Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

BMP4 administration induces differentiation of CD133$^+$ hepatic cancer stem cells, blocking their contributions to hepatocelluar carcinoma

Lixing Zhang[1], Hefen Sun[1], Fangyu Zhao[1], Ping Lu[1], Chao Ge[1], Hong Li[1], Helei Hou[1], Mingxia Yan[1], Taoyang Chen[2,6], Guoping Jiang[3,6], Haiyang Xie[3,6], Ying Cui[4,6], Xiaowu Huang[5], Jia Fan[5], Ming Yao[1], Jinjun Li[1*].

[1] State Key Laboratrory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

[2] Qi Dong Liver Cancer Institute, Qi Dong, Jiangsu Province, China

[3] Department of General Surgery, the First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

[4] Cancer Institute of Guangxi, Nanning, China

[5] Liver Cancer Institute, Zhong Shan Hospital, Fudan University, Shanghai, China

[6] These authors contributed equally to this work.

* Correspondence: jjli@shsci.org

Running Titel: High-dose exogenous BMP4 induces HCC CSC differentiation

Keywords: BMP4; CD133; differentiation; ABCG2; hepatocellular carcinoma

Contact Information: Jinjun Li, State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, 25/Ln 2200 Xietu Road, Shanghai 200032, China. Tel/Fax: 86-21-64432140; E-mail: jjli@shsci.org.

List of Abbreviations: ABCG2, ATP-binding cassette protein G2; BMP4, Bone morphogenetic protein 4; CD133 (also known as prominin 1), a pentaspan transmembrance glycoprotein; CSCs, Cancer stem cells; Erk1/2, p44/p42 MAP kinase; FACS, Fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; MAPK, Mitogen-activated protein kinase; qRT-PCR, quantitative reverse transcription polymerase chain reaction; siRNA, small interfering RNA; SMAD4, also known as DPC4, deleted in pancreatic carcinoma locus 4.

Grant Support: This work was supported in part by grants from the National Key Program for Basic Research of China (973) (2009CB521803), the National Key Sci-Tech Special Project of China (2008ZX10002-022), the Program of Shanghai Subject Chief Science (A) (09XD1403600) and Leading Academic Discipline Project of Shanghai Municipal Education Committee (J50208).

Disclosures: All authors have no financial, professional or personal conflicts to disclose.

Abstract

$CD133^+$ cancer stem cells (CSCs) 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 show 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 indicate that BMP4 may be a potent therapeutic agent in HCC that targets CSCs.

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 (CSCs), 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 demonstrated 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 co-expression 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 (BMPs) 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 (BMPR1A or BMPR1B) determine the specificity for the ligands eliciting different biological processes (12). The activated BMPRI

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 demonstrated 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 (Manassas, VA, USA); Huh7 was obtained from the Riken Cell Bank (Tsukuba, Japan); 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, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and incubated at 37°C in a humidified atmosphere with 5% CO_2. For differentiation-inducing experiments of CSCs *in vitro*, HCC cells were plated onto general six-well plates (NUNC, USA) in a monolayer culture with serum-free chemically defined medium (CDM) (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% BSA, 2 mM glutamine and 0.1 mM 2-mercaptoethanol, growth factors including 10 ng/ml BMP4 (Sigma-Aldrich), 10 ng/ml basic fibroblast growth factor (bFGF) (Millipore), 10 ng/ml epithelial growth factor (EGF) (Millipore), 20 ng/ml

hepatocyte growth factor (HGF) (Millipore), 20 ng/ml transforming growth factor alpha (TGFα) (Millipore), and 10^{-7} mol/L dexamethasone (Sigma-Aldrich) were added. The agents with no special indication were purchased from the Invitrogen Corporation, USA.

Cell isolation by fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS)

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 FACS 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 h and were then treated with BMP4 at the indicated concentrations for the indicated times. BrdU ELISA assay was performed according to manufacturer's manual (Roche Diagnostics). 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's *t*-test. $p < 0.05$ 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, two HCC cell lines, PLC/PRF/5 and Huh7, with a relatively high percentage of $CD133^+$ cells, were selected to further analyze the biological effects of growth factors on CSCs (Supplementary Fig. 1*A*). The stemness of the $CD133^+$ cell population was reported in previous studies or confirmed in preliminary experiments (Supplementary Fig. 1*C, D*), and the sorting purity was confirmed (Supplementary Fig. 1*B*). 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. 2*A*).

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 ng/ml 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. 1*A*). Subsequently, we found that a 50 ng/ml dose showed a sufficient

reduction effect and which 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 Figure 1*B*, 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. 1*D*).

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. 1*C*). The cell apoptosis was not significantly influenced after high-dose BMP4 treatment (Supplementary Fig. 2*B*).

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 $CD133^{+/-}$ 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 RT-PCR analysis showed that the expression of stem-cell-associated genes, including Oct4, Tert, Bmi1, beta-catenin, ABCG2 and tumor-sphere-related gene Ep300 (4), in $CD133^+$ cells were reduced following BMP4 treatment, while 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 $CD133^-$ cells (Fig. 2*A*), 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. 2*B*). Cytokeratin 8/18 (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. 2*B*). 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 demonstrated that the number of CK19-positive cells decreased, and CK8/18-positive cells increased after BMP4 treatment (Fig. 2*C*). These results

were confirmed in Huh7 (Supplementary Fig. 2*C*) and MHCC-97L (Supplementary Fig. 2*D*), 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 $CD133^+$ HCC cells first cultured with BMP4 possessed lower colony formation efficiency (CFE) than that of $CD133^+$ cells continually cultured in CDM (Fig. 3*A*). However, the CFE of $CD133^-$ HCC cells was not significantly affected by BMP4 treatment. We further tested the sphere-formation ability of $CD133^+$ cells with or without BMP4 treatments. As shown in Figure 3*B*, the tumor spheres obtained 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 $CD133^{+/-}$ cells with and without BMP4 treatment was analyzed in an immunodeficient mouse xenograft model. The orthotopical inoculation of freshly sorted $CD133^{+/-}$ PLC/PRF/5 cells was accompanied by

the co-injection 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. 3*C*), whereas the mice bearing $CD133^+$ cells with BMP4-releasing beads did not form visible tumors (Supplementary Fig. 3*A* and Fig. 3*D*). Although $CD133^-$ PLC/PRF/5 cells formed fewer (3 of 6 mice) and smaller tumors than $CD133^+$ cells (Fig. 3*D*), the tumorigenicity was not greatly affected by BMP4 co-transplantation (2 of 6 mice). Similar results were obtained with the MHCC-97L cell line, which exhibited a low percentage of $CD133^+$ cells (Fig. 3*C*, 3*D*, and Supplementary Fig. 3*B*). Thus, BMP4 also possessed anti-tumor effects *in vivo*, suggesting that BMP4 may be a useful component of the differentiation therapy for HCC.

BMP4 enhances the sensitivity of HCC CSCs to chemotherapeutic drugs *in vitro*

Another important property of CSC is their resistance to chemotherapeutic agents. In this study, two 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 Figures 4*A, C*$D133^+$ PLC/PRF/5 cells showed marked increases in their resistance to doxorubicin or vincristine compared to $CD133^-$ cells, and BMP4 treatment

consistently increased their sensitivity to these two agents.

Based on 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. 4*B*). In HCC, the expression and functional status of ABCG2 is reported to be closely associated with the side population (SP), a minor subset of cells with the unique capacity to extrude Hoechst 33342 (22). However, few SP cells could be detected in PLC/PRF/5 (unpublished data), we detected the alteration of the SP proportion in MHCC-97L cells. We found that ABCG2 expression was depressed in sorted or unsorted MHCC-97L cells after BMP4 treatment (Fig. 4*C*). As shown in Figure 4*D*, the SP proportion was decreased from 5.1% to 2.3% after BMP4 induction, suggesting that BMP4 may also possess a pro-differentiation effect on the SP 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. 5*A*, Supplementary Fig. 4*A*) by Western blot, and in human primary HCC tissues, the positive staining of BMPRIa and BMPRIb

proteins was 70% (165/236) and 57% (134/236), respectively, by IHC assay (Fig. 5*B*). Chiu *et al* 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. 5*C*).

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. 5*A*, Supplementary Fig. 4*B*). We also observed that SMAD6 overexpression was induced by BMP4 treatment in SMMC-7721 cells (Supplementary Fig. 4*A*).

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. 4*C*). As shown in Figure 5*D*, the downregulation of CD133 protein expression induced by BMP4 treatment was attenuated after SMAD4 knockdown, demonstrated that the BMP4 pro-differentiation effect was a consequence of its canonical signaling activation.

Endogenous BMP4 inversely contributes to CD133 expression in HCC

For the hepatic progenitor expansion medium (CDM) also contains a low concentration of BMP4, we then explored the effects of different doses,

especially low-dose BMP4 on CSC differentiation. As shown in Figure 1*A*, compared to 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. 6*A*) and in two primary cell lines (Supplementary Figure 5A). 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 lever in the $CD133^+$ CSCs culture supernatant then in their $CD133^-$ counterparts (Supplementary Figure 5B). Knockdown of BMP4 expression leaded to downregulation of CD133 and CK19 protein expression in PLC/PRF/5, but increased CK8/18 expression (Fig. 6*B,C*). These data suggested that although high-dose exogenous BMP4 possessed pro-differentiation effects on $CD133^+$ CSCs, low-dose exogenous or endogenous BMP4 may contribute to $CD133^+$ CSCs maintenance.

The protein expression of SMAD6, a BMP4 target gene, was detected in HCC tissue samples and adjacent non-cancerous 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. 5*C, D*). By IHC analysis, we found positive and diffused staining of SMAD6 in 62% (146/236) of HCC samples, and its expression positively correlated with CD133

expression (Fig. 6*D*). We also found that SMAD6 was differentially expressed in HCC cell lines and overexpressed in $CD133^+$ CSCs compared to the corresponding $CD133^-$ cells (Supplementary Fig. 5*E, F*). The knockdown of SMAD6 expression by siRNA could lead to a CD133 expression reduction (Supplementary Fig. 5*G, 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 show that SMAD and MAPK pathways communicate through signaling crosstalk (25). In this experiment, we found that BMP4 could induce Erk1/2 phosphorylation in a dose-dependent manner (Fig. 7*A*). 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 min, whereas at 5 min, Erk1/2 phosphorylation attained nearly a maximum concentration (Fig. 7*B*).

To further ascertain the relationship between CD133 expression and Erk1/2 phosphorylation, HCC cells were incubated with the 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. 7*C*). Continuous treatment with either MEK inhibitor nearly abolished the BMP4-induced CD133 depression (Fig. 7*D*). However, when the

inhibitors were added 24 hrs after the BMP4 treatment, the CD133 expression was still reduced (Fig. 7*D*), 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 in HCC cell lines, and the phosphorylation level of Erk1/2 was upregulated in $CD133^+$ HCC cells (Supplementary Fig. 6*A, 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 may 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 CSC's 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 pro-differentiation 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 anti-growth molecules (30). The mechanism of action of BMPs may be different at the molecular level in different cancerous environments (31). In hepatocellular carcinoma, Maegdefrau *et al.* reported that BMP4 expression increased in HCC samples and BMP4 suppression resulted in a strong reduction of migratory and invasive potential (32, 33). Chiu *et al.* reported that BMP4 and its receptor, BMPRIa are overexpressed in HCC and it promotes the growth and migration (23). Guo *et al* reported that BMP4 may be a marker for predicting the prognosis of HCC patients (34). We found that both BMP4 and SMAD6 were overexpressed in $CD133^+$ CSCs, and the secretary BMP4 was also at a higher lever 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 ESCs (36). In contrast, Li et al. 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 *et al.* report that $CD133^{+}$ HCC cells demonstrate 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. Additionally, 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 pro-differentiation effects. These data demonstrate that Erk signaling functions as an alternative pathway for BMP4-dependent CD133 repression.

The crosstalk 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). Base on 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 may participate in CSC self-renewal. Thus, endogenous BMP4 may serve to regulate the balance between the self-renewal and differentiation of $CD133^{+}$ HCC CSCs though MEK/ERK signaling. A similar phenomenon was reported and analyzed in mouse embryonic stem cell (ESC), Li *et al.* found that low-dose exogenous BMP4 could steadily attenuate ERK activity by upregulating ERK-specific DUSP9, and at the 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 utilization of BMP4 as an agent in the differentiation therapy of HCC.

References

1. Ye QH, Qin LX, Forgues M, He P, Kim JW, Peng AC, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat Med 2003; 9: 416-23.

2. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 2009; 138: 645-59.

3. Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. Hepatology 2006; 44: 240-51.

4. Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R, et al. Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines. BMC Gastroenterol 2011; 11: 71.

5. Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 2009; 136: 1012-24.

6. Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P, et al. Significance of CD90+ cancer stem cells in human liver cancer. Cancer Cell 2008; 13: 153-66.

7. Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. Cancer Res 2008; 68: 1451-61.

8. Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO. CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. Cell Stem Cell 2011; 9: 50-63.

9. Yin S, Li J, Hu C, Chen X, Yao M, Yan M, et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 2007; 120: 1444-50.

10. Zhu Z, Hao X, Yan M, Yao M, Ge C, Gu J, et al. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. Int J Cancer 2010; 126: 2067-78.

11. Si-Tayeb K, Lemaigre FP, Duncan SA. Organogenesis and development of the liver. Dev Cell 2010; 18: 175-89.

12. Chen D, Ji X, Harris MA, Feng JQ, Karsenty G, Celeste AJ, et al. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. J Cell Biol 1998; 142: 295-305.

13. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003; 425: 577-84.

14. Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, et al. Smad6 inhibits signalling by the TGF-beta superfamily. Nature 1997; 389: 622-6.

15. Lombardo Y, Scopelliti A, Cammareri P, Todaro M, Iovino F, Ricci-Vitiani L, et al. Bone morphogenetic protein 4 induces differentiation of colorectal cancer

stem cells and increases their response to chemotherapy in mice. Gastroenterology 2011; 140: 297-309.

16. Piccirillo SG, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 2006; 444: 761-5.

17. Li F, Liu P, Liu C, Xiang D, Deng L, Li W, et al. Hepatoblast-like progenitor cells derived from embryonic stem cells can repopulate livers of mice. Gastroenterology 2010; 139: 2158-69 e8.

18. Lemaigre FP. Mechanisms of liver development: concepts for understanding liver disorders and design of novel therapies. Gastroenterology 2009; 137: 62-79.

19. Ma S, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, et al. miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. Cell Stem Cell 2010; 7: 694-707.

20. Kim H, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, et al. Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. Hepatology 2011; 54: 1707-17.

21. Fiegel HC, Park JJ, Lioznov MV, Martin A, Jaeschke-Melli S, Kaufmann PM, et al. Characterization of cell types during rat liver development. Hepatology 2003; 37: 148-54.

22. Hu C, Li H, Li J, Zhu Z, Yin S, Hao X, et al. Analysis of ABCG2 expression and side population identifies intrinsic drug efflux in the HCC cell line

MHCC-97L and its modulation by Akt signaling. Carcinogenesis 2008; 29: 2289-97.

23. Chiu CY, Kuo KK, Kuo TL, Lee KT, Cheng KH. The Activation of MEK/ERK Signaling Pathway by Bone Morphogenetic Protein 4 to Increase Hepatocellular Carcinoma Cell Proliferation and Migration. Mol Cancer Res 2012: article in press.

24. Lee YC, Cheng CJ, Bilen MA, Lu JF, Satcher RL, Yu-Lee LY, et al. BMP4 promotes prostate tumor growth in bone through osteogenesis. Cancer Res 2011; 71: 5194-203.

25. Javelaud D, Mauviel A. Crosstalk mechanisms between the mitogen-activated protein kinase pathways and Smad signaling downstream of TGF-beta: implications for carcinogenesis. Oncogene 2005; 24: 5742-50.

26. Chou WC, Dang CV. Acute promyelocytic leukemia: recent advances in therapy and molecular basis of response to arsenic therapies. Curr Opin Hematol 2005; 12: 1-6.

27. Fan J, Shen H, Dai Q, Minuk GY, Burzynski FJ, Gong Y. Bone morphogenetic protein-4 induced rat hepatic progenitor cell (WB-F344 cell) differentiation toward hepatocyte lineage. J Cell Physiol 2009; 220: 72-81.

28. Vicente Lopez MA, Vazquez Garcia MN, Entrena A, Olmedillas Lopez S, Garcia-Arranz M, Garcia-Olmo D, et al. Low doses of bone morphogenetic protein 4 increase the survival of human adipose-derived stem cells maintaining their stemness and multipotency. Stem Cells Dev 2010; 20:

1011-9.

29. Langenfeld EM, Calvano SE, Abou-Nukta F, Lowry SF, Amenta P, Langenfeld J. The mature bone morphogenetic protein-2 is aberrantly expressed in non-small cell lung carcinomas and stimulates tumor growth of A549 cells. Carcinogenesis 2003; 24: 1445-54.

30. Buckley S, Shi W, Driscoll B, Ferrario A, Anderson K, Warburton D. BMP4 signaling induces senescence and modulates the oncogenic phenotype of A549 lung adenocarcinoma cells. Am J Physiol Lung Cell Mol Physiol 2004; 286: L81-6.

31. Singh A, Morris RJ. The Yin and Yang of bone morphogenetic proteins in cancer. Cytokine Growth Factor Rev 2010; 21: 299-313.

32. Maegdefrau U, Amann T, Winklmeier A, Braig S, Schubert T, Weiss TS, et al. Bone morphogenetic protein 4 is induced in hepatocellular carcinoma by hypoxia and promotes tumour progression. J Pathol 2009; 218: 520-9.

33. Maegdefrau U, Bosserhoff AK. BMP activated Smad signaling strongly promotes migration and invasion of hepatocellular carcinoma cells. Exp Mol Pathol 2011; 92: 74-81.

34. Guo X, Xiong L, Zou L, Zhao J. Upregulation of Bone Morphogenetic Protein 4 is Associated with Poor Prognosis in Patients with Hepatocellular Carcinoma. Pathol Oncol Res 2012.

35. Mangone FR, Walder F, Maistro S, Pasini FS, Lehn CN, Carvalho MB, et al. Smad2 and Smad6 as predictors of overall survival in oral squamous cell

carcinoma patients. Mol Cancer 2010; 9: 106.

36. Burdon T, Stracey C, Chambers I, Nichols J, Smith A. Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. Dev Biol 1999; 210: 30-43.

37. Li J, Wang G, Wang C, Zhao Y, Zhang H, Tan Z, et al. MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. Differentiation 2007; 75: 299-307.

38. Ding W, Mouzaki M, You H, Laird JC, Mato J, Lu SC, et al. CD133+ liver cancer stem cells from methionine adenosyl transferase 1A-deficient mice demonstrate resistance to transforming growth factor (TGF)-beta-induced apoptosis. Hepatology 2009; 49: 1277-86.

39. Tabu K, Kimura T, Sasai K, Wang L, Bizen N, Nishihara H, et al. Analysis of an alternative human CD133 promoter reveals the implication of Ras/ERK pathway in tumor stem-like hallmarks. Mol Cancer 2010; 9: 39.

40. Hayashida T, Decaestecker M, Schnaper HW. Cross-talk between ERK MAP kinase and Smad signaling pathways enhances TGF-beta-dependent responses in human mesangial cells. FASEB J 2003; 17: 1576-8.

41. Watanabe H, de Caestecker MP, Yamada Y. Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-beta-induced aggrecan gene expression in chondrogenic ATDC5 cells. J Biol Chem 2001; 276: 14466-73.

42. Zhou Q, Heinke J, Vargas A, Winnik S, Krauss T, Bode C, et al. ERK

signaling is a central regulator for BMP-4 dependent capillary sprouting. Cardiovasc Res 2007; 76: 390-9.

43. Li Z, Fei T, Zhang J, Zhu G, Wang L, Lu D, et al. BMP4 Signaling Acts via Dual-Specificity Phosphatase 9 to Control ERK Activity in Mouse Embryonic Stem Cells. Cell Stem Cell 2012; 10: 171-82.

Figure Legends

Figure 1. BMP4 decreases the CD133$^+$ CSCs population *in vitro*. *A*. Western blotting for CD133 in PLC/PRF/5 cells that were treated with different doses of BMP4. *B*. BMP4 initiates a CD133 protein decrease in a time-dependent manner. *C*. The BrdU ELISA assay results show that different concentrations of BMP4 showed different effect on PLC/PRF/5 cell proliferation. *D*. The flow cytometric analysis reveals that the percentage of CD133$^+$ cells is reduced after BMP4 treatment.

Figure 2. BMP4 induces the differentiation of HCC CSCs. *A*. The expression of stemness-related genes of sorted CD133$^{+/-}$ cells, which were cultured as spheres in CDM or in a monolayer with BMP4 treatment. The data are normalized to GAPDH expression and presented as magnitudes of change relative to freshly isolated CD133$^-$ cells. * p<0.05, *t*-test, *vs* CD133+ CDM. *B*. Western blotting shows that BMP4 upregulated CK8/18 expression and downregulated CK19 expression in a time-dependent manner. *C*. Immunocytochemistry for CK8/18 and CK19 in CD133$^+$ PLC/PRF/5 cells after BMP4 treatment. Representative images are shown along with the quantification of 5 randomly selected fields. Original magnification, 200×; * p<0.05, *t*-test.

Figure 3. BMP4 inhibits the self-renewal and tumorigenic capacities of CD133^{+} CSCs. *A*. CD133$^{+/-}$ PLC/PRF/5 cells were pretreated with BMP4 for 6 days. Subsequently, each group of cells was suspended in growth media containing 0.3% soft agar and seeded in 24-well plates to evaluate colony formation efficiency (CFE) (n=3). ***p*<0.01, **p*<0.05, *t*-test. Original magnification, 50×. *B*. BMP4 inhibits the capacity for CD133^{+} PLC/PRF/5 cell sphere formation. Original magnification, 200×. *C*. The weight of liver tissue with xenograft tumors. * *p*<0.05, *t*-test. *D*. The tumorigenicity of CD133$^{+/-}$ cells co-injected with BMP4 or vehicle-embedding beads.

Figure 4. BMP4 enhances the activity of chemotherapeutic agents on HCC CSCs. *A*. The cytotoxic effects of doxorubicin and vincristine on CD133$^{+/-}$ PLC/PRF/5 cells that had been pretreated with BMP4 were tested with the MTT assay. * *p*<0.05, *t*-test, *vs* CD133+ CDM. *B, C*. BMP4 inhibits the expression of ABCG2 in PLC/PRF/5 and MHCC-97L cells. *D*. Flow cytometric analysis of the SP percentage in MHCC-97L.* *p*<0.05, *t*-test.

Figure 5. The expression and activation of BMP receptors in HCC cell lines and human primary HCC tissues. PLC/PRF/5 and Huh7 cells were treated with BMP4 for the indicated time periods. *A*. BMP receptors (BMPRIa/b) and SMAD4 were constitutively expressed in these cells. BMP4 treatment results in dose-dependent SMAD1 phosphorylation. *B*. The IHC analysis of

BMPRIa/b in HCC samples. Representative images are shown. Original magnification, 200×. *C*. BMPRIa expression in $CD133^+$/$CD133^-$ cells showed no difference. *D*. PLC/PRF/5 cells were treated with SMAD4-siRNA-1, -2, and -3 for 48 h and then incubated with BMP4 for 3 d, CD133 expressions were analyzed by Western blot. NC represents for stable negative control siRNA, MOCK means for mock siRNA.

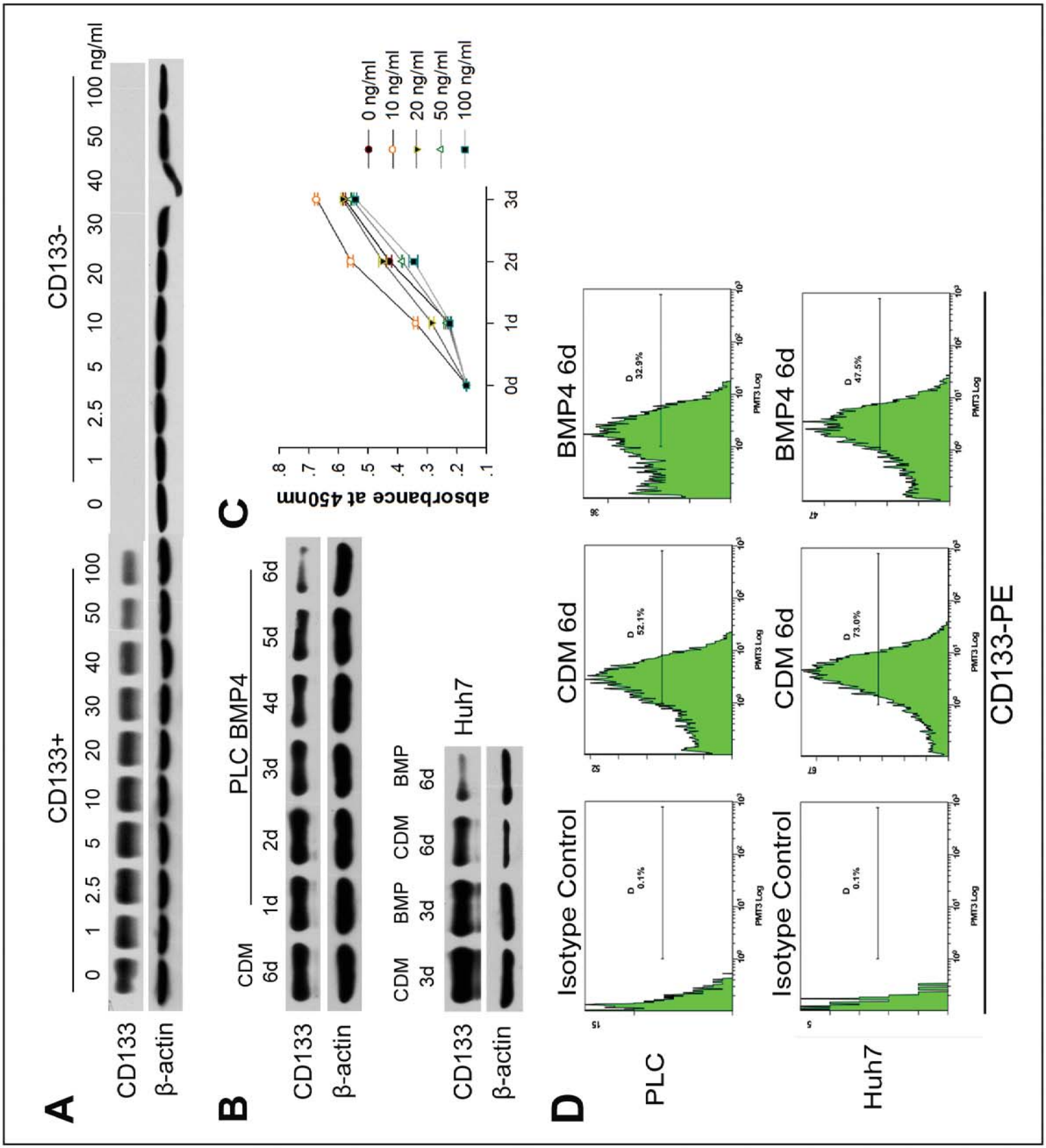
Figure 6. Endogenous BMP4 signaling activated in HCC CSCs. *A*. Endogenous BMP4 mRNA and protein were overexpressed in $CD133^+$ cells in HCC cell lines. *B*. BMP4 knockdown efficiency was confirmed by Realtime PCR assay in PLC/PRF/5 (siBMP4-1, -2, -3 are different siRNA fragments). *C*. BMP4 knockdown leaded to downregulation of CD133 expression in PLC/PRF/5. *D*. The IHC analysis of SMAD6 and CD133 in HCC samples. Representative images are shown. Original magnification, 200×.

Figure 7. Transient Erk1/2 phosphorylation facilitates the BMP4-induced CD133 expression reduction. *A*. BMP4 results in dose-dependent Erk1/2 phosphorylation. *B*. A time course of BMP4-activated Erk1/2 phosphorylation. *C*. The inhibition of Erk1/2 phosphorylation by MEK1/2 inhibitor U0126 (10 μM) and PD98059 (20 μM) upregulated CD133 protein expression. *D*. Western blotting for CD133 in HCC cells treated with BMP4 and Erk1/2 phosphorylation inhibitor. PLC/PRF/5 and Huh7 cells were treated with BMP4 for 3 days, and

the MEK1/2 inhibitor (U0126/PD98059) was added to the medium 2 hours before BMP4 treatment or 24 hrs later.

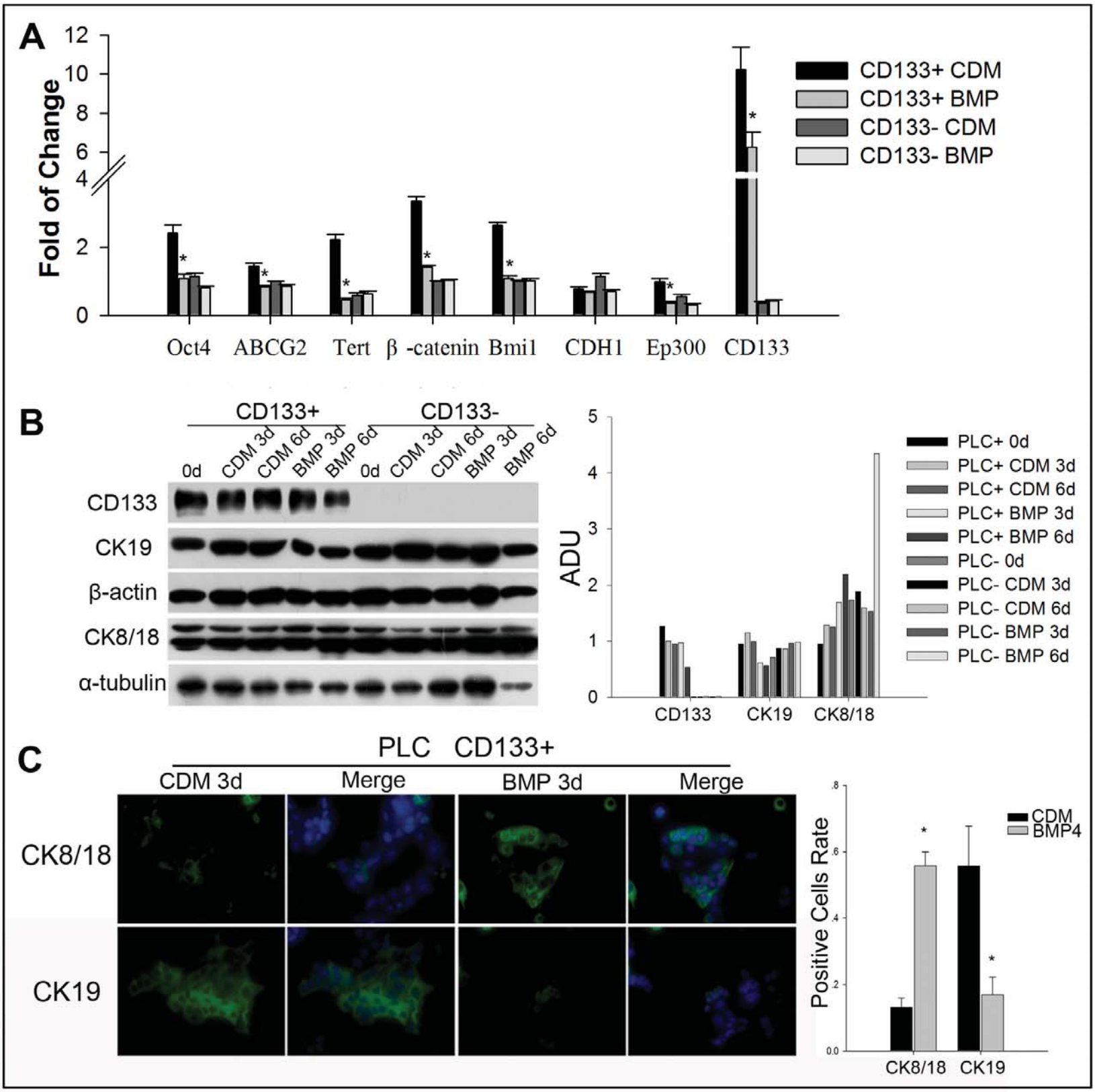
Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 1 by Zhang *et al.*



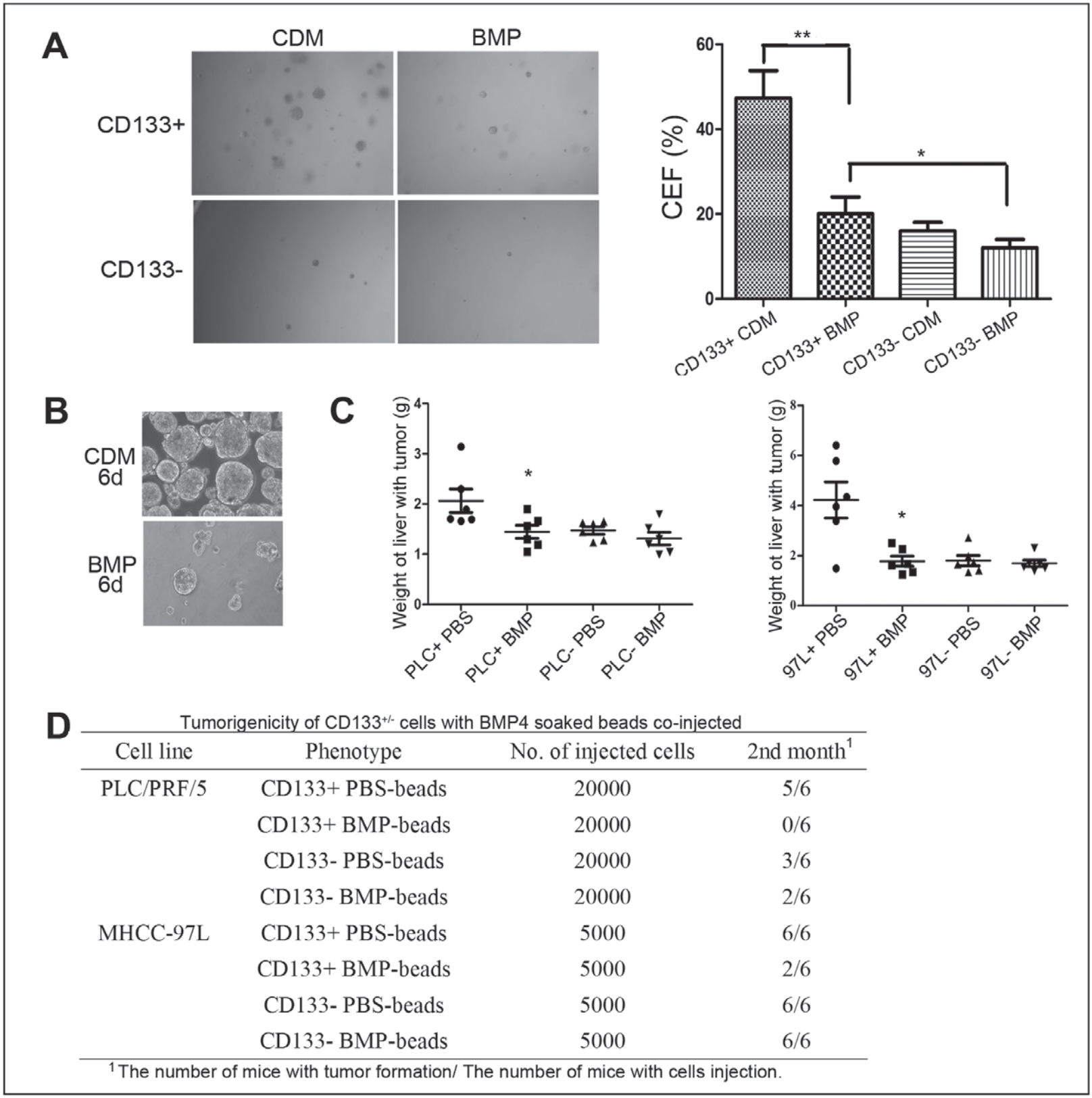
Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 2 by Zhang *et al.*



Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 3 by Zhang *et al.*



D

Tumorigenicity of CD133$^{+/-}$ cells with BMP4 soaked beads co-injected

Cell line	Phenotype	No. of injected cells	2nd month[1]
PLC/PRF/5	CD133+ PBS-beads	20000	5/6
	CD133+ BMP-beads	20000	0/6
	CD133- PBS-beads	20000	3/6
	CD133- BMP-beads	20000	2/6
MHCC-97L	CD133+ PBS-beads	5000	6/6
	CD133+ BMP-beads	5000	2/6
	CD133- PBS-beads	5000	6/6
	CD133- BMP-beads	5000	6/6

[1] The number of mice with tumor formation/ The number of mice with cells injection.

Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 4 by Zhang *et al.*

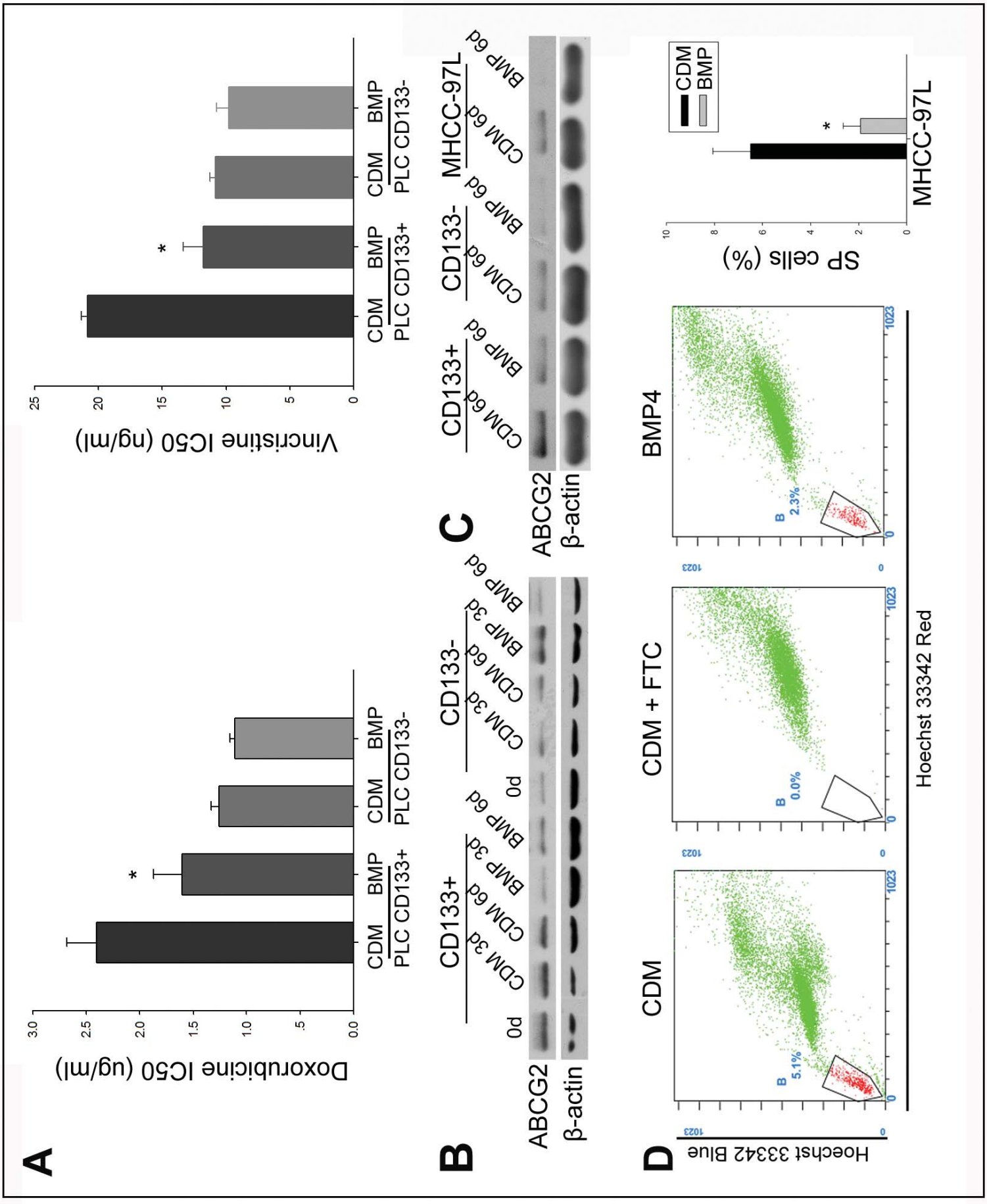
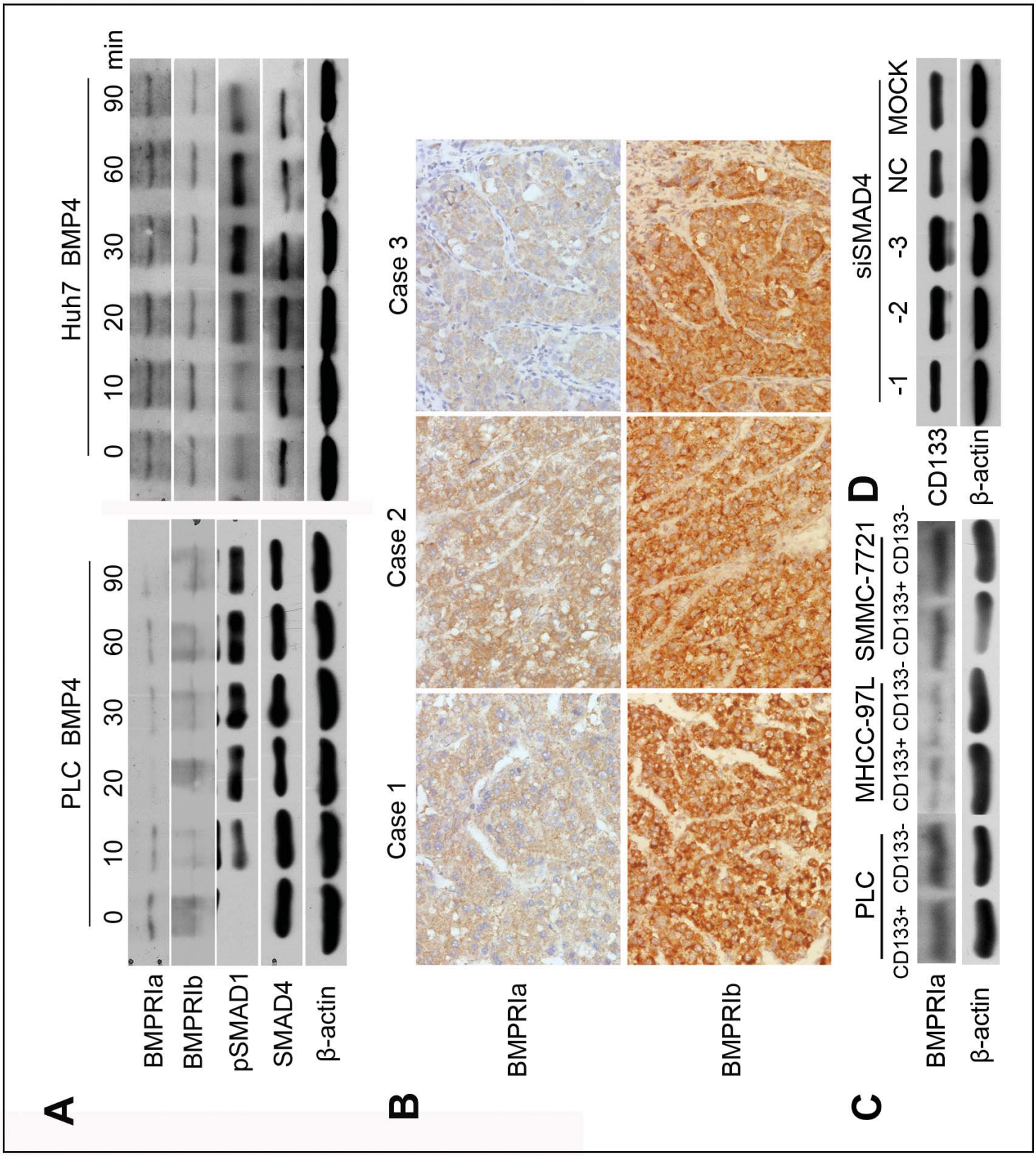
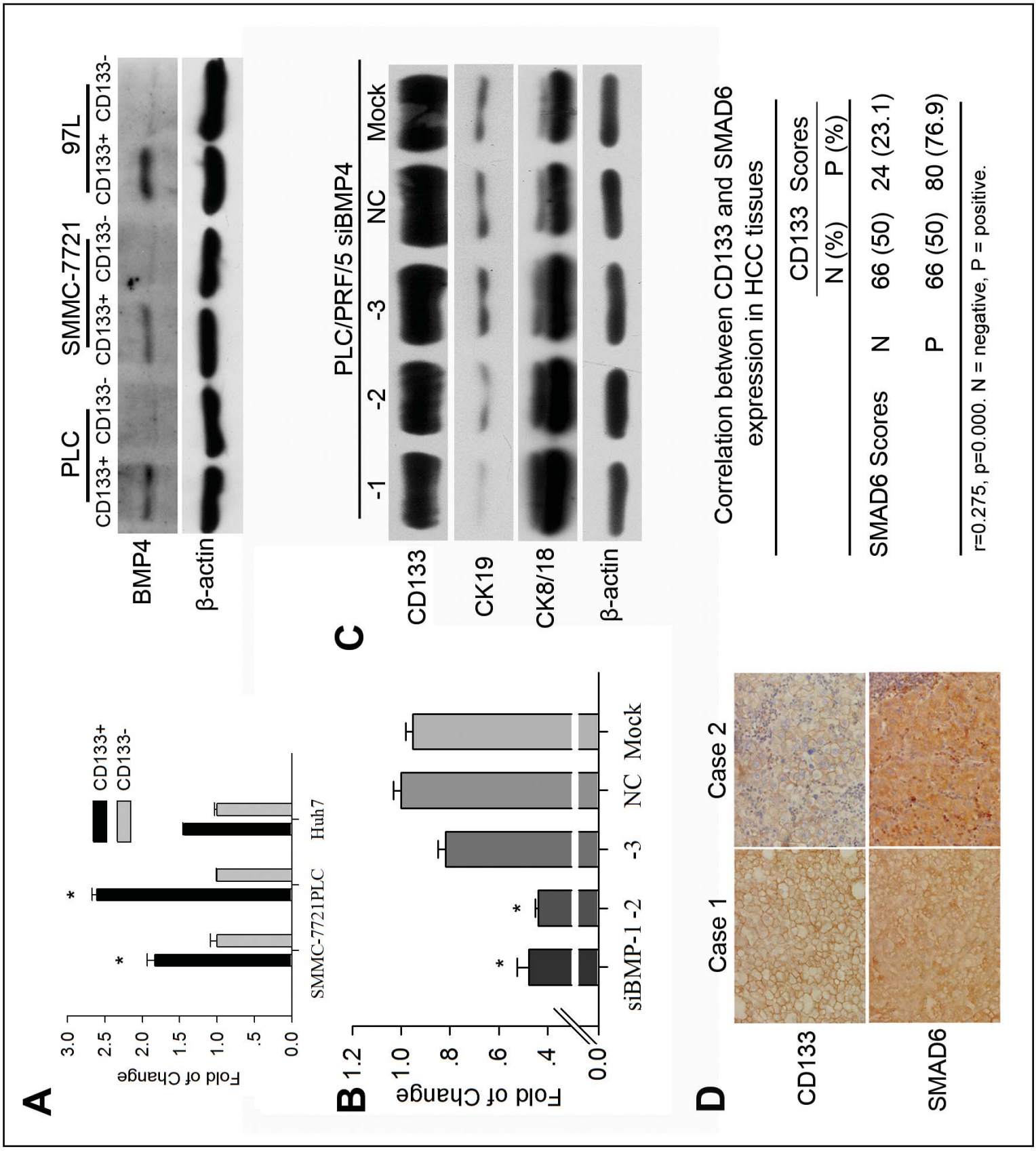


Figure 5 by Zhang *et al.*



Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 6 by Zhang *et al.*



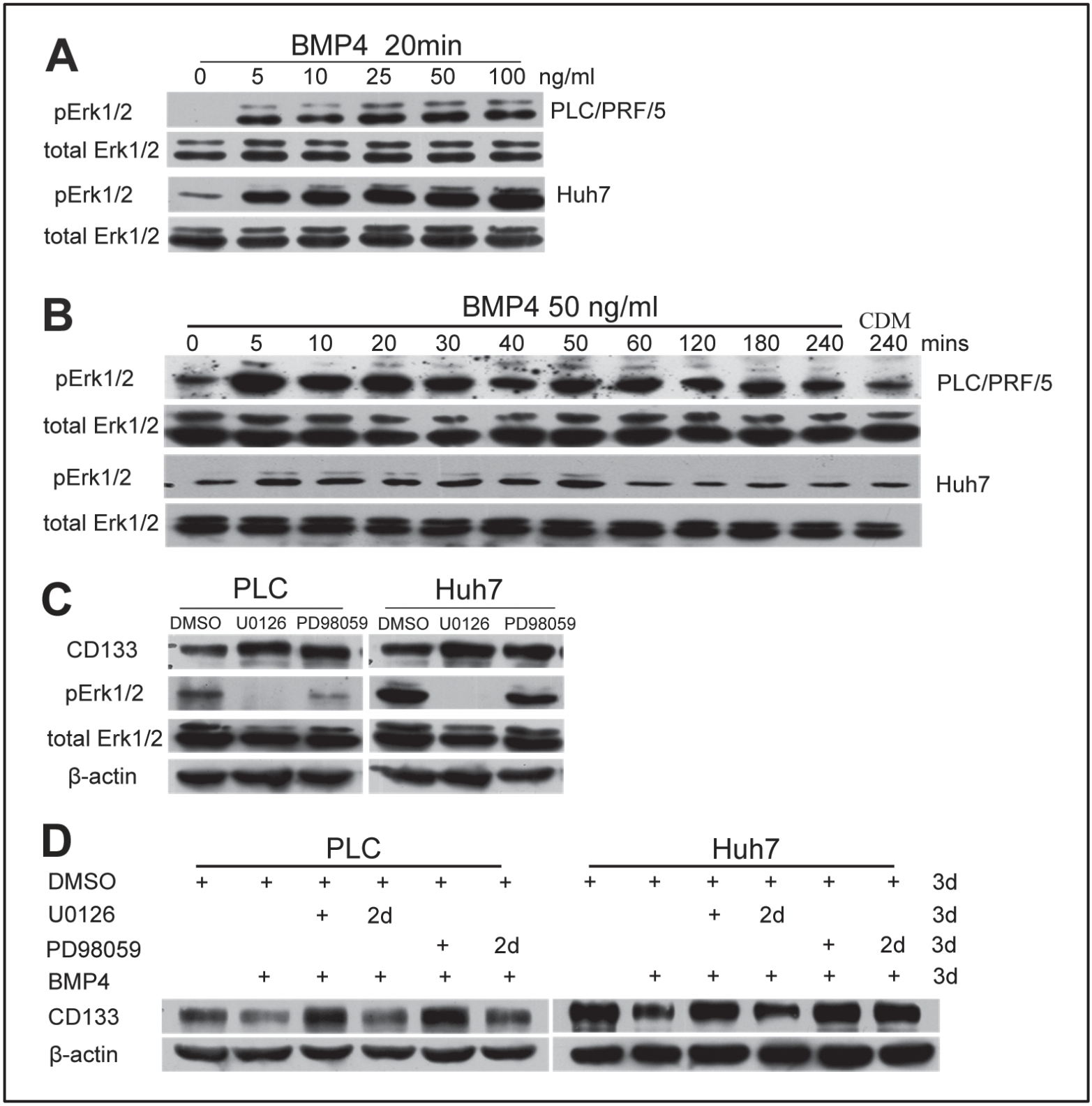
Correlation between CD133 and SMAD6 expression in HCC tissues

SMAD6 Scores	N	CD133 Scores N (%)	CD133 Scores P (%)
P	66 (50)	24 (23.1)	
	66 (50)	80 (76.9)	

r=0.275, p=0.000. N = negative, P = positive.

Author Manuscript Published OnlineFirst on July 5, 2012; DOI: 10.1158/0008-5472.CAN-12-1013
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 7 by Zhang *et al.*



Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

BMP4 administration induces differentiation of CD133+ hepatic cancer stem cells, blocking their contributions to hepatocelluar carcinoma

Lixing Zhang, Hefen Sun, Fangyu Zhao, et al.

Cancer Res Published OnlineFirst July 5, 2012.