Mitogen-activated protein kinase kinase 3 induces cell cycle arrest via p38 activation mediated Bmi-1 downregulation in hepatocellular carcinoma

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Abstract. The underlying molecular pathogenesis of hepatocellular carcinoma (HCC) remains poorly understood. Mitogen-activated protein kinase kinase 3 (MKK3), has been reported as a novel tumor suppressor in breast cancer. However, its potential suppressive role in HCC has not been evaluated. In the current study, the biologic functions of MKK3 in HCC were investigated and a previously unreported cell cycle regulation mechanism was observed. MKK3 overexpression suppressed HepG2 and PLC-PRF-5 cell proliferation and induced cell cycle arrest in the two cell lines. In addition, MKK3 overexpression upregulated the cyclin-dependent kinase inhibitors, p16 INK4A and p15 INK4B in HCC cells. Their negative regulator, Bim-1, was downregulated following MKK3 overexpression. Moreover, MKK3 activated p38 in HCC cells and SB203580, a p38 inhibitor, reversed the tumor suppressive effect of MKK3. In conclusion, the results identify MKK3 as a tumor suppressor and highlighted the significance of p38 pathway aberration in HCC.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent type of cancer and the third most frequent cause of cancer-related mortality (1). In 2014, the estimated number of novel cases and estimated number of fatalities of the disease in the United States were 33,190 and 23,000, respectively (2). The most common risk factors of HCC are chronic hepatitis B (HBV) and C (HCV) viral infection, chronic alcohol consumption and aflatoxin-B1-contaminated food (3-6). HCC often develops during the advanced stages of liver fibrosis and is the leading cause of mortality among patients with cirrhosis (7). Patients with cirrhosis are at the highest risk of developing HCC and should be monitored every 6 months (8). Hepatocarcinogenesis is a complex multistep process in which numerous signaling cascades are altered, leading to a heterogeneous molecular profile. The molecular analysis of human HCC has shown a number of genetic and epigenetic alterations that result in the deregulation of key oncogenes and tumor-suppressor genes, including TP53, β-catenin, ErbB receptor family members, MET and its ligand hepatocyte growth factor (HGF), p16 (INK4a), E-cadherin and cyclooxygenase 2 (COX2) (9). Despite this progress, the neoplastic evolution of HCC remains to be defined.

Mitogen-activated protein kinases (MAPKs) are signaling components that are important in converting extracellular stimuli into a wide range of cellular responses. ERK1/2 MAPKs are preferentially activated in response to growth factors and were found to be upregulated in human tumors (10). p38 kinases are more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation and have been found to be involved in inflammation, cell growth, cell differentiation, the cell cycle, and cell death (11,12). Moreover, p38 MAPK family members and their isoforms have been reported as tumor suppressors or oncoproteins in specific cell types (13,14). In addition, several negative regulators of p38 MAPK signaling have been found to be overexpressed in human tumors and cancer cell lines, including the phosphatases PPM1D and DusP26, and the inhibitors of the MAP3K apoptosis signal regulating kinase 1 (AsK1), glutathione S-transferase Mu 1 (gsTM1) and gsTM2 (13).

Each family of MAPKs is composed of a set of three evolutionarily conserved sequentially acting kinases: An MAPK, an MAPK kinase (MAPKK), and an MAPKK kinase (MAPKKK) (15). As to p38 (MAPK), MKK3 and MKK4 are two MAPK kinases. A recent study demonstrated its downregulated in HCC compared with normal liver tissue (16). However, whether it functions as a tumor suppressor in HCC is unclear. Therefore, in the present study, the role of MKK3 in HCC cell lines was investigated to determine whether MKK3 acts as a tumor suppressor in HCC, compared with normal liver tissue.

Materials and methods

Cell culture, reagents and plasmids. The HepG2 and PLC-PRF-5 hepatocellular carcinoma cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All hepatocellular carcinoma cell lines were maintained in

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Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS). p38 inhibitor, SB203580, was obtained from Sigma-Aldrich (St. Louis, MO, USA). The MKK3 expression plasmid (plasmid 14671) was obtained from Addgene (Cambridge, MA, USA). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For total RNA extraction, samples were processed using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA (1 μg) was reverse-transcribed using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen Life Technologies). Target genes and controls were analyzed by RT-qPCR using a StepOnePlus™ Real-Time PCR System (Invitrogen Life Technologies) and SYBR® Select Master mix (Invitrogen Life Technologies). β-actin was used as control; fold changes were calculated using the ΔΔCt method in Microsoft Excel. Primer sequences were as follows: Forward: CTTGGTGACCATCTCAGAACTGG and reverse: CTTCTGCTCCTGTGAGTTCACG for MKK3 and forward: CGTGACATTAAGGAGAAGCTG and reverse: CTAGAAGCATTTGCGGTGGAC for β-actin.

Immunoblot assay. Whole-cell extracts were obtained by lysis of cells in ice-cold radioimmunoprecipitation assay (RIPA) buffer. Cell lysates were separated on 10% SDS denatured polyacrylamide gel electrophoresis gels (Beyotime Institute of Biotechnologies, Haimen, China), transferred to nitrocellulose membranes (EMD Millipore, Temecula, CA, USA) and blocked in phosphate-buffered saline/Tween-20 containing 5% non-fat milk. Membranes were incubated with dilutions of primary antibodies against MKK3 (rabbit anti-human monoclonal antibody; cat. no. 8355; 1:1,000), Bmi-1 (rabbit anti-human monoclonal antibody; cat. no. .2830, 1:1000), cyclin D1 (rabbit anti-human monoclonal antibody, cat. no. 2978; 1:1,000), cyclin E (mouse anti-human monoclonal antibody; cat. no. 4129; 1:1,000), p21 Cip1 (rabbit anti-human monoclonal antibody, Cat. 2947, 1:1,000), p27 Kip1 (Rabbit anti-human monoclonal antibody; cat. no. 3686, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), p19 INK4D (rabbit anti-human polyclonal antibody; cat. no. ab80; 1:1,000), p18 INK4C (rabbit anti-human polyclonal antibody; cat. no. ab192239; 1:1,000), p16 INK4A (rabbit anti-human polyclonal antibody; cat. no. ab108349; 1:3,000), p15 INK4B (rabbit anti-human polyclonal antibody,; cat. no. ab126625; 1:1,000; Abcam, Cambridge, UK) and tubulin (mouse anti-human polyclonal antibody; cat. no. T6199; 1:10,000; Sigma-Aldrich), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, the targeted proteins were visualized by enhanced chemiluminescence and exposure to film (Fujifilm, Tokyo, Japan).

Cell cycle staging analysis. Cell cycle staging was analyzed by propidium iodide (PI) staining (BD Biosciences, Franklin Lakes, NJ, USA). Cells were treated with 1 mg/ml RNase A (BD Biosciences), fixed with 70% ethanol and then labeled with 20 mg/ml PI solution. The DNA content of cells was measured by flow cytometry (BD FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA). The proportions of cells in the G1, S, and G2/M phases were analyzed using ModFit Software (version 4.0; Verity Software House, Topsham, ME, USA).

MTS assay. HCC cells transfected with either MKK3 overexpression plasmid or vector were seeded in 96-well plates. 12 h later, MTS reagent (Promega Corporation, Madison, WI, USA) was added at a 1:10 dilution. Plates were read at 450 nm using an ELx800 microplate reader (BioTek Instruments, Inc.) 90 min later. The absorbance was recorded at day 0. Then at 24, 48 and 72 h, absorbance was also determined and recorded as day 1, 2 and 3, respectively.

BrdU incorporation and anaphase assay. The BrdU incorporation and anaphase assay were performed as a proliferation indicator. For the BrdU incorporation assay, a Cell Proliferation ELISA kit (Roche Diagnostics, Manheim, Germany) was applied and measurements were performed according to the manufacturer's instructions. For the anaphase assay, the number of cells, and the number of cells in anaphase were detected using DAPI and were counted in five visual fields per well.

Statistical analysis. GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analysis. A two-tailed unpaired Student's t test was used for statistical evaluation of data. $P<0.05$ was considered to indicate a statistically significant difference. Data are expressed as the mean ± standard deviation.

Results

MKK3 suppresses HepG2 and PLC-PRF-5 cell proliferation. To investigate whether MKK3 acts as a tumor suppressor in HCC, MKK3 was overexpressed in HepG2 and PLC-PRF-5 HCC cell lines. MKK3 overexpression was confirmed by RT-qPCR and immunoblotting (Fig. 1A and B). An MTS proliferation assay was then performed. HepG2 and PLC-PRF-5 cells transfected with the MKK3 expression plasmid exhibited impaired proliferation (Fig. 1C). Furthermore, a BrdU incorporation assay was also performed to examine proliferation alterations. In accordance with the results, MKK3 suppressed BrdU incorporation in the two cell lines (Fig. 1D). Moreover, the number of detectable anaphase cells, assessed in parallel as an indicator of active proliferation, was selectively reduced in MKK3-overexpressing HepG2 and PLC-PRF-5 cells (Fig. 1E). The results indicate that MKK3 may function as a tumor suppressor via inhibiting proliferation.

MKK3 induces cell cycle arrest in HepG2 and PLC-PRF-5 cells. Cell cycle deregulation is a common feature of human cancer, which leads to unscheduled proliferation, genomic instability and chromosomal instability (17). Given that MKK3 suppresses HCC cell proliferation, it was speculated that MKK3 may participate in cell cycle regulation. To test this hypothesis, cell cycle distribution in HepG2 and PLC-PRF-5 cells transfected with either MKK3 expression plasmid or vector was examined. As expected, compared with control, MKK3 overexpressing cells showed significant cell cycle arrest in the G1 phase (Fig. 2A and B). Together with the results of the proliferation study, these results indicate

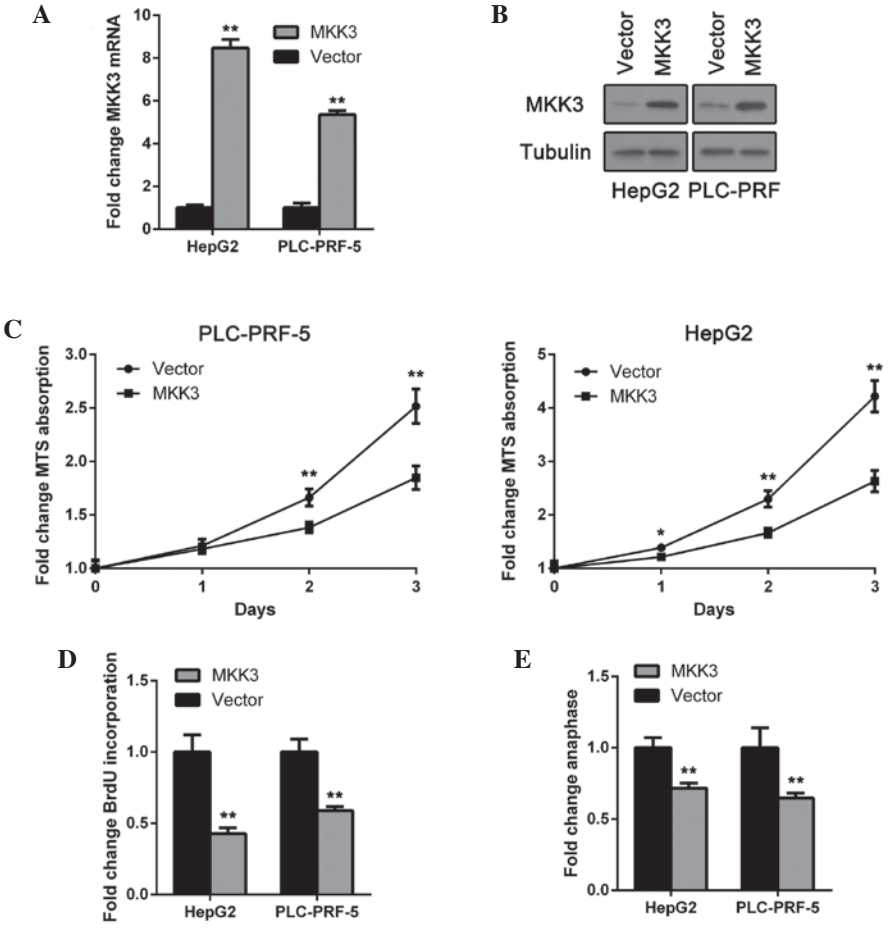

Figure 1. MKK3 overexpression suppresses hepatocellular carcinoma cell proliferation. HepG2 and PLC-PRF-5 cells were transfected with either MKK3 overexpression plasmid or vector plasmid. After 48 h, cells were harvested and subjected to (A) reverse transcription-quantitative polymerase chain reaction and (B) immunoblot assay. To investigate the impact of MKK3 impact on HCC cell proliferation the cells were then subjected to (C) an MTS assay, (D) BrdU incorporation assay and (E) anaphase analysis. The data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01, compared with vector. MKK3, mitogen-activated protein kinase kinase 3.

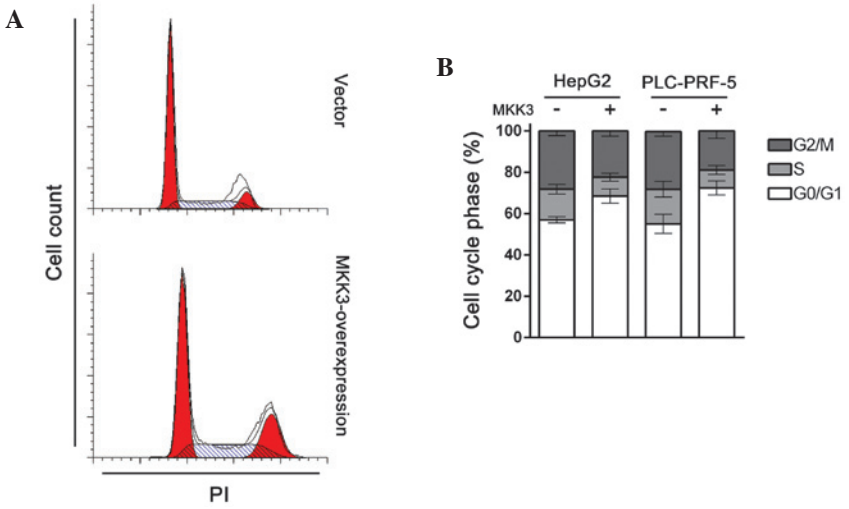

Figure 2. MKK3 overexpression induces cell cycle arrest in hepatocellular carcinoma cells. HepG2 and PLC-PRF-5 cells were transfected with either MKK3 overexpression plasmid or vector plasmid. Then cells were analyzed for cell-cycle progression staging by flow cytometry following propidium iodide staining. (A) Representative cell cycle staging of HepG2 cells. (B) Cell cycle distribution of HepG2 and PLC-PRF-5 cells. Data are presented as the mean ± standard deviation (n=3). MKK3, mitogen-activated protein kinase kinase 3.

that MKK3 regulates the tumor cell cycle and, thus, exhibits a tumor suppressive role in HCC.

MKK3 upregulates INK4A and INK4B in HepG2 and PLC-PRF-5 cells. The cell cycle is tightly controlled by

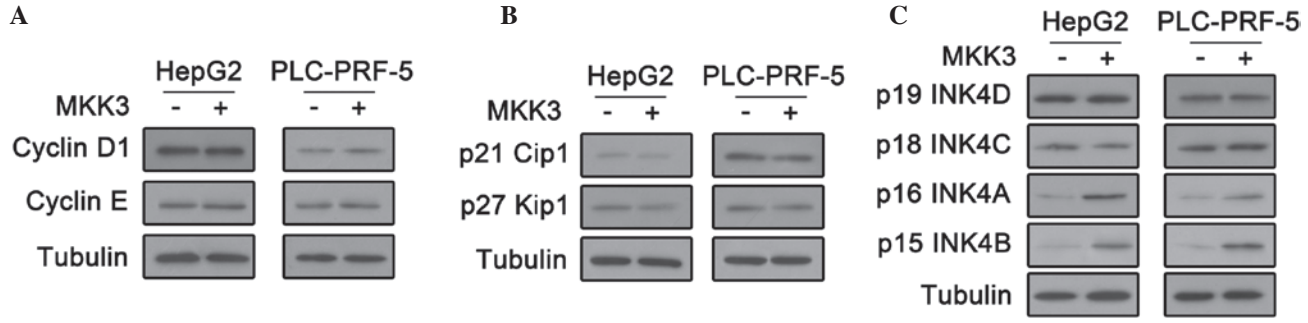

Figure 3. MKK3 upregulates CDK inhibitors, INK4A and INK4B, in hepatocellular carcinoma cells. HepG2 and PLC-PRF-5 cells were transfected with either MKK3 overexpression plasmid or vector plasmid. Then cells were harvested and subjected to immunoblot assay to detect expression alteration of (A) cyclin proteins, (B) CDK2 inhibitors and (C) CDK4/6 inhibitors. MKK3, mitogen-activated protein kinase kinase 3; CDK, cyclin-dependent kinase.

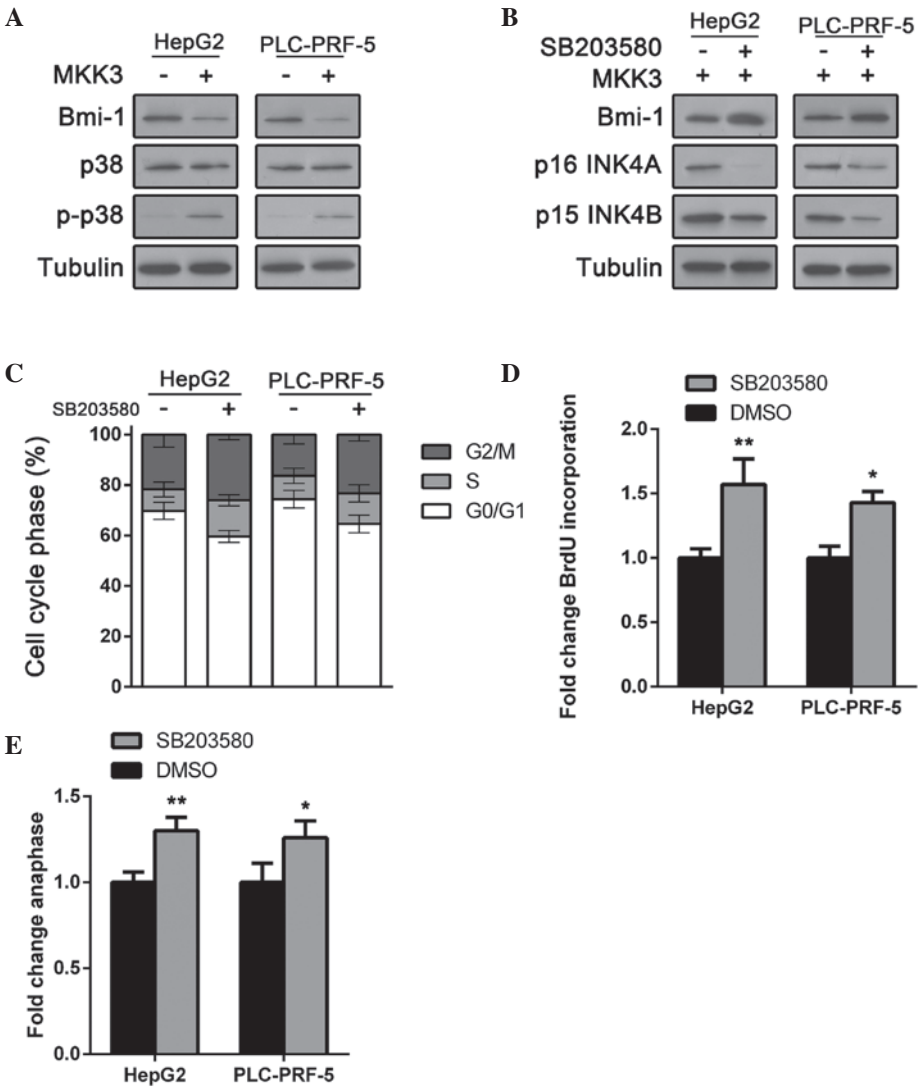

Figure 4. MKK3 tumor suppressive activity depends on Bmi-1 downregulation and p38 activation. (A) HepG2 and PLC-PRF-5 cells were transfected with either MKK3 overexpression plasmid or vector plasmid. Then Bmi-1 expression and p38 activation were analyzed by an immunoblot assay. (B) HepG2 and PLC-PRF-5 cells transfected with MKK3 overexpression plasmid were treated with either 10 μM SB203580 or vehicle (DMSO) and an immunoblot assay was performed to detect Bmi-1 and CDK4/6 inhibitor expression. (C) Cell cycle staging, (D) BrdU incorporation assay and (E) an anaphase assay were also performed. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01, compared with control. MKK3, mitogen-activated protein kinase kinase 3; DMSO, dimethyl sulfoxide.

cyclin-dependent kinases (CDKs) (18). CDK activity requires binding of regulatory subunits termed cyclins. In addition, There are two families of CDK inhibitors (CKIs), INK4 proteins and the Cip and Kip family (17,19,20). To investigate how MKK3 influences HCC cell cycle regulation, these cell cycle regulators were investigated. First, cyclin D1 and cyclin E

expression was analyzed by an immunoblot assay. As shown in Fig. 3A, there was no change in these levels following MKK3 overexpression. In addition, the immunoblot assay showed that MKK3 overexpression did not affect the expression of CDK2 inhibitors, p27 Cip1 and p27 Kip (Fig. 3B). Notably, p16 INK4A and p15 INK4B were upregulated in HepG2 and PLC-PRF-5 cells transfected with the MKK3 expression plasmid (Fig. 3C). These results indicate that MKK3 may affect cell cycle by upregulating the CDK4/6 inhibitors, p16 INK4A and p15 INK4B in HCC.

MKK3 tumor suppressor activity is dependent on Bmi-1 downregulation and p38 activation. Bmi-1 is a member of the polycomb group (PcG) of proteins and is important in the regulation of cell proliferation and senescence through repression of the p16 INK4A and p15 INK4B genes (21-24). A recent study also indicated its oncogenic role (25,26). Bmi-1 expression was examined in MKK-overerpressing cells and control cells. As expected, Bmi-1 was downregulated in HepG2 and PLC-PRF-5 cells following MKK3 overexpression (Fig. 4A). Furthermore, as MKK3 is an MAPK kinase that activates p38, p38 activation was investigated. As shown in Fig. 4A, p38 expression was not altered. However, phosphor-p38, the active form of p38, was significantly upregulated in MKK-overexpressing HCC cells. These results indicate that MKK3 may regulate Bmi-1 and cell cycle arrest by p38 activation.

To test whether the tumor suppressive role of MKK is p38 dependent, cells were treated with SB203580, a p38 inhibitor. As shown in Fig. 4B, Bmi-1 expression was rescued by SB203580 treatment in HCC cells. Furthermore, p16 INK4A and p15 INK4B were also downregulated to normal levels in MKK-overexpressing HCC cells (Fig. 4B). The impact of SB203580 on HCC cell cycle arrest was then determined. HepG2 and PLC-PRF-5 cells transfected with the MKK3 expression plasmid were treated with SB203580 or vehicle and cell cycle staging was conducted using flow cytometric analysis. Results showed that SB203580 suppressed MKK3-induced cell cycle arrest in HepG2 and PLC-PRF-5 cells (Fig. 4C). Moreover, the impact of SB203580 on HCC cell proliferation was also determined. The BrdU incorporation assay showed that SB203580 restored proliferation in MKK3-overexpressing cells (Fig. 4D). These results were confirmed by an anaphase cell count assay (Fig. 4E) and suggested that the tumor suppressive role of MKK3 is dependent on Bmi-1 downregulation and p38 activation.

Discussion

In the development of cancer, tumor cells acquire six capabilities, which are shared by the majority of/perhaps all types of human tumor. These six capabilities are the well-known hallmarks of cancer (27,28). Three of the six hallmarks, self-sufficiency in growth signals, insensitivity to growth-inhibitory signals and limitless replicative potential are associated with cell cycle control. Thus, understanding of this process is important for developments in cancer therapy. In the current study, a novel mechanism by which HCC cells control proliferation and cell cycle transition was determined. The results suggest that by activating p38, MKK3 suppresses the expression of a PcG protein, Bim-1, which is a negative regulator of the expression of CDK4/6 inhibitors p16 INK4A and p15 INK4B. In this way, MKK3 upregulates p16 INK4A and p15 INK4B and therefore, induces HCC cell cycle arrest. A recent study reported that MKK3 was downregulated in HCC (16). Together with our results, these data suggest that HCC may pass through the cell cycle checkpoint by down-regulating MKK3.

The role of p38 in cancer depends on the cell type and cancer stage. Certain studies have reported that p38 increases cell proliferation, whereas in others, the activation of the MAPK p38 pathway is described as tumor suppressive (29-34). In the present study, the p38 pathway was observed to exhibit a tumor suppressive role in HCC. MKK3-induced p38 activation impaired cancer cell growth. In addition, p38 inhibition by SB203580 restored HCC cell G1-S transition and proliferation. p38 MAPK targeting inhibitors and drugs are currently in development to treat cancer (13). However, a recent study reported the antitumor effect of another p38 inhibitor, SB202190 in colon adenocarcinoma (35). These conflicting results may reflect the differences in the effects of p38, depending on cell type. Furthermore, a recent study in breast cancer reported that MKK3 regulates cell cycle transition by p21 Cip1 and p27 Kip1. However, it was suggested that MKK3 regulates the cell cycle via p16 INK4A and p15 INK4B. Together, these results highlight the importance of understanding the cell type-specific differences of the effects of p38. It is important to carefully consider the type of tumor prior attempting to modulate this pathway for cancer therapy.

In conclusion, the present results indicate that MKK3 exhibits a critical suppressive role in hepatocarcinogenesis through the control of Bim-1 expression and p38 activation. It also reports a previously undescribed MKK3 dependent HCC cell cycle control mechanism. These results shed light on the regulation of HCC cell cycle and identify a novel target for HCC treatment.

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