RESEARCH PAPER

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BMP-2 inhibits the tumorigenicity of cancer stem cells in human osteosarcoma OS99-1 cell line

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Previously, based on high ALDH activity, we showed that cancer stem cells (CSCs) could be identified as ALDH^{br} cells from an aggressive human osteosarcoma OS99-1 cell line. In this study, we evaluate the impact of BMP-2 on CSCs. Three types of BMP receptors were expressed in freshly sorted ALDH^{br} cells. In vitro, growth of the sorted ALDH^{br} cells was inhibited by BMP-2. Using RT-PCR analysis, BMP-2 was found to downregulate the expression of embryonic stem cell markers Oct3/4, Nanog and Sox-2, and upregulate the transcription of osteogenic markers Runx-2 and Collagen Type I. In vivo, all animals receiving ALDH^{br} cells treated with BMP-2 did not form significant tumors, while untreated ALDH^{br} cells developed large tumor masses in NOD/SCID mice. Immunostaining confirmed few Ki-67 positive cells were present in the sections of tumor containing ALDH^{br} cells treated with BMP-2. These results suggest that BMP-2 suppresses tumor growth by reducing the gene expression of tumorigenic factors and inducing the differentiation of CSCs in osteosarcoma. BMP-2 or BMP-2-mimetic drugs, if properly delivered to tumor and combined with traditional therapies, may therefore provide a new therapeutic option for treatment of osteosarcoma.

Introduction

Osteosarcoma is the most common primary, nonhematologic malignant tumor of bone in children and young adolescents, accounting for about 5% of pediatric tumors overall.[1,2] Over preceding decades, the use of chemotherapeutics in combination with aggressive surgery has improved long-term survival in these patients to around 65%.[3] Approximately 10–20% of patients have metastases at initial diagnosis. Metastasis to the lung is common, and unless the pulmonary metastasis can be eradicated completely, nearly all patients succumb to death.[4] Therefore, to more effectively control this disease and improve patient survival rate, there is a pressing need to elucidate the molecular mechanism of human sarcomagenesis and to develop new treatment options for osteosarcomas.

Emerging evidence supports the notion that the capacity of a tumor to grow and propagate is dependent on a small population of cells termed cancer stem cells (CSCs) or tumor-initiating cells.[5] The discovery of CSCs has changed the view of how carcinogenesis propagates and the role of chemotherapy. CSCs have been shown to be more resistant to standard chemotherapeutic agents and radiotherapy. Traditional treatment may result in tumor shrinkage, but most tumors recur after treatment, likely because CSCs survive and regenerate tumor growth.[6,7] Treatment specifically targeting the CSC population has the potential to be a more effective therapy.

Recently, the existence of CSCs has also been reported in human osteosarcomas.[8-10] In addition, our recent study[11] showed that CSCs could be identified in the established human osteosarcoma OS99-1 cell line based on high aldehyde dehydrogenase (ALDH) activity. Cells with high ALDH activity (ALDH^{br} cells) from OS99-1 xenografts had high tumorigenicity compared to their counterparts with low ALDH activity (ALDH^{lo} cells), generating new tumors with as few as 100 cells in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice through serial transplantations. Eliminating the ALDH^{br} cells capable of initiating and maintaining osteosarcoma might be an alternative method of osteosarcoma treatment.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β superfamily, were originally identified as molecules that induce bone and cartilage formation and are now considered as multifunctional cytokines.[12] More than 20 BMPs have been shown to play important roles in cell differentiation, proliferation, morphogenesis, cellular survival and apoptosis, in which BMP-2 exhibits potent activity for inducing the formation of both cartilage and bone in vivo and in vitro through specific Type I and Type II receptors. In addition to bone formation, BMP-2 plays pivotal roles in cell proliferation, apoptosis and differentiation.[13,14] More recently, there have been a number of studies on the inhibitory effects of BMP-2 in tumorigenesis;[15-18] however, the role of BMP-2 in human osteosarcoma remains incompletely understood. This study explores the putative effects of BMP-2 on tumorigenic ALDH^{br} cells of the OS99-1 cell line.

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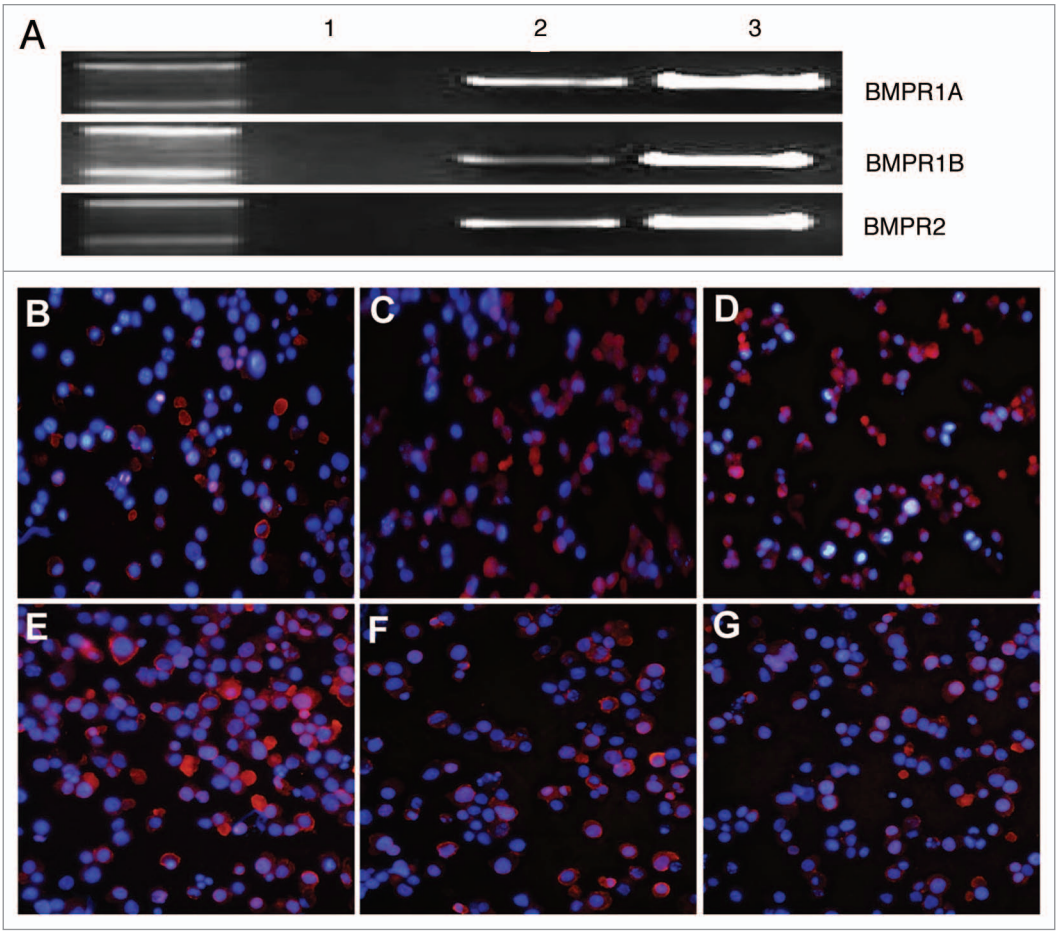

Figure 1. Expression of BMP receptors in human osteosarcoma tumorigenic ALDH^{br} cells. (A) Messenger RNA transcripts for BMP receptors. Lane 1: negative control; Lane 2: freshly sorted ALDH^{br} cells; Lane 3: MCF7 cells (positive control). Representative immunofluorescent staining of BMPR1A (B), BMPR1B (C) and BMPR2 (D) in freshly sorted ALDH^{br} cells. Representative immunofluorescent staining of BMPR1A (E), BMPR1B (F) and BMPR2 (G) in unsorted cells from OS99-1 xenografted tumors, and there was no difference on the expressions of those three BMP receptors between the ALDH^{br} cells and the unsorted cells. The nuclei counterstained with 4',6-diamidino-2 phenylindole (DAPI) (blue areas).

Results

Expression of BMP receptors in freshly sorted ALDH^{br} cells. To determine whether the BMP-2 pathway is perturbed in ALDH^{br} cells, we first sought to investigate the mRNA expression of BMPR1A, BMPR1B and BMPR2 in freshly sorted ALDH^{br} cells. PCR analysis revealed that BMPR1A, BMPR1B and BMPR2 were expressed in freshly sorted ALDH^{br} cells, as shown in **Figure 1A**. All three types of BMP receptors were further demonstrated to present in ALDH^{br} cells by immunofluorescent staining (**Fig. 1B–D**). To evaluate whether the ALDH^{br} cells might express BMP receptors at different levels compared with the unsorted cells from xenografted tumor, immunofluorescent staining of unsorted cells showed there was no difference on the expressions of those three BMP receptors between the ALDH^{br} cells and the unsorted cells, as shown in **Figure 1E–G**.

Effects of BMP-2 on ALDH^{br} cell growth. To test whether BMP-2 might inhibit tumorigenicity, we evaluated its effect on sorted tumorigenic ALDH^{br} cells. As shown in **Figure 2**, growth of the ALDH^{br} cells was significantly inhibited by the addition of 300 ng/mL of BMP-2 in the presence of 0.5% FBS for 48 h ($p < 0.05$) and ALDH^{br} cells with 300 ng/ml of BMP-2 treatment for 72 h showed growth inhibition although the significant was not obtained, whereas no growth inhibition was observed at the 300 ng/mL of BMP-2 for 24 h. Treatment of ALDH^{br} cells with 10 ng/ml at the three time points induced a stimulation of cell growth, to a lesser but significant extent, while no significant growth inhibition was observed at the 100 ng/mL of BMP-2 at the three time points.

BMP-2 downregulates the expression of embryonic stem cell markers in human osteosarcoma tumorigenic cells. Oct3/4A, Nanog and Sox-2 are important embryonic stem cell markers and have also been implicated in the tumorigenesis of many cancers.[19,20] We reported previously that the mRNA expression of Oct3/4A, Nanog and Sox-2 were all consistently higher in ALDH^{br} cells.[11] We therefore examined whether or not BMP-2 might inhibit the gene expression of Oct3/4A, Nanog and Sox-2 in tumorigenic ALDH^{br} cells. To test this, we chose 300 ng/mL of BMP-2 to treat ALDH^{br} cells for 48 h, since this dose consistently produced an inhibitory effect in cell growth. ALDH^{br} cells

treated with the same volume of vehicle were used as a control. As shown in **Figure 3**, Oct3/4A, Nanog and Sox-2 in ALDH^{br} cells were all consistently lower than those in control cells ($p < 0.05$; $p < 0.001$).

BMP-2 upregulates expression of osteogenic markers in human osteosarcoma tumorigenic cells. Blockage of stem cell differentiation may lead to tumorigenesis.[21] BMP-2 has been shown to act as a potent inducer of osteogenic differentiation.[22] We therefore sought to determine whether BMP-2 upregulates the transcription of osteogenic marker Runx-2 and Collagen Type I. Tumorigenic ALDH^{br} cells treated with BMP-2 at 300 ng/mL for 48 h were assayed for Runx-2 and Collagen Type I mRNA level by qRT-PCR. ALDH^{br} cells treated with the same volume of vehicle were used as a control. As shown in **Figure 4**, Runx-2 and Collagen Type I in ALDH^{br} cells were all consistently higher than those in control cells ($p < 0.05$).

BMP-2 inhibits tumor formation of human osteosarcoma tumorigenic cells in vivo. Based on previous findings, we reasoned that if BMP-2 inhibits embryonic stem cell marker expression, it might prevent tumor formation and growth in vivo. Therefore, we next injected freshly sorted tumorigenic ALDH^{br} cells with different concentrations of BMP-2 treatment or vehicle (control) subcutaneously into NOD/SCID mice. Experiments were performed in triplicate, as previously described in reference 23. Tumor growth of the ALDH^{br} cells was inhibited by the addition of 30 μg BMP-2 per animal (**Fig. 5A and B**) in all four animals. Hematoxylin and eosin staining of the tumor sections confirmed that tumors formed by ALDH^{br} cells without BMP-2 treatment contained malignant cells showing characteristic osteosarcoma features, including nuclear atypia, extensive neovascularization and high mitotic activity (**Fig. 5C**), whereas lesions in BMP-2-treated sites contained only residual large vesicle-like Affi-Gel blue beads, foreign-body giant cells and fibrocytes (**Fig. 5D**). No consistent growth inhibition was observed at the lower concentrations of BMP-2 at 1 and 10 μg/animal. To further confirm the tumor growth inhibitory effect of BMP-2 on ALDH^{br} cells, we performed the nuclear Ki-67 staining by immunohistochemistry. As shown in **Figure 6**, few Ki-67 positive cells were present in the sections of tumor from ALDH^{br} cells treated with BMP-2 (**Fig. 6A**), while most cells were Ki-67 positive in the sections of tumor from ALDH^{br} cells without BMP-2 treatment (**Fig. 6B**).

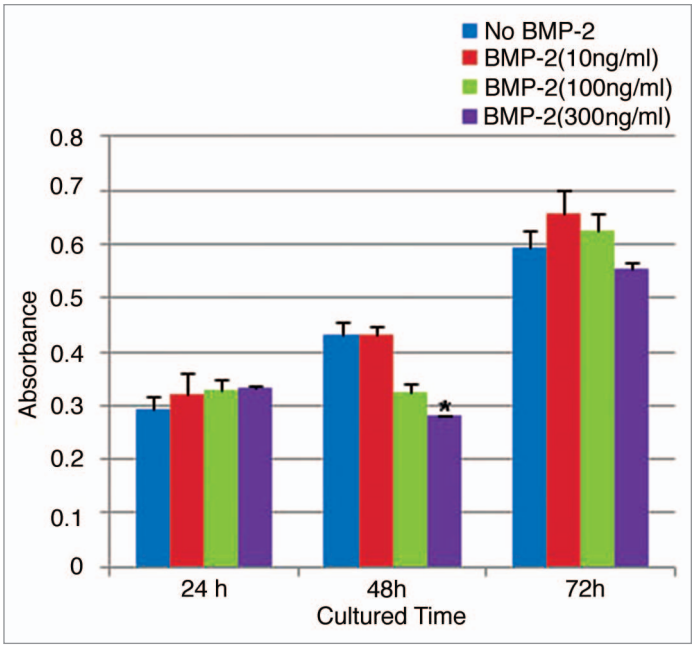

Figure 2. Effects of BMP-2 on ALDH^{br} cell growth. Freshly sorted ALDH^{br} cells were cultured for expansion and then inoculated in 96-well plates at a density of 1 x 10^4 cells/well for 24 h. Cells were then cultured in 0.5% serum-containing medium for another 24 h after washing in phosphate-buffered saline. Cells were treated with 10, 100 or 300 ng/mL BMP-2 diluted in 0.5% serum-containing medium or vehicle control for 24 , 48 and 72 h. Growth of the ALDH^{br} cells was significantly inhibited by the addition of 300 ng/mL of BMP-2 in the presence of 0.5% FBS for 48 h ($p < 0.05$). Each experiment was performed three times; representative examples are shown.

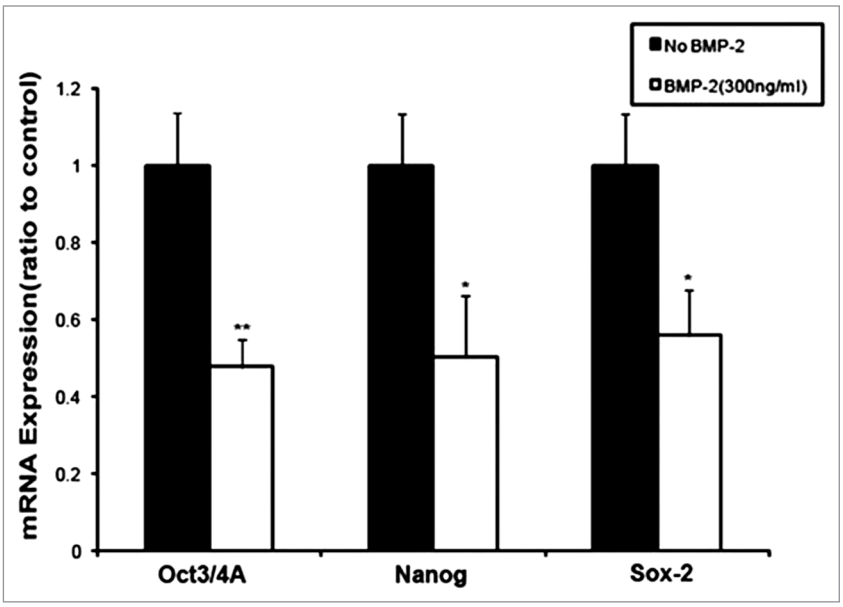

Figure 3. BMP-2 downregulates expression of embryonic stem cell markers in human osteosarcoma tumorigenic ALDH^{br} cells. Relative quantitative mRNA expression of Oct3/4A, Nanog and Sox-2 genes in ALDH^{br} cells freshly isolated from OS99-1 xenografts treated with 300 ng/mL of BMP-2 for 48 h. Gene expression levels were normalized to β-actin. Oct3/4, Nanog and Sox-2 in ALDH^{br} cells were all consistently lower than those in control cells (*$p < 0.05$; **$p < 0.001$). Each experiment was performed three times; representative examples are shown.

Discussion

Many studies[5,9,11,24-26] have demonstrated that most cancers originate from and are propagated by CSCs. The CSC theory postulates that most conventional cancer therapies are directed at the fast-growing tumor mass rather than the slow-dividing CSCs that lead to tumor recurrence or resistance to treatment.[27] Developing novel

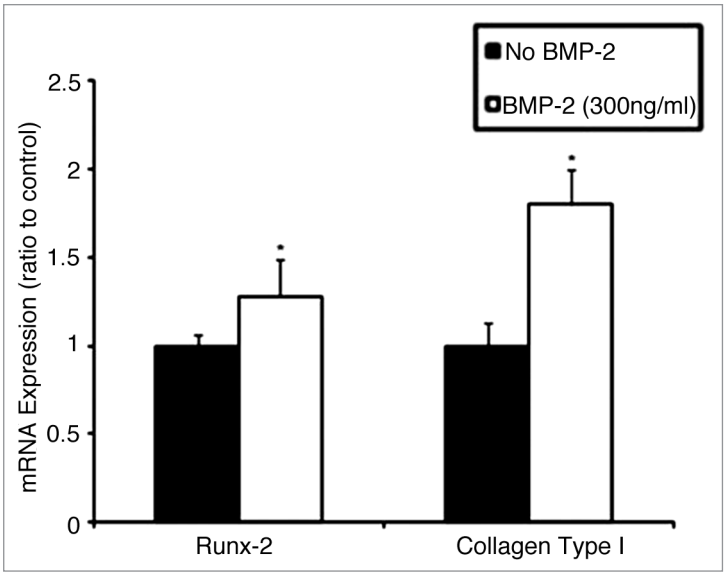

Figure 4. BMP-2 upregulates expression of osteogenic markers in human osteosarcoma tumorigenic ALDH^{br} cells. Relative quantitative mRNA expression of Runx-2 and collagen type I genes in ALDH^{br} cells freshly isolated from OS99-1 xenografts treated with 300 ng/mL of BMP-2 for 48 h. Gene expression levels were normalized to β-actin. Runx-2 and collagen type I in ALDH^{br} cells were all consistently higher than those in control cells (*$p < 0.05$). Each experiment was performed three times; representative examples are shown.

therapies is greatly needed to specifically target CSCs, which presumably will result in more effective cancer treatment.

BMPs are well-known members of the TGF-β superfamily that play an important role in cell activity in various tissues. There are currently three characterized BMP receptors: BMPR1A, BMPR1B and BMPR2. Activation of the BMP receptor complex initiates intracellular signaling transduction. Recent studies suggest that some BMPs and BMP signaling networks may be involved in tumorigenesis.[28] Treatment of human glioblastoma-derived brain tumor-initiating cells with BMPs, such as BMP-4, has been shown to result in inhibition of cell proliferation, induction of differentiation and importantly, a reduction in the ability to form tumors in immunodeficient mice.[18] CSCs have been identified as ALDH^{br} cells in human osteosarcoma;[11] however, the effect of BMPs on these putative CSCs has not yet been well-elucidated.

In the present study, we sought to examine the effect of BMP-2 on CSCs in the human osteosarcoma OS99-1 cell line. BMP-2 is approved by the United States Food and Drug Administration and is commercially available for clinical application. It has been reported that BMP signaling for the growth and differentiation of normal or neoplastic cells is dependent on its receptors.[29] Using regular PCR and immunostaining, we demonstrated that BMP receptors are expressed in tumorigenic ALDH^{br} cells, suggesting that BMP-2 could bind to its receptors and activate cell signaling to affect cell activities. Furthermore, we observed that BMP-2 had a significant inhibitory effect on tumorigenic ALDH^{br} cell proliferation at a concentration of 300 ng/mL as compared with much lower concentrations. This result is consistent with previous studies that have shown an inhibitory effect of BMP-2 on cancer cell growth, including prostate, breast, myeloma, gastric and colon cancers.[15,17,30,31] The response to BMP-2, however, is not uniform among all cancers. BMP-2 has been shown to stimulate the growth of pancreatic carcinoma cells.[32] In addition, Ide et al.[33] showed that BMP-2 inhibited the growth of prostate carcinoma cells in the presence of androgen, but stimulated cell growth in the absence of androgen. Thus, the biologic response of cancer cells to BMP-2 may depend not only on the particular cell type or concentration of BMP used, but may also depend on the presence of other factors that are not yet defined.

Oct3/4, Nanog and Sox-2 are essential transcription factors that regulate self-renewal and pluripotency of embryonic stem cells and play a critical role in the maintenance of self-renewal and pluripotency of embryonic stem cells. Recent studies showed that Oct3/4A, Nanog and Sox-2 have also been implicated in tumorigenesis.[19,20] Oct3/4A has been reported as a marker for adult stem cells and could be used to identify cancer stem cells.[34] Our previous investigation demonstrated that significantly elevated expression of embryonic stem cell marker genes Oct3/4A, Nanog and Sox-2 was shown in tumorigenic ALDH^{br} cells as compared to the non-tumorigenic ALDH^{lo} counterpart.[11] In this study using real-time PCR, we found BMP-2 decreased

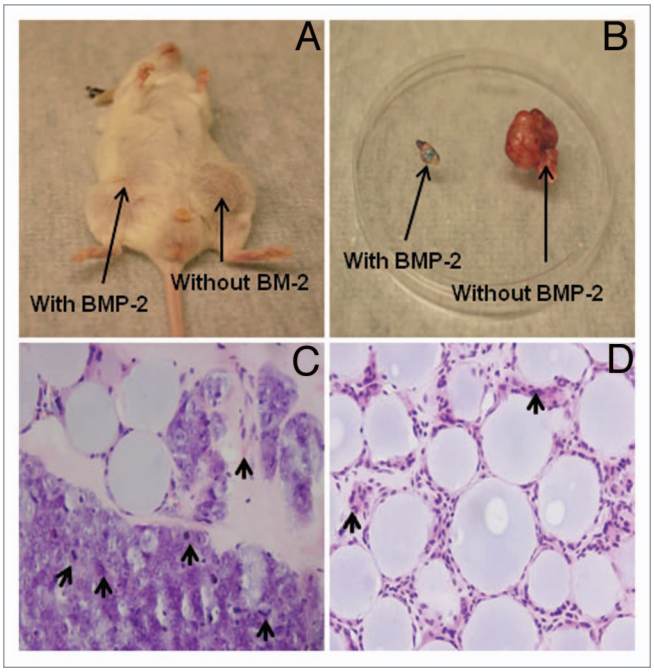

Figure 5. BMP-2 inhibits tumor formation by human osteosarcoma tumorigenic ALDH^{br} cells in vivo. (A and B) Representative tumor growth without BMP-2 treatment at the injection site in a NOD/SCID mouse. No significant tumor formation is seen at the injection site in BMP-2 treatment counterpart. (C) Representative hematoxylin and eosin staining of tumor generated from ALDH^{br} cells without BMP-2 treatment reveals presence of malignant cells showing characteristic osteosarcoma features, including nuclear atypia, extensive neovascularization and high mitotic activity (arrows). Large vesicle-like structures were Affi-Gel blue beads. (D) Injection site of ALDH^{br} cells treated with BMP-2 contained only residual large vesicle-like Affi-Gel blue beads, foreign-body giant cells (arrows) and fibrocytes.

the expression of stem cell marker genes Oct3/4A, Nanog and Sox-2 in tumorigenic ALDH^{br} cells treated with BMP-2 at a concentration of 300 ng/mL for 48 h compared to the control group without BMP-2. These results suggest that BMP-2 may reduce the gene expression of proteins associated with tumorigenesis.

Osteosarcoma may be caused by the disruption of osteogenic differentiation.[35] The induction of cell differentiation by BMP-2 has been reported for several cell types including myoblast cells, malignant fibrous histiocytoma cells and teratocarcinoma stem cells.[36-38] Analysis of the osteogenic marker Runx-2 and collagen type I in ALDH^{br} cells showed that BMP-2 increased the expression of the osteogenic marker gene Runx-2 and collagen type I in tumorigenic ALDH^{br} cells treated with BMP-2 at a concentration of 300 ng/mL for 48 h when compared with untreated controls. These findings suggest that BMP-2 might be able to activate osteogenic differentiation in human osteosarcoma CSCs and thus suppress their tumorigenicity.

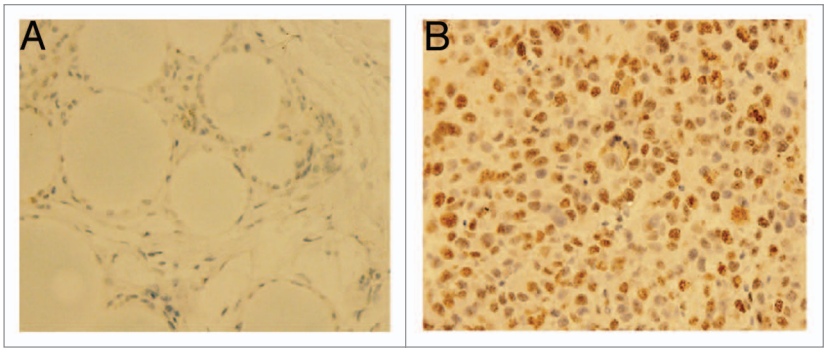

Figure 6. Nuclear Ki-67 staining by immunohistochemistry. (A) Few positive cells were seen in the sections containing ALDH^{br} cells treated with 30 μg of BMP-2. Large vesicle-like structures were Affi-Gel blue beads. (B) Representative tissue sections of tumor formed by ALDH^{br} cells without BMP-2 treatment showed most cells were Ki-67 positive.

Determining the effect of BMP-2 on putative osteosarcoma CSCs in vivo is essential for determining the potential use of BMP-2 clinically. In this study, we found that all animals receiving ALDH^{br} cells treated with 30 μg BMP-2 per animal did not form significant tumors. Conversely, untreated ALDH^{br} cells developed large tumor masses in NOD/SCID mice. Ki-67 immunostaining further confirmed the inhibitory effect of BMP-2 on ALDH^{br} cells. Thus, exposure to BMP-2 may deplete the CSC population and produce a significant decrease in the in vivo tumor-initiating ability of osteosarcoma cells. In contrast to the findings in our study, BMP-2 had previously been reported to promote tumor growth of human osteosarcomas in vivo.[22,35] In those studies, BMP-2 was secreted by transduced osteosarcoma cells with adenoviral vectors expressing BMP-2, but not exogenously as was done in our study. The different origin of BMP-2 may explain the contrasting results.

Although our report is the first to provide data indicating that BMP-2 has an inhibitory effect on CSCs in human osteosarcoma, the use of CSCs from one cell line provides limited evidence. Further research using more cell lines and primary tumor is therefore necessary to confirm the findings of this study.

In conclusion, our investigation revealed that BMP-2 inhibited the tumorigenic potential of CSCs in the human osteosarcoma cell line OS99-1. The inhibition may be due to decreased gene expression of proteins associated with tumorigenesis and/or an increased level of differentiation of osteosarcoma CSCs in response to BMP-2. The use of BMP-2 may therefore provide a new approach to human osteosarcoma treatment.

Materials and Methods

Cell culture. Human osteosarcoma OS99-1 cell line originally derived from a highly aggressive primary human osteosarcoma[39] was a generous gift from Dr. Sheila M. Nielsen-Preiss (Montana State University, Bozeman, MT). Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Gibco, Carlsbad, CA) supplemented with 10% FBS (Gibco) in a humidified atmosphere of 5% CO_2 in air at 37°C and used when in the log phase of growth.

Transplantation of human osteosarcoma OS99-1 cells into NOD/SCID mice. To obtain human osteosarcoma tumorigenic cells, subcutaneous injections of OS99-1 cells were performed in NOD/SCID mice (Harlan Laboratories Inc., Indianapolis, IN) to establish xenograft tumors as described previously in reference 11. Tumors formed were harvested for preparation of single-cell suspensions and histology. All animal studies were performed according to protocol approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Preparation of single-cell suspensions of xenograft tumor cells. Xenografted tumors formed by injection of OS99-1 cells were minced with scissors, mixed with 1 mg/mL Collagenase Type II (Sigma-Aldrich Co., St. Louis, MO), incubated for 3–4 h, passed through a 70 μm cell strainer and then washed twice with DMEM/F12/10% FBS medium for cell sorting.

ALDEFLUOR assay and isolation of tumorigenic cells from OS99-1 xenograft tumors by flow cytometry cell sorting. The ALDEFLUOR kit (Stemcell Technologies, Vancouver, BC, Canada) was used to isolate tumorigenic ALDH^{br} cells from OS99-1 xenografts, as described previously in reference 23. Incubation of cells from xenografts with ALDEFLUOR in the presence of the specific ALDH-inhibitor, dimethylaminobenzaldehyde (DEAB), which results in decreased fluorescence, was used as a negative staining control for the assay. During flow cytometry, mouse H-2K^b antibody (BD Biosciences Pharmingen, San Jose, CA) was used to eliminate cells of mouse origin from the xenograft tumors.

Cell proliferation assay. To investigate the effects of BMP-2 on cell growth, freshly sorted ALDH^{br} cells from OS99-1 xenografts were washed and cultured in 10% DMEM/F12 medium for expansion and then inoculated, at 1 x 10^4 cells per well, with 0.1 mL of the culture medium in a 96-well culture plate (Corning, New York, NY) and for 24 h. Cells were then cultured in 0.5% serum-containing medium for another 24 h after washing in phosphate-buffered saline. Cells were treated with 10,

100 or 300 ng/mL BMP-2 (GenScript Corporation, Piscataway, NJ) diluted in 0.5% serum-containing medium or vehicle control for 24, 48 and 72 h. Cell growth was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay according to the manufacturer's instructions (Promega, Madison, WI). Briefly, the culture medium was removed at different time points and 20 uL of MTS reagent mixed with 100 uL of Dulbecco phosphate-buffered saline was added to each well. Then, the cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere for 2 h and its absorbance at 490 nm was measured using Bio-TEK ELx 800 Plate Reader (Bio-Tek Instruments Inc., Wilrijk, Belgium).

Semiquantitative real-time polymerase chain reaction (PCR). To test the expression of BMP receptors in $ALDH^{br}$ cells, total RNA was extracted from freshly sorted $ALDH^{br}$ cells using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Reverse transcription was made using the SuperScript First-Strand Synthesis Kit (Invitrogen, Carlsbad, CA). For semiquantitative PCR, 1 μL of target cDNA conversion mixture was amplified using Hotstar Taq DNA Polymerase (Qiagen) for 35 cycles at 94°C for 30 s, at 56°C for 30 s and at 72°C for 1 min. The PCR primers included BMPR1A (forward, 5'-AAT GGA GTA ACC TTA GCA CCA GAG-3'; reverse, 5'-AGC TGA GTC CAG GAA CCT GTA C-3'), BMPR1B (forward, 5'-GGT TGC CTG TGG TCA CTT CTG G-3'; reverse, 5'-TAG TCT GTG ATT AGG TAC AAC TGG-3',), BMPR2 (forward, 5'-TCA GAT ATA TGG CAC CAG AAG TG-3'; reverse, 5'-GTG GAG AGG CTG GTG ACA CTT G-3',) and β-actin (forward, 5'-GCG GGA AAT CGT GCG TGA CAT T-3'; reverse, 5'-GGC AGA TGG TCG TTT GGC TGA ATA-3'). MCF-7 cell lines were used as positive controls for BMP receptors, as described previously in reference 18. PCR products were visualized by electrophoresis in agarose (1%) gels and stained with SYBR gel stain (Invitrogen).

Quantitative real-time polymerase chain reaction (qPCR). Freshly sorted $ALDH^{br}$ cells were washed and cultured for expansion and then inoculated, at 1 x 10^5 cells, with 2 mL of the culture medium in a 6-well culture plate. After 24 h incubation, the medium was replaced with 0.5% serum-containing medium for 24 h and then replaced with 0 and 300 ng/mL BMP-2 diluted in 0.5% serum-containing medium. After 48 h, total RNA was extracted as described above. Quantitative real-time PCR of embryonic stem cell marker Oct3/4A (Genbank accession number: NM_002701.4), Nanog (Genbank accession number: NM_024865.2), Sox-2 (Genbank accession number: NM_003106.2) and osteogenic marker Runx-2 (Genbank accession number: NM_001015051.2) and Collagen Type I (Genbank accession number: NM_000088.3) and β-actin gene expression were run in triplicate using Eppendorf Mastercycler Realplex Detection System (Eppendorf, Germany). Real-time quantitative primers were designed and purchased from Applied Biosystems. A positive standard curve for each primer was obtained using serially-diluted cDNA sample mixture. The quantity of gene expression was calculated using standard samples and normalized with β-actin.

In vivo co-treatment experiments. To examine the tumor inhibitory effect of BMP-2 on tumorigenic $ALDH^{br}$ cells, 1 x 10^4 freshly sorted $ALDH^{br}$ cells were mixed with Affi-Gel blue beads (Bio-Rad Laboratories, Philadelphia, PA) loaded with 1, 10 or 30 μg of BMP-2 at 37°C for 1 h. Sorted $ALDH^{br}$ cells treated with the same volume of vehicle were used as a control and then $ALDH^{br}$ cells with different concentrations of BMP-2 or vehicle mixed with Matrigel (BD Biosciences, San Jose, CA) (1:1 volume) were subcutaneously injected into right and left lower abdominal area of NOD/SCID mice.

Immunocytochemical and immunohistochemical staining. One million freshly sorted $ALDH^{br}$ cells and unsorted cells from OS99-1 xenografted tumors were put on a slide using the cytospin cytocentrifuge and spun for 5 min at 2,000 rpm. Immunocytochemistry was performed using three antibodies: BMPR1A, BMPR1B and BMPR2 (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA). The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA). Fluorescent cells were visualized and digital images were captured using an Olympus microscope (Olympus America Inc., Milville, NY). For immunohistochemistry, formalin-fixed paraffin sections of tumor samples were stained with mouse anti-Ki-67 antibody (1:100; Santa Cruz Biotechnology Inc.) using standard procedures.

Statistical analysis. Data were expressed as mean ± SD. Statistically significant differences were determined by two-tailed Student's t-test and SPSS 11 software (SPSS Inc., Chicago, IL) and defined as $p < 0.05$.

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