RESEARCH ARTICLE

Bone morphogenetic protein 2 inhibits hepatocellular carcinoma growth and migration through downregulation of the PI3K/AKT pathway

Ying Zheng · Xuemei Wang · Haidong Wang · Wei Yan · Quan Zhang · Xin Chang

Abstract Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Previous studies have suggested that abnormal expression of BMP-4, BMP-7, and BMP-9 is correlated with tumor progression in HCC, but the role played by BMP-2 in HCC has not yet been reported. To determine the role of BMP-2 in HCC, we first investigated the effect of exogenous BMP-2 on the growth of the cell lines HCC SK-Hep-1, Hep G2, and Hep 3B. Next, we studied the function of BMP-2 in SK-Hep-1 HCC cell line using a recombinant lentivirus vector to deliver BMP-2. We also used siRNA to silence endogenous BMP-2 expression in the HCC Hep 3B cell line. Then, cell growth and migration were assayed in vitro using WST-8, wound-healing, and transwell invasion assays. Cellular apoptosis and cell-cycle distribution were assessed using flow cytometry. We also investigated the effects of BMP-2 overexpression and knockdown on the expression of proliferating cell nuclear antigen (PCNA), matrix metallopeptidase-2 (MMP-2), phosphorylated AKT (p-AKT), phosphoinositide 3-kinase p85α (PI3Kp85α), Bax, Bcl-2, caspase-3, cleaved caspase-3, p21, and cyclin E. As a result, we observed that BMP-2 inhibited the proliferation of HCC cells. Furthermore, HCC cell proliferation and migration were significantly diminished by BMP-2 overexpression, as was indicated by WST-8, would healing, and transwell assays, while knockdown of BMP-2 led to an increase in proliferation and migration of Hep 3B cells. BMP-2 overexpression significantly increased the susceptibility of SK-Hep-1 cells to low-serum-induced apoptosis, while BMP-2 knockdown reduced the susceptibility of Hep 3B cells. Overexpression of BMP-2 induced G1 phase arrest through upregulation of p21. When BMP-2 expression was elevated in SK-Hep-1 cells, the expression of PI3Kp85α, p-AKT, PCNA, and MMP-2 declined. These results suggest that BMP-2 exerts an inhibitory effect on the growth and migration of HCC cells, possibly via a blockade of PI3K/AKT signaling.

Keywords Bone morphogenetic protein 2 · Hepatocellular carcinoma · Migration · PI3K · AKT

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in worldwide and accounts for approximately 7.9 % of all cancers [1]. Although therapeutic interventions for patients with HCC have improved over the past few decades, mortality is still high, and the 5-year survival rate is lower than 70 % [2]. One of the primary reasons for the poor prognosis for this disease is the lack of effective treatment options due to the complicated molecular pathogenesis of HCC. In recent years, improved knowledge of the single or multiple gene mutations that promote tumor cell proliferation, angiogenesis, invasion, and metastasis has led to the identification of potentially possible therapeutic targets. Thus, a better understanding of the molecular pathogenesis of HCC may stimulate the development of new therapeutic agents.

Bone morphogenetic proteins (BMPs) are regulatory genes known for their capacity to regulate bone and cartilage formation [3], but current evidences suggest that BMPs play a much wider role in metabolism than originally thought [4, 5]. In fact, BMPs exert both stimulatory and inhibitory effects on tumor growth that depends on dosage, the type of cells, and the tumor microenvironment. Mounting evidences demonstrated that aberrant expression of BMPs is positively correlated with disease progression and survival in lung cancer [6] and breast cancer [7]. BMP-2, an important regulator for bone formation, plays a pivotal role in cancer cell proliferation, apoptosis, and differentiation and thus may be considered as a oncogene. BMP-2 in breast cancer can promote vascularization possibly by stimulating the MAPK/p38 pathway [8]. Active endogenous BMP-2 signaling also promotes proliferation and migration of prostate cancer cells through the activation of AKT and ERK [9]. In addition, BMP-2 promotes cell migration in lung cancer through MAPK/Runx2/Snail signaling [10]. On the contrary, BMP-2 also functions as potent tumor suppressors in gastric carcinoma and renal cell carcinoma [11, 12]. Moreover, BMP-2 suppresses tumor growth by reducing the gene expression of tumorigenic factors and inducing the differentiation of cancer stem cells in osteosarcoma [13]. Taken together, these above studies demonstrate that BMP-2 play a pivotal role in cancer suppression and promotion. The biological response of cancer cells to BMP-2 may depend on the particular cell type and other factors that have not been yet defined. A recent study has confirmed that BMP-2 is expressed in normal adult rat liver and negatively regulates hepatocyte proliferation [14]. However, the effects and molecular mechanisms of BMP-2 in the progression of HCC cells have not yet been comprehensively explored.

In the present study, we examined endogenous expression of BMP-2 in HCC cell lines using real-time polymerase chain reaction (PCR) and Western blot analysis. We then determined the functional relevance of BMP-2 for HCC cell growth and migration in vitro by overexpressing BMP-2 in HCC SK-Hep-1, a cell line with relatively low BMP-2 expression, using a lentiviral vector encoding the BMP-2 gene. We also performed BMP-2 knockdowns using RNA interference in HCC Hep 3B, a cell line with relatively high BMP-2 expression. Then, we investigated the effects of BMP-2 knockdown and overexpression on the expression of AKT, p-AKT, PI3K, p-PI3K, proliferating cell nuclear antigen (PCNA), Bax, Bcl-2, caspase-3, cleaved caspase-3, p21, and cyclin E to explore the underlying signaling pathways involved in the progression of HCC.

Materials and methods

Cell culture One poorly differentiated (SK-Hep-1) and two well-differentiated (Hep G2, Hep 3B) human hepatocellular carcinoma (HCC) cell lines were used, which obtained from Institute of Biochemistry and Cell Biology (Shanghai, China); cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5 % CO_2 in air at 37 °C.

Cell proliferation assay To investigate the effects of BMP-2 on cell growth, HCC cells were seed on a 96-well culture plate (Corning, New York, NY) at 1×10^4 cells per well and then treated with 0.1, 1, and 10 μg/L BMP-2 for 48 h. The cell viability was determined using the WST-8 assay kit (Dingguo Biology, Shanghai, China) according to the manufacturer's protocol. Briefly, at indicated time points, the cells were added with 10 μL of WST reagent for each well. Then, the cells were incubated at 37 °C in a humidified, 5 % CO_2 atmosphere for 1 h, and measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, American).

Quantitative real-time polymerase chain reaction (qPCR) To quantitatively determine the mRNA expression levels of BMP-2 in HCC cell lines, qPCR was used. Total RNA of each sample was extracted with TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Reverse-transcription and cDNA amplification was carried out using SYBR Green Master Mix kit. The real-time quantitative primers were designed by Takara Biotechnology (Takara Dalian, China). The PCR primer sequences were as follows: BMP-2, (forward, 5'-TGCACCAAGATGAACACAG-3' and reverse, 5'-GCTGTTTGTGTTTGGCTTG-3'; β-actin (forward, 5'-GCGGGAAATCGTGCGTGACATT-3' and reverse, 5′-GGCAGATGGTCGTTTGGCTGAATA-3′). A positive standard curve was obtained using serially diluted cDNA sample mixture, and the quantity of gene expression was normalized with β-actin.

siRNA and transfected cells sorting The plasmid vector targeted at BMP-2 was designed by SiDanSai Biotechnology (SiDanSai, Shanghai, China). The sequences used were as follows: Human BMP-2 siRNA, 5'-CCUCGGAACAGUUG UUUAU-3'; a negative control siRNA, 5'-UUCUCCGAAG UCACGUdTdT-3'. RNA interference for knocking-down BMP-2 expression in Hep 3B was performed according manufacturer's protocol. Briefly, HCC cells were seeded on a 12-well plate and transfected with BMP-2 RNA interference plasmid vector (siBMP-2), or empty vector (siControl). Cells were sub-cultured at a 1:3 dilution in 500 μg/ml G418-containing medium to select the cells with stable transfectants. Subsequently, followed by 48 h recovery after transfection, the efficiency of BMP-2 knocking-down was tested by Western blot.

Transduction with lentiviral vectors SK-Hep-1 cell line was transfected with lentivirus vector encoding BMP-2 (Lenti-BMP-2) or GFP (Lenti-GFP). Transduction was performed according manufacturer's protocol. Briefly, SK-Hep-1 cells were plated in six-well dishes at a density of 1×10^6 cells in 2 ml medium per dish. Then the cells presented in 8 μg/mL Polybrene at MOI of 5 for Lenti-GFP and Lenti–BMP-2. After incubation for 24 h, the transduction medium was replaced with fresh DMEM. The SK-Hep-1 cells without transfection were used as control (CON). The infection efficiency was assessed by fluorescence microscopy.

Wound-healing assay HCC cells were plated in each well of a six-well culture plate and allowed to grow to 90 % confluence. At an indicated time, a wound was created using 10 μL micropipette tip. The migration of cells towards the wound was monitored daily, and images were captured at 24 and 48 h.

Transwell invasion assay Briefly, the cells were trypsinized with 0.25 % trypsin containing EDTA. Subsequently, cells were suspended and implanted with each transwell inserts with 8-μm pore size coated with 50 μL Matrigel (BD Biosciences), and 200 μL medium containing 10 % FBS was added in the bottom chamber. Undergoing migration for 24 h, a cotton swab was used to remove the non-migrated cells in the upper chamber then the filters were individually fixed with 4 % paraformaldehyde. The cell numbers were counted in five random fields of each chamber under the microscope.

Western blot assay HCC cells were harvested and extracted using lysis buffer. And then equal amount of cell extracts were separated on 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. Separated protein bands were transferred into polyvinylidene fluoride membranes, and the membranes were blocked in 5 % skim milk powder. The primary antibodies against BMP-2, PI3Kp85α, p-AKT, PCNA, Bax, Bcl-2, caspase-3, cleaved caspase3, p21, Cyclin E, and MMP-2 were diluted according to the instructions of antibodies and incubated overnight at 4ºC. Subsequently, horseradish peroxidase-linked secondary antibody was incubated at room temperature for 2 h. The membranes were washed with TBST for three times, and the immunoreactive bands were visualized using ECL-PLUS/Kit according to the kit's instruction. The relative protein level in different cell lines was normalized to β-actin concentration.

Measurement of apoptosis by flow cytometry Apoptosis incidence was detected by using the Annexin V-PE/PI apoptosis detection kit (Invitrogen, USA) according to the method of Yang et al. [15]. Apoptotic cells, including those staining positive for Annexin V-PE and negative for PI and those that were double positive, were counted.

Statistical analysis The results obtained were expressed as the mean±standard error from at least three independent experiments. One-way analysis of variance (ANOVA) was used to analyze the differences between groups. The LSD method of multiple comparisons was used when the probability for ANOVA was statistically significant. Statistical significance was $P<0.05$.

Results

Exogenous BMP-2 inhibits cell growth in HCC cell lines Previous reports have shown that BMP-2 is expressed in the liver and inhibits hepatocyte proliferation [14]. To expand upon these observations, we first analyzed the cellular growth response of the HCC SK-Hep-1, Hep G2, and Hep 3B cell lines to BMP-2. The effect of BMP-2 was checked in low serum conditions (0.1 % FBS) to avoid the bioactive concentrations of BMPs (BMP-2, BMP-4, BMP-6, and BMP-9) in FBS [16]. Therefore, the cell lines HCC SK-Hep-1, Hep G2, and Hep 3B were incubated in 0.1 % FBS media in combination with BMP-2 for 48 h. The WST-8 assay indicated that 10 μg/L of BMP-2 produced the strongest inhibition of proliferation in Hep G2 and Hep 3B cells at 31 % and 54 %, respectively (Fig. 1a). SK-Hep-1 growth was inhibited by 75 % in response to treatment with 10 μg/L of BMP-2. BMP-2 was also able to inhibit the proliferation of HCC cells, in agreement with results from Chen et al., who observed that BMP-2 inhibited the proliferation of MDA-MB-231 and MCF-7 breast cancer cells [17].

The endogenous expression of BMP-2 in HCC cell lines The endogenous expression of BMP-2 in HCC SK-Hep-1, Hep G2, and Hep 3B cell lines was evaluated using RT-PCR and Western blot analysis. As shown in Fig. 1b–c, there were different levels of mRNA and protein expression of BMP-2
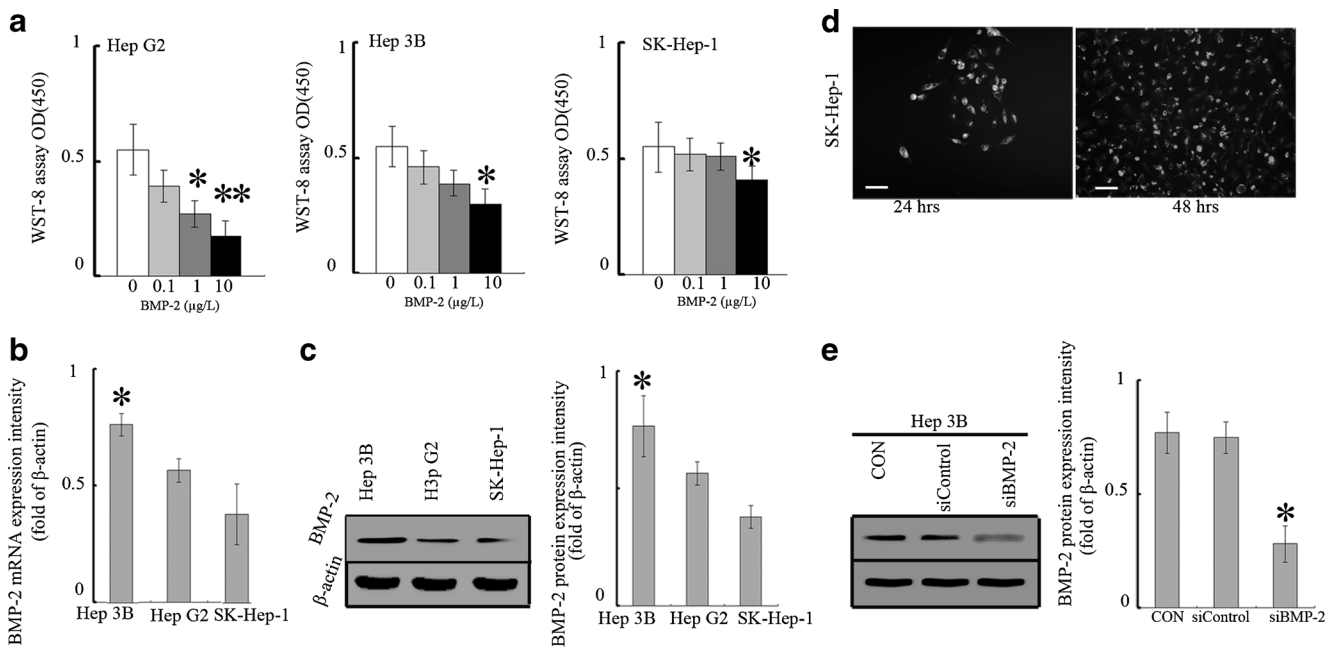
Fig. 1 Cell growth in vitro and cell lines construction. **a** Effects of BMP-2 on the proliferation of HCC SK-Hep-1, Hep G2, and Hep 3B cells revealed by the WST-8 assay. Cells were treated with 0.1, 1, and 10 μg/l BMP-2 for 48 h. Growth of the three cell lines was inhibited differently (*$P<0.05$; **$P<0.01$). **b, c** Expression of BMP-2 in SK-Hep-1, Hep G2, and Hep 3B cell lines were assessed by real-time PCR and Western blot assays. High level of BMP-2 expression mRNA and protein in Hep 3B and low level in SK-Hep were detected by real-time PCR and Western blot assays, respectively (*$P<0.05$). **d** A recombinant lentivirus vector encoding BMP-2 was constructed for infecting the SK-Hep-1 cell line. The infection efficiency of lenti-BMP-2 (MOI=5) was greater than 30 % at 24 h and 90 % at 48 h under fluorescence microscopy. **e** The protein levels of BMP-2 were determined by Western blot when Hep 3B cells transfected with siBMP-2 or siControl vectors (100 nM each) for 24 h. A significant reduction of BMP-2 expression was observed in siBMP-2 group (*$P<0.01$)

in SK-Hep-1, Hep G2, and Hep 3B cell lines, but the expression levels of BMP-2 were significantly high in Hep 3B cell line and low in SK-Hep-1 cell line. Thus, SK-Hep-1 cell line was chosen as the infective objects of lentivirus-mediated overexpression of BMP-2 (Lenti-BMP-2) and Hep 3B cell line as objects of knockdown by siRNA (siBMP-2). SK-Hep-1 cell line was transfected with lentivirus vector encoding BMP-2 (Lenti-BMP-2) or GFP (Lenti-GFP) and the SK-Hep-1 cells without transfection were used as CON. In pilot studies, the infection efficiency of Lenti-BMP-2 (MOI=5) in SK-Hep-1 cell line was greater than 90 % under fluorescence microscopy (Fig. 1d).

The endogenous expression of BMP-2 in HCC cell lines Endogenous expression of BMP-2 in the cell lines HCC SK-Hep-1, Hep G2, and Hep 3B was evaluated using real-time-PCR and Western blot analysis. As is shown in Fig. 1b and c, there were different levels of BMP-2 mRNA and protein expression in the SK-Hep-1, Hep G2, and Hep 3B cell lines, and expression levels of BMP-2 were significantly higher in Hep 3B and lower in SK-Hep-1. Therefore, SK-Hep-1 was chosen for lentivirus-mediated overexpression of BMP-2 (lenti-BMP-2), and Hep 3B was chosen for BMP-2 knockdown by siRNA (siBMP-2). SK-Hep-1 was transfected with a lentiviral vector encoding either BMP-2 (Lenti-BMP-2) or GFP (Lenti-GFP), and untransfected SK-Hep-1 cells were

used as a CON. In pilot studies, the infection efficiency of Lenti-BMP-2 (MOI=5) in the SK-Hep-1 cell line was greater than 90 %, as determined by fluorescence microscopy (Fig. 1d).

siRNAs targeting BMP-2 were successfully transfected into Hep 3B cells. As is shown in Fig. 1e, Western blot assays were performed after 48 h of recovery to measure the expression of BMP-2, and a significant reduction in BMP-2 expression was observed in the siBMP-2 group relative to the siControl and CON groups. No significant difference was found between the siControl and CON groups, however.

The effect of BMP-2 on HCC cell proliferation Deregulated cellular proliferation is a hallmark of cancer. To detect the effect of BMP-2 on HCC cell proliferation, we investigated the proliferation of the cell lines SK-Hep-1 and Hep 3B using the WST-8 assay. In this assay, overexpression of BMP-2 significantly reduced the proliferation of the SK-Hep-1 cell line in a time-dependent manner (Fig. 2a). However, knockdown of BMP-2 promoted cellular proliferation of the Hep 3B cell line, also in a time-dependent manner (Fig. 2a).

In addition, PCNA, a key factor in the cell cycle, is widely used to assay tumor progression and the outcome of anticancer treatment [18, 19]. To determine whether BMP-2 overexpression suppressed endogenous PCNA expression through translational repression, the expression of
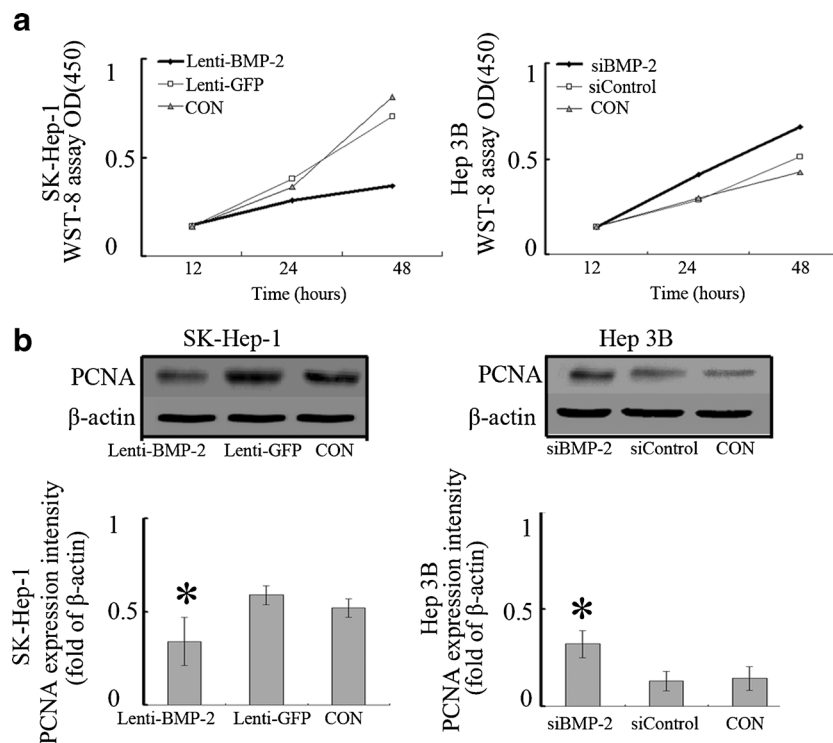
Fig. 2 Effect of BMP-2 on proliferation and its effect on PCNA expression. **a** WST-8 assay indicated that there was a significantly reduction of the proliferative activity in Lenti-BMP-2 group compared with those in Lenti-GFP group and CON group. But no difference was found between Lenti-GFP and CON groups. On the other hand, results of the WST-8 assay showed that the proliferative activity in siBMP-2 group was higher than those in siControl and CON groups. **b** The expression of PCNA was examined by Western blot assay. The results indicated that the amount of PCNA expression was significantly decreased in Lenti-BMP-2 group compared with Lenti-GFP group and CON group in SK-Hep-1 cell line (*$P<0.05$). By contrast, the expression of PCNA was higher in siBMP-2 group than those in siControl and CON groups

PCNA was examined using a Western blot. This assay demonstrated that PCNA expression was significantly reduced in the Lenti-BMP-2 group relative to the Lenti-GFP and CON groups in the SK-Hep-1 cell line (Fig. 2b). No difference was found, however, between the Lenti-GFP and CON groups. In contrast, knockdown of BMP-2 significantly enhanced the expression of PCNA (Fig. 2b). These results suggest that BMP-2 inhibits HCC cell proliferation via downregulation of PCNA expression.

The effect of BMP-2 on HCC cell invasion and metastasis To determine the effect of BMP-2 on SK-Hep-1 and Hep 3B cell migration, a wound-healing assay was performed. As is shown in Fig. 3a, the migration of SK-Hep-1 cells in the Lenti-BMP-2 group was markedly lower than in the Lenti-GFP and CON groups. There were no significant differences between the Lenti-GFP and CON groups, however. In contrast, knockdown of BMP-2 significantly promoted the migration of the Hep 3B cell line (Fig. 3a). Furthermore, a transwell assay was performed to determine the ability of cells to invade a matrix barrier, and representative micrographs of transwell filters are presented in Fig. 3b. These results demonstrated that invasive potential was significantly reduced in the Lenti-BMP-2 group relative to the Lenti-GFP and CON groups (**$P<0.01$). In addition, a significant increase in

invasive capacity was observed in the siBMP-2 group relative to the siControl and CON groups. These data demonstrate that BMP-2 expression inhibits HCC cell migration and invasiveness.

A Western blot assay was used to examine the effect of BMP-2 on MMP-2 expression. As shown in Fig. 3c, the expression of MMP-2 protein was significantly inhibited in the Lenti-BMP-2 group when compared with the Lenti-GFP and CON groups in the SK-Hep-1 cell line. No difference was observed between the Lenti-GFP and CON groups, however. Also, the expression of the MMP-2 protein in the siBMP-2 group was significantly higher than in the siControl and CON groups in the Hep 3B cell line. These data suggest that overexpression of BMP-2 inhibits HCC cell migration via downregulation of MMP-2 expression.

The effects of BMP-2 on HCC cell apoptosis and cell cycle distribution To determine whether BMP-2 affected the cell-cycle distribution of HCC cells, the cycle distribution of SK-Hep-1 and Hep 3B cells was analyzed using flow cytometry. As is shown in Fig. 4a, cell-cycle kinetics demonstrated that overexpression of BMP-2 resulted in an increase in the percentage of cells in the G0/G1 phases, with a concomitant decrease in the number of cells in the S phase. Furthermore, cell-cycle kinetics also showed that the G0/G1 phase of
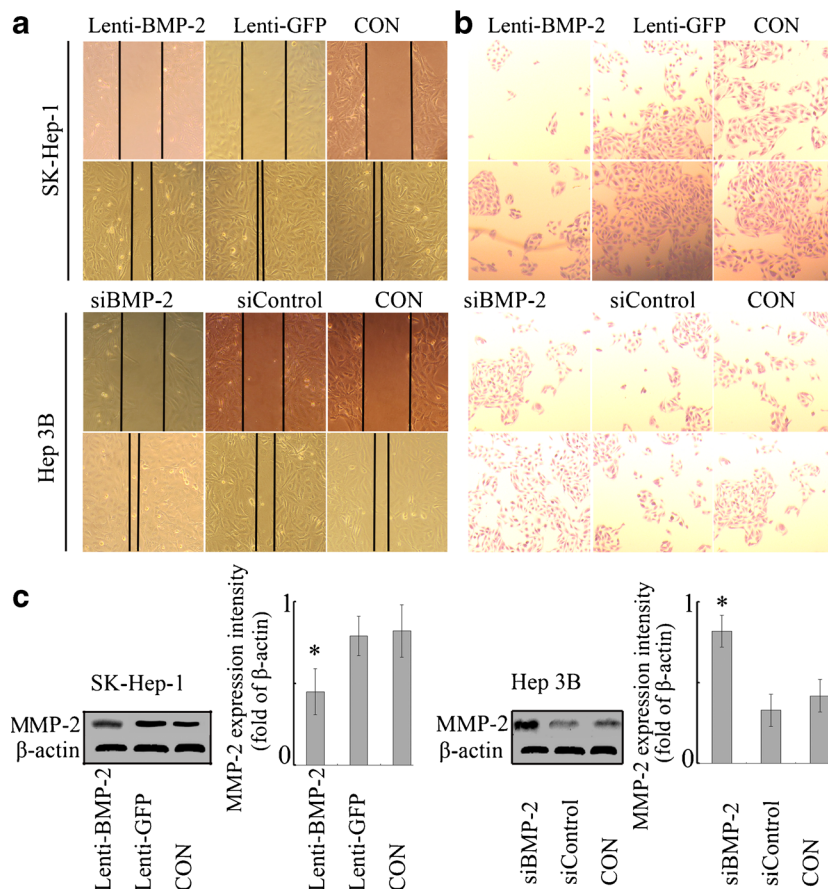
Fig. 3 Inhibition of HCC cell migration and invasiveness by BMP-2 in HCC cell lines. **a** Wound-healing assay showed the migration of SK-Hep-1 cells in the Lenti-BMP-2 group was markedly lower than in the Lenti-GFP and CON groups. By contrast, a significant increase of migration capacity of Hep 3B in siBMP-2 group as compared with siControl and CON groups. **b** Transwell invasion assay for BMP-2 overexpression and knockdown cells. The ability in Lenti-BMP-2 group was distinctly decreased as compared with Lenti-GFP and CON groups. But, a significantly increased invasive capacity was observed in siBMP-2 group as compared with siControl and CON groups. **c** Western blot assay was performed to examine the effect of BMP-2 on MMP-2. The expression of MMP-2 protein was significantly inhibited in Lenti-BMP-2 group as compared with the Lenti-GFP and CON groups in SK-Hep-1 cell line ($*P<0.05$). MMP-2 protein in siBMP-2 group was higher than those in siControl and CON groups in Hep 3B cell line

Hep 3B cells in the siBMP-2 group was not altered, while the S phase fraction was elevated (Fig. 4a). No significant difference was observed between the siControl and CON groups.

The inhibition of apoptosis is a critical factor for tumor progression. Therefore, we assayed Annexin V-PE/PI staining by flow cytometry. In the presence of 10 % serum, there were similar percentages of apoptotic cells in each group, indicating that BMP-2 did not change the incidence of apoptosis in the absence of stress (data not shown). Notably, after 48 h of low serum (0.1 %) treatment, the percentage of apoptotic cells significantly increased in the Lenti-BMP-2 group relative to the Lenti-GFP and CON groups ($**P<0.01$) (Fig. 4b). However, the incidence of apoptosis for Hep 3B cells in the siBMP-2 group was lower than in the siControl and CON groups. It has been reported previously that low serum triggers apoptosis in Hep 3B cells [20, 21], and a similar effect was also observed under our assay conditions in response to 0.1 %

FBS. Indeed, the above data demonstrated that BMP-2 increased the susceptibility of SK-Hep-1 cells to apoptosis induced by low serum.

Overexpression of BMP-2 and its effect on cell cycle regulators and pro- and anti-apoptotic gene expression As is shown in Fig. 4a, the inhibitory effect of BMP-2 on cell growth appears to be due to G1 phase arrest. p21 (a negative regulator) and cyclin E (a positive regulator) are two key regulators of progression from G1 to S [22]. Thus, proteins levels of p21 and cyclin E protein levels in SK-Hep-1 cells were measured by Western blot. Overexpression of BMP-2 induced a rapid increase in p21 expression while inhibiting cyclin E expression (Fig. 5a), which is in accordance with previous results that demonstrated that an increase in p21 and a decrease in cyclin E expression leads to G1 arrest [23, 24].

Furthermore, the observed increase in apoptosis may be correlated with the upregulation of the caspase signaling

Fig. 4 Cell cycle distribution and cell apoptosis under low serum condition. **a** Cell cycle distribution was analyzed using flow cytometry analysis. In SK-Hep-1 cell line, cell-cycle kinetics demonstrated that overexpression of BMP-2 resulted in an increase in the percentage of cells in the G0/G1 phases, with a concomitant decrease in the number of cells in the S phase, as compared with the Lenti-GFP group and CON groups. By contrast, cell cycle kinetics that the G_0/G_1 phase of Hep 3B cells in siBMP-2 group was altered, while S-phase population fraction was increased. But, no significant differences were observed between siControl and CON groups. **b** The apoptosis of SK-Hep-1 and HOS cells was analyzed by flow cytometry (AnnexinV-PE/PI). After low serum cultured for 48 h, the apoptosis incidence in Lenti-BMP-2 group was significantly higher than that in Lenti-GFP group and CON groups ($*P < 0.05$), but no difference was found between Lenti-GFP group and CON groups ($P > 0.05$). However, the results showed that the number of apoptotic cells decreased in the siBMP-2 group as compared with siControl and CON groups
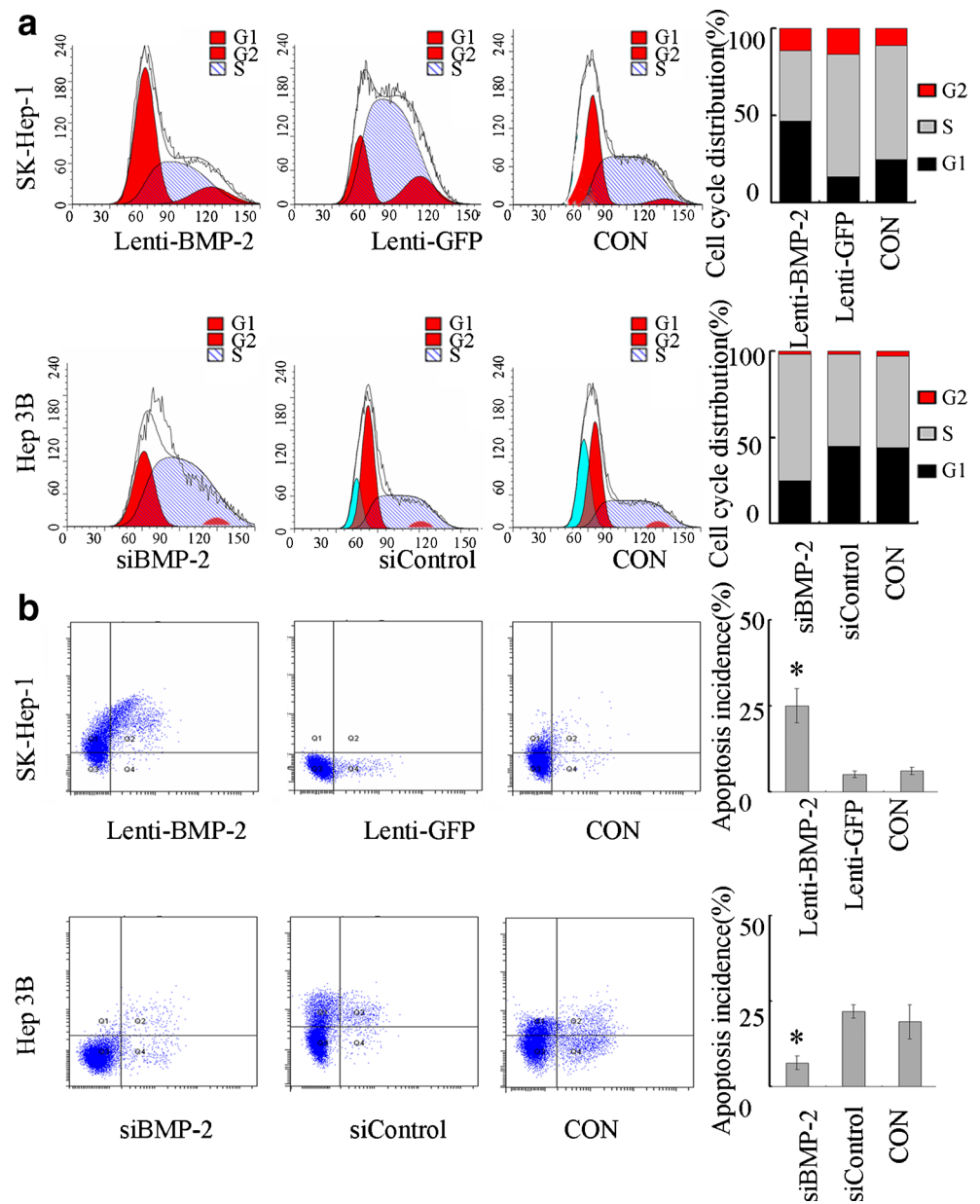
pathway or mitochondrial dysfunction. To confirm our hypothesis, a Western blot assay was performed to investigate the effect of BMP-2 overexpression on the endogenous expression of Bax, Bcl-2, caspase-3, and cleaved caspase-3 protein. As shown in Fig. 5b, expression of caspase-3 and cleaved caspase-3 was significantly increased in the Lenti-BMP-2 group ($*P < 0.01$ for each), although Bax and Bcl-2 expression was not altered, indicating that BMP-2 may induce apoptosis in HCC cells through upregulation of caspase-3 and cleaved caspase-3.

Effect of BMP-2 on p-AKT and PI3Kp85α expression Western blots were performed after 48 h of recovery to measure the expression of PI3Kp85α and p-AKT. As shown in Fig. 5c, Western blot revealed a marked decrease in PI3Kp85α and p-

AKT expression in the Lenti-BMP group relative to the Lenti-GFP and CON groups in the SK-Hep-1 cell line ($**P < 0.01$), while no difference was found between Lenti-GFP and CON groups in the SK-Hep-1 cell line ($P > 0.05$). However, knockdown of BMP-2 in Hep 3B cells resulted in an elevation in PI3Kp85α and p-AKT expression (Fig. 5c). These data indicated that BMP-2 inhibited cell HCC cell growth by blocking the PI3K/AKT signaling pathway.

Discussion

BMPs are multifunctional cytokines that regulate the growth, differentiation, and apoptosis of various cell types [25]. Importantly, recent studies also have demonstrated that BMP

Fig. 5 Overexpression of BMP-2 and its effect on cell cycle regulators and pro- and anti-apoptotic gene expression. **a** Western blot assay for cyclin E and p21 proteins in SK-Hep-1 cell line. The results showed that there was a decreased expression of cyclin E protein while increased expression of p21 in Lenti-BMP-2 group as compared with the Lenti-GFP and CON groups (*P<0.05). **b** Western blot assays for pro- and anti-apoptotic gene expression (Bax, Bcl-2, caspase-3, and cleaved caspase-3). The results showed that an obvious increases of caspase-3 and cleaved caspase-3 expression were observed in Lenti-BMP-2 group compared with the Lenti-GFP and CON groups (*P<0.05). But, no difference was found between Lenti-GFP and CON groups in SK-Hep-1 cell line (P>0.05). **c** Western blot assays for the exogenous expression of PI3Kp85α and p-AKT. The results showed that an obvious decrease of PI3Kp85α and p-AKT expression in SK-Hep-1 cells was observed in Lenti-BMP-2 group compared with Lenti-GFP and CON groups, while BMP-2 knockdown led to enhanced expressions of PI3Kp85α and p-AKT in Hep 3B cell line (*P<0.05)
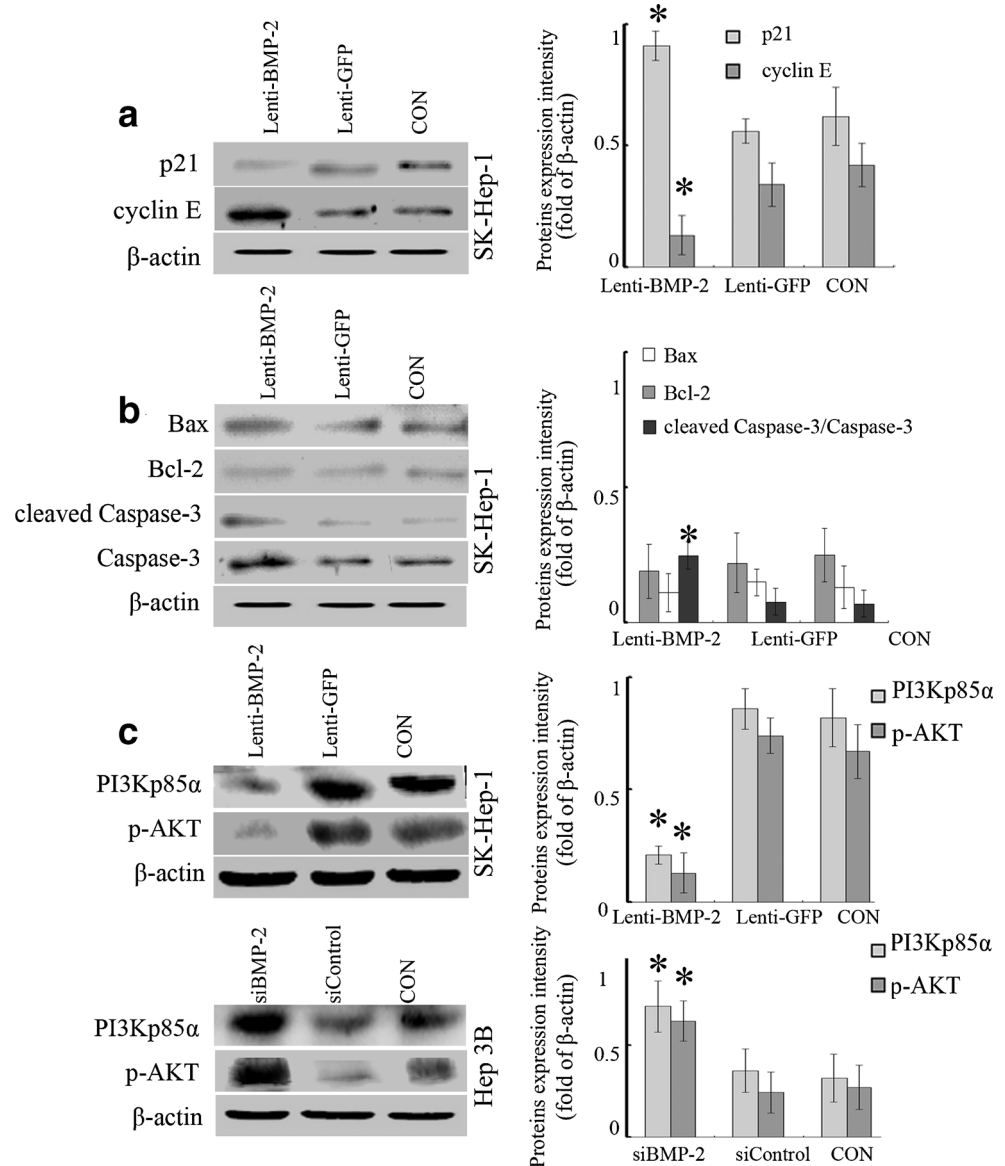
is one of the significant factors affecting the prognosis of bone tumor [26], lung cancer [6], and melanoma cancer [27]. Previous studies have suggested that abnormal expression of BMP-4 [28], BMP-7 [29], and BMP-9 [30] is correlated with tumor progress in HCC, but the role of BMP-2 in HCC has not yet been reported.

In the present study, we first analyzed the effects of exogenous BMP-2 on the proliferation of HCC cell lines. Based on previous research indicating that low doses of BMP-2 inhibit the tumorigenicity of breast cancer cells [17], we used similar doses of exogenous BMP-2 (0.1, 1, 10 μg/L) to analyze the effect of BMP-2 on the proliferation of HCC cell lines. These results consistently demonstrated that BMP-2 inhibited cancer cell growth in HCC cell lines in a dose-dependent manner. The inhibitory efficiency of BMP-2 was higher in the Hep G2 and Hep 3B cell lines than in the SK-Hep-1 cell line. This may

be because the expression of BMP receptors varies in different cell types.

We next assessed the endogenous expression of BMP-2 in the HCC SK-Hep-1, Hep G2, and Hep 3B cell lines. There was significantly high expression of BMP-2 in the Hep 3B cell line and low expression in the SK-Hep-1 cell line. Therefore, we successfully induced overexpression of BMP-2 in SK-Hep-1 and knocked down BMP-2 expression in Hep 3B. In our findings, we observed a significant reduction in the proliferative capacity of Lenti-BMP-2 SK-Hep-1 cells and an increase in proliferative capacity in the siBMP-2 Hep 3B cell line. Moreover, overexpression of BMP-2 inhibited the invasive and migratory capacity of SK-Hep-1 cells, while knockdown of BMP-2 promoted the invasive and migratory capacity of Hep 3B cells. We also noted a significant decrease in the expression of the cell proliferation marker PCNA in SK-Hep-

1 cells transfected with Lenti-BMP-2. Consistently, knockdown of BMP-2 gave rise to an increase in PCNA expression in the Hep 3B cell line.

Two previous studies found that BMP-2 increased the sensitivity of cancer cells to apoptosis by upregulation of Bax [31, 32]. In contrast to the findings in our study, BMP-2 did not change the levels of Bax or Bcl-2. However, BMP-2 significantly increased the expression of the pro-apoptotic proteins caspase-3 and cleaved caspase-3, which indicated that an increase in apoptosis might be correlated with upregulation of the caspase signaling pathway by BMP-2. All the above data suggest that BMP-2 could be considered a tumor suppressor, which is consistent with the results from studies on human osteosarcoma [13] and breast cancer [17].

PI3K/AKT is a major pathway for malignant progression in various tumors. It is reported that PI3K/Akt signaling pathway was associated with the anti-apoptotic role of FAM9C gene in human hepatocellular carcinoma [33]. Furthermore, doxorubicin induces apoptosis through inhibition of the PI3K/AKT signaling pathway in HCC cells [34]. Another study has shown that tyroserleutide exerts an anticancer effect on the PI3K/AKT pathway in HCC cells [35]. Interestingly, knockdown of BMP-2 promotes cell growth and resistance to low-serum treatment in HCC cell lines, with a concomitant increase of PI3Kp85α and p-AKT. Furthermore, there was a marked reduction in PI3Kp85α and p-AKT expression in overexpression BMP-2 cells, suggesting that BMP-2 might inhibit HCC cell growth and migration via PI3K/AKT signaling.

Based on our findings, we reasoned that BMP-2 inhibited HCC cells growth and migration via PI3K/AKT signaling and induced apoptosis via upregulation of the caspase signaling pathway. This result is consistent with previous studies that have shown an inhibitory effect of BMP-2 on cancer cell growth [13, 17]. Obviously, the response to BMP-2 is not accordant among all cancers. The biological response of cancer cells to BMP-2 may depend not only on the particular cell type and the dose, but may also depend on the presence of other factors that are not yet defined. Therefore, further research using more cell lines and primary tumor is indispensable to confirm the findings of this study. In conclusion, our investigation revealed that BMP-2 exerts inhibitory effects on growth and migration of HCC cells possibly via blockade of the PI3K/AKT signaling. BMP-2 as a tumor suppressor may provide a novel approach to HCC treatment.

Conflicts of interest None

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