Secreted phosphoprotein 24 kD (Spp24) inhibits growth of human osteosarcoma through the BMP-2/Smad signaling pathway

Hongfang Chen
Shanghai Jiao Tong University School of Medicine
Chenshuang Li
University of Pennsylvania School of Dental Medicine
Tangjun Zhou
Shanghai Jiao Tong University School of Medicine
Xunlin Li
Shanghai Jiao Tong University School of Medicine
Aimin Wu
Shanghai Jiao Tong University School of Medicine
Maria Eugenia L. Duarte
National Institute of Traumatology and Orthopaedics
Haijun Tian (haijuntianmd@gmail.com)
Jie Zhao
Shanghai Jiao Tong University School of Medicine
Michael D Daubs
University of Nevada School of Medicine
Zorica Buser
University of Southern California Keck School of Medicine
Elsa J. Brochmann
University of California Los Angeles
Jeffrey C. Wang
University of Southern California Keck School of Medicine
Samuel S. Murray
University of California Los Angeles

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Abstract

Background

Autocrine stimulation of tumors cells is an important mechanism for the growth of skeletal tumors. In tumors that are sensitive, growth factor inhibitors can dramatically reduce tumor growth. In this study, we aimed to investigate the effects of Secreted phosphoprotein 24 kD (Spp24) on the growth of osteosarcoma cells induced by BMP-2 both *in vitro* and *in vivo*.

Methods

Two osteosarcoma cell lines, 143B and MG63, were used in this study. MTT assay, flow cytometry, wound-healing assay, Matrigel invasion assay and immunohistochemical staining were used to evaluate the effects of Spp24 inhibition on the osteosarcoma cells induced by BMP-2 *in vitro*. Subcutaneous and intratibial tumor models in nude mice were used to evaluate the effects of Spp24 *in vivo*. Quantitative real-time PCR, western blotting and co-immunoprecipitation were used to investigate the underlying mechanisms.

Results

Our study demonstrated that Spp24 can inhibit proliferation and promote apoptosis of osteosarcoma cells as confirmed by MTT assays and immunohistochemical staining. We found that BMP-2 increased the mobility and invasiveness of tumor cells *in vitro* whereas Spp24 inhibited both effects either in the presence or absence of exogenous BMP-2. Intracellular binding of Spp24 and BMP-2 was confirmed by co-immunoprecipitation. Mechanically, phosphorylation of Smad1/5/8 was enhanced by treatment with BMP-2 but was significantly inhibited by treatment with Spp24. Subcutaneous and intratibial tumor models in nude mice demonstrated that BMP-2 promoted osteosarcoma growth *in vivo*, while Spp24 significantly inhibited tumor growth and reduced osteolytic lesions in the tibia induced by BMP-2.

Conclusions

From this study, we concluded that the BMP-2/Smad signaling pathway is involved in the pathogenesis of osteosarcoma growth, and that Spp24 inhibits the growth of human osteosarcoma induced by BMP-2 both *in vitro* and *in vivo*. These results validate the potential of Spp24 as a therapeutic agent for the treatment of osteosarcoma.

Introduction

Osteosarcoma (OS) is an aggressive malignancy that arises from osteoprogenitor cells (1). It is the most common type of primary bone tumor and features malignant osteoblasts that produce osteoid (2). It can

occur in any bone but most commonly arises in the metaphyseal portion of appendicular bones, especially in the knee region. The incidence of OS has a bimodal age distribution with the highest incidence during the second decade of life (3, 4). The vast majority of OS in adolescents are high-grade tumors and, therefore, have a poor prognosis. Surgery and chemotherapy, alone or in combination, are the mainstays in the treatment of OS. In spite of improvements in surgical techniques and the use of new chemotherapeutic regimens, the 5-year survival rate in OS patients has remained constant over the last 20 years, especially in the presence of metastatic disease (5). Furthermore, complete surgical excision is frequently not possible and, therefore, there is a great need for efficacious adjunctive therapies.

In addition to surgery, current management of skeletal tumors includes adjunctive modalities such as external beam irradiation; radioisotopes; bisphosphonates; RANK/RANKL system inhibitors (e.g. osteoprotegerin, Denosumab); hormonal therapy (for sensitive tumors); cell adhesion protein antagonists; growth factors or, more commonly, growth factor antagonists; and topical placement of cytotoxic materials such as betadine, phenol, and peroxide (6). Each of these modalities has significant clinical limitations and toxicities. Autocrine stimulation of tumor cells by growth factors is an important mechanism of tumor growth and, therefore, a logical target for the development of an efficacious and minimally toxic adjuvant therapeutic that can be applied directly to the bed of an excised tumor and deliver persistent tumor suppression at that site. Such agents could, theoretically, also be delivered systemically to address metastatic disease.

Another concern regarding the comprehensive therapy for osteosarcoma is the reconstruction of the bone defect after limb-salvage surgery. While bone morphogenetic proteins (BMPs) have been found to be helpful additions in skeletal reconstructive surgery, a conflict exists since they have also been found, in some cases, to possess tumor enhancing properties (7). Because of existing information supporting a role for BMPs as autocrine factors in OS cell growth (8), and because of the on-going concerns pertaining to the tumorigenic actions of exogenous BMPs, studies were undertaken to determine the effects of the BMP binding and neutralizing protein, Spp24, on tumor growth in a mouse model of osteosarcoma.

Materials And Methods

Production of Spp24

Recombinant bovine Spp24 was produced in a bacterial expression vector as described in detail previously (9). The expression protein is engineered in such a manner as to place a (His)6-Met at the amino terminus of the recombinant form of the mature protein (lacking the leader sequence) in order to provide for stability and to allow purification of the protein by means of IMAC (immobilized metal affinity chromatography).

Cell culture

The highly malignant 143B and moderately malignant MG63 cells were used in this study, both were obtained from ATCC (Manassas, VA). Cells were grown in minimum essential medium (MEM) and

Dulbecco's minimum essential medium (DMEM) respectively, supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) in a humidified atmosphere containing 5% CO_2 at 37°C. Media were changed every 3 days. Cells were passaged when cultures reached 80-90% confluence.

MTT assay

To investigate the effects of BMP-2 and Spp24 on cell growth, 143B or MG63 cells were seeded into 96-well microplates at 2×10^3 cells per well. Cells were cultured for 24 hours to achieve attachment, starved for 12 hours with 1% FBS medium, then treated with a different combination of BMP-2 or Spp24 (Control group: without BMP-2 or Spp24; BMP-2 group: 50 ng/mL BMP-2; Spp24 group: 50 μg/mL Spp24; BMP-2+ Spp24 group: 50 ng/mL BMP-2 + 50 μg/mL Spp24) for 24 h. Cell growth was evaluated using a 3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (ATCC Manassas, VA). Absorbances were read at 570 nm with a reference wavelength of 700 nm.

Wound-healing assay

143B and MG63 cells were seeded in 12-well plates and incubated for 24 h until 90% confluence was achieved, then starved overnight with 0.5% FBS medium. After changing back to normal culture media, a sterile 200μL pipette tip was used to scratch wounds across the monolayer of cells and the wells were gently washed twice with phosphate-buffered saline (PBS) to remove the detached cells. 143B and MG63 cells were cultured with 2% FBS to eliminate the influence of cell proliferation. Cells were treated with BMP-2, Spp24, and BMP-2 and Spp24 as previously described. The wounds were photographed at each time point using an inverted microscope (Leica, Wetzlar, Germany). Image J software (National Institutes of Health; http://rsb.info.nih.gov/ij/) was used to quantify the migration rate by measuring the distance between the wound edges. All experiments were repeated in triplicates.

Matrigel invasion assay

Transwell *in vitro* invasion 24-well chambers (Corning, USA) were used following the manufacturer's instruction. The membrane of each well was precoated with 500μL BD Matrigel (BD Biosciences, Bedford, MA). 2.5×10^4 143B or MG63 cells in 200μL serum-free medium were placed into the upper chamber, BMP-2 or Spp24 was added into the upper chamber as previously described. 500μL of medium containing 10% FBS was placed into the lower compartment. The transwell chambers were incubated for 24 h at 37°C in 95% air with 5% CO_2. The membranes were fixed with methanol and cell penetration through the membrane was detected using crystal violet staining. Cell penetration was quantified by counting the numbers of cells in five microscopic fields (at x200 magnification) per filter. The studies were conducted in triplicate.

Cell-cycle and apoptosis analysis by flow cytometry (FCM)

143B cells were seeded into 6-well plates at a density of 8×10^4 cells/well and cultured overnight. When a confluence of 70-80% was reached, wells were washed with PBS and both cell lines were exposed to culture media with BMP-2 (100 ng/mL), Spp24 (20 μg/mL), BMP-2+Spp24 (100 ng/mL, 20 μg/mL) for 24 h. Subsequently, the cells were collected and centrifuged in 15mL centrifuge tubes and washed twice with PBS. For cell-cycle analysis, 1mL DNA Staining solution and 10μL permeabilization solution were added to all samples and then incubated at room temperature for 30 min in the dark according to the cell cycle staining Kit manufacturer's instruction (Multiscience Biotech, Hangzhou, China). All samples were immediately analyzed by flow cytometry.

For apoptosis analysis, 5μL of annexin V-PE and 5μL of 7-amino-actinomycin D (7-AAD) were added to all samples, which were then incubated at room temperature for 15 min in the dark according to the manufacturer's instruction (Thermo Scientific, Waltham, MA, USA)- and the fluorescence intensity of the samples was immediately analyzed by flow cytometry.

Quantitative Real-time PCR

To examine the effects of BMP-2 and Spp24 on Smad signaling in osteosarcoma cells at the gene level, 143B and MG63 cells were seeded and grouped as Control, BMP-2 (100 ng/mL), Spp24 (20 μg/mL), BMP-2 + Spp24 (100 ng/mL, 20 μg/mL) in 6-well plates at a density of 1×10^5 cells/well and cultured for 24 hours. Total RNA was isolated by E.Z.N.A.® HP Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA). 1 μg RNA was subjected to reverse transcription using the Prime-Script RT reagent kit (Takara, Shiga, Japan). Real-time PCR was performed with a 7300 Real-Time PCR system with SYBR® Premix Ex Taq™ (Takara, Shiga, Japan). The 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 (10).

Table 1
Sense and anti-sense primers for Real-time PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
Smad1	TTTACAAGTCCAGCTGTGAAGA	AAAGCATCAACAGCTTTCTCTG
Smad4	ACAAGTAATGATGCCTGTCTGA	CTCCCATCCAATGTTCTCTGTA
Smad5	ACAATTGAAAACACTAGGCGAC	AGTTGCAGTTCCTACTCTGTAC
Smad8	GAAGTGTGCATTAACCCTTACC	ATATTCACTGTGTCTTGGCACG
GAPDH	CAGCGACACCCACTCCTC	TGAGGTCCACCACCCTGT

Western blotting analysis

143B and MG63 cells were seeded and grouped as Control, BMP-2 (100 ng/mL), Spp24 (20 μg/mL), BMP-2+Spp24 (100 ng/mL, 20 μg/mL) in 6-well plates at a density of 1×10^5 cells/well and cultured for 24 h. The cells were washed three times with PBS and lysed with RIPA buffer (150mM NaCl, 1% sodium

deoxycholate, 0.1% SDS, 50mM Tris-HCl pH7.4, 1mM EDTA, 1mM PMSF, and 1% TritonX-100) supplemented with protease inhibitors and phosphatase inhibitors for 30 min at 4 °C. For western blotting analysis, a total of 20-30 mg of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto nitrocellulose membranes. The primary antibodies used were rabbit monoclonal anti-Smad4 (Cell Signaling Technology catalog #: 46535S); rabbit monoclonal anti-Smad1/5/8 (Abcam, catalog #: ab80255); and rabbit monoclonal anti-phospho-Smad1/5/8 (Cell Signaling Technology, catalog #: 13820S). For normalization of protein loading, GAPDH antibody (Cell Signaling Technology, catalog #: 5174S) was used. The protein bands were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

Plasmid construction and transfection

BMP-2 and Spp24 cDNA was purified by Genechem (Shanghai, China) and cloned into the plvx-ires vector to generate plvx-ires-BMP-2-FLAG and plvx-ires-Spp24-GFP recombinant plasmids. Transient transfection was performed using the Lipofectamine™ 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. Scrambled lentiviral particles were used as the negative control. After 48 h of incubation, the medium was replaced with DMEM containing 5 μg/mL puromycin. After maintenance culture for 2-3 weeks in selection media, puromycin-resistant colonies were screened and selected for BMP-2 or Spp24 expression.

Co-immunoprecipitation (Co-IP)

Co-immunoprecipitation assays were performed using a co-immunoprecipitation kit (Thermo Scientific, Waltham, MA, USA). 143B cells were lysed in SDS lysis buffer on ice for 30 min, and after centrifugation at 4°C and 14, 000 ×*g* for 15 min, the supernatants were collected. The protein lysates were then incubated with anti-FLAG and anti-GFP antibody on a rotator overnight at 4°C. Then, the mixtures were incubated with immobilized protein A/G beads (Thermo Scientific) on a rotator for 2 h at 4°C. The beads were collected by centrifugation at 3000 × *g* for 2 min and washed five times with 0.5 mL of IP wash buffer. SDS loading buffer was then added to the beads, and the samples were denatured at 95°C for 10-12 min. Finally, the supernatants were collected and immediately analyzed by western blotting.

Transfection of Lenti-CMV-Firefly luciferase (FL)

143B cells were seeded at 2×10^5 cells per well in 6-well plates (Corning, USA) and cultured at 37°C overnight in mediums supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Transduction was performed in 1 mL of medium with Lenti-CMV-Fluc2 at a multiplicity of inflection of 5 (MOI = 5), including lentiviral vector supernatant and 1mg/ml of protamine sulfate. The cells were then washed with PBS 24h post-transduction and incubated in regular culture medium for another 48 h. A luminometer (Berthold Detection Systems, Pforzheim, Germany) was used to measure the FL luciferase activity following the manufacturer's instructions (Promega). Each value was normalized to cell number or protein amount and calculated as the average of triplicate samples.

Implantation of tumor cells and test materials

All animal experiments were approved by the Animal Ethics Committee of Shanghai Ninth People's Hospital. Forty-eight 8-week-old male BALB/c nude mice were purchased from Shanghai Super - B&K laboratory animal Corp. Ltd. Twenty-four nude mice received a subcutaneous injection of a total of 5.0×10^5 143B osteosarcoma cells in a volume of 200μL that contained 50μL of culture medium, 40 μL of growth factor reduced Matrigel (BD Biosciences, San Jose, CA, USA), and specified combinations of target proteins dissolved in PBS. The mice were divided into four groups as follows: Group 1, Control group with vehicle only; Group 2, 15 μg BMP-2; Group 3, 1.0 mg Spp24; Group 4, 15 μg BMP-2+1.0 mg Spp24. Mice were anesthetized with isoflurane delivered in oxygen and 70% ethanol was used to sterilize the overlying skin of the back. A 27.5-gauge needle was used to perform the subcutaneous injection on the lower left quarter of the back. Another twenty-four nude mice received a tibial injection of 1.0×10^5 143B osteosarcoma cells in a total volume of 50μL of culture medium with vehicle only (Group 1, control group); BMP-2 15 μg (Group 2); Spp24 0.5 mg (Group 3) and BMP-2 15 μg +Spp24 0.5 mg (Group 4). Mice were anesthetized with isoflurane delivered in oxygen and 70% ethanol was used to sterilize the overlying skin of the lower limb. Then 50 μL of mixtures containing cells were injected into the cavity of the proximal tibia through the proximal tibial plateau using a 27.5-gauge needle.

Imaging of tumors

Mice were anesthetized with 2% isoflurane delivered in oxygen and then subjected to intraperitoneal injection of 150 mg/kg of D-luciferin (Caliper LifeSciences, Hopkinton, MA). Twenty minutes after the injection, images were photographed using an IVIS cooled CCD camera (Xenogen, Alameda, CA). Images were analyzed with IGOR-PRO Living Image Software (Xenogen). The mice with subcutaneous xenografts were imaged on days 0 and 14 before excessive tumor growth could result in data outflow. The mice with intratibial xenografts were imaged on days 0 and 28 and the injected limbs were imaged with X-ray as well on day 28.

Measurement of tumor size and weight

For the subcutaneous tumors, tumor size was measured using an external caliper on days 0, 4, 7, 14 and 21, and the tumor volume was calculated according to the modified ellipsoidal formula (7). Mice receiving subcutaneous implantations were euthanized on day 21 and the tumor specimens were dissected out immediately. The tumor weight was measured using a precision electronic balance. Mice receiving tibial implantations were euthanized on day 28.

Histology and immunohistochemistry

143B cells were seeded at 1×10^4 cells per dish in 10mm confocal dishes. After cell attachment, all dishes were treated with BMP-2 or Spp24 as previously described and incubated for 24 hours. Cells were then rinsed with PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature, and permeabilized with 0.2% Triton X-100 followed by blocking with 0.5% BSA and 1% FBS.

Cells were then stained with anti-PCNA, anti-Caspase6, or anti-phospho-Smad1/5/8, followed by Alexa 488 conjugated secondary antibody. Images were observed and photographed under fluorescent microscopy (Nikon Eclipse CI, Tokyo, Japan). The subcutaneous tumor specimens were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) 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. Immunohistochemistry to detect phospho-Smad1/5/8 in nude mouse xenograft tumor specimens was performed as previously described (7).

Statistical analysis

The data were presented as means ± standard deviation (SD) and were analyzed with SPSS version 22.0 (IBM, Armonk, NY). A value of $P < 0.05$ was considered statistically significant. Comparisons of two or more data sets were performed using one-way analysis of variance (ANOVA) with subsequent Tukey-Kramer comparisons. Student's t-test was used for comparison involving two groups.

Results

Spp24 inhibits the in vitro growth of osteosarcoma cells

BMP-2 did not affect the proliferation of tumor cells significantly but increased migration and increased invasiveness in both 143B and MG63 cells. This effect was attenuated by the addition of Spp24 and, furthermore, the administration of Spp24 alone yielded dramatic inhibitory effects on proliferation, migration, and invasiveness of osteosarcoma cells. The results of the MTT analysis of 143B and MG63 cells after treatment with BMP-2, Spp24 for 24 h are shown in Fig. 1A, B. The proliferation of 143B and MG63 cells were significantly inhibited in Spp24 and BMP-2 + Spp24 groups. Figure 1C-F demonstrates that cell migration was inhibited in Spp24 and BMP-2 + Spp24 groups of 143B and MG63 cells. Fig. G-I shows that the invasiveness of 143B and MG63 cells were inhibited in Spp24 and BMP-2 + Spp24 groups.

Spp24 inhibits in vitro osteosarcoma cell growth by inducing tumor cell apoptosis

The effects of Spp24 on 143B osteosarcoma cell cycle and apoptosis were assessed using flow cytometry assays and immunohistochemistry. Figure 2A, B shows a representative sample and calculated data for a flow cytometry cell apoptosis analysis using an Annexin V-PE/7AAD method. BMP-2 inhibited the apoptosis initially at 24 hours, although, this was not statistically significant. The addition of Spp24, with or without BMP-2, dramatically induced cell apoptosis with the Spp24 + BMP-2 group having the highest percentage of apoptotic cells. The cell cycle distribution of all treatment groups showed no differences at 24 hours (Fig. 2C, D). Immunohistochemistry of *in vitro* cultures of the osteosarcoma cells showed that the proliferation marker PCNA was slighted upregulated in the BMP-2 group and downregulated in the Spp24 and BMP-2 + Spp24 groups (Fig. 2E), whereas, more dramatically, the apoptosis marker Caspase-6 was downregulated by BMP-2 and significantly upregulated by Spp24 (Fig. 2F). Altogether, this suggests that BMP-2 promoted tumor growth by inhibition of cell apoptosis and

that Spp24 dramatically induced tumor cell apoptosis, which inhibited tumor growth and obviated the effects of BMP-2.

Spp24 inhibits osteosarcoma growth induced by BMP-2 in vivo

The intensity of luminescence for the subcutaneous tumors was recorded on day 14. After this time the intensity became too high in the BMP-2 group for reliable data to be obtained. The intensity of luminescence for the subcutaneous tumors in the other groups were also recorded on day 28. For the subcutaneous tumor model in the nude mice, BMP-2 led to an increase in tumor weight and volume, whereas the Spp24 group and BMP-2 + Spp24 group, showed a remarkable inhibition of tumor weight and tumor volume (Fig. 3A-C). This finding was confirmed with the luminescence assay (Fig. 3D, E). The dissection of representative tumors is depicted in Fig. 3A and shows the obvious difference between the treatment groups. A smaller contour of tumor size in the Spp24 group compared to that of the control or BMP-2 groups was also demonstrated by histological examination with H&E staining (Fig. 3F). It is apparent that there is central necrosis in tumors in the BMP-2 group due to rapid tumor growth.

Spp24 also inhibited tumor growth in the intratibial injection model as shown in a lower luminescence intensity (Fig. 3G-I). The Spp24 group demonstrated less cortical bone destruction and fewer osteolytic lesions in the tibia as seen in the X-ray images compared to the BMP-2 and control groups (Fig. 3J). Histological examination with H&E staining also demonstrated a smaller contour of tumor size in the Spp24 group (Fig. 3K) and central necrosis in tumors from the BMP-2 group.

Spp24 binds to BMP-2 and down-regulates the BMP-2/Smad signaling pathway by inhibition of phosphorylation of Smad1/5/8

Co-immunoprecipitation assays confirmed the intracellular binding of Spp24 and BMP-2 (Fig. 4A). Gene expression studies revealed that the expression level of Smads was affected by the administration of BMP-2 and Spp24, especially for the case of Smad8, which was upregulated by the BMP-2 and downregulated by Spp24 (Fig. 4B, C). Western blotting showed that pSmad1/5/8 was significantly upregulated by BMP-2 and somewhat blocked by Spp24 (Fig. 4D and E). Immunohistochemical staining of the *in vitro* culture of cells, as well as the specimens from the animal studies demonstrated significant inhibition of phosphorylation of Smad1/5/8 by Spp24, confirming that Spp24 inhibits BMP-2 induced up-regulation of the Smad pathway by binding BMP-2 to its specific binding domain (Fig. 4F, G).

Discussion

Autocrine stimulation is important for tumor growth in the skeleton for both primary tumors and for metastases. The process of metastasis to bone is a complex, sequential process in which a malignant cell detaches from its primary site of origin (e.g. lung, prostate, or breast), enters the vasculature, comes to rest in and escapes from a distant capillary bed, and begins to proliferate in bone (11). While over-expression of genes that control the cell cycle, angiogenesis, chemotaxis, and invasion (e.g. matrix metalloproteinases and cell adhesion molecules) is critically important for the survival of cancer cells

and their subsequent affinity for bone (11), over-expression of pro-osteogenic growth factors play a central role in promoting the growth and extension of tumor cells once they have localized to the bone. Pertinent growth factors include platelet-derived growth factor, insulin-like growth factor, adrenomedullin, endothelin-1, parathyroid hormone-related peptide fragments (12), and, most importantly, members of the BMP/TGF-beta superfamily of cytokines and their receptors (13–21). BMPs-2, -4, -6, and – 7 and their receptors are up-regulated in osteosarcoma, breast, prostate, and lung cancer cell lines (12, 22–25). Because of their central roles in the development, growth, and remodeling of bone, members of the TGF-beta/BMP family of proteins are regarded as the most promising targets for therapeutic intervention aimed at growth factor systems in bone tumors (26). Extension of primary bone tumors is driven by the same factors that enhance the spread of metastatic foci once they have become established in bone. Therefore, a therapeutic agent that impinges upon an autocrine system, for example, the TGF/BMP family of proteins, would be expected to be efficacious both in the case of the primary tumor and the case of metastasis in the bone.

In this study, we showed that BMP-2 promoted tumor growth not by affecting the proliferation of tumor cells significantly but by increasing migration and invasiveness in both 143B and MG63 cell types. BMP-2 is a member of the TGF-beta family of growth factor proteins and it has been studied extensively with respect to tumorigenesis. It has both positive and negative trophic effects in a large spectrum of tumor types (27). For example, it has been shown that BMP-2 can promote carcinogenesis, tumor growth, invasion and metastasis in oral carcinoma and gastric carcinoma cells (28, 29). Similarly, BMP-2 is expressed in the human non-small cell lung cancer cell line A549 and stimulates A549 tumor growth *in vivo* (30) while TGF-beta initiates signal transduction in A549 cells (18). The prostate cancer cell line PC-3 was derived from a human skeletal metastasis and produces mixed osteolytic/osteoblastic lesions when implanted into the bones of animals (31).The BMP receptor was shown to be expressed on the cell surface of the PC-3 cells and BMP increases cell migration and invasiveness of these cells (31). TGF-beta and BMP-2 also promote angiogenesis, immune-escape or metastasis in other tumors and tumor cells (32, 33). More pertinent to this study, Sotobori et al. reported that the expression of BMP-2 and its receptors (BMPR1, BMPR2) was greatly increased in Dunn osteosarcoma cells (34). Additionally, Mohseny et al. have shown that, compared to osteoblastoma, phosphorylated Smad1 was highly expressed in human osteosarcoma samples by immunohistochemical analysis (35). Furthermore, Yang et al. demonstrated that increased BMP-2 induces over-expression of tumorigenic factors such as VEGF, EMMPRIN, and MMP-9 both intra- and extra-cellularly in osteosarcoma mesenchymal stem cells (36). In terms of a more generalized mechanistic view, Tian et al. demonstrated that BMP-2 stimulates the proliferation, motility, invasiveness and the epithelial-to-mesenchymal transition in osteosarcoma cells in tissue culture and promoted proliferation and invasiveness of 143B osteosarcoma cells in mouse xenograft models (7).

Secreted phosphoprotein 24 kD (Spp24) is a bone matrix protein that is likely to have a number of varied functions in the natural bone environment-related not only to its ability to bind several members of the TGF-beta/BMP family of proteins (37, 38), but also related to its (or, more precisely, truncated fragments of it) little explored ability to stimulate intracellular signaling pathways (39). We have engineered several

protein and peptide products that, while they are thought to function by a single mechanism (BMP binding), operationally have seemingly paradoxically opposite outcomes along a spectrum from the enhancement of BMP activity (“slow-release”) to inhibition of BMP activity (“sequestration”) (37). For example, bone morphogenetic protein binding peptide (BBP) is a synthetic cyclic 19 amino acid peptide that is based on the BMP binding domain of Spp24 which has a similarity to the TGF-beta receptor(40). When BBP was applied to a collagen sponge to which BMP-2 or BMP-7 was subsequently added, the material enhanced the bone healing effects of BMP in models of ectopic bone formation (40), spinal fusion (41, 42), and long bone healing (43). On the other hand, full-length Spp24, when applied to a collagen sponge to which BMP-2 was subsequently applied, dramatically inhibited the activity of BMP-2 in models of ectopic bone formation (44) and spinal fusion (45). The latter observation suggested a role for Spp24 in the suppression of BMP/TGF autocrine systems.

Several previous studies have demonstrated the potential of BMP binding materials to sequester BMP, interrupt BMP autocrine systems and reduce tumor growth. Feeley et al. (13, 31, 46, 47) demonstrated that the addition or over-expression of noggin (an extracellular, high-affinity BMP-binding BMP antagonist) inhibited the proliferation, and invasive potential of A549 lung cancer cells, PC-3 prostate cancer cells and other tumor cell lines are drive by BMP-2 autocrine stimulation. More recently, we have demonstrated that Spp24 can suppress pancreatic cancer (48), hepatocellular carcinoma (49), prostate cancer (50) and lung cancer (51) growth induced by exogenous or, more importantly, endogenous BMP-2. Thus, it appears that Spp24 may have potential as a therapeutic agent for use in the management of not only primary bone tumors and skeletal metastases, but also for a number of other tumors that are not orthopedic in nature.

Conclusion

In this study, we have shown that Spp24 can bind to BMP-2 and inhibit its function in osteosarcoma cells, both *in vitro* and *in vivo*. *In vitro* cell proliferation, migration and invasiveness of osteosarcoma cells were all inhibited by Spp24. Treatment with Spp24 reduced tumor growth *in vivo*. Our studies strongly suggest that Spp24 inhibits *in vitro* osteosarcoma growth by inducing tumor cell apoptosis. Treatment with Spp24 in 143B cells resulted in down-regulation of the BMP-2/Smad signaling at both gene and the protein expression levels. We cannot state that the sequestration of BMP-2 is the only mechanism of action for the effects that we observed. Spp24 could also be acting by sequestering other members of the TGF-beta family of proteins. Furthermore, there may be completely different biological mechanisms involved such as receptor binding and inhibitory signaling activation. While more research will be required to investigate these unknown factors, the fact remains that, empirically, Spp24 has shown great potential as an anti-cancer therapeutic.

Abbreviations

Spp24: Secreted phosphoprotein 24 kD

BMP: bone morphogenetic protein

Co-IP: co-immunoprecipitation

OS: Osteosarcoma

IMAC: immobilized metal affinity chromatography

MEM: minimum essential medium

DMEM: Dulbecco's minimum essential medium

FCM: flow cytometry

7-AAD: 7-amino-actinomycin D

PBS: phosphate-buffered saline

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

FL: Firefly luciferase

H&E: Hematoxylin and eosin

SD: standard deviation

ANOVA: one-way analysis of variance

Declarations

Ethics approval and consent to participate

All Procedures performed in studies involving animals were reviewed and granted by the Ethical Committee of Shanghai Ninth People's Hospital in Shanghai, China.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

ZB - Consultancy: Cerapedics, Xenco Medical (past), AO Spine (past); Research Support: SeaSpine (past, paid to the institution), Next Science (paid directly to institution); North American Spine Society: committee member; Lumbar Spine Society: Co-chair Research committee, AOSpine Knowledge Forum Degenerative: Associate member; AOSNA Research committee- committee member

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JCW - Royalties: Biomet, Seaspine, Amedica, DePuy Synthes; Investments/Options - Bone Biologics, Pearldiver, Electrocore, Surgitech; Board of Directors - North American Spine Society, AO Foundation (20,000 honorariums for board position, plus travel for board meetings), Cervical Spine Research Society; Editorial Boards - Spine, The Spine Journal, Clinical Spine Surgery, Global Spine Journal; Fellowship Funding (paid directly to institution): AO Foundation

Other authors declare that they have no competing interests.

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Authors' contributions

Design: HT, JZ, JW, MD, SM and EB. Experimental operation: HC, CL, TZ, XL and HT. Acquisition of data and Analysis: HC, HT, CL, JZ, TZ and EB. Writing, review, and/or revision of the manuscript: HT, HC, CL, JZ, ML, ZB, MD, JC and SM. All authors read and approved the final manuscript.

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Figures

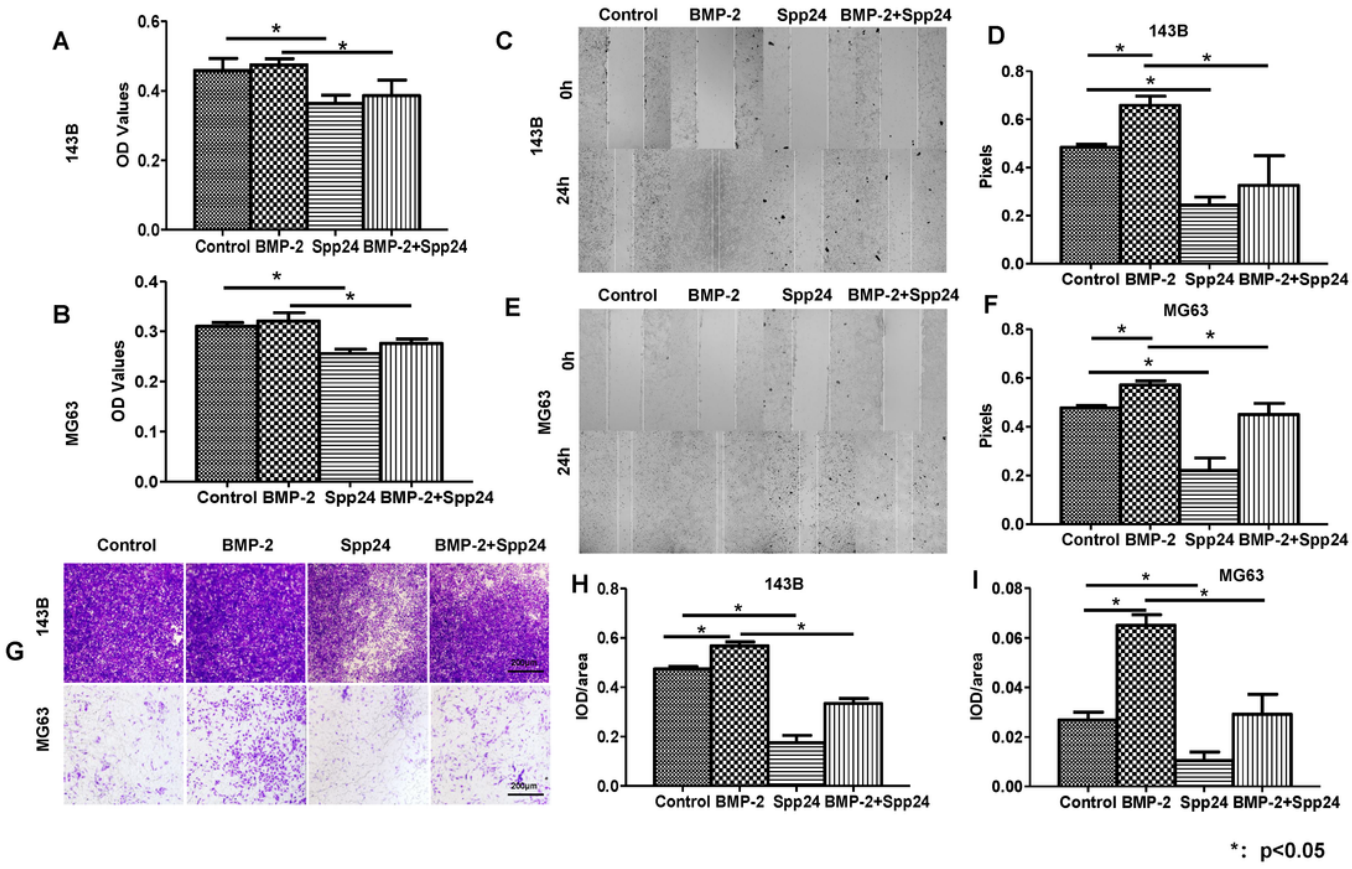

Figure 1

The changes of proliferation, migration and invasion abilities after treatment with BMP-2, Spp24 in 143B and MG63 cells. A-B. The quantitative results of the MTT analysis of 143B and MG63 cells after treatment with BMP-2, Spp24 for 24h. BMP-2 did not affect the proliferation, and Spp24 inhibited tumor growth with or without BMP-2. C-F. Cell migration image and data analysis of the wound healing assay in 143B cells (E, F). BMP-2 increased cell migration in both cell lines, while Spp24 (alone or combined with BMP-2) inhibited cell migration in both cell lines. G. Transwell invasion by 143B and MG63 cells after

treatment with BMP-2, Spp24 for 24 h. H and I shows the quantitative results of the transwell test of 143B and MG63 cells respectively. Spp24 (alone or combined with BMP-2) inhibited cell invasiveness in both cell lines.

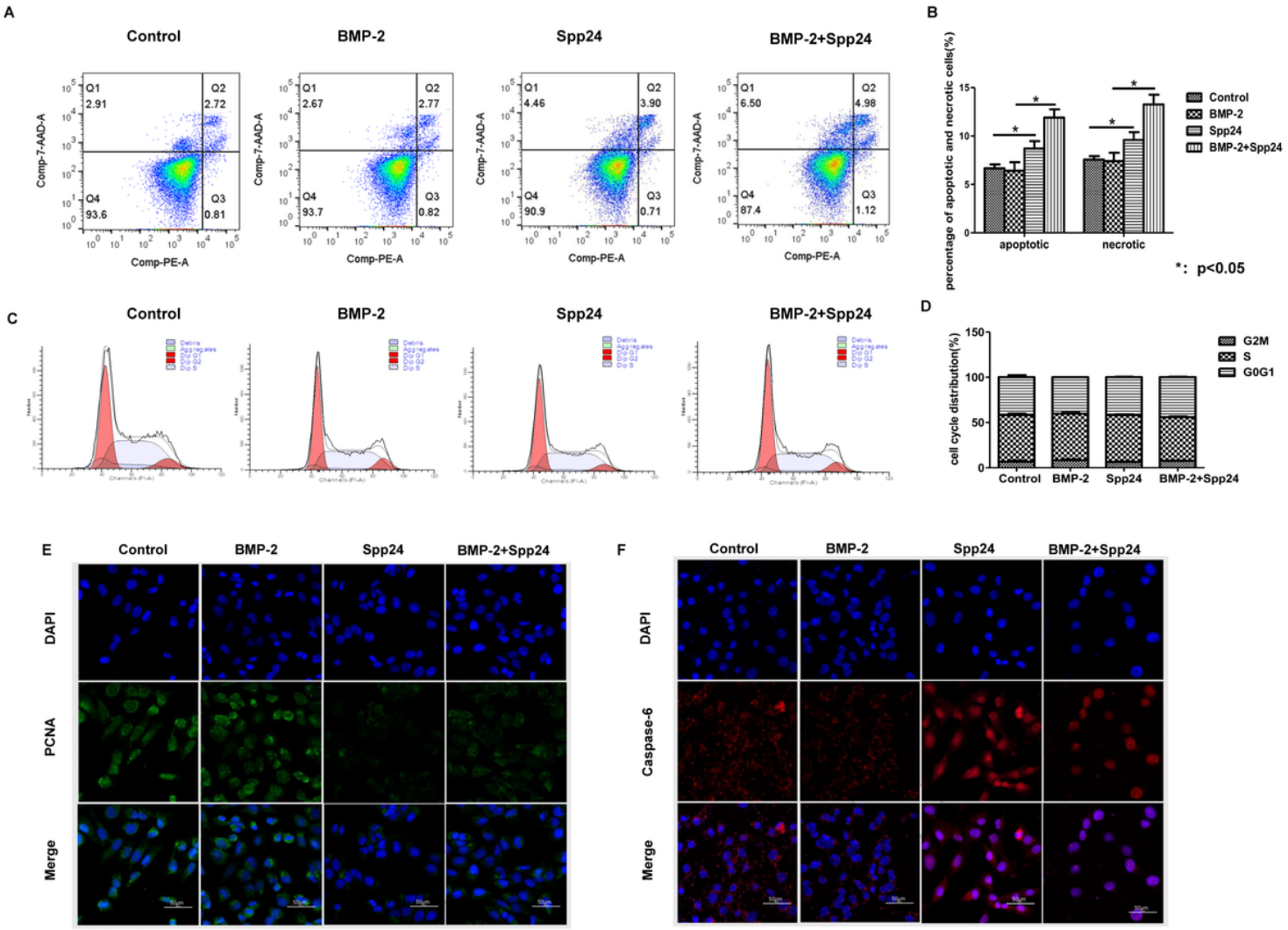

Figure 2

The apoptosis and cell cycle analysis of 143B cells. A. The total apoptotic rates of 143B cells after treatment with BMP-2, Spp24 for 24 h. B. The quantitative results of the apoptosis analysis of 143B cells after treatment with BMP-2, Spp24 for 24 h. Spp24 with or without BMP-2 significantly induced apoptosis of osteosarcoma cells. C. The cell cycle analysis of 143B cells after treatment with BMP-2, Spp24 for 24 h. The quantitative results were calculated in figure D, showing no difference between groups. E. Expression of cell proliferation marker PCNA on 143B cells after treatment with BMP-2 and/or Spp24 for 24h. BMP-2 has a minimal effect on cell proliferation, while Spp24 with or without BMP-2 significantly inhibited PCNA expression. F. Expression of marker for apoptotic cell death caspase-6 on 143B cells after

treatment with BMP-2 and/or Spp24 for 24 h. BMP-2 seem to downregulate caspase-6 activity that is significantly upregulated by Spp24.

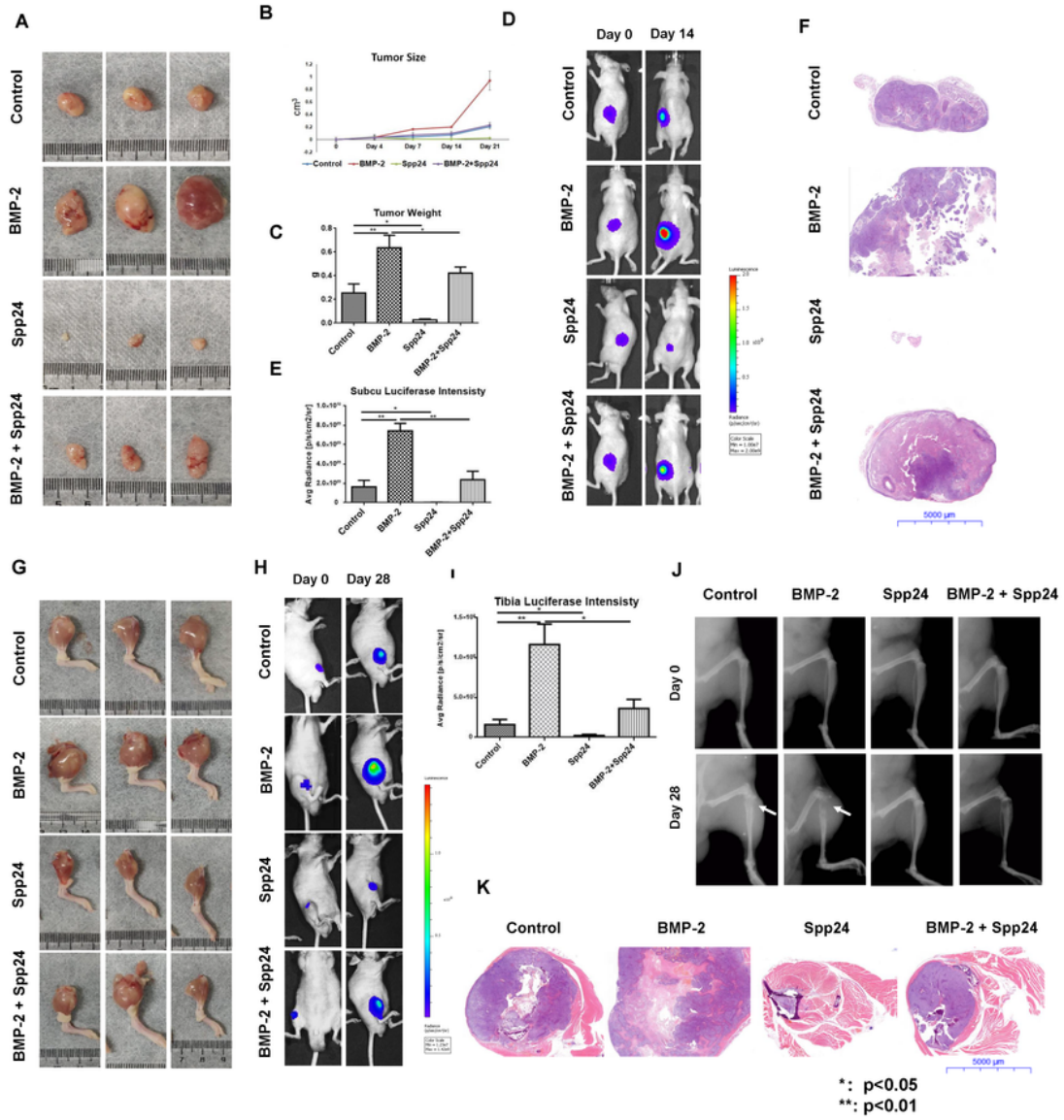

Figure 3

In vivo study of the effects of Spp24 on osteosarcoma growth using subcutaneous or intratibial xenograft of 143B cells in SCID mice. A. Three representative subcutaneous tumors per group on day 21. B. Calculation of tumor volume measured with a caliper on days 0, 4, 7, 14 and 21. C. The mean weights

of subcutaneous tumors 21 days after the injection of 5.0×105 143B osteosarcoma cells. D. In vivo luciferase expression of the subcutaneous tumor signal on day 0 and day 14. Luminescence signal grew too strong afterwards in the BMP-2 group that resulted in overflow of data (not recorded). E. The relative quantitative results of the subcutaneous luciferase intensity on day 14. F. H&E staining of representative subcutaneous tumor specimens. Note area of central necrosis due to rapid tumor growth in animal from BMP-2 group. G. Three representative intratibial tumors per group on day 28. H. In vivo luciferase expression of the intratibial tumor signal on day 0 and day 28. I. The relative quantitative results of the intratibial luciferase intensity on day 28. J. X-ray images of the limb injected with 143B cells on day 0 and day 28. Aggressive osteolytic lesion in the proximal tibia with extensive cortical bone destruction and soft-tissue invasion in animal from BMP-2 group. K. H&E staining of representative intratibial tumor specimens. Note area of central necrosis due to rapid tumor growth in control and in animal from BMP-2 group.

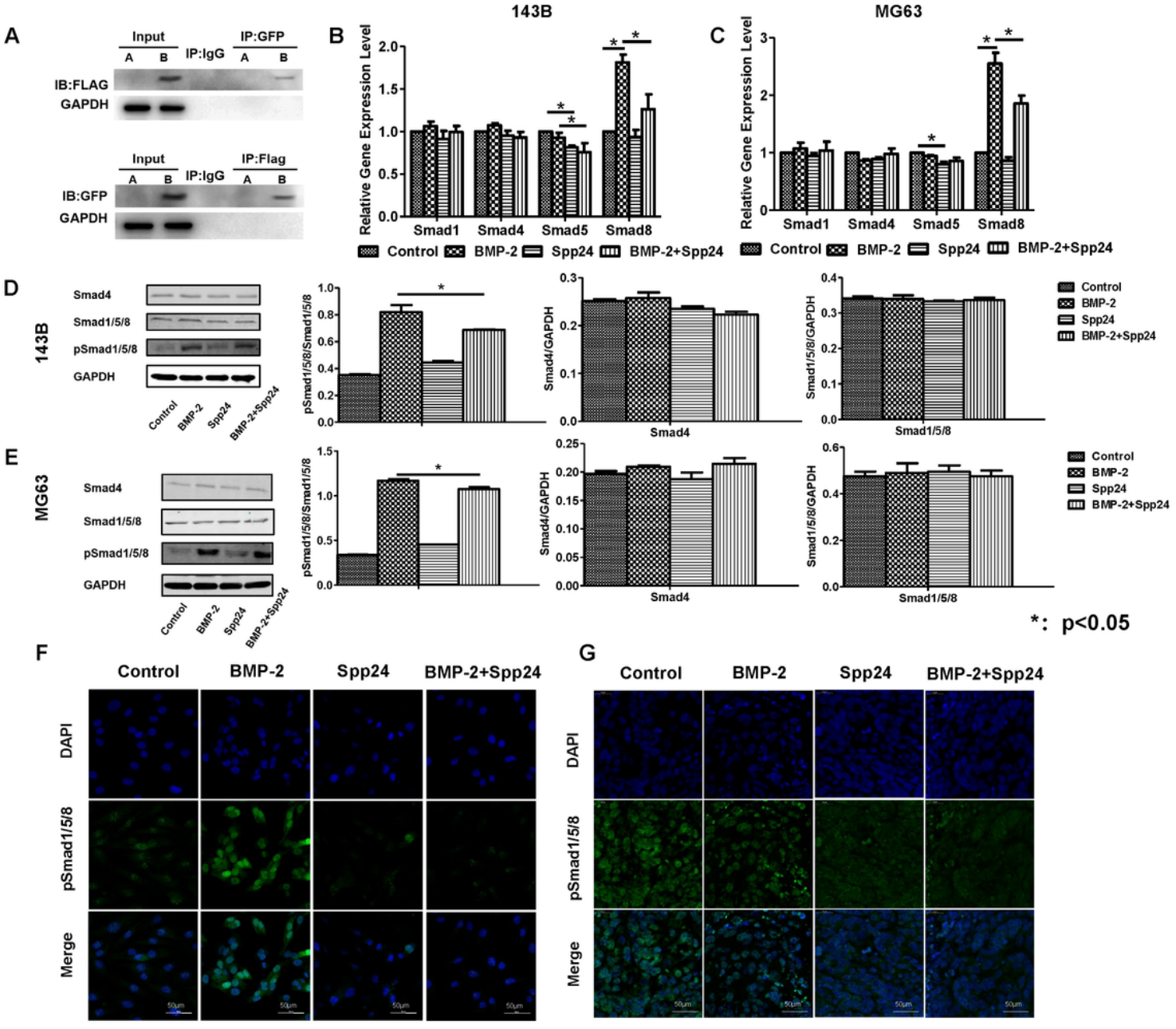

Figure 4

The interaction between BMP-2 and Spp24 and changes in the protein and the mRNA expression levels of the BMP/Smad pathway. A. Panel A (Upper panel) represents untreated 143B control cells, and Panel B (Lower panel) shows the 143B cells transfected with Spp24-GFP and BMP-2-FLAG together. Total protein was extracted from untreated 143B cells and 143B cells expressing both Spp24-GFP and BMP-2-FLAG. The Spp24-GFP protein was immunoprecipitated with anti-GFP antibody, and the presence of BMP-2-

FLAG protein was detected by immunoblot analysis with anti-FLAG antibody. The BMP-2-FLAG protein was immunoprecipitated with anti-FLAG antibody, and the presence of Spp24-GFP protein was detected by immunoblot analysis with anti-GFP antibody. B, C. The RNA transcript levels of Smad1, Smad4, Smad5 and Smad8 in 143B and MG63 cells after treatment with BMP-2, Spp24 for 24 h using quantitative RT PCR. D. The protein expression levels of the BMP-2/Smad pathway and the quantitative results of phospho-Smad1/5/8, Smad4 and Smad1/5/8 in 143B cells after treatment with BMP-2, Spp24 for 24 h. E. The protein expression levels of the BMP-2/Smad pathway and the quantitative results of phospho-Smad1/5/8, Smad4 and Smad1/5/8 in MG63 cells after treatment with BMP-2, Spp24 for 24 h. F. The expression of phospho-Smad1/5/8 of 143B cells after treatment with BMP-2, Spp24 for 24 h analyzed by immunofluorescence. G. The expression of phospho-Smad1/5/8 in nude mouse subcutaneous xenograft tumor specimen analyzed by immunofluorescence.