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MECP2 PROMOTES CELL PROLIFERATION BY ACTIVATING ERK1/2 AND INHIBITING p38 ACTIVITY IN HUMAN HEPATOCELLULAR CARCINOMA HEPG2 cells

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and currently represents the leading cause of death amongst cirrhotic patients, but the mechanisms remain unknown. In this experiment, we investigated the expression of Methyl CpG-binding protein 2 (MeCP2) in HCC, the effect of MeCP2 on the proliferation of human HCC HepG2 cells, and the activation of mitogen-activated protein kinases (MAPKs) signaling pathways. The results showed that MeCP2 expression levels was higher in human HCC tissue than normal hepatocellular tissue, and MeCP2 siRNA reduced the proliferation of HCC HepG2 cells by decreasing cell activity and cell division in vitro. After MeCP2 siRNA treatment, the proportion of G1/G0 phase cells increased, but the proportion of S and G2/M phase cells decreased, indicative of G1/G0 cell cycle arrest. Furthermore, the proportions of early and late apoptosis in HCC HepG2 cells were enhanced after MeCP2 siRNA treatment. It was also found that activation of extracellular signal-regulated protein kinase (ERK) and p38 signaling pathways were involved in the proliferation of HepG2 cells. After MeCP2 siRNA treatment, p-ERK1/2 levels decreased, but p-p38 levels increased. Our findings demonstrated that MeCP2 promoted the proliferation of human HCC HepG2 cells with activation of ERK1/2 signaling pathways, suggesting a novel mechanism for pharmacological study of treatment for human HCC.

Key words: Hepatocellular carcinoma, Methyl CpG-binding protein , proliferation, cell cycle, mitogen-activated protein kinases.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide and currently represents the leading cause of death amongst cirrhotic patients (7). About 600,000 patients suffer from HCC annually. The highest incidence is in Southeastern and Eastern Asia, with a rate of 18.3-35.5 per 100,000 population, and the lowest is in central America, with a rate of 2.1 per 100,000 population. Now, surgical treatments including hepatic resection and liver transplantation are considered as the most effective treatment of HCC. Unfortunately, less than 20-25% of HCC patients have been treated surgically. Therefore, the search for better therapeutic agents of HCC is very important. The neoplastic evolution of hepatocellular carcinoma proceeds through a multistep biological process. The molecular analysis of premalignant dysplastic nodules and hepatocellular carcinoma has shown many genetic and epigenetic alterations that result in the deregulation of key oncogenes and tumor suppressor genes (8). However, the precise mechanisms underlying HCC are not well defined.

Methyl CpG-binding protein 2 (MeCP2) is one of the methyl-CpG-binding domain proteins (MBDs), which are important constituents of the DNA methylation machinery, since they are directly involved in the mediation of the epigenetic signal. There are 5 MBD proteins, MeCP2 being the best studied member of the MBD family. MeCP2 contains 2 functional domains, an 85-amino-acid MBD essential for its binding to 5-methylcytosine and a 104-amino-acid transcriptional repression domain that interacts with histone deacetylase. MeCP2 has a number of molecular functions including chromatin organization (12), RNA splicing (34), and transcription regulation (33). MeCP2 is widely studied in neuronal systems since it acquired biomedical importance with the discovery that mutations in its gene, located at Xq28 in human, determined a profound neurodevelopmental autism spectrum disorder, the Rett syndrome (RTT) (10). Recently, a non-neuronal role for MeCP2 has emerged in fibrosis (17), cells growth (9), lung development (14) and tumorigenesis (27). It is reported that MeCP2 mutation determines growth disadvantage in glial cells (19), lymphocyte cells (32), mesenchymal stem cells (26) and prostate cancer cells (2). The mitogen-activated protein kinases (MAPKs) signalling cascade involves membrane-to-nucleus signaling modules that are involved in multiple physiological processes (35). Extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinases (MAPKs) are main members of the MAPK family. The ERK pathway are activated in response to many different extracellular and intracellular signals including those originating at growth factor receptors, integrins, src and fyn, and G-protein coupled receptors, such as EGF and PDGF activate ERK cascade by binding to their respective transmembrane receptor tyrosine kinases. Then, ERK pathway transduce these signals into enduring changes and regulate cell proliferation by regulating cellular activities and gene transcription (28).

The p38 MAPK pathway is activated by different signals, it indirectly affects gene transcription or translation of specific proteins and regulates cell apoptosis by interacting with transcription factors and kinases (16).

The role of MeCP2 in regulating proliferation of prostate cancer cells (2) and gastric cancer (30) is soundly evidenced. However, little is known about its biological characteristics and molecular mechanisms in human hepatocellular carcinoma. In this experiment, we examined the effects of MeCP2 on proliferation of human hepatocellular carcinoma HepG2 cells and investigated its mechanisms related to cell signaling system.

MATERIALS AND METHODS

Preparation of human hepatocellular tissue and HCC tissue

Human normal hepatocellular tissue and HCC tissue were prepared from the First Affiliated Hospital, Xi'an Jiaotong University. Informed consent was obtained before specimen collection. The specimen collection was conducted in accordance with the guidelines of National Institutes of Health. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine and followed the guidelines of the Declaration of Helsinki.

Cell culture

Human hepatocellular carcinoma cell lines (HepG2) were provided by Environment and Genes Related to Diseases Key Laboratory of Education Ministry, Xi'an Jiaotong University College of Medicine, China. HepG2 cells (1.0×105 cells/mL) were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum(Gibco BRL, Grand Island, NY, USA), 100 kU/L penicillin, 0.1 g/L streptomycin, 0.3 g/L L-glutamine and 0.85 g/L NaHCO3 in 5% CO2 at 37°C.

siRNA transfection

siRNA was pre-designed for MeCP2 gene silencing. Human MeCP2 siRNA (sense 5′-GCU UAA GCA AAG GAA AUC UUU-3′, antisense 5′-AGA UUU CCU UUG CUU AAG CUU-3′)and negative siRNA (NC-siRNA, sense 5′-UUC UCC GAA CGU GUC ACG UTT-3′, antisense 5′-ACG UGA CAC GUU CGG AGA ATT-3′) were chemically synthesized by Shanghai GenePharma Corporation (SGC, Shanghai, China). Lipofectamine™-2000 (Invitrogen, Carlsbad, CA, USA) was used to optimize siRNA transfection. Lipofectamine and siRNA were diluted separately in serum-free RPMI-1640 and incubated for 5 min at room temperature. Then the two solutions were softly mixed and incubated for 15 min at room temperature. Lipofectamine–siRNA complexes were initially formed with 500 nM siRNA, and the complexes would be diluted to desired transfection concentrations and added to the plated cells in future experimental procedures.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay was applied to estimate the effect of MeCP2 on HepG2 cell proliferation. Cells were seeded into 96-well plates (5,000 cells/well in 200 µl medium) and incubated for 1 day. Then, HepG2 cells were treated with different concentrations of Lipofectamine-siRNA complexes (25, 50 and 75 nM) for 1, 2 and 3 days, respectively. Cells cultured with complete medium were used as control, and those treated with negative siRNA (NC-siRNA, 50 nM) were used as negative control. At the end of culture, 20 µl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 h. Supernatants was removed, and formazan crystals were dissolved in 150 µl of dimethylsulfoxide (Sigma, St Louis, MO, USA). Optical density was determined at 490 nm using multi-microplate test system (POLARstar OPTIMA, BMG Labtechnologies, Germany). In each assay, six parallel wells were made. The results were collected as the mean of four independent experiments.

Flow cytometry analysis for cell cycle

DNA content and cell cycle distribution were analyzed using FACSort Cellquect software (BD Biosciences, San Jose, CA, USA). Cells were cultured in six-well plates for 1 day and treated by 50 nM siRNA for 2 days. The cells dissociated into single-cell suspension, and fixed in 75% ice-cold ethanol overnight at 4 °C. The fixed cells were stained with 50 µg/ml propidium iodide (PI) containing 50 µg/ml RNase A (DNase free) for 20 min at room temperature in the dark and determined by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA, USA). The cells were excited at 488 nm, and the emission was collected through a 630-nm filter. Totally 20,000 cells were collected from each sample. The cell cycle distribution was evaluated by calculating the proportion of cells in G0/G1, S, and G2/M stages. In each independent experiment, three parallel wells were made, and the procedures were carried out in triplicate. Data obtained were presented as mean±SEM.

Apoptosis analysis

HepG2 cells were treated with 50 nM siRNA at 37 ºC for 2 days, then harvested and washed twice with PBS. The cells were labeled by incubation with 5 µL FITC-Annexin V and 10 µL PI at 250 µg/ml for 15 min in the dark at room temperature. The cells were washed with PBS and examined using flow cytometry. Quantification of apoptosis was determined by counting the number of cells stained by FITC-labeled Annexin V. The apoptosis of cells was detected using the Annexin V/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) by FACS. Early apoptotic cells were identified with PI negative and FITC Annexin V positive; cells that were in late apoptosis or already dead were both FITC Annexin V and PI positive.

Real-Time PCR

Total RNA samples were extracted from normal hepatocellular tissue, hepatocellular carcinoma tissue, HepG2 cells, and HepG2 cells treated with 50 nM siRNA for 2 days. The RNA was reversely transcribed to cDNA using a reverse transcriptase kit (PrimeScript™ RT reagent Kit, TaKaRa Bio. Co. Ltd., USA). Relative abundance of each mRNA sample was quantitated by Q-PCR using specific primers and the SYBR® Premix Ex Taq™II (TaKaRa. Bio. Co. Ltd., USA). Primers for human MeCP2 (forward 5'- GCCGAGAGCTATGGACAGCA -3'; reverse 5'- CCAACCTCAGACAGGTTTCCAG -3') and human β-actin (forward 5'-TGGCACCCAGCACAATGAA-3';

reverse 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3') were designed and synthesized by TaKaRa Biotechnology. Real-time PCR reactions were carried out using an iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA). Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad, USA). Data were analyzed using the ΔΔCt method and β-actin served as an internal control. The results were presented as mean±SEM of three separate experiments.

Western blot analysis

To analyze the expression of MeCP2, phosphorylated ERK and p38, HepG2 cells were treated by 50 nM siRNA for 1 day. Normal hepatocellular tissue, hepatocellular carcinoma tissue and the HepG2 cells were lysed in RIPA lysis buffer. Protein were subjected to electrophoresis using 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% non-fat dry milk in TBST (10 mM Tris-HCl and 0.05% Tween-20). The membrane was incubated with primary antibodies overnight at 4°C and then incubated with secondary antibody for 2 hr at room temperature. The primary monoclonal antibodies included mouse monoclonal anti-MeCP2 (1:2000, Santa Cruz, CA, USA), mouse monoclonal anti-β-actin (1:5000, Santa Cruz, CA, USA), rabbit monoclonal anti-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-P-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-p38 (1:2000, Cell Signaling, Danvers, MA, USA) and rabbit polyclonal anti-P-p38 (1:1000, Cell Signaling, Danvers, MA, USA). The membranes were incubated in the dark with ECL (Amersham) for chemiluminescence detection. The luminescent signal was detected by CCD camera, recorded and quantified with Syngene GBox (Syngene, UK). The experiment was carried out in triplicate for each group, and results were presented as mean±SEM.

Statistical Analysis

The quantitative data were presented as as mean ± SEM and analyzed by one-way ANOVA. All analyses were performed using SPSS13.0 software (version 13.0). Student's t-tests were used to assess the difference between groups; $P < 0.05$ was considered statistically significant.

RESULTS

Expression of MeCP2 in human HCC

Using real-time PCR and Western blotting, we analyzed the change of mRNA and protein of MeCP2 in human HCC. The results from real-time PCR showed that MeCP2 mRNA was higher levels in human HCC than normal hepatocellular tissue (Fig. 1A) ($P < 0.05$). MeCP2 protein expression in western blot examinations showed the same changing trend as MeCP2 mRNA (Fig. 1B) ($P < 0.05$).

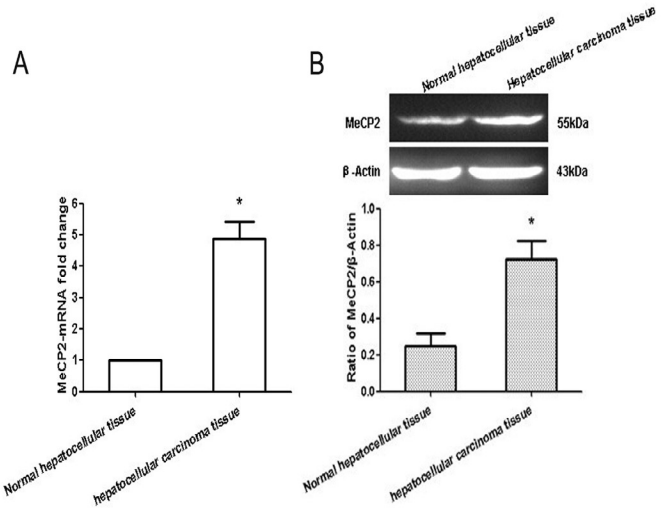

Figure 1. Expression of MeCP2 in human hepatocellular carcinoma. (A) Real-time PCR products. (B) Western blotting analysis. β-Actin was used as a loading control (* $P < 0.05$, compared with normal hepatocellular tissue, n=28).

Effect of MeCP2 on the proliferation of HepG2 cells

To determine whether MeCP2 plays an important role in the proliferation of HepG2 cells, cells were treated with MeCP2 siRNA (25, 50 and 75 nM) for 1, 2 and 3 days. MTT assay was employed to analyze the cell activity. The results showed that MeCP2 siRNA at the concentration of

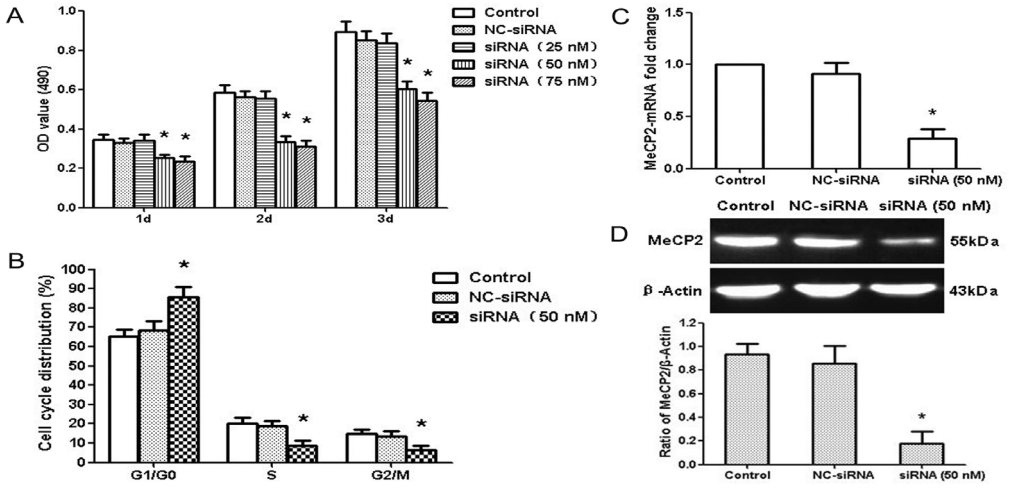

Figure 2. Effects of MeCP2 on the growth of HepG2 cells. (A) MTT assay showed that MeCP2 siRNA decreased the activity of HepG2 cells at 1, 2, and 3 days. (B) The results of flow cytometry analysis of the cell cycle in HepG2 cells were visualized via PI staining. The data show the percentage of cells in the G1/G0, S and G2/M phases. G1/G0 phase cells significantly increased after siRNA (50 nM) treatment, but S and G2/M phases cells remarkably decreased. (C) Real-time PCR results showed that knockdown efficiency of siRNA in HepG2 cells. (D) Western blotting analysis results showed that knockdown efficiency of siRNA. β-Actin was used as a loading control (NC-siRNA represent negative control siRNA; * $P < 0.05$, compared with control group, n=3).

50 and 75 nM significantly decreased the cell activity (Fig. 2A) ($P < 0.05$). Due to the significant differences on cell activity in the treatment groups, MeCP2 siRNA (50 nM) group was chosen to be used in the following experiments. Because the cell cycle are involved in the regulation of cell growth, we examined the processes using a flow cytometer 2 days after treatment. The cell cycles were arrested significantly at G1/G0, the S and G2/M phase cell number significantly decreased in siRNA group (Fig. 2B) ($P < 0.05$). After MeCP2 siRNA had been transfected into HepG2 cells, we analyzed knockdown efficiency of MeCP2 siRNA in mRNA and protein levels. The mRNA and protein expression of MeCP2 of HepG2 cells decreased significantly to 29.31±8.42% and 19.32±7.25% in siRNA groups compared with control (Fig. 2C, D) ($P < 0.05$).

Effect of MeCP2 on apoptosis of HepG2 cells

We also observed that the possible effects of MeCP2 on cell death, the measurement of apoptosis was examined by Annexin-V/PI staining. We found that the proportion of early apoptotic (13.38±3.14%) and late apoptotic (22.16±3.79%) increased significantly after siRNA treatment, the proportion of non affected cells decreased in siRNA group (Fig. 3) ($P < 0.05$). These findings showed that MeCP2 may interrupt apoptosis of HepG2 cells.

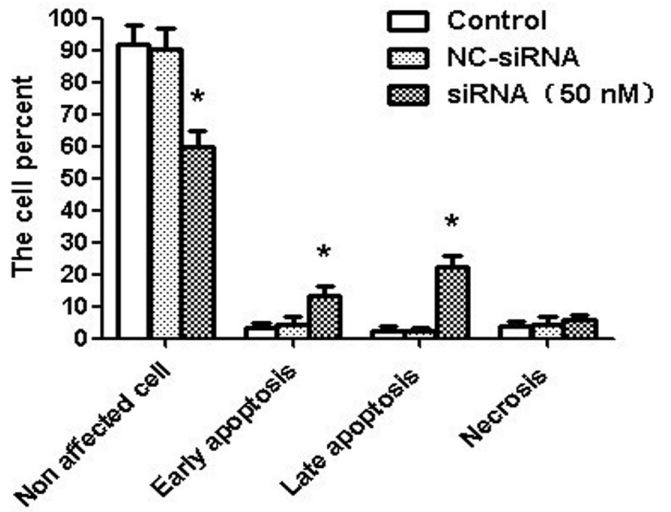

Figure 3. MeCP2 silencing enhanced apoptosis in HepG2 cells. The results of flow cytometry analysis of apoptosis were visualized using Annexin-V/PI staining. The data show the percentage of non-affected, early apoptotic, late apoptotic, and necrotic (NC-siRNA represent negative control siRNA; * $P < 0.05$, compared with control group, n=3).

MeCP2 promoted the proliferation of HepG2 cells with activiating ERK1/2 signaling pathways

To explore the potential molecular mechanisms of MeCP2 in the proliferation of HepG2 cells, the cells were treated by 50 nM siRNA for 1 day, and then the expressions of phosphorylated ERK and p38 were measured. No significant change was observed in the total protein expression of ERK and p38, but the expression of phosphorylation of ERK and p38 evidently changed in treatment groups. The ratio of p-MAPK/total MAPK was used to indicate the phosphorylation and activation of the proteins. It was found that the ratio of p-ERK1/2 to ERK1/2 was significantly decreased 2.16-fold after treatment with siRNA, when compared to that in the control group (Fig. 4A) ($P < 0.05$). By contrast, the ratio of p-p38 to total p38 was remarkably increased by nearly 2.42-fold in the siRNA group compared to the control group (Fig. 4B) ($P < 0.05$).

DISCUSSION

The MBD family consists of five isoforms, including MeCP2, MBD1, MBD2, MBD3, and MBD4. With the exception of MBD4, which is primarily a thymine glycosylase involved in DNA repair, all MBPs are implicated in the transcriptional repression mediated by DNA methylation. MeCP2, MBD1, and MBD2 have been demonstrated to be involved in chromatin structure (12) and transcription regulation (33). MeCP2 is originally considered to be a transcriptional repressor in conjunction with Sin3A and histone deacetylase (5), but was found later to also have a significant role as a transcriptional activator (12), as well as in the regulation of chromatin architecture and RNA splicing (24). Therefore, there is considerable complexity in the possible mechanisms by which MeCP2 can regulate gene expression. MeCP2 involves a wide range of cellular processes such as fibrosis (27), proliferation and tumorigenesis (9). Babbio et al. found that MeCP2 can promote cell proliferation in mammalian cells (1). MeCP2 is highly expressed in gastric cancer (30), breast cancer (21) and prostate cancer (2). In this experiment, we found that MeCP2 expression was also higher levels in human HCC than normal hepatocellular tissue. In addition, it is reported that MeCP2 can promote cell proliferation and play important roles in carcinogenesis in gastric cancer (30) and prostate cancer (2). Tao et al. reported that MeCP2 provide molecular mechanisms for perpetuated fibroblast activation and fibrogenesis in the liver (27). Gene

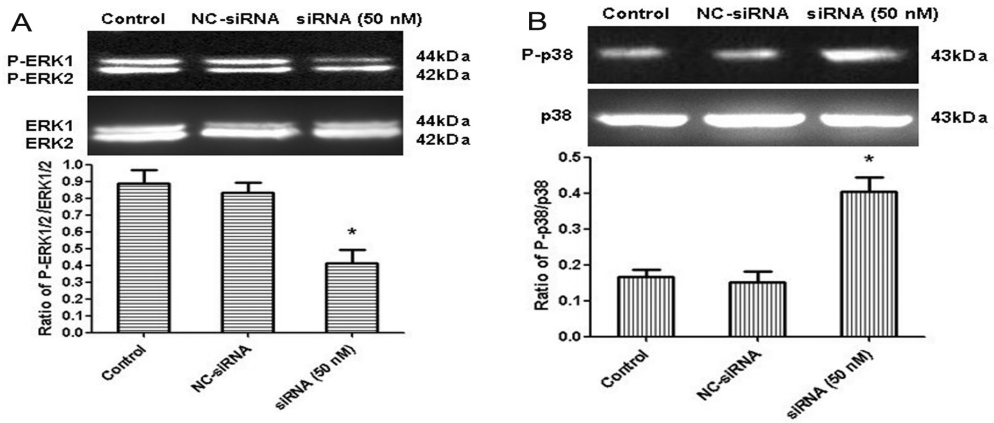

Figure 4. Expressions of phosphorylated ERK and p38 after treatments. (A) Phosphorylated and total ERK1/2. (B) Phosphorylated and total p38. Data were expressed as a ratio of the normalized percentage of p-MAPKs and MAPKs. (NC-siRNA represent negative control siRNA; * $P < 0.05$, compared with control group, n=3).

silencing by RNA interference is a powerful method for analyzing gene function which inhibits gene expression through sequence-specific degradation of a target mRNA. In our experiments, siRNA was adopted to specifically silence MeCP2, we successfully transfected NC-siRNA and MeCP2-siRNA into human HCC HepG2 cells. The results showed that 50 nM MeCP2-siRNA inhibited MeCP2 expression and decreased cell proliferation in HCC HepG2 cells.

The first gap (G1) phase of the mammalian cell cycle is a unique period when cells respond to environmental signals to determine cell fate such as survival, proliferation, differentiation and cellular senescence (25). There are many important cell cycle regulators, including cyclinD, cyclinE, Cdk2, Cdk4 and Cdk6 protein kinase complexes, which can govern the cellular progression through the G1 phase of the cell cycle. In this experiment, we demonstrated that MeCP2 knockdown induced G1 cell cycle arrest in HepG2 cells. These results suggest that MeCP2 may drive more cells crossing G1/S node and entering into cell cycle, resulting in cell proliferation in human HCC HepG2 cells.

The RAS MAPK signaling pathway includes membrane-to-nucleus signaling modules that are involved in the regulation of multiple biological and physiological processes such as proliferation, survival, differentiation, transformation and apoptosis. ERK and p38 MAPKs are main members of the MAPK family. ERK pathway are involved in the regulation of proliferation (3), differentiation and migration (23), but p38 MAPKs pathway participate in regulating apoptosis (20). The ERK cascade can be activated by growth factors and transmit signals to promote cell survival and proliferation (11,18). ERK interacts with many proteins or substrates, and lead to precise biological outcomes (22,29). The ERK1/2 pathway has been considered essential in regulating cell proliferation and survival (15). The activation of ERK1/2 signaling pathway may promote cell proliferation and survival in gastric cancer, colon cancer (4), cervical cancer (13) and lung cancer (31). In addition, RAS MAPK signaling pathway hyperactivity play a prominent role in hepatic fibrogenesis (6). MeCP2 can promote activation of RAS MAPK thought inhibiting the expression of RASAL1 (an inhibitor of RAS MAPK) in the hepatic fibrosis (27). Our studies showed that MeCP2 siRNA suppressed the phosphorylation and activation of ERK1/2 in human HCC HepG2 cells, suggested that MeCP2 might promote cell proliferation thought activation of ERK1/2 signaling pathway. However, the ERK signaling cascades seem to be far more complex. More work is needed to uncover the mechanisms of MeCP2 and ERK pathways on HCC cell proliferation.

Phosphorylation of p38 MAP kinase signal can induce cell apoptosis, and inhibition of p38 MAP kinase with selective inhibitor SB203580 can reduce the cell death (16). In particular, the study of Zheng et al. suggests that p38 MAPK signaling acts as a negative regulator of HCT116 colorectal cancer cell proliferation (36). In this experiment, phosphorylation of p38 MAPK is found to have increased in HCC HepG2 cells after the treatment with MeCP2 siRNA. These results suggest that MeCP2 may reduce apoptosis of HepG2 cells by regulating the phosphorylation of p38 MAPK signaling. Cell proliferation is involved in cell growth and cell death, the expression of p-ERK and p-p38 resulted in a new balance between cell growth and cell death after MeCP2 knockout.

In conclusion, our study provides evidence that MeCP2 expression was higher levels in human HCC tissue than normal hepatocellular tissue, and MeCP2 promoted the proliferation of human HCC HepG2 cells and reduce the apoptosis of the cells, and MeCP2 knockout may decreased the expression of phosphorylation of ERK1/2 and increased phosphorylation of p38 MAPK. The results suggest a potential cure target for treatment of human HCC. However, more work is needed to uncover the mechanisms of MeCP2 promoting the proliferation of HCC cells.

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