

RESEARCH ARTICLE

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

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Abstract

Autocrine stimulation of tumor 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, our aim was to investigate the effects of Secreted phosphoprotein 24 kD (Spp24) on the growth of osteosarcoma (OS) cells in the presence and absence of exogenous BMP-2 both in vitro and in vivo. Our study demonstrated that Spp24 inhibited proliferation and promoted apoptosis of OS cells as confirmed by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay and immunohistochemical staining. We found that BMP-2 increased the mobility and invasiveness of tumor cells in vitro whereas Spp24 inhibited both of these processes alone and in the presence of exogenous BMP-2. Phosphorylation of Smad1/5/8 and Smad8 gene expression was enhanced by treatment with BMP-2 but inhibited by treatment with Spp24. Subcutaneous and intratibial tumor models in nude mice demonstrated that BMP-2 promoted OS growth in vivo, while Spp24 significantly inhibited tumor growth. We conclude that the BMP-2/Smad signaling pathway is involved in the pathogenesis of OS growth and that Spp24 inhibits the growth of human OS induced by BMP-2 both in vitro and in vivo. Interruption of Smad signaling and increased apoptosis appear to be the

Dr. Hongfang Chen and Dr. Chenshuang Li contributed equally to this study.

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primary mechanisms involved. These results confirm the potential of Spp24 as a therapeutic agent for the treatment of OS and other skeletal tumors.

KEYWORDS

autocrine, BMP-2, osteosarcoma, Smad, Spp24

1 | 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 The incidence of OS has a bimodal age distribution with the highest incidence during the second decade of life.^{3,4} 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.⁶

Autocrine stimulation of tumor cells by growth factors is an important mechanism of tumor growth. Pertinent growth factors include platelet-derived growth factor, insulin-like growth factor, adrenomedullin, endothelin-1, parathyroid hormone-related peptide fragments,⁷ and, most importantly, members of the BMP/TGF- β superfamily of cytokines and their receptors.^{8–16} BMPs-2, -4, -6, and -7 and their receptors are upregulated in OS, breast, prostate, and lung cancer cell lines.^{7,17–20} Because of their central roles in the development, growth, and remodeling of bone, members of the TGF- β /BMP family of proteins are regarded as the most promising targets for therapeutic intervention aimed at growth factor systems in bone tumors.²¹

Another concern regarding the comprehensive therapy for OS 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.²² Because of existing information supporting a role for BMPs as autocrine factors in OS cell growth,²³ and because of the ongoing concerns pertaining to the tumorigenic actions of exogenous BMPs, studies were undertaken to determine the effects of the BMP binding and neutralizing protein, secreted phosphoprotein 24 kD (Spp24), on tumor growth in a mouse model of OS.

2 | MATERIALS AND METHODS

2.1 | Production of Spp24

Recombinant bovine Spp24 was produced in a bacterial expression vector as described in detail previously.²⁴ 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) to provide for stability and to allow purification of the protein by means of immobilized metal affinity chromatography (IMAC).

2.2 | Cell culture

The highly malignant 143B and moderately malignant MG63 cell lines used in this study were obtained from ATCC. 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% CO₂ at 37°C. Media were changed every 3 days. Cells were passaged when cultures reached 80%–90% confluence.

2.3 | MTT assay

To investigate the effects of BMP-2 and Spp24 on cell growth, 143B or MG63 cells were seeded into 96-well microtiter plates at 2×10^3 cells/well. Cells were cultured for 24 h to achieve attachment, starved for 12 h with 1% FBS medium, then treated with a different combination of BMP-2 and Spp24 (Control group: without BMP-2 or Spp24; BMP-2 group: 100 ng/mL BMP-2; Spp24 group: 20 μ g/mL Spp24; BMP-2 + Spp24 group: 100 ng/mL BMP-2 + 20 μ g/mL Spp24) for 24 h. Cell growth was evaluated using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (ATCC). Absorbances were read at 570 nm with a reference wavelength of 700 nm.

2.4 | Wound-healing assay

143B and MG63 cells were seeded in 12-well microtiter 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 200 μ L 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 minimize the influence of cell proliferation. Cells were treated with vehicle, BMP-2, Spp24, or BMP-2 plus Spp24 as described above. The wounds were photographed at each time point using an inverted

microscope (Leica). ImageJ 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 performed in triplicate.

2.5 | Matrigel invasion assay

Trans-well in vitro invasion 24-well chambers (Corning) were used following the manufacturer's instructions. The membrane of each well was precoated with 500 μ L BD Matrigel (BD Biosciences). 2.5×10^4 143B or MG63 cells in 200 μ L serum-free medium were placed into the upper chamber and then vehicle, BMP-2, Spp24, or BMP-2 plus Spp24 was added into the upper chamber. Five hundred microliters of medium containing 10% FBS was placed into the lower compartment. The trans-well chambers were incubated for 24 h at 37°C in 95% air with 5% CO₂. 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 $\times 200$ magnification) per filter. The studies were conducted in triplicate.

2.6 | Apoptosis analysis by flow cytometry (FCM)

143B cells were seeded into six-well microtiter 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 cells were exposed to culture media with vehicle, BMP-2 (100 ng/mL), Spp24 (20 μ g/mL), or BMP-2 + Spp24 (100 ng/mL, 20 μ g/mL) for 24 h. For apoptosis analysis, 5 μ L of annexin V-PE and 5 μ L 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 instructions (Thermo Scientific) and the fluorescence intensity of the samples was immediately analyzed by FCM.

2.7 | Quantitative real-time polymerase chain reaction (PCR)

To examine the effects of BMP-2 and Spp24 on Smad signaling in OS cells at the gene level, 143B and MG63 cells were seeded and

grouped as Control, BMP-2 (100 ng/mL), Spp24 (20 μ g/mL), BMP-2 + Spp24 (100 ng/mL, 20 μ g/mL) in six-well microtiter plates at a density of 1×10^5 cells/well and cultured for 24 h. Total RNA was isolated by E.Z.N.A.® HP Total RNA Kit (Omega Bio-Tek). One microgram RNA was subjected to reverse transcription using the Prime-Script RT reagent kit (Takara). Real-time PCR was performed with a 7300 Real-Time PCR system with SYBR® Premix Ex Taq™ (Takara). 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 $\Delta\Delta C_T$ method.²⁵

2.8 | Western blotting analysis

143B and MG63 cells were seeded and grouped as Control, BMP-2 (100 ng/mL), Spp24 (20 μ g/mL), or BMP-2 + Spp24 (100 ng/mL, 20 μ g/mL) in six-well microtiter 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 (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.4, 1 mM EDTA, 1 mM PMSF, and 1% Triton X-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, the GAPDH antibody (Cell Signaling Technology, catalog #: 5174S) was used. The protein bands were visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.9 | Plasmid construction and transfection

BMP-2 and Spp24 cDNA was purified by Genechem 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

TABLE 1 Sense and antisense primers for real-time PCR.

Gene	Forward (5'–3')	Reverse (5'–3')
Smad1	TTTACAAGTCCAGCTGTGAAGA	AAAGCATCAACAGCTTTCTCTG
Smad4	ACAAGTAATGATGCCTGTCTGA	CTCCCATCCAATGTTCTCTGTA
Smad5	ACAATTGAAAACACTAGGCGAC	AGTTGCAGTTCCTACTCTGTAC
Smad8	GAAGTGTGCATTAACCCCTTACC	ATATTCAGTGTCTTGGCACG
GAPDH	CAGCGACACCCACTCCTC	TGAGGTCCACCACCTGT

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction.

(Invitrogen) following the manufacturer's instructions. Lentiviral particles with scrambled DNA sequences 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.

2.10 | Co-immunoprecipitation (Co-IP)

Co-IP assays were performed using a Co-IP kit (Thermo Scientific). 143B cells were lysed in SDS lysis buffer on ice for 30 min, and after centrifugation at 4°C and 14,000g for 15 min, the supernatants were collected. The protein lysates were then incubated with anti-FLAG and anti-GFP antibodies 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 3000g 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.

2.11 | Transfection of Lenti-CMV-firefly luciferase (FL)

143B cells were seeded at 2×10^5 cells/well in six-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 infection of 5 (MOI = 5), including lentiviral vector supernatant and 1 µg/mL of protamine sulfate. The cells were then washed with 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). Each value was normalized to cell number or protein amount and calculated as the average of triplicate samples.

2.12 | Implantation of tumor cells and test materials

All experiments involving animal subjects 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 OS cells in a volume of 200 µL that contained 50 µL of culture medium, 40 µL of growth factor reduced Matrigel (BD Biosciences), 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; and 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 24 nude mice received a tibial injection of 1.0×10^5 143B OS cells in a total volume of 50 µL 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.

2.13 | 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). FL expressed by the tumor cells catalyzes the oxidation of D-luciferin while causing light emission. Twenty minutes after the injection, images were photographed using an IVIS-cooled CCD camera (Xenogen). 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 resulted 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.

2.14 | 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.²² 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.

2.15 | Histology and immunohistochemistry

143B cells were seeded at 1×10^4 cells/dish in 10 mm confocal dishes. After cell attachment, all dishes were treated with BMP-2 or Spp24 as previously described and incubated for 24 h. Cells were then rinsed with PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich) 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-Caspase-6, anti-Caspase-3, or anti-phospho-Smad1/5/8, followed by Alexa 488 conjugated secondary antibody. Images were observed and photographed under

fluorescent microscopy (Nikon Eclipse CI). The subcutaneous tumor specimens were fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C overnight before paraffin embedding. Hematoxylin and eosin (H&E) staining was performed on 5- μ m sections for histological analyses. Immunohistochemistry to detect phospho-Smad1/5/8 in nude mouse xenograft tumor specimens was performed as previously described.²²

2.16 | Statistical analysis

The data were presented as means $\pm$ standard deviation and were analyzed with SPSS version 22.0 (IBM). 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 with subsequent Tukey-Kramer comparisons. Student's *t* test was used for comparison involving two groups. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$).

3 | RESULTS

3.1 | Spp24 inhibits proliferation, migration, and invasiveness of OS cells in vitro

BMP-2 did not significantly affect the proliferation of 143B or MG63 OS cells but, Spp24 did inhibit proliferation, even in the presence of BMP-2 (Figure 1A,B). On the other hand, treatment with BMP-2 increased migration in both cell types whereas Spp24 inhibited migration, either alone or in combination with BMP-2 (Figure 1C-F). Furthermore, BMP-2 increased invasiveness while Spp24 decreased the invasiveness, even in the presence of BMP-2, of both cell types (Figure 1G-I).

3.2 | Spp24 inhibits in vitro OS cell growth by inducing tumor cell apoptosis

The effects of Spp24 on 143B OS apoptosis were assessed using FCM assays and immunohistochemistry. Figure 2A,B shows a representative sample and calculated data for an FCM cell apoptosis analysis using an Annexin V-PE/7AAD method. BMP-2 inhibited the apoptosis initially at 24 h, although, this was not statistically significant. The addition of Spp24, with or without BMP-2, induced cell apoptosis with the Spp24 + BMP-2 group having the highest percentage of apoptotic cells (Q1: necrotic cells. Q2: early apoptotic cells. Q3: late apoptotic cells. Q4: live cells). Western blotting demonstrated that the protein levels of Caspase-6 and Caspase-3 were higher in the Spp24 and BMP-2 + Spp24 groups (Figure 2C). Immunocytochemistry of in vitro cultures of the OS cells showed that the proliferation marker PCNA was slightly upregulated in the BMP-2 group and downregulated in the Spp24 and BMP-2 + Spp24 groups (Figure 2D,F), whereas the apoptosis marker Caspase-6 was downregulated by BMP-2 and upregulated by Spp24 (Figure 2E,F).

Altogether, this suggests that Spp24 affected tumor cell apoptosis, which inhibited tumor growth and obviated the effects of BMP-2.

3.3 | Spp24 inhibits OS growth in vivo

Coimplantation of Spp24 with OS cells resulted in a marked inhibition of tumor growth in vivo (Figure 3). This occurred both in the presence and absence of exogenously added BMP-2 as co-implantation of BMP-2 with tumor cells resulted in dramatic tumor growth that was attenuated by the presence of Spp24. However, it is especially noteworthy that Spp24 inhibited tumor growth in animal subjects into which no BMP-2 had been implanted. For the subcutaneous tumor model, intensity of luminescence, a reflection of in vivo volume, was recorded up to Day 14 for all groups. After that time point, the intensity for the BMP-2 group became too high to provide reliable data. However, the values for the other groups were recorded up to the final time point of Day 21. For the subcutaneous tumor model in nude mice, co-implantation of BMP-2 with tumor cells resulted in an increase in tumor volume, weight, and luminescent activity whereas the Spp24 treatment group showed a diminution in these parameters compared with vehicle control and the Spp24 plus BMP-2 group showed an inhibition of the growth induction when compared to the BMP-2 only group (Figure 3A-E). Histological examination of specimens from each group confirmed OS tumor growth (Figure 3F). Similar results were found with the intratibial tumor injection model as reflected in tumor size and luminescent activity as well as radiological and histological appearance (Figure 3G-K). Osteolytic fracture was noticed in the BMP-2 and control group, indicating a fast and aggressive growth of OS.

3.4 | Spp24 binds to BMP-2 and downregulates the BMP-2/Smad signaling pathway by inhibition of phosphorylation of Smad1/5/8

While extracellularly binding of BMP-2 and Spp24 has been proved by the authors in a previous study²⁶ and in the supplementary data by immunoprecipitation using cell supernatants, to further test the hypothesis that Spp24 inhibits the tumor-promoting activities of BMP-2 by binding to and sequestering BMP-2 and, therefore, inhibiting the downstream Smad pathway, gene expression studies were undertaken. It was revealed that the expression levels of Smads were affected by the administration of BMP-2 and Spp24, especially in the case of Smad8, which was upregulated by the BMP-2 and downregulated by Spp24 (Figure 4A,B). Furthermore, Western blotting showed that pSmad1/5/8 was significantly upregulated by BMP-2 and somewhat blocked by Spp24 (Figure 4C,D), indicating that BMP-2 induced phosphorylation of Smad1/5/8. These findings were confirmed in living cells and tissues in that immunohistochemical staining of the in vitro culture of 143B cells as well as specimens from the subcutaneous tumor model demonstrated significant inhibition of phosphorylation of Smad1/5/8 by Spp24,

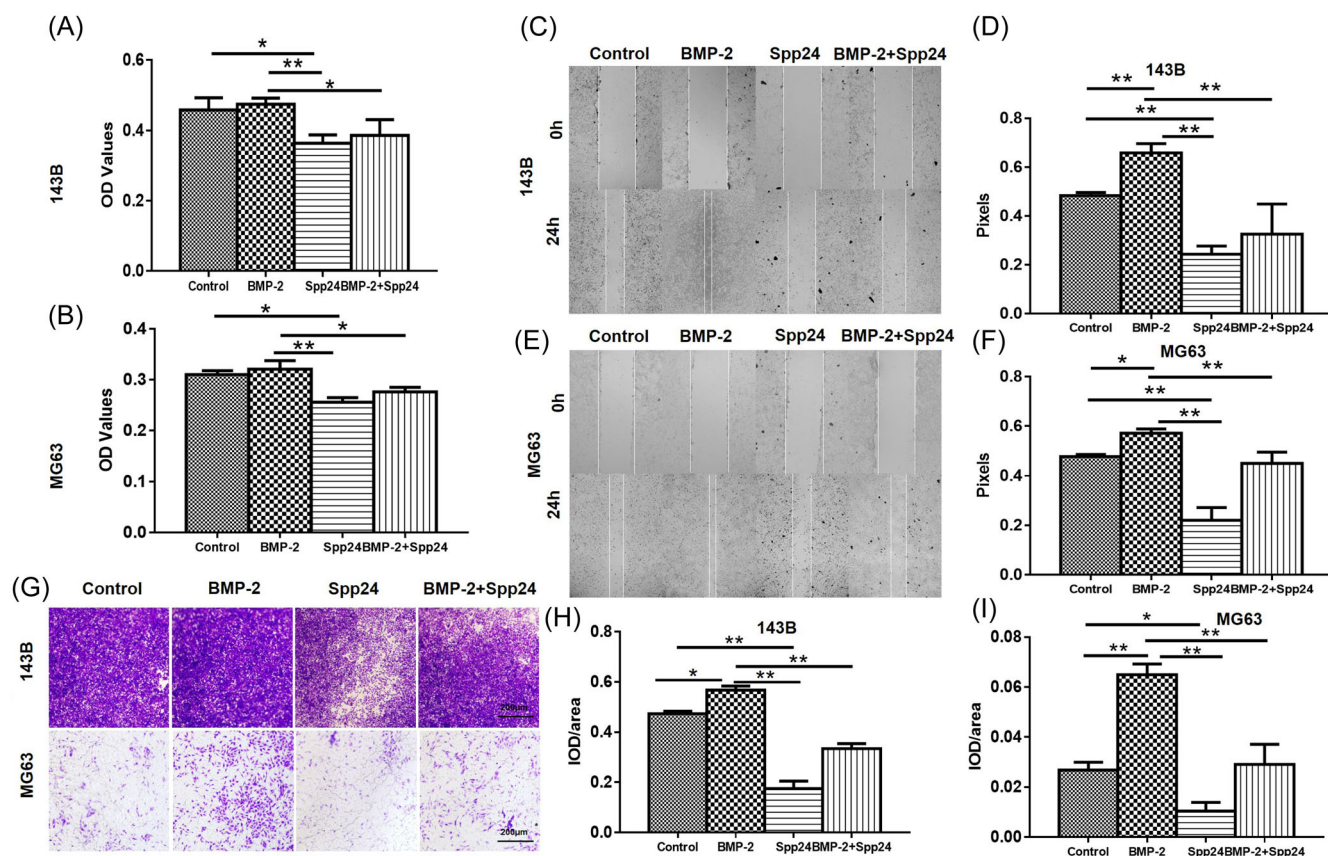


FIGURE 1 Changes in proliferation, migration, and invasiveness after treatment with combinations of BMP-2 and Spp24 in 143B and MG63 osteosarcoma cells. Quantitative results of a 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide assay for 143B cells (A) and MG63 cells (B) after treatment with BMP-2 and Spp24 for 24 h. BMP-2 did not affect proliferation whereas Spp24 inhibited tumor cell growth with or without BMP-2. Cell migration images and data analyses for the wound healing assay in 143B cells (C, D) and MG63 cells (E, F) following treatment with BMP-2 and Spp24. BMP-2 increased migration whereas Spp24 inhibited migration in both cell types. Transwell invasiveness of 143B cells and MG63 cells. (G) Data analyses for transwell invasiveness in 143B cells (H) and MG63 cells. (I) BMP-2 increased invasiveness whereas Spp24 decreased invasiveness in both cell lines. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$). All the quantitative analyses were calculated as the average of triplicate samples ($n = 3$). MTT, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide; Spp24, secreted phosphoprotein 24 kD.

supporting our contention that Spp24 inhibits BMP-2 induced upregulation of the Smad pathway by binding to BMP-2 through the specific binding domain (Figure 4E,F).

4 | DISCUSSION

Autocrine stimulation is important for tumor growth in the skeleton for both primary tumors and metastases. 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.⁶ While overexpression 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,⁶ overexpression of pro-osteogenic growth factors plays a central role in promoting the growth and extension of tumor cells once they have localized to the bone.

It would be ideal to develop an efficacious and minimally toxic adjuvant therapeutic that can be applied directly to the bed of an excised tumor as local chemotherapy. In this regard, we are working to develop the bone matrix protein, Spp24, that avidly binds proteins of the TGF- β /BMP family of proteins, as a potential bone tumor therapeutic. Spp24 is likely to have a number of varied functions in the natural bone environment, not only due to its ability to bind several proteins of the TGF- β /BMP family,^{27,28} but also related to its (or, more precisely, truncated fragments of it) little explored ability to stimulate intracellular signaling pathways.²⁹ 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").²⁷ 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- β receptor.³⁰ When BBP was applied to a

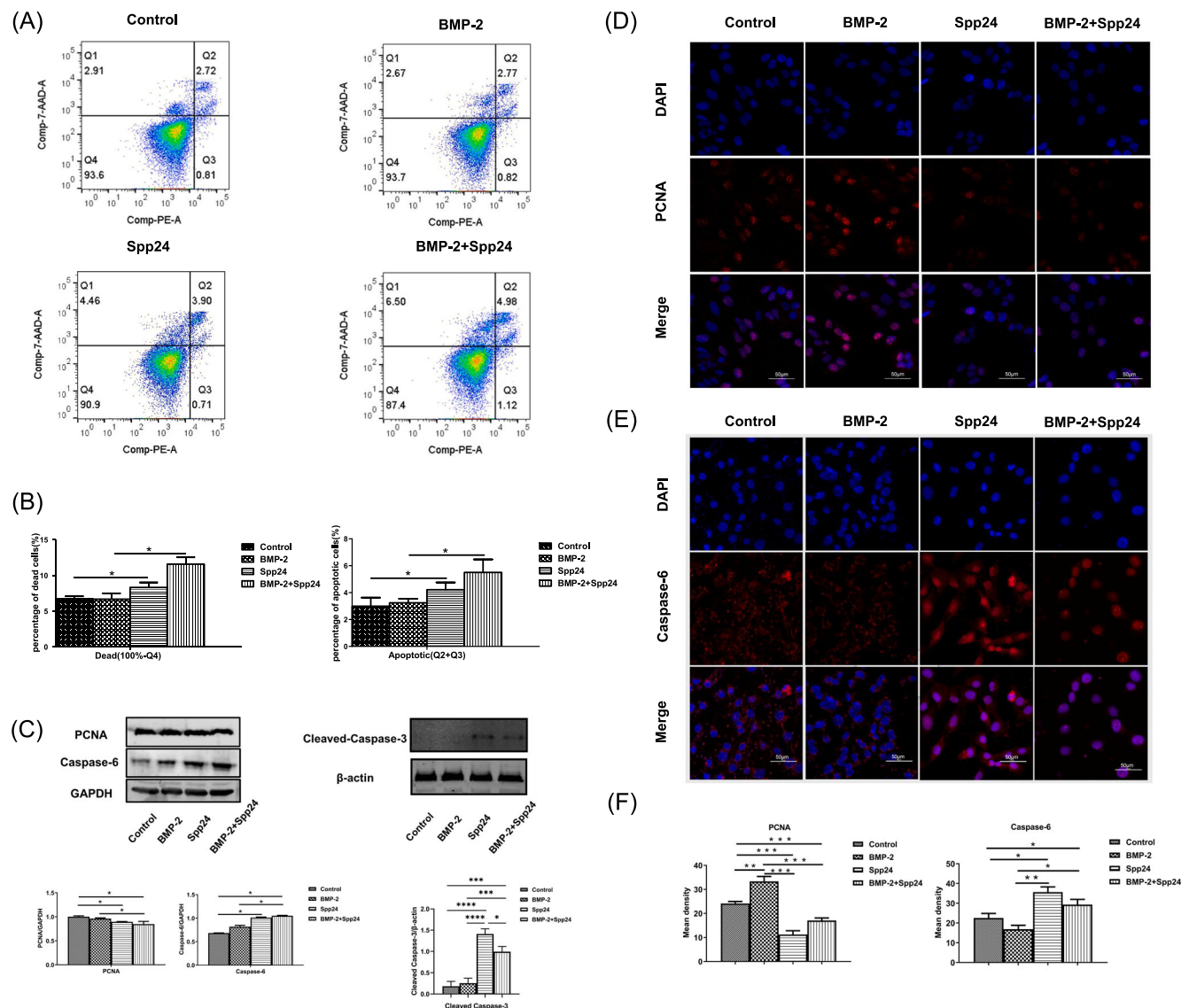


FIGURE 2 The apoptosis of 143B cells. The flow cytometry of 143B cells after treatment with combinations of BMP-2 and Spp24 for 24 h (A) and the quantitative results of the apoptosis analysis of 143B cells. (B) Note that Spp24, with or without BMP-2, induced apoptosis of osteosarcoma cells. Q1: necrotic cells. Q2: early apoptotic cells. Q3: late apoptotic cells. Q4: live cells. (C) Protein expression levels for the proliferation marker PCNA and the apoptotic cell death markers Caspase-3 and Caspase-6 in 143B cells treated with combinations of BMP-2 and Spp24 for 24 h. Quantified data are standardized for levels of GAPDH and β -actin. Immunofluorescence images for expression of PCNA (D) and Caspase-6 (E) in 143B cells treated with combinations of BMP-2 and Spp24 for 24 h and the quantitative analysis of fluorescence intensity of PCNA and Caspase-6. (F) Note that Caspase-6 activity was upregulated by Spp24 with/without BMP-2. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$). All the quantitative analyses were calculated as the average of triplicate samples ($n = 3$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Spp24, secreted phosphoprotein 24 kD.

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,³⁰ spinal fusion,^{31,32} and long bone healing.³³ 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 formation²⁶ and spinal fusion.³⁴ The latter observation suggested a role for Spp24 in the suppression of BMP/TGF autocrine systems.

We employed the bone matrix protein Spp24 to test the hypothesis that the interruption of a key autocrine system could be

used to arrest tumor growth both in vitro and in vivo. Spp24 functions, at least in part, by binding to and sequestering TGF- β /BMP family proteins, though other mechanism such as direct interactions with receptors by Spp24 cannot be completely discounted. Irrespective of the mechanism, our studies showed that treatment of tumor cells both in vitro and in vivo with Spp24 resulted a dramatic reduction in tumor growth.

BMP-2 is a member of the TGF- β 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

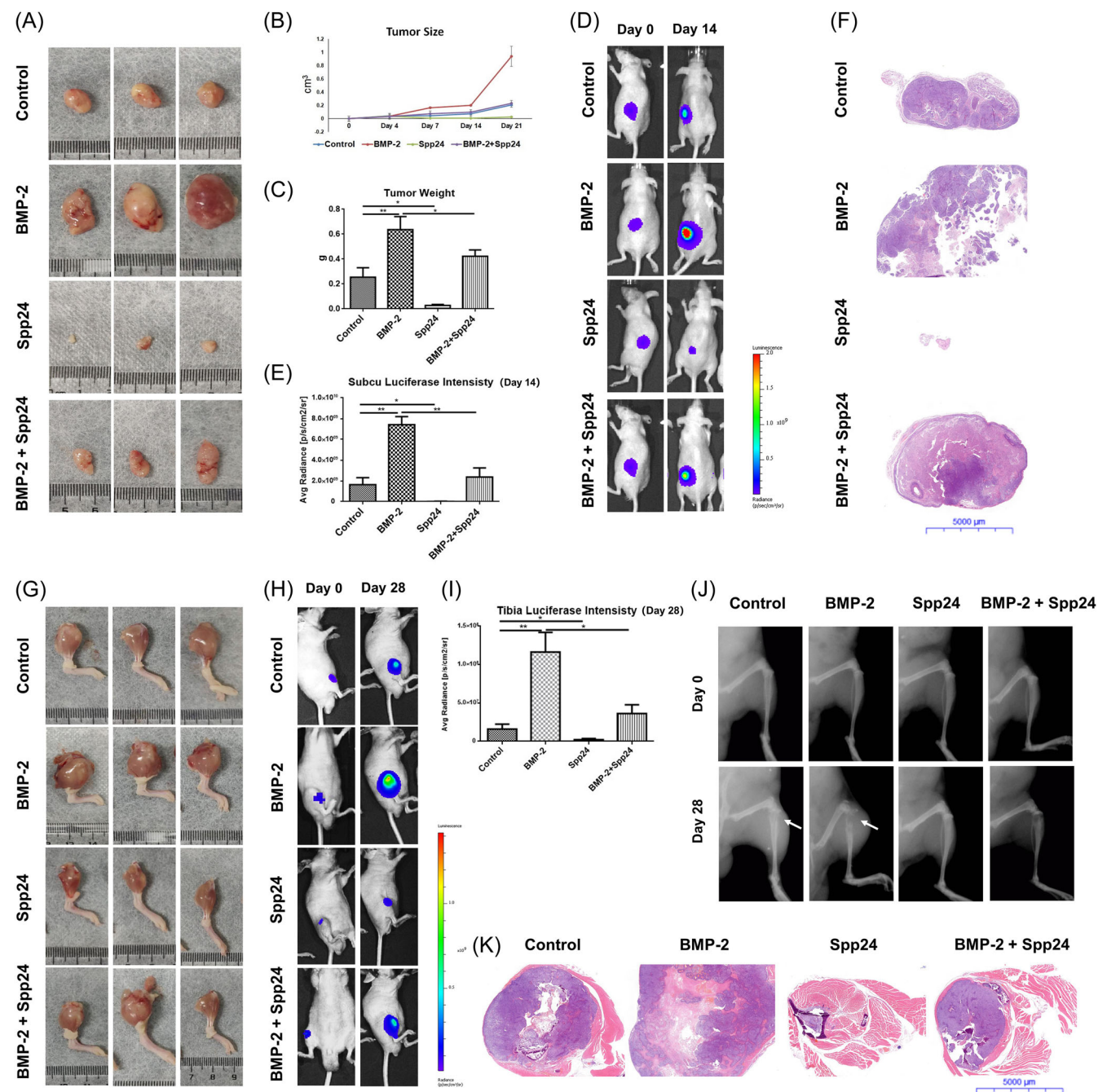


FIGURE 3 In vivo study of the effects of Spp24 on osteosarcoma (OS) growth using both subcutaneous and intra-tibial injections of 143B OS cells into SCID mice. Subcutaneous tumor model: (A) Images of three representative tumors excised from subjects from each treatment group on Day 21. (B) Externally measured mean tumor volumes from Days 0, 4, 7, 14, and 21. (C) Mean weights of tumors excised on Day 21. (D) luminescent images from representative specimens from each treatment group for Days 0 and 14. (E) Numerical values for mean luminescence for specimens from each group other than the BMP-2 alone group on Day 21. For the BMP-2-only group, data from Day 14 were used because after that time the luminescence became too intense to provide reliable data. (F) Hematoxylin and eosin (H&E)-stained histological specimens from representative tumors from each treatment group. Note the extensive central necrosis in the specimen from the BMP-2 group which resulted from rapid tumor growth. Note also the minimal size of the tumor from the Spp24-only group. Intratibial tumor model: (G) Gross appearance of three representative tumors from each group excised on Day 28. (H) Luminescent images from representative specimens from each treatment group for Days 0 and 14. (I) Numerical values for mean luminescence for specimens from each group on Day 28. (J) Radiological images of intra-tibial tumors on Days 0 and 28. Note the aggressive osteolytic lesion in the proximal tibia with extensive cortical bone destruction and soft tissue invasion in the specimen from the BMP-2 treatment group. (K) H&E stained histological specimens from representative tumors from each treatment group. Note the areas of central necrosis due to rapid tumor growth in the specimens from the control and BMP-2 groups. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$). H&E, hematoxylin and eosin; OS, osteosarcoma; Spp24, secreted phosphoprotein 24 kD.

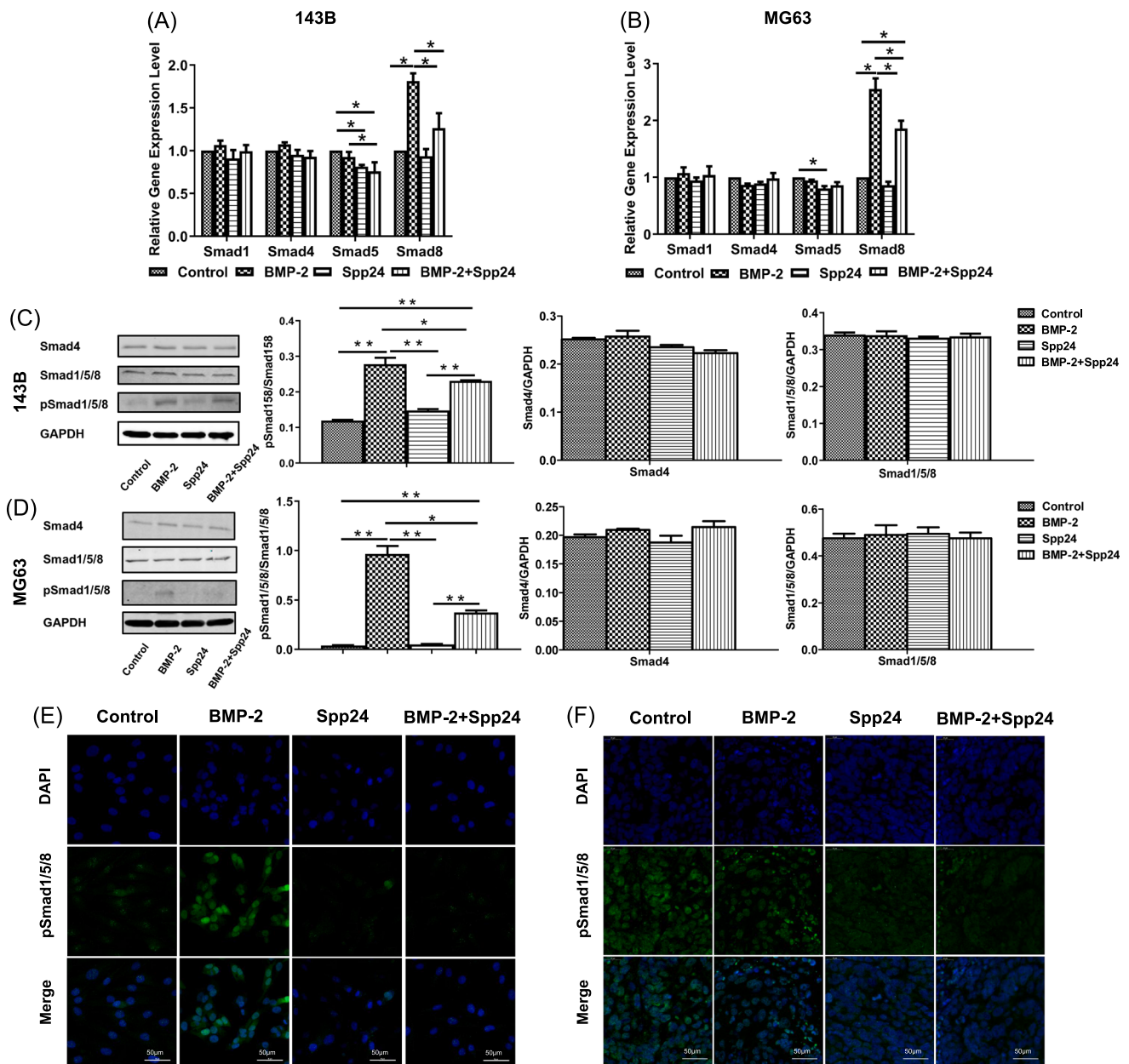


FIGURE 4 The interaction between BMP-2 and Spp24 and changes in the protein and the mRNA expression levels of proteins in the BMP/Smad pathway. RNA transcript levels for Smad1, Smad4, Smad5, and Smad8 in 143B cells (A) and MG63 cells (B) after 24 h of treatment with combinations of BMP-2 and Spp24. Images and quantified data for protein expression of Smad pathway proteins after 24 h of treatment with combinations of BMP-2 and Spp24 in 143B cells (C) and MG63 cells. (D) Immunofluorescence to detect phospho-Smad1/5/8 after 24 h of treatment with combinations of BMP-2 and Spp24 in 143B cells (E) and after 28 days of implantation of combinations of BMP-2 and Spp24 in the nude mouse subcutaneous tumor model. (F) Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$). All the quantitative analyses were calculated as the average of triplicate samples ($n = 3$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Spp24, secreted phosphoprotein 24 kD.

large spectrum of tumor types.^{35–37} More pertinent to this study, Sotobori et al.³⁸ reported that the expression of BMP-2 and its receptors (BMPR1, BMPR2) was greatly increased in Dunn OS cells. Additionally, Mohseny et al.³⁹ have shown that, compared with osteoblastoma, phosphorylated Smad1 was highly expressed in human OS samples by immunohistochemical analysis. Furthermore, Yang et al.⁴⁰ demonstrated that increased BMP-2 induced

overexpression of tumorigenic factors such as VEGF, EMMPRIN, and MMP-9 both intra and extracellularly in OS mesenchymal stem cells. In terms of a more generalized mechanistic view, Tian et al.²² demonstrated that BMP-2 stimulates the motility, invasiveness, and the epithelial-to-mesenchymal transition in OS cells in tissue culture and promoted growth and invasiveness of 143B OS cells in mouse xenograft models.

We observed that Spp24 inhibited tumor cell proliferation while BMP-2 did not significantly enhance proliferation. This could be because the proliferation mechanism of the cells could be already saturated with respect to endogenous BMP-2 or because Spp24 is affecting other growth factors or mechanistically distinct systems. Although BMP-2 did not significantly enhance proliferation, it did increase migration and invasiveness in both 143B and MG63 cell types. In addition, treatment of tumor cells with Spp24, either with or without exogenous BMP-2, enhanced the process of apoptosis.

The Smad pathway is the most central mechanism for the intracellular transmission of BMP signaling. That the treatment of OS tumor cells with Spp24 in the presence or absence of exogenously added BMP-2 strongly affected this pathway supports our contention that the primary mechanism of action for Spp24 in this application is through the sequestration of BMP-2. Furthermore, the pattern of the affects with the greatest magnitude, which is to say, the inhibition by Spp24 of BMP-2-induced Smad8 gene expression and phosphorylation of Smad1/5/8, also supports this contention. On the other hand, based on the data that Spp24 alone downregulated the expression of Smad5, and that Spp24 partially eliminate the upregulation of Smad8 caused by BMP-2, it is possible that Spp24 might not function solely by reducing the activity of BMP-2. Caspase-3 and Caspase-6 are categorized as executioner Caspases,⁴¹ and they promote the activation of programmed cell death pathways including pyroptosis, apoptosis, and necroptosis.⁴² Activation of Caspase-6 is upstream of Caspase-3/-7 activation and decreased expression of proapoptotic proteins (Bax, Apaf-1, Caspase-3, Caspase-6, and Caspase-7) results in cancer growth, angiogenesis, and metastasis.⁴³ In this study, we found that Spp24 induced apoptosis in 143B cells by upregulating Caspase-3 and Caspase-6, and further study should focus on connecting the Smad pathway with Caspase-3 and Caspase-6.

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.^{8,44–46} demonstrated that the addition or overexpression 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. More recently, we have demonstrated that Spp24 can suppress pancreatic cancer,⁴⁷ hepatocellular carcinoma,⁴⁸ prostate cancer,⁴⁹ and lung cancer⁵⁰ 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.

In this study, we have shown that Spp24 can bind to BMP-2 and inhibit its function in OS cells, both in vitro and in vivo. In vitro cell proliferation, migration, and invasiveness of OS cells were all inhibited by Spp24. Treatment with Spp24 reduced tumor growth in vivo. Our studies strongly suggest that Spp24 inhibits in vitro OS growth. 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 definitively 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- β family of proteins or through completely different biological mechanisms 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 anticancer therapeutic.

AUTHOR CONTRIBUTIONS

Design: Haijun Tian, Jeffrey C. Wang, Michael D. Daubs, Samuel S. Murray, and Elsa J. Brochmann. *Experimental operation:* Hongfang Chen, Chenshuang Li, Tangjun Zhou, Xunlin Li, and Haijun Tian. *Acquisition of data and analysis:* Hongfang Chen, Haijun Tian, Chenshuang Li, Tangjun Zhou, and Elsa J. Brochmann. *Writing, review, and/or revision of the manuscript:* Haijun Tian, Hongfang Chen, Chenshuang Li, Maria Eugenia L. Duarte, Zorica Buser, MD, Jeffrey C. Wang, Samuel S. Murray, and Li Jiao. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

Zorica Buser—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. Michael D. Daubs—Royalties and Consultancy: Dupuy-Synthes. Research or institutional support: Stryker. Jeffrey C. Wang—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. The remaining declare no conflict of interest.

ETHICS STATEMENT

All Procedures involving animal subjects were reviewed and approved by the Ethical Committee of Shanghai Ninth People's Hospital in Shanghai, China.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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