**BMP4 Administration Induces Differentiation of CD133⁺ Hepatic Cancer Stem Cells, Blocking Their Contributions to Hepatocellular Carcinoma**

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Abstract

CD133⁺ cancer stem cells (CSC) contribute to hepatocellular carcinoma (HCC) progression and resistance to therapy. Bone morphogenetic protein BMP4 plays an important role in hepatogenesis and hepatic stem cell differentiation, but little is known about its function in hepatic CSCs. In this study, we showed that high-dose exogenous BMP4 promotes CD133⁺ HCC CSC differentiation and inhibits the self-renewal, chemotherapeutic resistance, and tumorigenic capacity of these cells. Interestingly, we found that low-dose exogenous BMP4 upregulated CD133 protein expression in vitro, and endogenous BMP4 was preferentially expressed in CD133⁺ HCC CSCs, suggesting that low doses of BMP4 may facilitate CSC maintenance. A reduction in endogenous BMP4 levels decreased CD133 protein expression in vitro. In HCC tissues, expression of the BMP4 signaling target gene SMAD6 was positively correlated with CD133 expression. Activation of the Erk1/2 signaling pathway led to BMP4-mediated reduction in CD133 expression, which was reversed by treatment with MEK inhibitors. Taken together, our findings indicated that BMP4 might be a potent therapeutic agent in HCC that targets CSCs.

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Introduction

Although surgery, radiation, and chemotherapy all result in reducing the bulk of the tumor mass, tumor recurrence and metastases are still universal (1). Recently, cancer progression has been thought to be driven by cancer stem cells (CSC), which have the ability to self-renew, show therapeutic resistance, and give rise to relatively differentiated cells (2). In hepatocellular carcinoma (HCC), several markers, such as EpCAM, OV6, CD90, CD24, and a side population fraction have been identified for the enrichment of hepatic CSCs (3–8). In our previous studies, we have shown that CD133⁺ CSCs in HCC cell lines were distinctive for their high clonogenicity in vitro and high tumorigenicity in an immunodeficient mouse xenograft model. In addition, this cell population could be further characterized by the coexpression of CD133 and CD44 (9, 10). The CD133⁺ CSCs in HCC exhibited a preferential expression of stem cell–related genes and were more resistant to chemotherapeutic agents as a result of the upregulation of ATP-binding cassette (ABC) superfamily transporters.

Bone morphogenetic proteins (BMP) have been linked to several aspects of embryonic liver development (11). BMPs are a subgroup of the TGF-β superfamily members, which elicit their cellular effects via specific membrane receptors. Different combinations of type II receptors (BMPRII) with type I receptors (BMPRIA or BMPRIB) determine the specificity for the ligands eliciting different biologic processes (12). The activated BMPRII phosphorylates receptor-regulated SMAD1/5/8 proteins, which can assemble into heteromeric complexes with SMAD4 and subsequently translocate into the nucleus to regulate the transcription of target genes (13). SMAD6 is one of the inhibitory SMADs, and its expression is induced by BMP-activated SMAD1/5/8, creating a negative feedback loop that interferes with BMP signaling (14).

BMP signaling has been shown to promote CSCs differentiation in the brain and colon and to facilitate brain or colon xenograft tumor eradication (15, 16). Therefore, BMPs have been proposed as a treatment option for glioblastoma and colorectal tumors. Here, we show that high-dose exogenous BMP4 exhibited potent differentiation therapy activity against CSCs in HCC; however, low-dose or endogenous BMP4 contributed to promote CD133 protein expression. Endogenous BMP signaling target gene, SMAD6 protein expression is positively correlated to CD133 protein expression in HCC. We also found that BMP4 could induce Erk1/2 activation in...
a time- and dose-dependent manner, and short-term exposure to high-level Erk1/2 phosphorylation is sufficient for BMP4-dependent CD133 expression reduction.

Materials and Methods

Cell culture

PLC/PRF/5 was obtained from the American Type Culture Collection; Huh7 was obtained from the Riken Cell Bank; SMMC-7721 was provided by the Cell Bank of the Institute of Biochemistry and Cell Biology, China Academy of Sciences (Shanghai, China). MHCC-97L was provided from the Liver Cancer Institute of Zhongshan Hospital, Fudan University (Shanghai, China). All cell lines used in this study were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich) containing 10% heat-inactivated FBS (HyClone) and incubated at 37 °C in a humidified atmosphere with 5% CO₂.

For differentiation-inducing experiments of CSCs in vitro, HCC cells were plated onto general 6-well plates (NUNC) in a monolayer culture with serum-free chemically defined medium (CDM; ref. 17). For BMP4 treatment, different amounts of BMP4 were added to the CDM to reach the indicated final concentration.

The CDM consisted of a 1:1 mixture of neurobasal medium and DMEM/F12 medium supplemented with 0.5× N2, 0.5× B27 supplements, 0.1% bovine serum albumin, 2 mmol/L glutamine, and 0.1 mmol/L 2-mercaptoethanol, growth factors including 10 ng/mL BMP4 (Sigma-Aldrich), 10 ng/mL basic fibroblast growth factor (Millipore), 10 ng/mL EGF (Millipore), 20 ng/mL hepatocyte growth factor (Millipore), and TGFβ (Millipore), and 10⁻⁷ mol/L dexamethasone (Sigma-Aldrich) were added. The agents with no special indication were purchased from the Invitrogen Corporation.

Cell isolation by fluorescence-activated cell sorting or magnetic activated cell sorting

For PLC/PRF/5 and Huh7, cells were labeled directly with PE-conjugated anti-human CD133/1 antibody (AC133; Miltenyi Biotec) according to the manufacturer’s instruction and sorted by fluorescence-activated cell sorting to obtain CD133⁺ and CD133⁻ cell subpopulations. For SMMC-7721 and MHCC-97L, which only contain fewer than 1% CD133⁺ cells, cells were magnetically isolated CD133⁺ and CD133⁻ cells with the corresponding antibodies using the EasySep PE Selection Kit (StemCell Technologies), according to the manufacturer’s instructions. The purity for sorted cells was evaluated by flow cytometry, and more than 90% of cells with viability determined by the trypan blue staining were acceptable for the following experiments.

Cell proliferation assay

Three thousand cells were plated in 96-well culture plates for 24 hours and were then treated with BMP4 at the indicated concentrations for the indicated times. Bromodeoxyuridine (BrdUrd) ELISA assay was carried out according to manufacturer’s manual (Roche Diagnostics). Optical density (OD) values were measured by an ELISA reader (Multiskan MK3; Thermo Scientific) at 450 nm.

Statistical analysis

The experimental data were presented as the mean ± SD and analyzed using the Student t test. P value less than 0.05 was considered statistically significant.

Results

High-dose exogenous BMP4 induces the reduction of CD133 expression in HCC

Liver development is regulated by several growth factors that may contribute to the differentiation of hepatic CSCs (18). Here, 2 HCC cell lines, PLC/PRF/5 and Huh7, with a relatively high percentage of CD133⁺ cells, were selected to further analyze the biologic effects of growth factors on CSCs (Supplementary Fig. S1A). The stemness of the CD133⁺ cell population was reported in previous studies or confirmed in preliminary experiments (Supplementary Fig. S1C and D), and the sorting purity was confirmed (Supplementary Fig. S1B).

Several growth factors related to liver development were screened to treat CD133⁺ PLC/PRF/5 cells for 3 days, and the percentage of CD133⁺ cells was determined by flow cytometry in the preliminary experiments. We found that the percentage of CD133⁺ cells decreased after BMP4 treatment (Supplementary Fig. S2A).

We then explored the effects of different doses of BMP4 on the differentiation of CSCs. CD133⁺ and CD133⁻ PLC/PRF/5 cells were cultured in a monolayer with serum-free CDM added with different doses of BMP4 ranging from 0 to 100 ng/mL for 6 days. We found that in CD133⁺ cells, 30 ng/mL of BMP4 could induce the downregulation of CD133 expression, and the downregulation was most obvious after stimulation with 100 ng/mL BMP4 (Fig. 1A). Subsequently, we found that a 50 ng/mL dose showed a sufficient reduction effect and was selected for further investigation. The BMP4 treatment exhibited no special effect on the CD133⁻ cell population. We investigated the effects of BMP4 on these cell lines over time. As shown in Fig. 1B, CD133 expression began to decrease on day 3 in PLC/PRF/5 and reached its lowest level on day 6. A similar result was obtained in Huh7 cells. These data indicated that BMP4 induced CD133 depression in HCC cell lines in a time- and dose-dependent manner. Flow cytometric analysis also revealed that the percentage of CD133⁺ cells had decreased by nearly 40% after BMP4 treatment for 6 days, with an initial 52.1% in PLC/PRF/5 that decreased to 32.9%, and 73.0% in Huh7 that decreased to 47.5% (Fig. 1D).

To determine the effect of exogenous BMP4 on the cell proliferation and apoptosis in HCC, we incubated the cells with different doses of BMP4 in the media and found that although lose-dose BMP4 (10 ng/mL) increased the cell proliferation in PLC/PRF/5, high-dose BMP4 (50–100 ng/mL) inhibited cell growth (Fig. 1C). The cell apoptosis was not significantly influenced after high-dose BMP4 treatment (Supplementary Fig. S2B).

BMP4 could induce the differentiation of HCC CSCs

Cancer cells cultured in suspension may form spheroids that have been shown to be an efficient way to enrich CSCs. The number and size of the spheres may reflect the self-renewal capability of these cell populations (19). Here, sorted CD133⁻/CD133⁺
PLC/PRF/5 cells were cultured in suspension with serum-free medium CDM to maintain stemness or in monolayers with the addition of BMP4 to induce differentiation. HCC cell spheres were obtained by growing undifferentiated cells in the CDM, suggesting that they maintained the capacity for self-renewal in vitro (data not shown). Real-time reverse transcriptase PCR analysis showed that the expression of stem cell–associated genes, including Oct4, Tert, Bmi1, β-catenin, ABCG2, and tumor sphere–related gene Ep300 (4), in CD133+ cells were reduced following BMP4 treatment, although the E-cadherin protein coding gene CDH1 expression was not significantly affected. Conversely, the expression of these genes was not affected or was only slightly reduced in CD133−/C0 cells (Fig. 2A), indicating that BMP4 mainly targeted the CD133+ CSCs pool of HCC.

Cytokeratin 19 (CK19) has been used as a marker for biliary epithelial cells, and CK19 is closely correlated with the clinicopathologic features of HCC aggressiveness (20). After BMP4 treatment, PLC/PRF/5 CD133+ CSCs displayed a time-dependent downregulation of CK19 expression (Fig. 2A). CK8/18 are hepatocyte-specific markers (21). We found that BMP4 treatment could also induce a time-dependent enhancement of CK8/18 expression in the CD133+ CSCs (Fig. 2B). In addition, CK19 expression in the CD133− cells was largely unaffected, whereas CK8/18 was clearly upregulated after BMP4 treatment for 6 days. Immunofluorescence staining results showed that the number of CK19-positive cells decreased, and CK8/18-positive cells increased after BMP4 treatment (Fig. 2C). These results were confirmed in Huh7 (Supplementary Fig. S2C) and MHCC-97L (Supplementary Fig. S2D), which indicated that high-dose exogenous BMP4 induced CD133+ PLC/PRF/5 CSC differentiation and increased their expression of hepatocyte lineage marker.

**BMP4 inhibits the self-renewal and tumorigenic capacity of HCC CSCs**

Sorted CD133+/− PLC/PRF/5 cells were cultured in suspension in CDM or in monolayers, and BMP4 was added to observe their differentiation for 6 days. To evaluate the alteration of the self-renewal capability of these cells, we first compared their clonogenicity by anchorage-independent growth assays in soft agar. The results showed that the number of CK19-positive cells decreased, and CK8/18-positive cells increased after BMP4 treatment (Fig. 2C). These results were confirmed in Huh7 (Supplementary Fig. S2C) and MHCC-97L (Supplementary Fig. S2D), which indicated that high-dose exogenous BMP4 induced CD133+ PLC/PRF/5 CSC differentiation and increased their expression of hepatocyte lineage marker.
from treated CD133\(^+\) cells were fewer in number and smaller than those of CD133\(^+\) cells continually cultured in CDM. These results indicated that the self-renewal capability of CD133\(^+\) HCC CSCs was impaired after differentiation.

The tumorigenicity of CD133\(^+\)/C\(_0\) cells with and without BMP4 treatment was analyzed in an immunodeficient mouse xenograft model. The orthotopical inoculation of freshly sorted CD133\(^+\)/C\(_0\) PLC/PRF/5 cells was accompanied by the coinjection of vehicle (control)- or BMP4-saturated polyacrylic beads [releasing BMP4 for 1 week (16)] at the time of cell implantation. And to ensure the xenograft tumors generation, large cell number was used. In these experiments, 5 of 6 animals that received CD133\(^+\) PLC/PRF/5 cells with control beads developed large tumors (Fig. 3C), whereas the mice bearing CD133\(^+\) cells with BMP4-releasing beads did not form visible tumors (Supplementary Fig. S3A and D). Although CD133\(^+\)/C\(_0\) cells formed fewer (3 of 6 mice) and smaller tumors than CD133\(^+\) cells (Fig. 3D), the tumorigenicity was not greatly affected by BMP4 cotransplantation (2 of 6 mice). Similar results were obtained with the MHCC-97L cell line, which exhibited a low percentage of CD133\(^+\) cells (Fig. 3C and D, and Supplementary Fig. S3B). Thus, BMP4 also possessed antitumor effects \textit{in vivo}, suggesting that BMP4 might be a useful component of the differentiation therapy for HCC.

BMP4 enhances the sensitivity of HCC CSCs to chemotherapeutic drugs \textit{in vitro}

Another important property of CSC is their resistance to chemotherapeutic agents. In this study, 2 structurally and functionally unrelated drugs, doxorubicin and vincristine, were used to evaluate the drug resistance attributes of BMP4-treated and untreated HCC cells. As shown in Fig. 4A, CD133\(^+\) PLC/PRF/5 cells showed marked increases in their resistance to doxorubicin or vincristine compared with CD133\(^+\)/C\(_0\) cells, and BMP4 treatment consistently increased their sensitivity to these 2 agents.

On the basis of our previous findings that HCC CSCs were more resistant to chemotherapeutic agents as a result of the upregulation of the superfamily of ABC transporters, we analyzed ABCG2 expression in PLC/PRF/5. We found clear reductions in ABCG2 expression after BMP4 treatment (Fig. 4B). In HCC, the expression and functional status of ABCG2 is reported to be closely associated with the side population, a minor subset of cells with the unique capacity to extrude Hoechst 33342 (22). However, few side population cells could be detected in PLC/PRF/5 (unpublished data), we detected the alteration of the side population proportion in MHCC-97L cells. We found that ABCG2 expression was depressed in sorted or unsorted MHCC-97L cells after BMP4 treatment (Fig. 4C). As
shown in Fig. 4D, the side population proportion was decreased from 5.1% to 2.3% after BMP4 induction, suggesting that BMP4 may also possess a prodifferentiation effect on the side population population in HCC.

**Exogenous BMP4 induces the activation of the canonical BMP/SMAD signaling pathway in HCC**

To investigate the effect of canonical BMP signaling in BMP4-induced differentiation, we first examined the activation of the BMP4 signaling pathway. Nearly all of the HCC cell lines analyzed were found to express BMPRIa and BMPRIb proteins (Fig. 5A, Supplementary Fig. S4A) by Western blot, and in human primary HCC tissues, the positive staining of BMPRIa and BMPRIb proteins was 70% (165 of 236) and 57% (134 of 236), respectively, by immunohistochemical assay (Fig. 5B).

Chiu and colleagues reported that BMPRIa is overexpressed in HCC and BMPRIa knockdown HCC cells were less tumorigenic (23), though we found that BMPRIa expression showed no difference between CD133+ and CD133− cells (Fig. 5C).

After BMP4 treatment, different HCC cells showed constitutively active BMP/SMAD canonical signaling, as revealed by the high levels of both pSMAD1/5/8 and SMAD4 intermediate molecules. Furthermore, SMAD1/5/8 phosphorylation induced by BMP4 is time dependent (Fig. 5A, Supplementary Fig. S4B). We also observed that SMAD6 overexpression was induced by BMP4 treatment in SMMC-7721 cells (Supplementary Fig. S4A).

In many cases, the heterotetramer SMAD1/5/8-SMAD4 complex is required for BMPs signaling. siRNA oligonucleotides specifically targeting SMAD4 were synthesized, and the knockdown efficiency was confirmed (Supplementary Fig. S4C). As shown in Fig. 5D, the downregulation of CD133 protein expression induced by BMP4 treatment was attenuated after SMAD4 knockdown, which showed that the BMP4 prodifferentiation effect was a consequence of its canonical signaling activation.

**Endogenous BMP4 inversely contributes to CD133 expression in HCC**

Because the hepatic progenitor expansion medium (CDM) also contains a low concentration of BMP4, we explored the effects of different doses, especially low-dose BMP4 on CSC
Differentiation. As shown in Fig. 1A, compared with low-dose BMP4 groups (2.5–10 ng/mL), CD133 expression was also downregulated with no exogenous BMP4 present in vitro.

Endogenous BMP4 mRNA and protein were preferentially expressed in CD133⁺ CSCs in HCC cell lines (Fig. 6A) and in 2 primary cell lines (Supplementary Fig. S5A). As BMP4 is a secretary growth factor (24), secretary BMP4 in the culture supernatant was also analyzed; the results showed that the quantification of BMP4 was at a higher level in the CD133⁺ CSCs culture supernatant than in their CD133⁻ counterparts (Supplementary Fig. S5B). Knockdown of BMP4 expression lead to downregulation of CD133 and CK19 protein expression in PLC/PRF/5, but increased CK8/18 expression (Fig. 6B and C). These data suggested that although high-dose exogenous BMP4 possessed prodifferentiation effects on CD133⁺ CSCs, low-dose exogenous or endogenous BMP4 might contribute to CD133⁺ CSCs maintenance.

The protein expression of SMAD6, a BMP4 target gene, was detected in HCC tissue samples and adjacent noncancerous tissue samples by Western blot. Of the 28 pairs of HCC samples detected, SMAD6 expression was found to be upregulated in 22 cases in tumor tissues, indicating that BMP4 signaling is active in most hepatic cancer tissues (Supplementary Fig. S5C and D). By immunohistochemical analysis, we found positive and diffused staining of SMAD6 in 62% (146 of 236) of HCC samples, and its expression positively correlated with CD133 expression (Fig. 6D) . We also found that SMAD6 was differentially expressed in HCC cell lines and overexpressed in CD133⁺ CSCs compared with the corresponding CD133⁻ cells (Supplementary Fig. S5E and F). The knockdown of SMAD6 expression by siRNA could lead to a CD133 expression reduction (Supplementary Fig. S5G and H), which suggested that SMAD6 may contribute to the regulation of CD133 expression.

**Transient Erk1/2 phosphorylation facilitates the BMP4-induced CD133 depression**

Recent reports showed that SMAD and mitogen-activated protein kinase pathways communicate through signaling cross-talk (25). In this experiment, we found that BMP4 could induce Erk1/2 phosphorylation in a dose-dependent manner (Fig. 7A). Furthermore, when we incubated cells with a particular concentration of BMP4 for different periods of time, Erk1/2 phosphorylation reached a peak at approximately 30 minutes, whereas at 5 minutes, Erk1/2 phosphorylation attained nearly a maximum concentration (Fig. 7B).

To further ascertain the relationship between CD133 expression and Erk1/2 phosphorylation, HCC cells were incubated with the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitors PD98059 and U0126. Treatment with either of the MEK inhibitors for 3 days induced the inhibition of Erk1/2 phosphorylation, which promoted CD133 protein expression (Fig. 7C). Continuous treatment with either MEK inhibitor nearly abolished the BMP4-induced CD133 depression (Fig. 7D). However, when the inhibitors were added 24 hours after the BMP4 treatment, the CD133 expression was still reduced (Fig. 7D), indicating that a short-term exposure to a high level of Erk1/2 phosphorylation plays a very important role in the BMP4-induced reduction of CD133 protein expression. However, after long-term (6 days) treatment of BMP4, Erk1/2 phosphorylation was inhibited in a dose-dependent manner.
manner in HCC cell lines, and the phosphorylation level of Erk1/2 was upregulated in CD133⁺ HCC cells (Supplementary Fig. S6A and B). Considering that cell growth was repressed after treatment with high-dose exogenous BMP4 or MEK inhibitors, we speculated that an adequate level of Erk1/2 phosphorylation might participate in the self-renewal of CSCs.

Discussion

CSCs are thought to be responsible for the resistance of hepatic carcinoma to conventional therapies. Differentiation therapy could result in a loss of the CSCs self-renewal ability and the induction of terminal differentiation. The most successful application of differentiation therapy is the use of all-trans retinoic acid in acute premyelocytic leukemia, which is applied as a prodifferentiation inducer to enhance the chemotherapeutic effects (26).

BMP4 plays an important role in the hepatogenesis, and BMP4 has been shown to induce rat hepatic progenitor cell differentiation (27). Here, we screened several growth factors involved in hepatic development and found that BMP4 could induce a reduction in the percentage of CD133⁻ CSCs. BMP canonical signaling can be switched on by the exogenous administration of BMP4. With a mode of action similar to which has been reported in glioblastoma and colorectal cancer (15, 16), high-dose exogenous BMP4 treatments could induce the differentiation of CD133⁻ CSCs and simultaneously inhibit the self-renewal, chemotherapeutic resistance, and tumorigenesis of these cells. And as the report in adipose-derived stem cells (28), we found that although high-dose exogenous BMP4 reduced cell proliferation, low-dose BMP4 increased the cultured cell content and upregulated CD133 expression in HCC.

In addition, we found that endogenous BMP4 is required for CD133⁺ CSCs maintenance.

The reported function of BMPs in cancers is inconsistent, and they are described as both growth stimulators (29) and antigrowth molecules (30). The mechanism of action of BMPs may be different at the molecular level in different cancerous environments (31). In hepatocellular carcinoma, Maegdefrau and colleagues reported that BMP4 expression increased in HCC samples and BMP4 suppression resulted in a strong reduction of migratory and invasive potential (32, 33). Chiu and colleagues reported that BMP4 and its receptor, BMPRIa are overexpressed in HCC, and it promotes the growth and migration (23). Guo and colleagues reported that BMP4 might be a marker for predicting the prognosis of HCC patients (34). We found that both BMP4 and SMAD6 were overexpressed in CD133⁻/CD133⁺ CSCs, and the secretary BMP4 was also at a higher level in the CD133⁺ CSCs culture supernatant, suggesting that BMP signaling may be constitutively activated by endogenous BMP4 in CSCs and in HCC in an autocrine or a paracrine way, which may aid in maintaining their CD133 expression. However, BMPRIa expression showed no difference between CD133⁺/CD133⁻ cells. We also found that SMAD6 expression positively correlates with CD133 in HCC.
samples, and SMAD6 knockdown led to CD133 protein expression depression, indicating that SMAD6 may contribute to the CD133 expression regulation in HCC. Although SMAD6 was reported as a predictor of overall survival in oral squamous cell carcinoma (35), our results did not identify correlation between SMAD6 expression and clinical features in HCC tissues (data not shown). The relationship between SMAD6 and CSC differentiation needs to be further investigated.

Erks are involved in a variety of cellular processes, but the effects of Erk signaling in stem cells are contradictory. For example, blocking Erk1/2 signaling through treatment with a MEK inhibitor promotes the growth of undifferentiated mouse embryonic stem cells (ESC; ref. 36). In contrast, Li and colleagues reported that high basal MEK/ERK activity was required for maintaining human ESCs in an undifferentiated state (37). In HCC, the constitutive activation of Erk1/2 has been shown to be required for cancer cell proliferation and invasion. And Ding and colleagues report that CD133+ HCC cells show a substantial increase in Erk1/2 signaling activation (38). Our results showed that short-term treatment with BMP4 could induce Erk1/2 activation in a time- and dose-dependent manner. In addition, the inhibition of Erk1/2 phosphorylation by a MEK1/2 inhibitor may lead to CD133 protein overexpression in HCC cell lines, which is distinct from the observation of CSCs in colon carcinoma (39). The MEK inhibitor nearly abolished the CD133 expression reduction resulting from the BMP4 treatment, and a short-term exposure to a high level of Erk1/2 phosphorylation plays an important role in BMP4 prodifferentiation effects. These data showed that Erk signaling functions as an alternative pathway for BMP4-dependent CD133 repression.

The cross-talk between BMP/SMAD and Erk signaling has been reported in many cell types (40, 41). In human umbilical vein endothelial cells, Erk is essential for efficient transduction of BMP signals and serves as a positive feedback mechanism in capillary sprouting, and stimulation of SMAD6 inhibits Erk activation and thus results in a negative feedback loop to fine-tune BMP signaling (42). However, the Erk pathway has been variably reported to enhance or inhibit SMAD activity (40). On the basis of our results, we speculate that high level of Erk phosphorylation may enhance the effect of BMP canonical pathway on the depression of CD133 expression in CSCs, and SMAD6 might function as a feedback inhibitor for them, though the relationship between them need to be verified in our future work.

Moreover, we find that after long-term BMP4 treatments, CD133+/− PLC/PRF/5 cells displayed a dose-dependent reduction of Erk1/2 phosphorylation. The Erk1/2 phosphorylation level increased in CD133+ CSCs, which is coincident with the expression phenotype of BMP4 in CD133+ and CD133− HCC cells. Considering that cell growth was repressed after treatment with high-dose BMP4 and MEK inhibitors, we speculated that a required level of Erk1/2 phosphorylation...
might participate in CSC self-renewal. Thus, endogenous BMP4 may serve to regulate the balance between the self-renewal and differentiation of CD133\(^+\) HCC CSCs through MEK/ERK signaling. A similar phenomenon was reported and analyzed in ESCs. Li and colleagues found that low-dose exogenous BMP4 could steadily attenuate ERK activity by upregulating ERK-specific DUSP9 and, meanwhile, BMP signaling reinforces the self-renewal status of ESCs together with LIF (43).

In conclusion, BMP4 signaling plays a critical role in CSCs in HCC. Although high-dose exogenous BMP4 promotes differentiation of CSCs, low-dose or endogenous BMP4, in contrast, may contribute to CSCs maintenance. Therefore, further evaluation is necessary to determine the clinical safety and usefulness of BMP4 as an agent in the differentiation therapy of HCC.

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

References

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhang, J. Li
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