′-GGGACCUAUGUAUGAUGACTT-3′ (sense) and 5′-GUCAUCAUACAUAGGUCCCTT-3′ (anti-sense); si-control with scrambled sequence (negative control siRNA having no perfect matches to known rat genes), 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACG UUCGGAGAATT-3′ (antisense). Transfection was allowed to proceed for various times and cells were processed for different assays. The siRNA transfection efficiency of Lipofectamine™2000 in cells was determined by the BLOCK-iT Alexa FluorR Red Fluorescent Oligo protocol (Invitrogen).

2.9. Western blotting

Rat liver tissues and cells were lysed with RIPA lysis buffer (Beyotime, China). Whole extracts were prepared, and protein concentration were detected using a BCA protein assay kit (Boster, China). Total protein (30 or 50 mg) from samples were separated by SDS-PAGE and blotted onto a PVDF membrane (Millipore Corp., Billerica, MA, USA). After blockade of nonspecific protein binding, nitrocellulose blots were incubated for 1 h with primary antibodies diluted in TBS/Tween20 (0.075%) containing 3% Marvel. Rabbit polyclonal antibody recognizing MeCP2 (ab-2828, Abcam PLc) was used 1:1000, mouse monoclonal anti-α-SMA (Boster) was diluted 1:200, mouse monoclonal antibody directed against RASAL1 (Santa Cruz, CA, USA) was used at 1:500 as was anti-β-actin (Boster, China). Horseradish peroxidase conjugated anti-goat, anti-mouse and anti-rabbit antibodies were used as secondary antibodies correspondingly. After extensive washing in TBS/Tween-20, the blots were processed with distilled water for detection of antigen using the enhanced chemiluminescence system. Proteins were visualized with ECL-chemiluminescent kit (ECL-plus, Thermo Scientific).