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Abstract

The correlation between BMP-2 and osteosarcoma growth has gained increased interest in the recent years, however, there is still no consensus. In this study, we tested the effects of BMP-2 on osteosarcoma cells through both in vitro and in vivo experiments. The effect of BMP-2 on the proliferation, migration and invasion of osteosarcoma cells was tested in vitro. Subcutaneous and intratibial tumor models were used for the in vivo experiments in nude mice. The effects of BMP-2 on EMT of osteosarcoma cells and the Wnt/β-catenin signaling pathway were also tested using a variety of biochemical methods. In vitro tests did not show a significant effect of BMP-2 on tumor cell proliferation. However, BMP-2 increased the mobility of tumor cells and the invasion assay demonstrated that BMP-2 promoted invasion of osteosarcoma cells in vitro. In vivo animal study showed that BMP-2 dramatically enhanced tumor growth. We also found that BMP-2 induced EMT of osteosarcoma cells. The expression levels of Axin2 and Dkk-1 were both down regulated by BMP-2 treatment, while β-catenin, c-myc and Cyclin-D1 were all upregulated. The expression of Wnt3α and p-GSK-3β were also significantly upregulated indicating that the Wnt/β-catenin signaling pathway was activated during the EMT of osteosarcoma driven by BMP-2. From this study, we can conclude that BMP-2 significantly promotes growth of osteosarcoma cells (143B, MG63), and enhances mobility and invasiveness of tumor cells as demonstrated in vitro. The underlying mechanism might be that BMP-2 promotes EMT of osteosarcoma through the Wnt/β-catenin signaling pathway. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 37:1638–1648, 2019. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

Keywords

bone morphogenetic protein-2, epithelial-mesenchymal transition, osteosarcoma, Wnt/β-catenin, Animals, Bone Morphogenetic Protein 2, Bone Neoplasms, Cell Line, Tumor, Cell Movement, Cell Proliferation, Epithelial-Mesenchymal Transition, Humans, Male, Mice, Mice, SCID, Osteosarcoma, Wnt Signaling Pathway, axin2 protein, beta catenin, bone morphogenetic protein 2, cyclin D1, dickkopf 1 protein, glycogen synthase kinase 3beta, Myc protein, protein, unclassified drug, Wnt protein, Wnt3a protein, bone morphogenetic protein 2, animal experiment, animal model, animal tissue, Article, cancer growth, cell invasion, cell migration, cell motility, cell proliferation, down regulation, epithelial mesenchymal transition, human, human cell, in vitro study, in vivo study, male, mouse, nonhuman, nude mouse, osteosarcoma, osteosarcoma cell, priority journal, signal transduction, tumor model, tumor volume, tumor weight, upregulation, animal, bone tumor, cell motion, drug effect, epithelial mesenchymal transition, osteosarcoma, SCID mouse, tumor cell line, Wnt signaling

Disciplines

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Comments

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Bone Morphogenetic Protein-2 Promotes Osteosarcoma Growth by Promoting Epithelial-Mesenchymal Transition (EMT) Through the Wnt/β-Catenin Signaling Pathway

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ABSTRACT: The correlation between BMP-2 and osteosarcoma growth has gained increased interest in the recent years, however, there is still no consensus. In this study, we tested the effects of BMP-2 on osteosarcoma cells through both in vitro and in vivo experiments. The effect of BMP-2 on the proliferation, migration and invasion of osteosarcoma cells was tested in vitro. Subcutaneous and intratibial tumor models were used for the in vivo experiments in nude mice. The effects of BMP-2 on EMT of osteosarcoma cells and the Wnt/β-catenin signaling pathway were also tested using a variety of biochemical methods. In vitro tests did not show a significant effect of BMP-2 on tumor cell proliferation. However, BMP-2 increased the mobility of tumor cells and the invasion assay demonstrated that BMP-2 promoted invasion of osteosarcoma cells in vitro. In vivo animal study showed that BMP-2 dramatically enhanced tumor growth. We also found that BMP-2 induced EMT of osteosarcoma cells. The expression levels of Axin2 and Dkk-1 were both down regulated by BMP-2 treatment, while β-catenin, c-myc and Cyclin-D1 were all upregulated. The expression of Wnt3α and p-GSK-3β were also significantly upregulated indicating that the Wnt/β-catenin signaling pathway was activated during the EMT of osteosarcoma driven by BMP-2. From this study, we can conclude that BMP-2 significantly promotes growth of osteosarcoma cells (143B, MG63), and enhances mobility and invasiveness of tumor cells as demonstrated in vitro. The underlying mechanism might be that BMP-2 promotes EMT of osteosarcoma through the Wnt/β-catenin signaling pathway. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 37:1638–1648, 2019.

Keywords: bone morphogenetic protein-2; osteosarcoma; epithelial-mesenchymal transition; Wnt/β-catenin

Osteosarcoma is one of the most common skeletal tumors affecting children and adolescents and historically was associated with a poor survival rate.[1] With the development of adjuvant chemotherapy, limb-salvage surgery has not only become possible but is now preferred over amputation in the surgical management of this disorder.[2] Currently, 80–90% of cases are being treated with limb-salvage procedures instead of amputation without compromising oncological principles.[3] In spite of a relative satisfactory survival rate following limb-salvage surgeries, reconstruction of the bone defect left by tumor resection is still a major concern.[4]

Since approved for clinical use by FDA in 2002, BMP-2 has been used as a substitute for bone auto graft or allograft, yielding superior fusion results.[5] The relationship between BMP-2 and tumor growth has gained increasing interest in the recent years, especially after Carragee et al. raised concerns with respect to tumor formation in association with the use of BMP-2 in spine surgery,[6] However, there is still no consensus regarding either the safety of exogenously delivered BMP-2 or the role of endogenous BMP-2 in autocrine stimulation of tumor growth.[7] The effects of BMP-2 on osteosarcoma are of particular interest to orthopaedic surgeons. The excellent osteogenic effect of BMP-2 imparts to it obvious potential for application in postoperative reconstruction following limb-salvage surgeries. Some authors have even suggested using BMP-2 as a therapeutic option for the treatment of osteosarcoma.[8] Although there have been some reports describing the use of BMP-2 in the treatment of mandibular sequelae after resection of ameloblastoma, most orthopaedic surgeons maintain a more cautious attitude with respect to the use of BMP-2 following surgery involving osteosarcoma.[9]

The role of endogenous BMP-2 in autocrine stimulation or suppression of tumors is especially complex because of the heterogeneity of tumor types and the marked differences in the many tumor models, even

Drs. Haijun Tian, Tangjun Zhou, and Hongfang Chen contributed equally to this study.

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those representing the same disease. Such heterogeneity suggests that BMP-2 related therapeutics will need to be employed in an individualized manner. The role of BMPs in osteosarcoma in particular has been extensively studied previously, and, for example, both BMPs and BMP receptors have been shown to be expressed in many tumor samples.[10] However, both stimulatory and inhibitory effects on different osteosarcoma-derived tumor cell lines have been documented and many of the results are patently contradictory. On the one hand, Sotobori et al. reported that BMP signaling enhanced osteosarcoma cell migration in vitro,[11] Yang et al. found that BMP-2 induced faster proliferation in osteosarcoma mesenchymal stem cells compared to control bone marrow mesenchymal stem cell,[12] and Luo et al. reported that BMP-2 failed to induce bone formation from human osteosarcoma cells but rather effectively promoted osteosarcoma tumor growth in an orthotropic model.[13] On the other hand, Rici et al. treated canine mesenchymal stem cells with BMP-2 and then added them to cultures of canine osteosarcoma cells and their findings suggested that treatment of bone marrow cells with rhBMP-2 may have therapeutic potential for osteosarcoma treatment.[14] Furthermore, Park et al. found that treatment with BMP-2 combined with a MEK inhibitor inhibited the proliferation of MG-63 osteosarcoma cells,[15] and Geller et al. reported that the application of BMP-2 did not increase local recurrence of osteosarcoma in an orthotopic xenograft murine model.[16]

This study was aimed to test the effects of BMP-2 on osteosarcoma through both in vitro experiments and in vivo studies, hoping to serve as a reference for further clinical practice.

MATERIALS AND METHODS

Reagents and Antibodies

Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN). Anti-phospho GSK-3β, anti-GSK-3β, anti-Wnt3α were purchased from Cell Signaling (Beverly, MA). Anti-vimentin and anti-fibronectin were purchased from Invitrogen (Carlsbad, CA). Anti-β-tubulin and anti-β-catenin were purchased from Sigma (St. Louis, MO). Anti-E-cadherin and anti-N-cadherin were purchased from BD Bioscience (San Diego, CA). Anti-Snail was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and DAPI was purchased from Molecular Probes (Carlsbad, CA).

Cell Culture

The human osteosarcoma cell line 143B and MG63 was obtained from ATCC (Manassas, VA). The 143B cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C, and the MG63 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 5% CO_2 at 37°C.

MTT Assay

To investigate the effects of BMP-2 on cell growth, 143B and MG63 osteosarcoma cells were seeded into 96 well micro plates at 2×10^3 cells per well. Cells were cultured for 24 h to allow for attachment, starved for 12 h with 1% FBS medium, and then treated with different concentrations of BMP-2 (10, 50, 100, and 500 ng/ml) for 12, 24 and 48 h. Cell growth was evaluated using a 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay following the manufacturer's instructions (ATCC Manassas, VA).

CFSE Cell Proliferation Assay

A second proliferation assay was performed using the CellTrace™ CFSE Cell Proliferation Kit (Life Technologies Inc, Grand Island, NY). 143B and MG63 osteosarcoma cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) following the manufacturer's instructions. Cells were resuspended in pre-warmed PBS/0.1% BSA at a final concentration of 1×10^6 cells/ml, and CFSE was added to the cell suspension to achieve a final concentration of 10 μM. After incubation at 37°C for 10 min, ice-cold culture media were added to quench the staining and the cells were incubated for an additional 5 min on ice. The cells were pelleted and resuspended in fresh media for a total of three washes. Cells were again cultured until sufficient cells could be harvested. The stained cells were then seeded into 96 well micro plates at 3×10^3 cells per well and cultured for 24 h to allow for attachment. The attached cells were starved for 12 h with 1% FBS medium and then treated with different concentration of BMP-2 (10, 50, 100, 500 ng/ml) for 48 h. Flow cytometry was performed using a FACSCalibur™ instrument (BD Biosciences, San Jose, CA) and the data output analyzed using FlowJo software (Tree Star Inc., Ashland, OR) as previously described.[17] Cells were analyzed for CFSE dilution and percent suppression was determined based on the percentage of dividing CFSE-labeled cells in the co-culture as compared to untreated CFSE-labeled cells.

Wound Healing Assay

The wound healing/cell migration assay was performed as previously described.[18] Briefly, 143B and MG63 cells were seeded in a 12-well plate and cultured for 24 h to allow for attachment and then starved for 12 h with 1% FBS medium. After changing back to normal culture conditions, a scratch (wound) was produced across the monolayer of cells using the tip of a pipette and the adherent monolayer was gently washed twice with PBS to remove non-adherent cells. Cells were then grown in culture medium with 5% FBS to which was added either PBS (control) or different concentration of BMP-2(0, 10, 50 ng/ml). Images of the wound were obtained at 0, 2, 4, and 8 h with a microscope with the 5x objective. The migration rate was quantified by measuring the distance between the wound edges by using the Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij/). All experiments were repeated at least three times.

Matrigel Invasion Assay

Invasion assays were performed using a 24-well invasion chamber system (BD Biosciences, Bedford, MA). 143B or MG63 cells were trypsinized and counted with a hemocytometer using trypan blue and viable cells were seeded in the upper chamber at 1×10^4 cells/well in serum-free MEM or EMEM. MEM or EMEM supplemented with 10% FBS containing BMP-2 or control (used as a chemoattractant) was

placed in the bottom well. Incubation was carried out for 36 h at 37°C in humidified air with 5% CO_2. Non-migratory cells in the upper chamber then were removed with a cotton-tip applicator. Migrated cells on the lower surface were fixed with methanol and stained with hematoxylin. The number of migrating cells was determined by cell counting. Cells were assayed in triplicate for each experiment and mean values were calculated.

Realtime PCR

To examine effects of BMP-2 on Wnt/β-catenin signaling in osteosarcoma cells at the gene level, 143B cells were seeded in 6-well plates at a density of 2×10^4 cells/well and cultured, with or without BMP-2(0, 100 ng/ml), for 96 h. Total RNA was isolated by TRIzol® Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) followed by DNase treatment (Invitrogen, Life Technologies). 1 μg RNA was subjected to reverse transcription with the SuperScript II Reverse Transcriptase Kit (Invitrogen, Life Technologies) per the manufacturer's instruction. Real-time PCR was performed with a 7300 Real-Time PCR system with SYBR Green Mastermix (Invitrogen, Life Technologies). All primer sequences used are listed in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was evaluated in separate tubes for each RT reaction as a standard. Relative gene expression was analyzed by the ΔΔCT method.[19]

Western Blotting Analysis

Western blotting analysis was performed as previously reported.[20] Whole cell extracts were prepared by lysing the cells in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH7.4, 1 mM EDTA, 1 mM PMSF, and 1% TritonX-100) containing a cocktail of protease inhibitors and phosphatase inhibitors. Protein concentrations were determined using the BCA protein assay (Thermo Scientific, IL). A total of 20–30 mg of protein was separated by SDS–PAGE and transferred to a nitrocellulose membrane. The proteins were detected with primary antibodies. The protein bands were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Transfection of Lenti-CMV-Firefly Luciferase (FL)

143B or MG63 Cells were seeded at 2×10^5 cells per well in 6-well plates (Corning) and incubated at 37°C overnight in MEM or EMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Transduction was carried out in 1 ml of medium with Lenti-CMV-Fluc2 at a multiplicity of inflection of 5 (MOI = 5), including lentiviral vector supernatant and 1ug/ml of protamine sulfate. The cells were then washed with phosphate-buffered saline (PBS) 24 h post-transduction and incubated in regular growth medium for an additional 48 h. FL luciferase activity was measured following the manufacturer's instructions (Promega) using a luminometer (Berthold Detection Systems, Pforzheim, Germany). Each value was normalized to cell number or protein amount and calculated as the average of triplicate samples.

Table 1. List of Primers for Quantitative Realtime PCR

Gene	Sequence
GAPDH	5′-AGG GCT GCT TTT AAC TCT GGT-3′
	5′-CCC CAC TTG ATT TTG GAG GGA-3′
β-catenin	5′-AAACGGCTTTCAGTTGAGC-3′
	5′-CTGGCCATATCCACCAGAGT-3′
Axin2	5′-CTCCTTGGAGGCAAGAGC-3′
	5′-GGCCACGCAGCACCGCTG-3′
Dkk-1	5′-GACTGTGCCTCAGGATTGTGT-3′
	5′-CAGATCTTGGACCAGAAGTGTCT-3′
c-myc	5′-CAG CTG CTT AGA CGC TGG ATT-3′
	5′-GTA GAA ATA CGG CTG CAC CGA-3′
Cyclin D1	5′-ACA AAC AGA TCA TCC GCA AAC AC-3′
	5′-TGT TGG GGC TCC TCA GGT TC-3′

Implantation of Tumor Cells and Test Materials

All animal experiments were approval in advance by the UCLA Chancellor's Animal Research Committee. Sixteen 8-week-old male Crl 474 SCID mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed and maintained at the UCLA vivarium under the care of veterinarians and in accordance with the regulations set forth by the UCLA Office of Protection of Research Subjects. All the mice received subcutaneous injections of 5×10^5 143B osteosarcoma cells in a total volume of 200 μl that contained 40 μl of growth factor reduced Matrigel (BD Biosciences, San Jose, CA), 50 μl of culture medium, and specified combinations of test proteins. The mice are divided into two groups as follows: Group 1 (control group), vehicle only, no BMP-2; Group 2, 15 μg BMP-2. Mice were anesthetized with isoflurane delivered in oxygen, and the overlying skin of the back was sterilized using 70% ethanol. Subcutaneous injections were made on the lower left quarter of the back using a 27.5-gauge needle. Subcutaneous tumors were measured externally on days 0, 4, 7, 10, 14 and 21, by using a tumor meter, and tumor volume was estimated as described previously.[21]

Another 16 SCID mice received tibial implantation of 5×10^5 143B osteosarcoma cells in a total volume of 50 μl of culture medium with (Group 1, 15 μg BMP-2) or without BMP-2 (Group 2, control group). Mice were anesthetized with isoflurane delivered in oxygen, and the overlying skin of the lower limb was sterilized using 70% ethanol. A 27.5-gauge needle was introduced through the proximal tibial plateau and into the proximal tibia, and the 50 μl of mixtures containing cells was injected into the cavity. X-ray images of the injected limb were obtained every week.

Imaging of Tumors

Under 2% isoflurane anesthesia, mice were imagedtwenty minutes after intraperitoneal injection of 150 mg/kg of D-Luciferin (Caliper LifeSciences, Hopkinton, MA) using an IVIS cooled CCD camera (Xenogen, Alameda, CA). Images were analyzed using IGOR-PRO Living Image Software (Xenogen), as described previously.[22] The mice with subcutaneous injection of tumor cells were imaged on days 0, 4, 7, 10, and 14, and the mice with tibia injection of tumor cells were imaged on days 0, 7, 14, 21, and 28.

Measurement of Tumor Size and Weight

For the subcutaneous tumor, an external caliper was used for the measurement of tumor size on days 0, 4, 7, 10, 14, and 21, and the tumor volume was calculated based on the modified ellipsoidal formula.[23] All the mice were sacrificed on day 21 and the tumor specimens were dissected immediately in order to measure the tumor weight using a precision electronic auto balance as previously described.[24] For the

intratibial tumor, mice were sacrificed on day 28, and both lower limbs were cut at the same level of hip joint, and the weight of specimens were measured using the precision electronic auto balance. The approximate tumor weight was calculated as the weight of tumor bearing limb—the weight of tumor free limb.

Histology and Immunohistochemistry

The subcutaneous tumor specimens were fixed in 4% paraformaldehyde (Sigma–Aldrich, St. Louis, MO) at 4°C overnight before paraffin embedding. The intratibial tumor specimens were decalcification by submersion in Cal-Ex (Thermo Fisher; Waltham, MA) and then were washed with tap water. Hematoxylin and eosin (H&E) staining was performed on 5-μm sections for histological analyses.

Statistical Analysis

Data were expressed as mean ± standard error (mean, S.E.) and SPSS version 13.0 (IBM, Armonk, NY) was employed for statistical analysis. Means of values for multiple groups were compared by one-way ANOVA with subsequent Tukey-Kramer comparisons tests and Student's t-test was performed for comparisons involving just two groups.

RESULTS

BMP-2 Does Not Affect Proliferation of Osteosarcoma Cells In Vitro

The effect of BMP-2 on osteosarcoma cell proliferation in vitro was assessed using two standard methods, MTT and CFSE, with different mechanism. Neither of the two experimental methods identified any difference between the BMP-2 group and control group in either 143B cells or MG63 cells (Fig. 1). The two groups showed similar cell counts as measured by MTT and cell division rates as measured by CFSE for up to 48 h of incubation when the cell culture reached near-confluence.

BMP-2 Promotes Migration and Invasion of Osteosarcoma Cells In Vitro

On the other hand, in the in vitro wound-closure/migration assay, BMP-2 promoted the migration of 143B cells and MG63 cells in a dose-dependent manner, with higher concentrations of BMP-2 correlated with higher cellular motility (Fig. 2A). The difference between groups could be seen as early as four hours after the initiation of treatment (Fig. 2B). Similarly, treatment of osteosarcoma cells with BMP-2 was observed to increase cell invasiveness, as assessed by migration through Matrigel, in a dose-dependent manner (Fig. 2C and D).

BMP-2 Promotes Osteosarcoma Growth In Vivo

To examine the effect of BMP-2 on osteosarcoma growth in vivo, we implanted tumor cells in SCID mice with or without BMP-2. Luciferase expression was used to track tumor growth for a period of 14 days (subcutaneous model) or 28 days (intratibial model). The studies were discontinued at this point for humane reasons relating to the huge size of the tumors in the BMP-2 treated animal subjects.

For the subcutaneous injection model, co-implantation of BMP-2 with the osteosarcoma cells resulted in a remarkable acceleration of tumor growth as manifest by luminesce (Fig. 3A and B) and tumor volume (Fig. 3C). Dissection of two representative tumors is depicted in Figure 3D and shows the obvious difference between the two treatment groups. The mean weights for the tumors from the two groups are shown in Figure 3E. Histological examination with H&E staining also demonstrated a larger contour of tumor size in the BMP-2 group compared with the control group. Tumors from both groups contained areas of central necrosis because of the fast tumor growth but the BMP-2 group showed more such areas related to the faster growth rate caused by BMP-2 stimulation (Fig. 3F).

BMP-2 also promoted osteosarcoma tumor growth in the intratibial injection model. The BMP-2 group showed more obvious osteolytic lesions in the tibia, and larger contour of tumor mass as seen in the X-ray images (Fig. 4A). It also resulted in a higher luminescence intensity (Fig. 4B and C) and tumor weight (Fig. 4D and E). Histological examination with H&E staining also demonstrated a larger contour of tumor size in the BMP-2 group. While the control group contained little area of central necrosis, the BMP-2 group showed more such areas related to the faster tumor growth (Fig. 4F).

BMP-2 Promotes EMT of Osteosarcoma Through Wnt/β-Catenin Signaling Pathway

Based on the finding that BMP-2 mainly affected migration and invasiveness, rather than proliferation, we examined EMT marker expression in OS cells, as EMT is often implicated in cancer progression, invasiveness, metastasis, and drug resistance. We found that BMP-2 treatment did result in a cadherin switch in a dose-dependent fashion, with significant downregulation of E-cadherin and upregulation of N-cadherin. Moreover, expression of two other EMT markers, Vimentin, and Snail, was also positively correlated with BMP-2 treatment (Fig. 5A).

Since Wnt/β-catenin signaling is clearly related to osteosarcoma progression and to EMT in other cancer cells, we further tested the activation of the Wnt/β-catenin signaling pathway. The levels of β-catenin, Wnt inhibitors Axin2 and Dkk-1, and downstream c-myc, Cyclin-D1 gene expression were quantified using Realtime PCR. Wnt3α, β-catenin, GSK-3β as well as p-GSK-3β were also examined with Western blotting analysis. The expression levels of Axin2 and Dkk-1 were both down regulated by BMP-2 treatment while β-catenin, c-myc and Cyclin-D1 were all upregulated (Fig. 5B). The expression of Wnt3α and p-GSK-3β were also significantly upregulated (Fig. 5C). Taken together, these findings indicate that the Wnt/β-catenin signaling pathway was activated during the EMT of osteosarcoma driven by BMP-2.

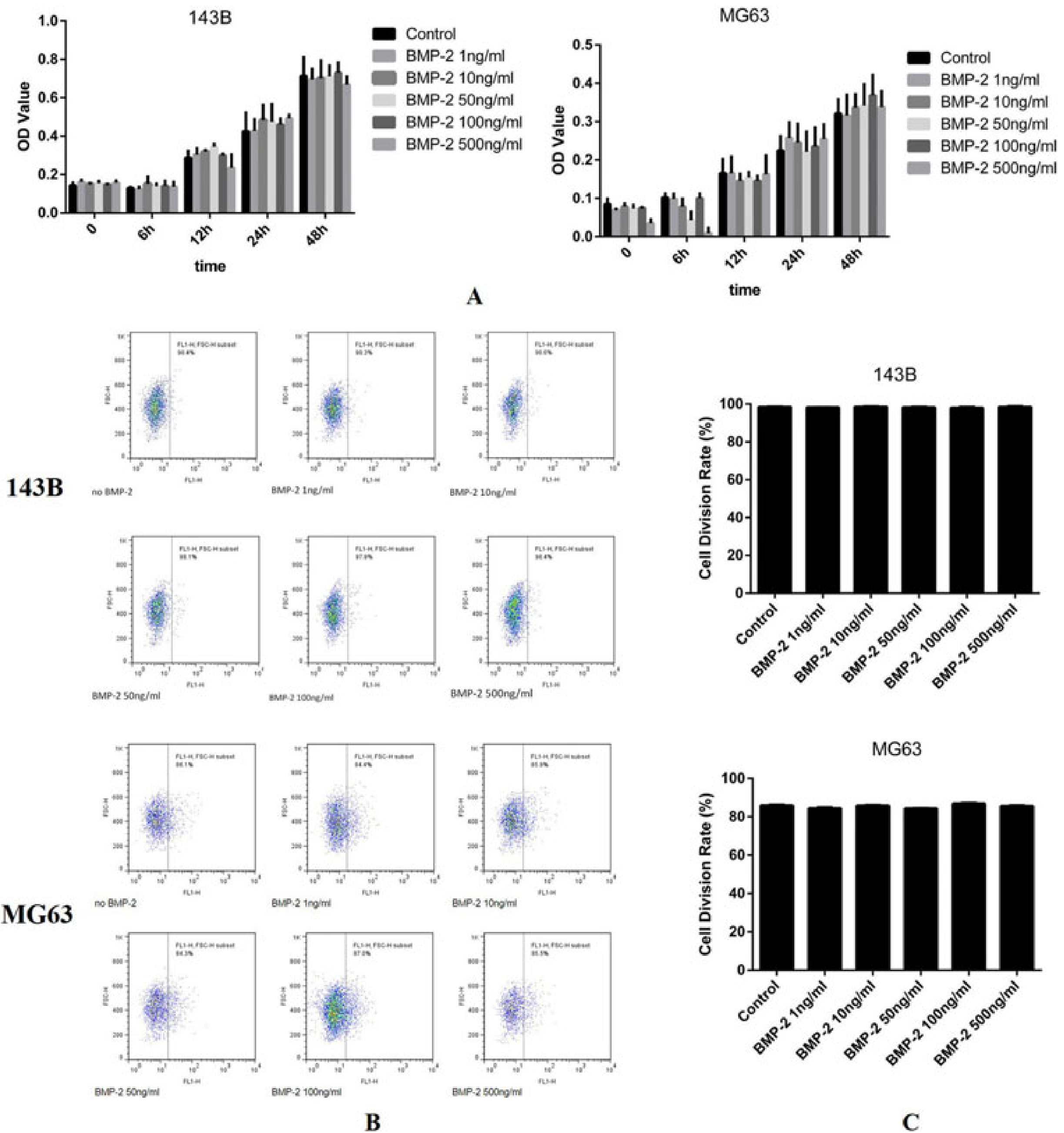

Figure 1. In vitro proliferation tests showing the effects of BMP-2 on 143B and MG63 osteosarcoma cells. A, MTT test; B, CFSE test; C, Cell division rate calculated by CSFE. Differences between the BMP-2 groups and control group could not be identified by either of the two experimental methods.

DISCUSSION

In an effort to provide a clear and practical summary of the roles of BMP-2 so that clinicians can make informed decisions regarding the use of exogenous BMP-2 and pursue the use of BMP agonists and antagonists as potential therapeutics, our group has recently published a comprehensive review.[25] A prominent finding in our review was that those works that hypothesize an inhibitory effect for BMP-2 most often examined only the proliferative properties of the tumor cells, whereas the papers that hypothesized a promotive effect were often more comprehensive and examined the effects of BMP-2 on invasiveness and migration of tumor cells as well.[7] Many authors reported opposing results pertaining to the effects of BMP-2 on tumor growth when proliferation testing alone was employed. On the other hand, a number of authors concluded that BMP-2 enhanced tumorigenesis when the results of proliferation assays were de-emphasized in favor of more sophisticated testing.[25] Thus, it is apparent that the analysis of proliferation alone is insufficient to assess the overall tumorigenic properties of BMP-2.

Orui et al. reported that the growth of osteosarcoma cell lines was not promoted by BMP-2 at different

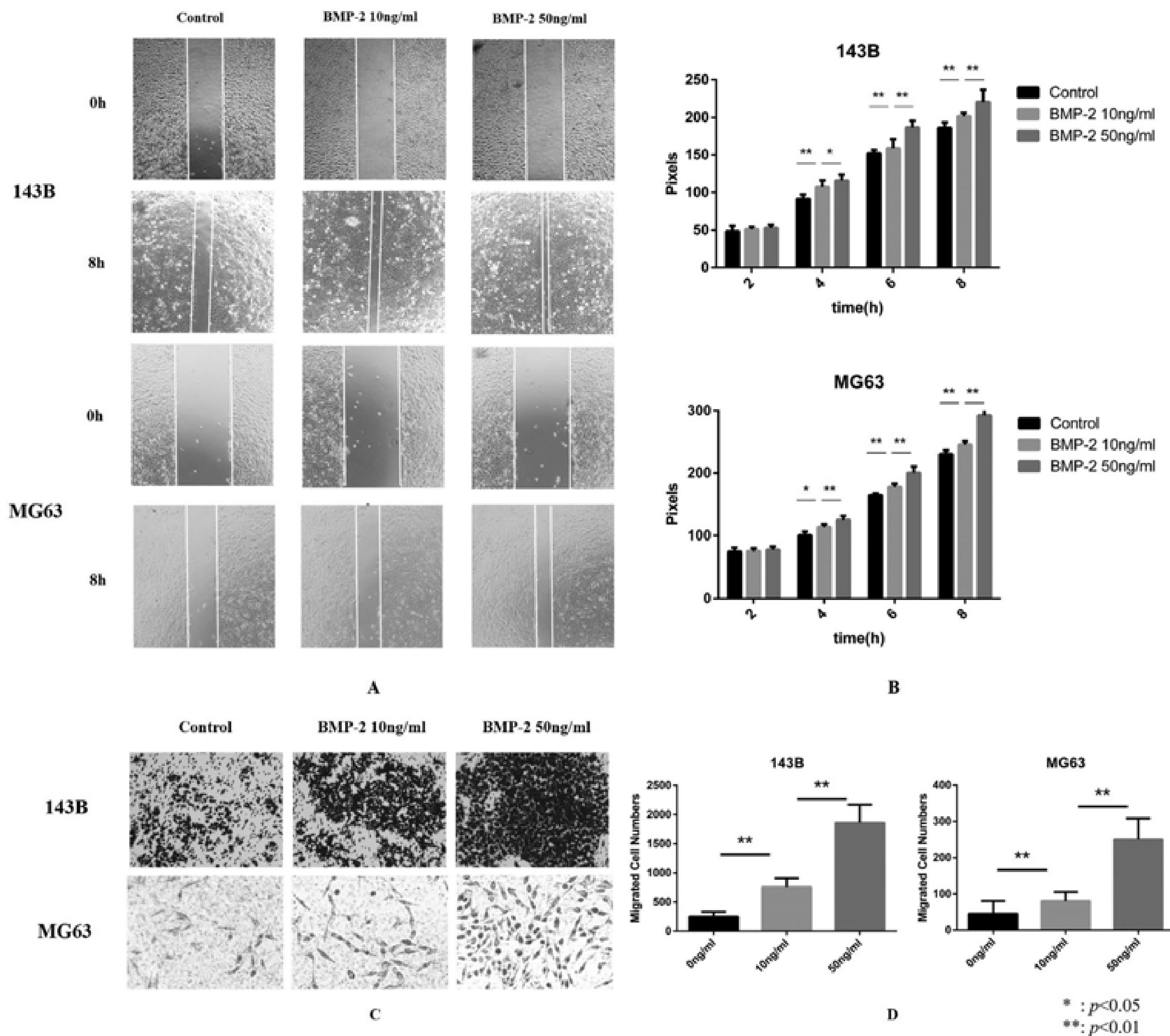

Figure 2. A and B, Wound-closure assay showing that BMP-2 promoted the migration of osteosarcoma cells and that 50 ng/ml BMP-2 had a significantly greater effect than did 10 ng/ml. C and D, Invasion assay showing that an increased number of cells migrated through Matrigel following BMP treatment and this also occurred in a dose-dependent manner.

concentrations as evaluated by the MTT assay.[26] This is consistent with our current findings. In addition, we repeated the studies using the CSFE assay which assesses proliferation through a different mechanism and we observed similar results. Obviously, tumor growth in situ is a much more biologically complex process than is cell division in vitro. In an effort to address this increased complexity in vitro we also assessed cell motility and invasiveness, two properties that are central to tumor growth. We observed that BMP-2 promoted both migration and invasiveness of osteosarcoma cells in vitro.

Invasion and metastasis are important biological characteristics of malignant tumors. The processes are quite complicated and involve multiple molecular mechanisms, including the adhesion, migration and invasion.[27] Cadherins are a family of transmembrane glycoproteins that play an indispensable role in intercellular adhesion. One member of this family, E-cadherin, which is expressed predominantly on epithelial cells, is considered to be an important suppressor of tumor metastasis.[28] The cytoplasmic domain of E-cadherin binds to β-catenin and the E-cadherin-β-catenin complexes promote cell-cell adhesions and maintain the polarity of the same cells.

Epithelial-mesenchymal transition (EMT) is defined by conversion of cells with an epithelial phenotype into cells with a mesenchymal phenotype. It is critical for embryonic development and in adults, EMT occurs during wound healing, tissue regeneration, organ fibrosis and tumor progression.[29] EMT is characterized by the loss of cell polarity, altered cell-cell and cell-matrix adhesion, and the acquisition of a migratory, mesenchymal phenotype. Other reported changes include down-regulation of E-cadherin, induction of N-cadherin, and up-regulation of Snail and Vimentin.[30] TGF-β family members are considered to be major triggers of EMT. In our study, we found that BMP-2

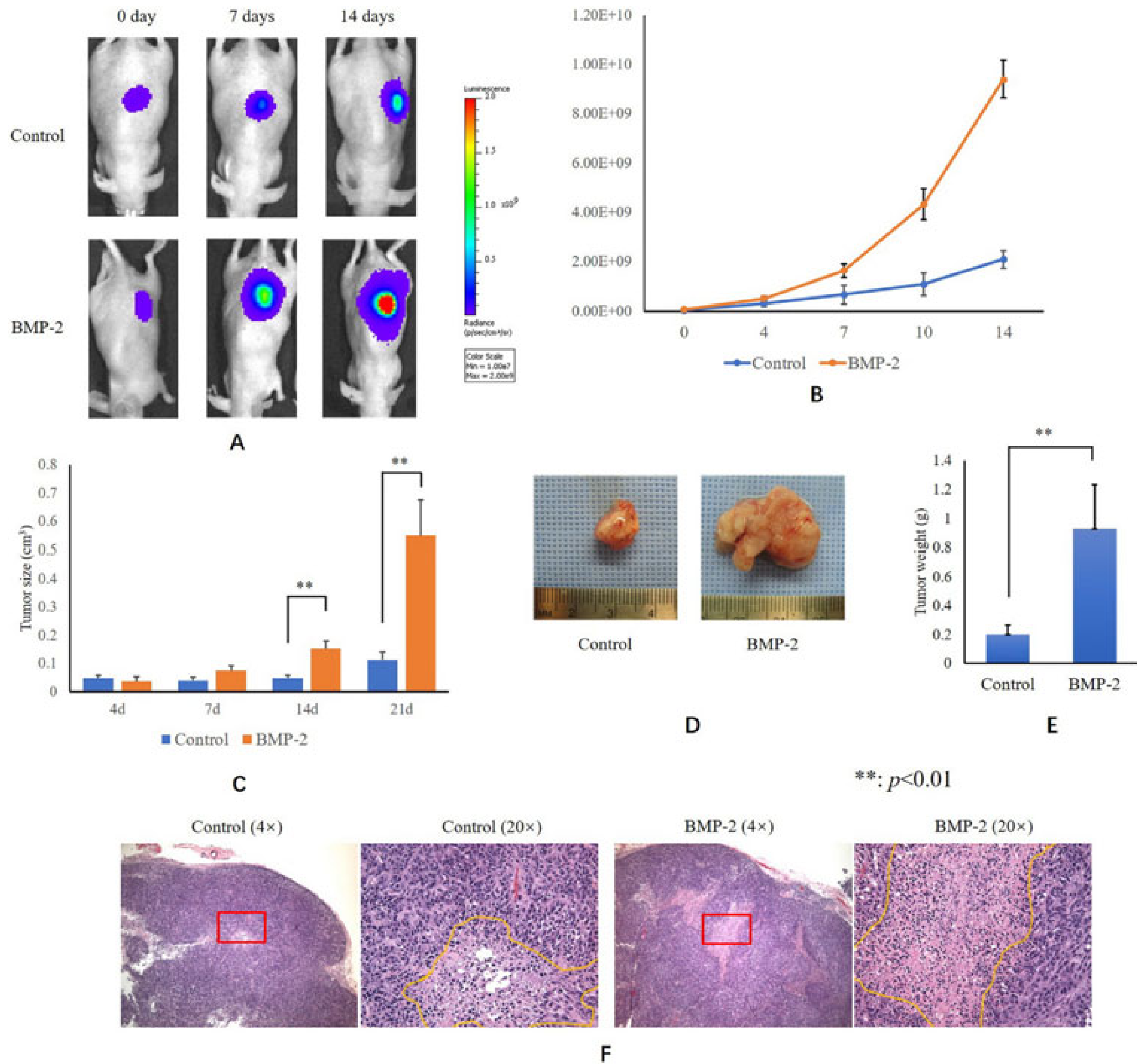

Figure 3. In vivo study of the effects of BMP-2 on osteosarcoma growth using a xenograft SCID mice model. A and B, Luciferase expression showing that co-implantation of BMP-2 with the osteosarcoma cells resulted in a remarkable acceleration of tumor growth. After 14 days the data for the BMP-2 group overflowed and could not be properly collected. C, An external caliper was used for the measurement of tumor size and tumor volume was then calculated, further demonstrating that BMP-2 treatment significantly promoted tumor growth. The studies had to be discontinued at day 21 for humane reasons relating to the huge size of the tumors in the BMP-2 treated animal subjects. D, Dissection of two representative tumors is depicted showing the obvious difference between the two treatment groups. E, The mean weights for the tumors from the two groups. F, H&E staining of representative histological specimens from the in vivo study. A larger contour of tumor size is apparent in the BMP-2 group in the lower magnification images. Both groups contain areas of central necrosis as delineated by the yellow boxes. The BMP-2 group shows more such areas than the control group indicating a faster growth rate caused by BMP-2 stimulation.

induced EMT of osteosarcoma cells. Cells treated with BMP-2 showed downregulation of E-cadherin and upregulation of N-cadherin, Vimentin, and Snail in a dose-dependent fashion.

Contact inhibition being unlocked by EMT could be a possible explanation of the disparity between in vitro proliferation testing and in vivo tumor growth. Chen et al.[31] reported that the proliferative status of retinal pigment epithelium cells assessed by the BrdU became abruptly negative from day 7 onward post-confluence. However, the contact inhibition could be unlocked by EGTA with EGF + FGF-2 and was accompanied by the gain of a mesenchymal cell phenotype suggestive of EMT associated with activation of the Wnt/β-catenin system. A similar phenomenon was observed in cancer cells as well. Lee et al.[32] studied the effects of transmembrane four superfamily (TM4SF) protein five on human hepatocarcinoma. These investigators demonstrated that TM4SF5 induced EMT associated with the loss of contact inhibition in that TM4SF5-expressing cells showed overlapping nuclei in the middle of cell division even in a subconfluent condition, whereas control cells showed well established cell-cell contacts and did not pile up. This effect was inhibited by the

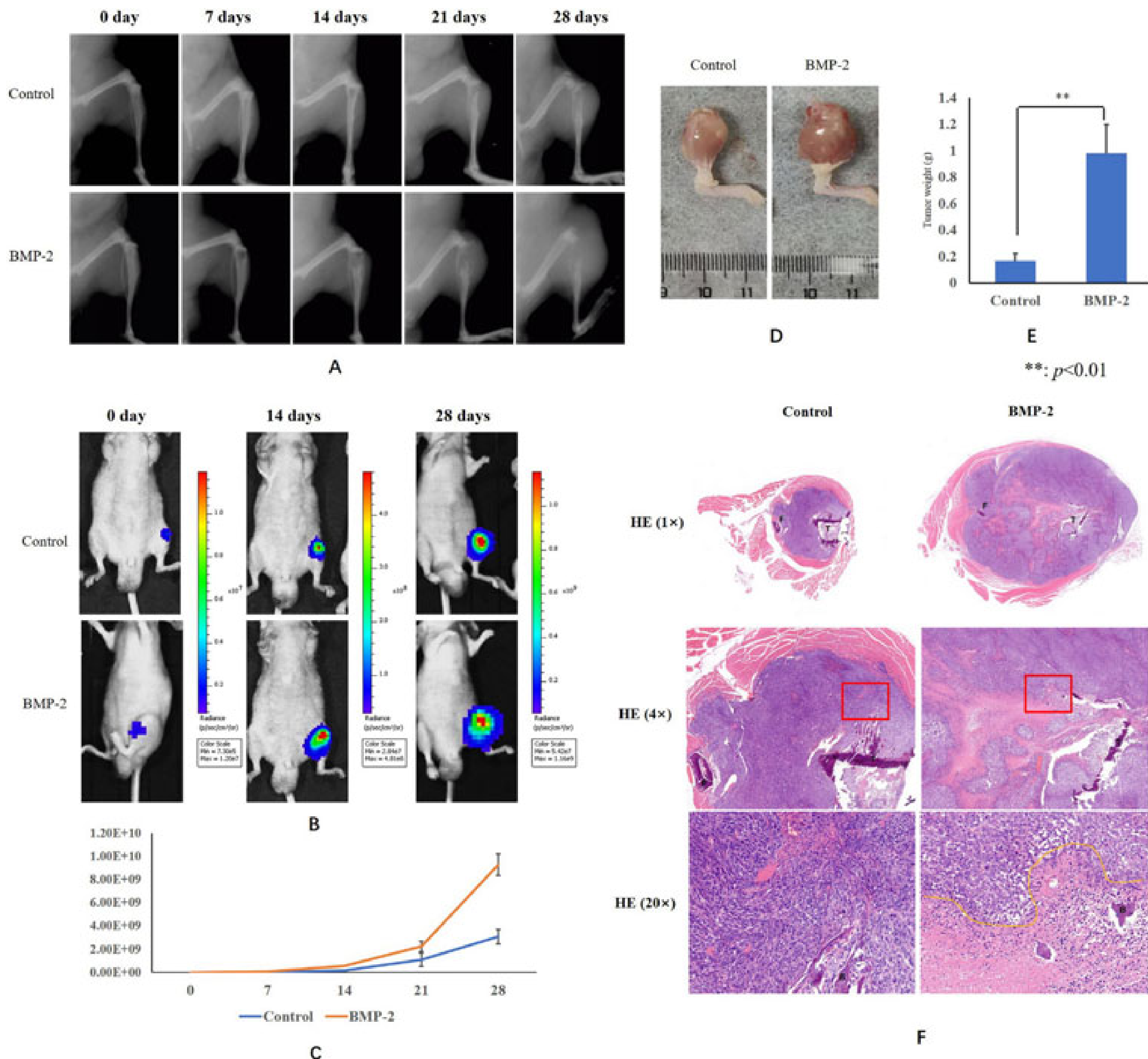

Figure 4. In vivo study of the effects of BMP-2 on osteosarcoma growth using an intratibial injection model. A, X-ray images of the limb injected with 143B osteosarcoma cells. The BMP-2 group showed more obvious osteolytic lesions in the tibia, and larger contour of tumor mass. B and C, Luciferase expression showing that the BMP-2 group had a higher luminescence intensity indicating an acceleration of tumor growth. D, Dissection of two representative tumors showing the difference between the two treatment groups. E, The mean weights for the tumors. F, H&E staining of representative histological specimens demonstrating a larger contour of tumor size in the BMP-2 group. The control group contained little area of central necrosis, and the BMP-2 group showed more such areas related to the faster tumor growth. Osteolytic lesion was also more obvious in the BMP-2 group (Fig. 4F).

blockage of EMT though reexpression of E-cadherin. Anchorage-independent growth of cancer cells was also investigated, and after 27-day culture in soft agar, TM4SF5-expressing cells clearly formed more foci than did control cells. These observations all indicate that EMT mediated loss of contact inhibition promoted tumor growth. In our study, and in all other reports pertaining to the effects of BMP-2 on in vitro osteosarcoma proliferation, a 2D cell culture plate will be insufficient to show the difference before cell confluence. An anchorage-independent cell growth assay or in vivo testing are the preferred models in which to examine this phenomenon.

While the term "carcinoma" refers to malignant tumors of epithelial origin, the term "sarcoma" refers to malignant tumors arising from cells that descend from the mesenchyme. For epithelial type tumor cells, documentation of epithelial to mesenchymal transformation (EMT) is considered to be a powerful means of assessing overall tumorigenicity. It should be noted that tumor cell migration and invasiveness are subsidiary components of the EMT process.[33] Obviously, a mesenchymal type tumor, such as osteosarcoma, cannot undergo EMT per se. However, because EMT is a dynamic and gradual process, the existence of intermediate EMT states or incomplete EMT has become widely accepted.[34] It is reasonable to expect that, depending on their cell type, sarcomas can shuttle in their phenotype through EMT related processes, which can, in turn, contribute to their progression and

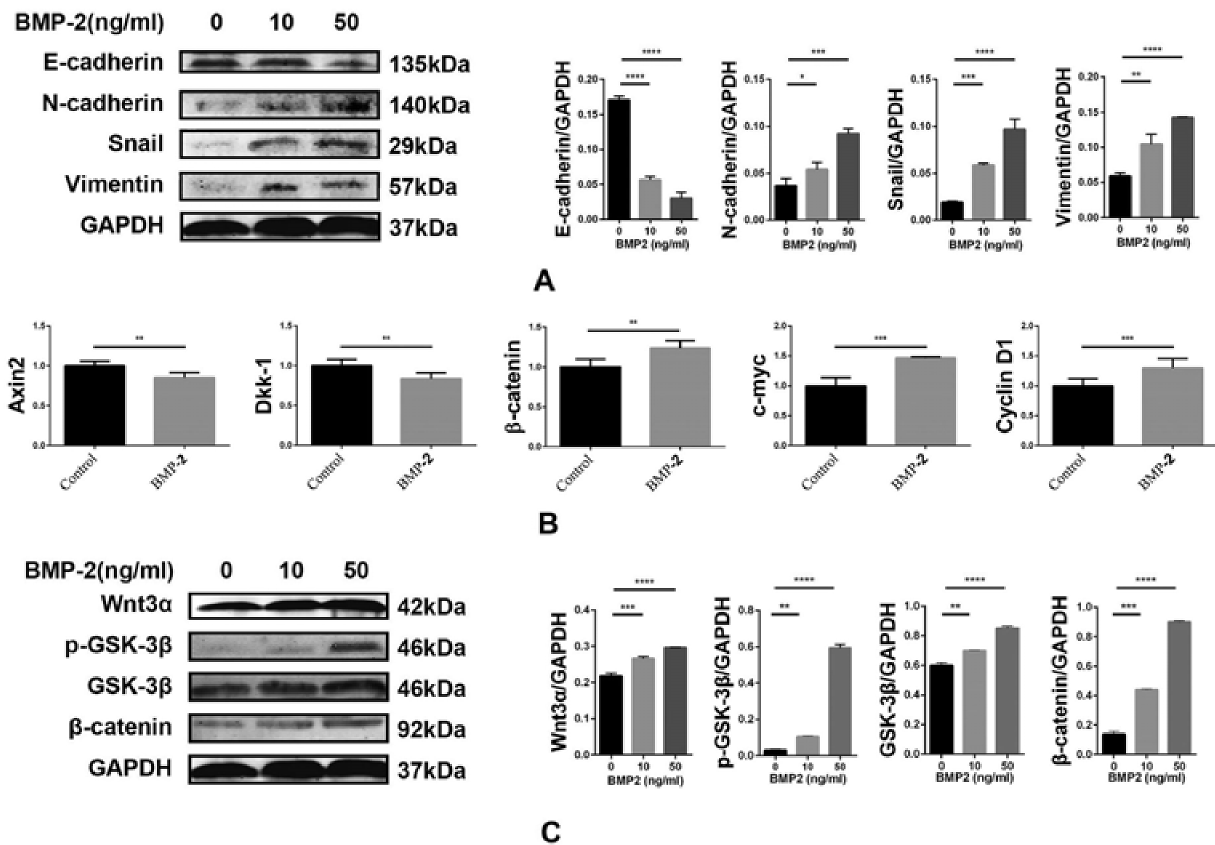

Figure 5. A, EMT marker expression tested by Western Blotting. E-cadherin was significantly downregulated with upregulation of N-cadherin at the same time, indicating a typical "cadherin switch." Expression of the two other EMT markers, Vimentin and Snail, were also positively correlated with BMP-2 treatment. All of the changes exhibited a dose-dependent pattern. B, Gene expression of Wnt/β-catenin signal pathway components analyzed by Realtime PCR. The expression levels of the Wnt inhibitors Axin2 and Dkk-1 were down regulated by BMP-2 treatment while β-catenin as well as downstream c-myc, and Cyclin-D1 were upregulated. C, Western blotting analysis showing that the expression of Wnt3α, β-catenin and p-GSK-3β were also significantly upregulated, indicating an activation of Wnt/β-catenin signaling pathway during the EMT of osteosarcoma driven by BMP-2.

aggressiveness. Researchers have found that changes in the expression of EMT-related genes or proteins is correlated with osteosarcoma growth,[35] and have speculated that inhibitors of this process could be therapeutic for osteosarcoma.[36] Our study further supports this hypothesis.

Since, under normal conditions, E-cadherin binds to β-catenin, during EMT, decreased E-cadherin levels could lead to an accumulation of β-catenin which would activate the Wnt/β-catenin signaling pathway. The concept that canonical Wnt signaling and EMT depend on the same pool of β-catenin has been demonstrated by genetic and overexpression experiments in embryos and cultured cells.[37] Aberrant Wnt/β-catenin signaling plays a role in multiple cancers such as colon, gastric, lung, breast, prostate, skin cancers as well as osteosarcoma.[38] The main components of this signaling pathway are Wnt proteins, frizzled proteins (Fzls), low density lipoprotein receptor related protein5/6 (LRP5/6), GSK-3β, disheveled proteins (Dsh), Axin, adenomatous polyposis coh (APC), β-catenin, T-cell factor/lymphocyte enhancer factor (TCF/LEF) and its downstream target genes such as c-myc.[39] In normal circumstances, free β-catenin is always maintained in a low concentration and abnormally high expression of β-catenin causes aberrant Wnt/β-catenin signaling, leading to the prompt occurrence of a wide variety of cancers. Rubin et al.63 reported that overexpression of the Wnt inhibitor, WIF-1, significantly decreased tumor growth rate in nude mice. WIF-1 overexpression also markedly reduced the number of lung metastasis in vivo in an orthotopic mouse model of osteosarcoma.[40] We were able to also document the activation of the Wnt/β-catenin system by BMP-2 treatment.

It is well accepted that the typical signaling pathway of BMP-2 is the BMP-2/Smad pathway. However, cross talk between the BMP-2 and the Wnt pathways has been implicated in many studies. Fischer et al. performed a series studied and showed that BMP-2 treatment of C3H10T1/2 cells resulted in an increased protein level of β-catenin,[41] and that expression of Wnt-3a was also upregulated strongly, suggesting that functional crosstalk occurred between the two pathways.[42] Although our study supported the association between BMP-2 and the Wnt/β-catenin signaling pathway, there are still much work to be done to reveal the

details as to how BMP-2 regulates the Wnt/β-catenin signaling pathway.

CONCLUSION

From this study we can conclude that BMP-2 significantly promotes growth of osteosarcoma cells (143B, MG63),and enhances mobility and invasiveness of tumor cells as demonstrated in vitro. The underlying mechanism might be that BMP-2 promotes EMT of osteosarcoma through the Wnt/β-catenin signaling pathway. Based on our study, we suggest that it is not appropriate at this time to employ BMP-2 in efforts of reconstruction of bone defects resulting from osteosarcoma tumor resection. On the contrary, use of BMP antagonists may provide some benefit, but that remains to be experimentally documented. Furthermore, application of BMP-2 in sites distant from the primary tumor, for example in conjunction with spinal fusion, should be undertaken with great caution.

AUTHORS' CONTRIBUTIONS

Study design: HT, JZ, JW, MD and SM. Study conduct: HT, TZ, HC, CL, ZJ, SK. Data collection: TZ, HC, CL, LL. Data analysis: HT, JZ, HC, CL and EB. Data interpretation: ZB and MED. Drafting manuscript: HT, JZ, CL and SM. Revising manuscript content: JZ, HC and AF. Approving final version of manuscript: HT, TZ, JZ, JW, SM. HT takes responsibility for the integrity of the data analysis.

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