The Activation of MEK/ERK Signaling Pathway by Bone Morphogenetic Protein 4 to Increase Hepatocellular Carcinoma Cell Proliferation and Migration

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Abstract
Hepatocellular carcinoma (HCC) is one of the most common visceral malignancies worldwide, with a very high incidence and poor prognosis. Bone morphogenesis protein 4 (BMP4), which belongs to the TGF-β superfamily of proteins, is a multifunctional cytokine, which exerts its biologic effects through SMAD- and non-SMAD-dependent pathways, and is also known to be involved in human carcinogenesis. However, the effects of the BMP4 signaling in liver carcinogenesis are not yet clearly defined. Here, we first show that BMP4 and its receptor, BMPR1A, are overexpressed in a majority of primary HCCs and that it promotes the growth and migration of HCC cell lines in vitro. We also establish that BMP4 can induce HCC cyclin-dependent kinase (CDK1) and cyclin B1 upregulation to accelerate cell-cycle progression. Our study indicates that the induction of HCC cell proliferation is independent of the SMAD signaling pathway, as Smad4 knockdown of HCC cell lines still leads to the upregulation of CDK1 and cyclin B1 expression after BMP4 treatment. Using mitogen-activated protein/extracellular signal-regulated kinase (MEK) selective inhibitors, the induction of CDK1, cyclin B1 mRNA and protein were shown to be dependent on the activation of MEK/extracellular signal-regulated kinase (ERK) signaling. In vivo xenograft studies confirmed that the BMPR1A-knockdown cells were significantly less tumorigenic than the control groups. Our findings show that the upregulation of BMP4 and BMPR1A in HCC promotes the proliferation and metastasis of HCC cells and that CDK1 and cyclin B1 are important SMAD-independent molecular targets in BMP4 signaling pathways, during the HCC tumorigenesis. It is proposed that BMP4 signaling pathways may have potential as new therapeutic targets in HCC treatment. Mol Cancer Res; 10(3); 415–27. ©2012 AACR.

Introduction
Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide and the third most common cause of cancer-related deaths. Diagnosis of advanced stage of HCC is a devastating experience for both patients and family. More than 80% of HCC cases originate in developing countries (1, 2). Chronic infections with hepatitis B virus or hepatitis C virus are responsible for the majority of HCC cases in those countries, especially in Asia (3, 4). Other risk factors include prolonged dietary exposure to aflatoxin and cirrhosis associated with genetic liver diseases (5, 6). Although much is known about the biochemical and morphologic features of changes in normal hepatocytes which lead to HCCs in humans, histologic findings suggest that HCC involves a multistep carcinogenesis process. However, the molecular pathogenesis of HCC is still not fully understood, and many key issues still remain unresolved in regard to this disease (7–9).

Bone morphogenesis proteins (BMP), a large subgroup in the TGF-β superfamily, have been shown to mediate a variety of biologic functions, which regulate cell proliferation, differentiation, and the determination of cell fate (10, 11). In particular, BMP signaling has been implicated in the regulation of early liver development and has been shown to play a vital role in the determination of liver bud morphogenesis and to promote hepatoblast migration, proliferation, and survival (12, 13). BMP4 is made up of glycosylated and secreted cytokine, which can form a ligand-receptor complex with type I and type II receptors, but which favors type I (BMPRIA and BMPRIIB) rather than the type II receptors (BMPR2 and ACVRF2B; ref. 14). BMP4, which is also part of the TGF-β family, is a vital regulatory cytokine that functions throughout development in mesoderm induction to activate downstream response genes, primarily through the canonical SMAD signaling pathway (15). When dimeric
BMP4 binds to BMPR receptor complex, the type II receptors phosphorylate the type I receptors in their intracellular kinase domain and the receptor-specific SMAD1/5/8 are recruited onto the receptor complex and phosphorylated. The phosphorylated SMAD1/5/8 then binds to a co-mediator, SMAD4, to convey signals to the nucleus. This SMAD complex translocates to the nucleus and activates or suppresses BMP4-responsive gene expression (14–16).

Recent findings have revealed that TGF-β and BMPs are linked to a wide variety of clinical disorders and have been shown to regulate proliferation, migration, or invasion of carcinoma cells, derived from several organs (17). Recent studies have suggested that BMP2 is highly upexpressed in human lung cancer cells and stimulates tumor development and motility (18). The overexpression levels of BMP4, as evaluated in breast cancer and malignant melanoma cells, contribute to the promotion of cell invasion and migration (19–21). In addition, BMP9 has been reported to promote ovarian cancer cell proliferation through an autocrine mechanism (22). In primary HCCs, BMP4 has been reported to be upregulated in cancerous liver tissues by hypoxia (23); however, there is insufficient details of the molecular mechanisms of BMP4 prooncogenic functions, with regard to HCC.

In this study, we provide significant molecular and cellular evidence to support the assumption that the upregulation of BMP4 in HCCs promotes tumor proliferation and metastasis. First, we focused our attention on the expression of BMP4 and its function during hepatic carcinogenesis and showed that BMP4 activates the mitogen-activated protein/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, instead of the SMAD pathway, to promote the proliferation and migration of human HCC cells. In particular, the downstream signaling effectors cyclin-dependent kinase (CDK)1 and cyclin B1, which mediate the increase in HCC cell growth stimulated by BMP4, were identified. These findings provide a better understanding of the effects of BMP4 signaling in clinically relevant diseases and could be crucial in the development of effective diagnostic and therapeutic strategies for HCC.

Materials and Methods

Cell culture, tumor tissues, inhibitors, RNA isolation, and cDNA synthesis

The HCC cell lines, HepG2 and Hep3B, were provided by K.-K. Kuo (Kaohsiung Medical University, Kaohsiung, Taiwan) and 293T cells were obtained from Dr. W.C. Hung (National Sun Yat-sen University, Kaohsiung, Taiwan). The cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), and supplemented with 10% FBS, nonessential amino acids, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO2 incubator. Seventy-one pairs of HCC tumors and the adjacent benign livers were available for the study. The tumor samples, with the matched adjacent benign tissue, were collected during surgical resections at the Kaohsiung Chung-Ho Hospital Clinic, between 2008 and 2011. The surgically resected tumor samples were immediately snap-frozen and shipped within 24 hours in dry ice and subsequently stored in liquid nitrogen. The study was approved by the Institutional Review Board at the Kaohsiung Medical University. Sections from each specimen were examined by pathologists and graded histologically. Inhibitors treatment, RNA isolation, and cDNA synthesis from the cell lines and tumor samples were carried out using previously described procedures (24, 25).

Lentivirus production and short hairpin RNA for gene knockdown

All the plasmids required for short hairpin RNA (shRNA) lentivirus production were purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. The pLKO.1-shRNA vectors used for knockdown of SMAD4, BMPR1A are as follows: TRCN0000010332 and TRCN0000010312 (SMAD4, NM_005359); TRCN0000194749, TRCN000000454 and TRCN0000000795 (BMPR1A, NM_004329). The pLKO.1-shEGFP control plasmid is TRCN0000072190 (enhance GFP, EGFp). The Lipofectamine 2000 reagent (Invitrogen) was used for lentiviral production in 293T cells, with a packaging construct (pCMV-ΔR8.91), an envelope construct (pMD.G) and different shRNA or rescue constructs, according to the protocol on the RNAi Core website (http://rnti.genmed.sinica.edu.tw/protocols).

Western blot analysis and immunofluorescence

For Western blot analysis, cells were harvested in RIPA lysis buffer [150 mmol/L NaCl, 10 mmol/L Tris, pH 7.5, 1% NP40, 1% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)]. The proteins from total cell lysates were resolved by the 10% Bis-Tris gradient gel (Invitrogen), transferred to the polyvinylidene fluoride membrane, blocked in 5% non-fat milk in PBS/Tween-20, and blotted with the antibodies. The following primary antibodies were used: BMPR1A (SC-20736), Smad4 (SC-7966), Smad1/5/8 (SC-6031-R), p-Smad1/5/8 (#9511), p-p44/42 (#9101), cyclin B1 (SC-752), and CDK1 (SC-54; Santa Cruz and Cell Signal); BMP4 (NCL-BMP4l 8); Smad1/5/8 are recruited onto the receptor complex and phosphorylated. The phosphorylated SMAD1/5/8 then binds to a co-mediator, SMAD4, to convey signals to the nucleus. This SMAD complex translocates to the nucleus and activates or suppresses BMP4-responsive gene expression (14–16).
BMP4 Increases HCC Cell Proliferation and Migration

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<th>Table 1. Comparison of clinicopathologic features and the status of BMP4 mRNA expression in 71 patients with HCC</th>
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*Categorized parameters were compared using Fisher’s exact test.*

Cell proliferation assay

For cell growth assay, 2 × 10^3 cells were seeded in 24-well plates and incubated overnight. The cells were treated with or without BMP4 (10–30 ng/mL; R&D) and incubated for 1 to 5 days. Twenty-five microliters of MTT (5 mg/mL; PROTECH) in 500 μL medium was then added and incubated for another 2 hours for reaction. The medium was removed and crystal was completely dissolved with 200 μL dimethyl sulfoxide (Sigma). The OD_{570} reading was then detected with a BioTek ELISA reader (Level).

Colonies formation assay

A total of 10 × 10^3 cells were grown in 60-mm tissue culture dishes. After 14 days, the cells were washed with PBS and fixed with methanol and 0.1% crystal violet. The colonies were manually counted and then photographed.

**In vitro migration and invasion assay**

For Transwell migration assays, 1 × 10^5 to 2 × 10^5 cells were plated in the top chamber, with the noncoated filter membrane (6-well insert; pore size, 8 μm; BD Biosciences). Cells were plated in medium without serum or growth factors and the bottom medium supplemented with 10% FBS. PBS was used as a chemoattractant in the lower chamber. The cells were incubated for 24 hours and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were counted under the microscope (100×) and stained with the crystal violet, at which point, the representative images were captured. The crystal violet was further dissolved in 10% acetic acid, and the absorbance was measured at 540 nm for quantitative analysis.

**Fluorescence-activated cell-sorting analysis**

Cells that were subjected to designated treatments were trypsinized, harvested and collected, washed 3 times in PBS, and then resuspended in PBS. Cells were then fixed in 70% ice-cold ethanol, for a minimum of 2 hours, and were ethanol eliminated by washing 3 times in PBS. Cell pellets were resuspended in PBS to adjust the cell concentration to about 1 × 10^6/mL and RNase to 20 μg/mL and propidium iodide (PI) to 50 μg/mL. Incubation was then carried out for 2 hours at room temperature, in the dark, for staining. Finally, the cells were analyzed by FACSscan (Becton Dickinson), using the Cell Quest Program.

**SCID mice and injection**

Specific pathogen-free, 8-week-old female C.B17/Scid-SCID mice were purchased from BioLASCO Taiwan Co., Ltd, for the *in vivo* tumorigenicity study. The animals were maintained in the animal center at the Department of Medical Research, Kaohsiung Medical University Hospital, and treated according to the institutional guidelines for the care and use of experimental animals. They were housed under controlled lighting (14 hours of light, 10 hours of darkness) at temperatures of 21°C to 22°C and provided with water and NIH-31 laboratory mouse chow ad libitum. The mice were injected subcutaneously into the left and

experiments were independently repeated 3 times and each treatment consisted of triplicate samples.

**Transient transfections and luciferase reporter assays**

Cells of 60% confluence in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen). Firefly luciferase reporter gene construct (200 ng) and 1 ng of the pRL-SV40 Renilla luciferase construct (for normalization) were cotransfected per well. The transfected cells were allowed to grow overnight, prior to BMP4 (R&D Systems) treatments, and the cell extracts were then prepared 24 to 48 hours after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay Kit (Promega), according to the manufacturer’s protocol, using OrionII Microplate Luminometer (Berthold Technologies) at 570 nm. Expression was calculated as the ratio of arbitrary firefly luciferase units, normalized to Renilla luciferase, and were described in detail previously (26). These experiments were independently repeated 3 times and each treatment consisted of triplicate samples.

**Wound-healing assay**

Cells were grown to 90% of confluency and a wound was introduced using a sterile Q-tip. The ability of cells to migrate was monitored at different time points using a light microscope under different conditions. Images were captured using a Zeiss Axiocam digital camera to monitor the cell migration rate.
right flank with $1 \times 10^6$ cells in 0.1 mL and raised during the following 2 to 3 months. The mice were then monitored for tumor volume, overall health, and total body weight. The size of the tumor was determined by caliper measurement of the subcutaneous tumor mass. Tumor volume was calculated according to the formula $4/3\pi r_1^2 r_2$ ($r_1 < r_2$). Each experimental group contained more than 3 mice. All mice were killed at the end of 3 months, and the tumor volumes and weight were then measured.

**Mice surgery, necropsy, histopathology, and immunohistochemistry**

Tissue samples were fixed in 10% buffered formalin for 12 hours, followed by a wash with PBS, a transfer to 70% ethanol, and then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Immunohistochemical (IHC) analysis for BMP4 and BMPR1A was conducted, as previously described (25).

**Statistical analysis**

Data are presented as mean ± SEM. Student $t$ test (2-tailed) was used to compare the 2 groups ($P < 0.05$ was considered significant), unless otherwise indicated ($\chi^2$ test).

**Results**

**BMP4 and BMPR1A are upregulated, whereas BMPR1B is downregulated in HCC**

We first investigated the relative mRNA expression level of BMP4 in 71 pairs of human HCC specimens, using real-time quantitative PCR (qPCR) analysis, and found that mRNA levels of BMP4 were significantly upregulated in more than 60% of HCC specimens compared with those of corresponding noncancerous liver tissues (Fig. 1A). All tumor diagnosis, and further subclassification, were confirmed by light microscopic examination of the H&E-stained sections by pathologists at Kaohsiung Medical University Hospital (Table 1). To confirm the results of real-time qPCR, BMP4 protein expression in HCC in situ was further evaluated using IHC staining, with a specific antibody against BMP4. The results also reveal that BMP4 protein is overexpressed in the epithelial cells of HCC specimens compared with the corresponding noncancerous livers [Fig. 1B, (iii) and (vi)].

The type I receptors of BMPs, BMPR1A (ALK3, BRK1) and BMPR1B (ALK6, BRK2), have been identified as being involved in conducting the BMP4 signaling to the cell. We further evaluated the expression pattern of BMPR1A and BMPR1B in HCC specimens using real-time qPCR analysis, and our results showed that upregulation of BMPR1A was observed in 30 of the 71 (43%) examined HCC specimens; however, the relative expression level of BMPR1B was decreased in 71 human HCC specimens, in comparison with that of corresponding noncancerous livers (Fig. 1A, bottom and Supplementary Fig. S1). Additional IHC analysis confirmed the upregulation of BMPR1A protein in human HCC specimens with a specific antibody against BMPR1A, and the IHC sections revealed that the immunosignal of BMPR1A was highly detectable in many HCC specimens in situ but mildly detectable in the corresponding noncancerous livers [Fig. 1B (iv) and (vi)].

**BMP4 induced CDK1 and cyclin B1 expression to promote HCC proliferation**

Many studies, including our reports, have shown that BMP4 overexpression occurs in many cancers, including HCC, and may be associated with tumor stages and poor prognosis (25). However, the exact molecular mechanism for BMP4-induced HCC tumor promotion is not yet fully understood.

Human HCC cells, HepG2 and Hep3B, were treated with recombinant BMP4 protein (10–30 ng/mL) to test the effects of BMP4 on HCC cells. Using a light microscope, it was observed that HCC cell populations showed an increase in absolute numbers, during 10 ng/mL BMP4 treatment for 48 hours. The growth rate was increased after BMP4 treatment compared with controls during a period of 5 days of treatment for cell proliferation analysis (Fig. 2A and B). The expression of cell-cycle-associated genes that may be involved in BMP4-induced HCC cell growth was then screened, using real-time qPCR analysis. Western blot analysis was used to evaluate and confirm the protein expression of targets (Fig. 2C).

The results indicate that the expression levels of cyclin B1 and CDK1 were increased in both HepG2 and Hep3B cells, after BMP4 treatment (Fig. 2C). Cyclin B1 and CDK1 specifically control the G2–M phase transition of the cell cycle and are key components in the control of cell-cycle progression. Several studies have shown that high levels of cyclin B1 promote cell-cycle progression and division. We then conducted immunostaining analysis with the anti-p-histone3 antibody to analyze the mitotic index in HepG2 and BMP4-treated HepG2 cells, as the histone, H3, is phosphorylated at Ser10 by active CDK1 during cell mitosis. p-Histone3-stained cells were photographed, and their numbers estimated by counting the number of p-histone–positive signals by using a fluorescence microscope, as p-H3–stained positive cells can present as mitotic cells. An increase in the number of p-histone3+ cells was observed in BMP4-treated HepG2 cells (Fig. 3A). An additional study, using Ki-67 immunofluorescence staining, supports the findings of the p-histone3 assays (data not shown). The cell-cycle progression was also confirmed, using multiple parameters, via flow cytometric analysis. Our results confirm an increase in the number of cells in S-phase and a decrease in the percentage of viable G1 phase in the cell cycle, after recombinant BMP4 treatment (Fig. 3B).

Further analysis of the protein expression patterns of cyclin B1 and CDK1 was undertaken. To determine the patterns of cyclin B1 and CDK1 expression at different times during the cell cycle, the cells were tracked and collected for up to 24 hours after serum-starved (asynchronous) treatment. The representative pattern of cyclin B1 and CDK1 protein expression was analyzed by Western blot analysis, as shown in Fig. 3C. Our results show
that BMP4 induces the earlier expression of cyclin B1 and CDK1 to promote cell-cycle progression in HCC. This implies that BMP4 promotes cell division and accelerated cell-cycle progression, by upregulation and activation of cyclin B1 and CDK1.

**Smad4 deficiency leads to increased expression of CDK1 and cyclin B and enhances cell proliferation, especially in the presence of BMP4**

To determine the molecular pathway involved in BMP4-stimulated HCC cell proliferation, we first investigate the effect of canonical SMAD-dependent signaling on BMP4-induced HCC cell proliferation. Intact BMP4-induced SMAD signaling in HCC cells was first examined. We then conducted Smad-binding element-driven reporter plasmid (SBE4-Luc) assays on the HCC model cell lines to determine whether SMAD-dependent signaling is activated by treatment with BMP4. The results show that luciferase activity was significantly increased, by 2- to 3-fold, in HCC cells after stimulation with recombinant BMP4 (10 ng/mL) for 18 hours. To further confirm that the SMAD signaling pathway was intact and could respond to BMP4 treatment, we conducted Western blot analysis for detecting the status of phospho-Smad1/5/8 proteins in HCC cell lines to examine the BMP4-induced serine/threonine kinase receptors to phosphorylated Smad1/5/8 response, which lead to intracellular activated Smad1/5/8 binding to Smad4. The results confirm that treatment with BMP4 increases the level of phosphorylation of Smad1/5/8 in HCC cells, which indicates the intact functioning of the BMP4/SMAD signaling pathway in our HCC cell lines (Supplementary Fig. S2).

To evaluate whether the status of BMP4/SMAD-mediated signaling has an effect on BMP4-induced HCC cell proliferation using established shRNA techniques, we successfully generated stable SMAD4-knockdown HepG2 clones for comparison with wild-type parental cells. The effects of SMAD4 shRNAs were verified by Western blot analysis, which showed that SMAD4 protein was suppressed by 80% in Smad4 shRNA HepG2 stable clones (Fig. 4A). The results showed that there were increased levels of expression of cyclin B1 and CDK1 upon SMAD4 knockdown, as verified with real-time qPCR and Western blot analysis of those HepG2 cells [Fig. 4B (i)]. Similar

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**Figure 1.** The increased expression of BMP4 and BMPR1A in patients with HCC. A, real-time qPCR analysis of mRNA expression in liver tumor tissues and normal controls, BMP4 (top) and BMPR1A (bottom). B, H&E staining in liver tissues. i, normal tissue (N); ii, tumor tissue (T). Representative IHC staining in normal liver tissues and HCC. iii and v, BMP4; iv and vi, BMPR1A (magnification, 200×).
observations were confirmed in experiments that depleted SMAD4, using different SMAD4 shRNAs that corresponded to a different coding sequence of the SMAD4 gene (data not shown). In further tests, we added recombinant BMP4 protein to SMAD4-knockdown HepG2 clones. It was observed that the expression of cyclin B1 and CDK1 were still upregulated in those SMAD4-knockdown cells compared with those SMAD4-knockdown groups not subjected to treatment with recombinant BMP4 [Fig. 4B (ii)].

Next, we examined the cell growth rates of HepG2 SMAD4-knockdown clones, and the corresponding mock control, with or without recombinant BMP4 treatment, by cell proliferation assay during the 5 days of treatment. The data are consistent with the molecular studies, which clearly indicate that the BMP4/SMAD-dependent signaling cascade is not involved in the BMP4 stimulation of HCC tumor proliferation (Fig. 4C, top, middle, and bottom).

Inhibition of MEK/ERK signaling pathway efficiently decreases the action of BMP4, which induced the increased expression of CDK1 and cyclin B1 in HCC

TGF-β and BMP signaling are composed of conical SMAD-dependent pathways and SMAD-independent pathways, including those involving MEK/ERK, c-jun-NH2-kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways. To investigate the effect of the MEK/ERK signaling pathway on BMP4-induced HCC cell proliferation, we used the selective inhibitor, PD98059, to inhibit MEK/ERK signaling transduction in HCC cells. We first showed that the activation of p42/44 (ERK1/2) was detected, using Western blot analysis for phospho-p42/44 (p-ERK1/2). The results show that the expression levels of phospho-p42/44 (ERK1/2) in cells treated with recombinant BMP4 were higher than those for the controls, as shown in Fig. 5A, which indicates that BMP4 can stimulate the phosphorylation of p42/44 (ERK1/2) in HCC cells.
We then added the selective MEK/ERK inhibitor, PD98059, to cells combined with BMP4 treatment. The results of real-time qPCR analysis imply that the selective MEK/ERK inhibitor, PD98059, inhibited the level of the BMP4-induced overexpression of cyclin B1 and CDK1 in HepG2 and Hep3B cells [Fig. 5B and C (i) and (ii)]. The protein expression of cyclin B1 and CDK1 was further determined by Western blot analysis [Fig. 5C (iv)]. We also examined the influence of the p38 MAPK pathway inhibitor on BMP4-induced HCC cell proliferation. Using p38 MAPK inhibitor, SB203580, we aimed to determine whether the p38 MAPK pathway was involved in BMP4-induced HCC cell-cycle progression and cell proliferation. Our data suggested that the p38 MAPK pathway was not required for the BMP4-induced upregulation of CDK1 and cyclinB1 in HCCs (Supplementary Fig. S3).

The results indicate an important role for the MEK/ERK pathway in BMP4-induced upregulation of cyclin B1 and CDK1 in HCCs and increases in HCC cell proliferation. The inhibition of MEK/ERK, but not of the p38 MAPK pathway, strongly impaired the BMP4-induced upregulation of cyclin B1 and CDK1 and inhibited HCC cell proliferation.

**BMPR1A-knockdown HCC cells are significantly less tumorigenic in vitro and in vivo**

To study the effects of BMP4 signaling in HCC, we conducted a colony formation assay, which is considered to be a stringent assay for the measurement of cellular transformation and tumorigenic ability in vitro. First, we produced BMPR1A-knockdown HepG2 cell clones, using shRNA knockdown techniques, to examine
their effect on tumorigenicity in HepG2 cells in vitro. The knockdown efficiency was confirmed by Western blot analysis (Fig. 6A). The BMPR1A-knockdown HepG2 cells showed a significant reduction in the number of colonies formed on the Petri dish compared with scrambled shRNA-EGFP-transfected controls, confirming the requirement of active BMP4 signaling for HCC growth (P < 0.01; Supplementary Fig. S4).

To further investigate the effect of BMP4 signaling on HCC carcinogenesis in vivo, BMPR1A-knockdown HepG2 cells were injected into the flanks of 2 severe combined immunodeficient (SCID) mice subcutaneously. Lentiviral vector bearing shRNA (shEGFP) were used as controls to investigate the overall effect of BMP4 signaling on HCC progression. As expected, BMPR1A-knockdown HepG2 cells showed less tumorigenicity in vivo compared with the control groups. Our results showed that tumor size and weight were clearly reduced compared with those of the controls (Fig. 6B, left and right). The Western blot analysis from those tumor samples verified that BMPR1A protein expression was reduced in the knockdown tumor samples compared with the controls (Fig. 6C).

BMP4 type IA receptor knockdown inhibits the MEK/ERK signaling pathway and attenuates BMP4-induced HCC cell migration

A recent study has reported that hypoxia can induce BMP4 to promote the migration of HCC cells. It confirmed the inhibitory effect of cell migration of HCC cells treated...
with BMP4 siRNA compared with cells treated with control siRNA (23). We also observed that BMP4 signaling stimulates HCC cell migration, possibly through the MEK/ERK signaling cascade.

To investigate the molecular mechanisms of BMP4-induced activation of HCC cell migration, BMPR1A-knockdown HepG2 clones were chosen to examine the extent of cell migration by wound-healing assays. We

Figure 5. Inhibition of MEK-ERK signaling pathway attenuates BMP4-induced increased expression of CDK1 and cyclin B1. A, Western blot analysis for p-p42/44 (p-ERK1/2) to confirm the MEK/ERK signaling cascades. B, using selective inhibitors, SB203580 (p38; 10 μg/mL) and PD98059 (MEK/ERK; 10 μg/mL), to identify the effective pathways after BMP4 (30 ng/mL) treatment. Real-time qPCR analysis for detecting the expression of CDK1 mRNA in HepG2 cells after treatment with different inhibitors. C, the PD98059 inhibits the BMP4-mediated CDK1 and cyclin B1 expression. i and iii, HepG2; ii and iv, Hep3B cells. C, control.

Figure 6. BMPR1A-knockdown HepG2 cells are significantly less tumorigenic in vivo xenograft studies. A, Western blot analysis confirmed the efficiency of BMPR1A-knockdown in HepG2 cells. Three shRNA-BMPR1A HepG2 stable clones were analyzed for BMPR1A protein expression and selected the knockdown clone no. 3 to inject into SCID mice (n = 3) subcutaneously for in vivo tumor formation study. B, top, gross tumor formation of shRNA-BMPR1A knockdown and control shEGFP HepG2 cells. Bottom left, average tumor weights of shRNA-BMPR1A groups were significantly less than the control groups. Bottom right, average tumor volumes of shRNA-BMPR1A groups were smaller than the control (P < 0.01). C, control. C, the decreased BMPR1A protein expression was detected in knockdown of BMPR1A in xenograft tumors by Western blot analysis.
observed that the time taken for BMPR1A-knockdown HepG2 cells to migrate into the cell free areas, and completely close the wound, was longer than 48 hours, whereas the migration rate of HepG2 shEGFP cells was faster, regardless of the presence of BMP4 (Fig. 7B). The SMAD4 knockdown was also found to accelerate the motility of HCC cells (data not shown).

Using the MEK pathway selective inhibitor, PD98059, to block MEK/ERK signaling transduction in HepG2 and Hep3B cells, our data showed that selective MEK/ERK inhibitor efficiently blocked BMP4-triggered cell migration for 72 hours compared with that of control cells (Fig. 7A, left and right). It is hypothesized that BMPR1A knockdown may diminish the BMP4-induced MEK/ERK pathway and attenuate the effect of cell migration, triggered by BMP4 in HCC cells, and that the BMP4-induced MEK/ERK pathway might be involved in the migratory and invasive properties of HCC (Fig. 7C). However, a detailed understanding of BMPR1A action requires identification of molecular alterations and downstream genes regulated by BMPR1A receptor.

Discussion

The upregulation of BMP family members has been found in various human malignancies, including pancreatic, skin, and liver cancer (21, 23, 27). Several studies, of various cancer models, have indicated the importance of the activation of the BMP signaling pathway in tumor tissues during carcinogenesis, through autocrine and paracrine mechanisms, for the modulation of tumor cell growth, invasion, and metastasis (28–30). This study showed that BMP4 and BMPR1A were overexpressed in the majority of primary HCC specimens. Although there was no overexpression of BMP4 protein in the case of every HCC, we observed that BMP4-overexpressed patients accounted for a high proportion (60%) of our patients with HCC and that BMPR1A was overexpressed in more than 40% of patients, regardless of upregulation of their BMP4 levels. Immunohistochemistry reveals that the upregulation of BMP4 and BMPR1A in HCCs is primarily localized in the cytoplasmic compartment of tumor cells, in primary human HCCs. Our results also indicated that the upregulation of BMP4 protein may correlate with HCC tumor stages and microscopic peritumoral satellites, which are recognized as a risk factor for distant dissemination (Supplementary Table S2). Therefore, BMP4 and BMPR1A are ideal prognostic markers for HCC, as some cases of HCC progression and aggression are associated with this cytokine, BMP4, or its specific receptor, BMPR1A. In the case of BMPR1B expression, however, our results suggest that there is downregulated expression of BMPR1B in HCCs.

In general, BMPR1A and BMPR1B are similar in structure and are preferentially bound by BMP2 and BMP4, with differing affinities. Several studies also
suggest that BMPR1A and BMPR1B may have distinct functions in embryonic development. The loss of BMPR1A leads to embryonic lethality (31, 32), but it is still not possible to distinguish between unique or redundant functions of BMPR1A and BMPR1B in the later stages of human development. Germ line mutations of BMPR1A have been associated with juvenile polyposis in humans and produce a significantly higher risk of gastrointestinal cancer (33). BMPR1B homozygous null mice are viable; however, mutants exhibit skeletal defects (34), and BMPR1B is epigenetically silenced in some patient-derived tumor-initiating glioblastoma cell lines (35). A recent study indicated that lower BMPR1B expression was observed in breast cancer tissues with poor prognosis, and the decreased expression of BMPR1B resulted in increased cell proliferation in breast cancer cells in vitro, which suggested that BMPR1B mediated a repressive effect on breast cancer cells (36). The role of the downregulation of mRNA of BMPR1B in liver cancer cells, as determined by real-time-qPCR, is still unclear, and presents an opportunity for future research.

The role of BMP4 in tumorigenesis and cell-cycle progression in HCCs was unclear, prior to our studies. To determine whether the upregulation of BMP4 protein plays a critical role that favors tumor malignancy and a propensity for metastasis of HCC, we selected HCC cell lines model systems to investigate both the molecular and cellular alterations of HCCs in response to an increase in BMP4 levels. Our results indicated that, as with other BMP family members, BMP4 can function as an activator in cell-cycle progression in epithelial cancer cells and may be essential for the aggressive metastasis of tumor cells. We further identified that a function of BMP4 signaling can induce cyclin B1 and CDK1 overexpression in HCC cells. The complex kinase of cyclin B1 and CDK1 is an important mediator of the cell-cycle regulator, which accelerates the progress of mitosis (37–39). It was observed, through Ki-67 staining, that BMP4 triggered the synthesis of DNA, which is required for cell division, and promoted the phosphorylation of ser10 residue of histone3 known as a marker for mitotic progression (40). This study shows that BMP4 accelerates the growth of HCC cells by the upregulation of cyclin B1 and CDK1 to promote cell-cycle progression and cell division. Using BMPR1A shRNA, we also confirmed that the HepG2 cells significantly reduced the tumorigenicity in vitro, by colony formation assays and in vivo SCID mouse xenograft models. The migratory ability of HepG2 cells was also decreased by the BMPR1A knockdown in HepG2 cells, as verified by wound-healing assays. The overexpression of constitutively active BMPR1A (ALK-3 QD) receptor enhances HepG2 cells proliferation and migration, as well as exhibiting an increased cyclin B1 and CDK1 protein expression (Supplementary Fig. S5). It is proposed that the enforced BMP4 may contribute to progression and malignancy of HCC.

BMP4 belongs to the TGF-β superfamily. It is common knowledge that BMP4 regulates gene expression not only through the classical SMAD-dependent signaling pathway but also by the activation of many SMAD-independent signaling pathways. We determined the predominant pathway that mediates BMP4-induced HCC cell proliferation by knockdown of Smad4 protein expression to reduce the SMAD-dependent pathway in HepG2 cells and then confirmed that BMP4 induced phosphorylation of Smad1/5/8, and subsequently activated Smad4 downstream transcriptional activation in a SMAD reporter activity assay. Next, we used the selective MEK/ERK signaling pathway inhibitor, PD98059, to attenuate the BMP4-activated MEK/ERK pathway. On the basis of these studies, we found that further upregulation of the expression of cyclin B1 and CDK1 could result from Smad4 deficiency in HepG2 cells, whereas inhibition of the MEK/ERK signaling pathway efficiently attenuates BMP4-induced upregulation and maintained basal levels of cyclin B1 and CDK1 in HepG2 cells. MEK/ERK signaling is believed to play an important role in the regulation of cell proliferation and its signaling pathway is activated by different growth factors, including BMP4 cytokines (41, 42). Growth factor–induced MEK/ERK cascade is considered to be an inducer for the regulation of multiple phosphorylating proteins and their target proteins and to promote the interaction between retinoblastoma (Rb) protein and E2F-1 and to affect transcriptional regulation of target genes involved in cell-cycle control (43–45). E2F family–regulated genes are required for cell-cycle progression, depending on the activation status of MEK/ERK. The E2F-regulating genes include CDK1, Cdc25A, and cyclin B1, which are associated with cell-cycle progression (46, 47). Therefore, our findings suggest that BMP4 induces the increased expression of cyclin B1 and CDK1 to accelerate cell-cycle progression through the MEK/ERK pathway but not through the SMAD-dependent pathway in HCCs (Fig. 7C). It was also noted that the BMP4-induced phospho-level of Smad1/5/8, at 24 hours after BMP4 treatment, was lower than that at 1 hour after treatment. It is presumed that there was cross-talk between the 2 pathways in BMP4 activation, the MEK/ERK signaling pathway and SMAD-dependent pathway, during the 24 hours of the study. Recent studies have indicated that Smad1 is targeted to proteasome for degradation in response to BMP type I receptor activation and that p-Smad1 targeted for degradation to the centrosome is associated with MEK/ERK activation (48, 49). This may explain how the MEK/ERK pathway overcomes the SMAD-dependent signaling to become the predominant pathway during BMP4 activation in liver carcinogenesis. Our study of the BMP4–induced MEK/ERK pathway in HCC is consistent with the results of our previous work, which described the TGF-β1–mediated activation of MEK/ERK pathways, combined with Smad4 deficiency, to promote colorectal cancer malignancy (26, 50). We also determined that the MEK/ERK signaling pathway has a potential role in regulating the proliferation and migration of HCC cells.
In conclusion, our findings further support the notion that a BMP4 → BMPR1A → SMAD1/5/8 signaling axis acts as a tumor suppressor in HCC cells (16, 25, 26), and that the BMP4-induced MEK/ERK signaling pathway facilitates proliferation and migration of liver cancer cells and the upregulation of BMP4, as an aggressive enhancer for hepatocarcinogenesis, by stimulating the overexpression of cyclin B1 and CDK1 proteins in HCC cells. The results of the study suggest that the effects of BMP4 signaling, which contribute to HCC, are worthy of further investigation. They also show that the BMP4-induced MEK/ERK cascade in HCC tumors is an ideal therapeutic target for HCC treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References

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