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A Carboxy Terminal BMP/TGF-β Binding Site in Secreted Phosphoprotein 24 kD Independently Affects BMP-2 Activity

Haijun Tian,[1,2]* Chen-Shuang Li,[3] Ke-Wei Zhao,[4] Jeffrey C. Wang,[2] M. Eugenia L. Duarte,[5] Cynthia L. David,[6] Kevin Phan,[2] Elisa Atti,[7] Elsa J. Brochmann,[4,8,9] and Samuel S. Murray[4,8,9]

[1]*Department of Orthopaedic Surgery, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai 200003, P.R. China*

[2]*Department of Orthopaedic Surgery, University of California, Los Angeles, California 90024*

[3]*Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing 100081, P.R. China*

[4]*Research Service, VA Greater Los Angeles Healthcare System, North Hills, California 91343*

[5]*National Institute of Traumatology and Orthopaedics, Federal University of Rio de Janeiro, Rio de Janeiro 21941, Brazil*

[6]*Center for Toxicology, University of Arizona, Tucson, Arizon 85721*

[7]*School of Dentistry, University of California, Los Angeles, California 90024*

[8]*Geriatric Research, Education and Clinical Center, VA Greater Los Angeles Healthcare System, North Hills, California 91343*

[9]*Department of Medicine, University of California, Los Angeles, California 90024*

ABSTRACT

Secreted phosphoprotein 24 kD (spp24) is a bone matrix protein isolated during attempts to identify osteogenic proteins. It is not osteogenic but performs other important roles in the regulation of bone metabolism, at least in part, by binding to and affecting the activity of members of the BMP/TGF-β family of cytokines. Spp24 exists in a number of forms that preserve the N-terminus and are truncated at the C-terminus. The hypothesized cytokine binding domain is present within the cystatin domain which is preserved in all of the N-terminal products. In this report, we describe a C-terminal fragment that is distinct from the cystatin domain and which independently binds to BMP-2 and TGF-β. This fragment inhibited BMP-2 activity in an ectopic bone forming assay. A shorter C-terminal product did not inhibit BMP-2 activity but improved bone quality induced by BMP-2 and produced increased calcium deposition outside of bone. Spp24 has been used to develop several potential therapeutic proteins. These results provide more information on the function of spp24 and provide other materials that can be exploited for clinical interventions. J. Cell. Biochem. 116: 667–676, 2015.
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Secreted phosphoprotein 24 kD (spp24; UniProtKB accession number for bovine spp24: Q27967; UniProtKB accession number for human spp24: Q13103) is a bone matrix protein the function of which is only now being elucidated. While investigators working several decades ago isolated this protein, or fragments of this protein, in extracts of bone matrix proteins that were described

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*Correspondence to: Haijun Tian, MD, PhD, 415 Fengyang Rd, Department of Orthopaedic Surgery, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai, 200003, P.R. China.
E-mail: haijuntianmd@gmail.com

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as "osteogenic," the only function documented for the protein to date is to bind to and inhibit the actions of BMPs (bone morphogenic proteins) and TGF-β (transforming growth factor-β) [Brochmann et al., 2010; Tian et al., 2013]. Several lines of evidence strongly supported the hypothesis that the interaction between spp24 and BMP/TGF-β proteins was mediated by a region in the middle of the molecule (amino acids 110–128 of the 203 amino acid bovine protein) that bore some similarity to the TRH-1 (TGF-β Receptor II Homology-1 domain) region of fetuin [Tian et al., 2013]. In the physiological environment, spp24 is proteolytically truncated to four major size forms (spp24, spp18.1, spp16.0, and spp14.5). These forms maintain the N-terminus of the mature parental protein and are truncated with respect to the C-terminus. However, subsequent observations suggested that the interactions of spp24 and BMP/TGF-β proteins might be more complex. Full-length spp24 binds BMP-2 more avidly than do the smallest truncation products [Brochmann et al., 2010]. In addition, the shapes of the curves for surface plasmon resonance (SPR) kinetic analyses suggested binding kinetics that were not first order. In order to clarify the biological function of the protein and to define properties that can be exploited to engineer biotherapeutics, we have undertaken a series of studies on the binding properties and biological actions of the various N-terminal size forms and the corresponding released C-terminal products. In this report, we describe the results of studies on the properties of two C-terminal products, one that corresponds to the C-terminal fragment removed to form spp18.1 (C-terminal fragment complementary to bovine spp18.1; C-term c/t 18.1) and one that corresponds to the fragment removed to form spp14.5 (C-terminal fragment complementary to human spp14.5; C-term c/t 14.5). The various proteolytic fragments of spp24 are illustrated in Figure 1.

MATERIALS AND METHODS

MATERIALS

Human BMP-2, TGF-β1, and TGF-β2 used for binding studies were obtained from R&D Systems (Minneapolis, MN) whereas BMP-2 used for in vivo studies was obtained from Medtronic (Minneapolis, MN).

PRODUCTION OF PROTEINS AND PEPTIDES

Two C-terminal proteins were generated. The first corresponds to the fragment of the full-length protein that would remain after the production of spp18.1. This peptide was designated as C-terminal complementary to 18.1 (C-term c/t 18.1) and is comprised of amino acids 177–203 of the bovine molecule. The complete sequence of this peptide is (Acetyl-EMRRNFPLGNRRYSNPWPRARVNPGFE-amide) and it was synthesized by LifeTein (South Plainfield, NJ). The second protein corresponds to the fragment of the full-length protein that would remain after the production of spp14.5. This protein was designated as C-terminal complementary to 14.5 (C-term c/t 14.5). In the bovine protein this would correspond to amino acids 144–203. However, in the studies presented here, the human protein was used and this protein corresponds to amino acids 151 to 211 in the human protein. This protein was generated as a recombinant protein using pET20b as previously described [Murray et al., 2007]. As in the case of previously described recombinant proteins, this protein contains six histidine residues at the N-terminus which facilitate isolation by immobilized metal affinity chromatography. In distinction to previously described recombinant proteins, this protein also retains the N-terminal PelB leader sequence of the pET20b vector which increases the yield of the recombinant protein. The complete sequence of the recombinant protein is (MKYLLPTAAAGLLLLAAQPAMAADHHHHH-HFGDMLGSHKWRNNYLFGLISDESISEQFYDRSLGIMRRVLPPGNRRYP-NHRHRARINTDFE). The relationships of the various size forms of spp24 and the C-terminal fragments are shown in Figure 1. A control peptide with the same amino acid composition as C-term c/t 18.1 but with the amino acid order randomly scrambledwas also generated. The sequence of that peptide is (FENRAGRLVNGRRSNPRFMPRPWNEPY).

SURFACE PLASMON RESONANCE

Surface plasmon resonance analyses of protein interactions were performed on a Biacore T100 optical biosensor with CM5 sensor chips (G.E. Healthcare; Piscataway, NJ). 10 μg of BMP-2 were dissolved in 100 μl of 10 mM NaOAc pH 5.0 and dialyzed against the same buffer for 4 hr at 4°C using a mini-dialysis unit, MWCO 3.5 K (Pierce Biotechnology, Thermo Fisher Scientific; Rockford, IL). TGF-β1 and TGF-β2 were dissolved in 10 mM NaOAc pH 4.0 to a final concentration of 20 μg/ml. Spp24 C-terminal peptide C-term c/t 14.5 and C-term c/t

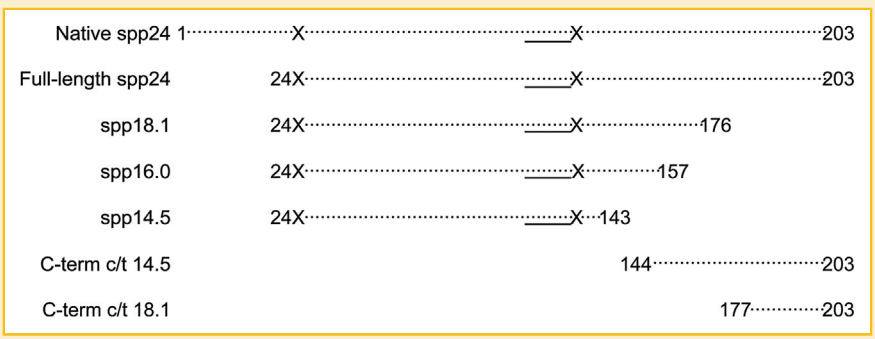

Fig. 1. Schematic representation of bovine spp24, spp24 proteolytic fragments, and the corresponding complementary C-terminal peptides. Amino acids 1 through 23 comprise a leader sequence which is removed upon secretion. The cystatin-like region (amino acids 24–130) is demarcated by two X symbols. The TRH-1 domain (amino acids 110–128) is indicated by underlining. The C-terminal peptides do not contain any of the cystatin-like region or the TRH-1 domain. The C-terminal protein C-term c/t 14.5 used in the experiments described in this paper is the human homolog which has slightly different amino acid numbering.

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18.1 were solubilized with running buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% Tween 20). The solutions were centrifuged at 16,000*g* for 2 min at RT. Optical absorbances at 280 nm of the spp24 C-terminal protein and peptide supernatants were measured and the final concentrations were calculated using the molar extinction coefficients of 10558 M^{-1} cm^{-1} and 1490 M^{-1} cm^{-1}, respectively. Serial dilutions of C-term c/t 14.5 were made in running buffer from 895 to 56 nM and dilutions for C-term c/t 18.1 and "scrambled" C-term c/t 18.1, were 111 to 6.94 μM. BMP-2, TGF-β1, and TGF-β2 were immobilized on a CM5 chip using standard amine coupling. Briefly, carboxy groups on the chip surfaces were activated with an injection of 0.05 M N-hydroxysuccinimide/0.2 M 1-Ethyl-3-[dimethyamino-propyl] carbodiimidehydrochloride (Pierce Biotechnology, Thermo Fisher Scientific; Rockford, IL) at a flow rate of 10 μl/min for 7 min. BMP, diluted in 10 mM NaOAc pH 5.0 to 30 μg/ml was pulsed over the surface at a flow rate of 1 μl/min until 4211 RU (resonance units) were achieved. A similar procedure was followed for the immobilization of TGF-β1 and TGF-β2 except that the sample buffer was 10 mM NaOAc pH 4.0 and the concentrations were 20 and 10 μg/ml, respectively. Final RU values for TGF-β1 and TGF-β2 were 5023 and 3888. Any remaining active esters were blocked by injecting 1 M ethylenediamine pH 8.5 (Pierce Biotechnology, Thermo Fisher Scientific; Rockford, IL) for 7 min. The chip surfaces were washed three times with 30 s pulses of 10 μM glycine pH 2.0 at a flow rate of 30 μl/min. A blank immobilization procedure was used on the reference surface. Analyses were conducted at 25°C using the running buffer defined above. Each concentration was repeated three times and each experiment was repeated at least twice. Raw data were processed by subtracting the responses in the reference flow cell and in buffer blanks (double referencing). For the kinetic analysis, data were fit to a 1:1 binding model using a global fit algorithm (Biacore T100 Evaluation Software). Some sensograms did not have sufficient curvature to calculate kinetic K_Ds thus, affinity K_Ds were calculated by plotting the analyte concentration versus the biosensor response (RU).

IN VIVO ASSAY FOR BONE FORMATION

All research involving animals was reviewed and approved by the VA Greater Los Angeles Healthcare System Institution Animal Care and Use Committee (IACUC) prior to the initiation of any experiments. The effects of the test proteins on BMP activity was assessed using the mouse hindquarter ectopic bone formation assay. The designated amounts of test material (usually 1 mg) were dissolved in PBS at a concentration of 1 mg/0.150 ml and placed onto 10 mm × 10 mm × 5 mm pieces of collagen sponge (Helistat; Integra LifeSciences, Plainsboro, NJ). The sponge fragments were dried and then lyophilized. If BMP-2 was added, this was done in a second application where 10 μg of rhBMP-2 was added, the sponge saturated with 0.1 ml of PBS, and the sponge dried and lyophilized again. Dried sponges were packed into number 5 gelatin capsules (Torpac; Fairfield, NJ) and sterilized by exposure to chloroform vapor for 4 hr. Capsules were subsequently implanted into the hindquarter muscles of 4–6 week old male Swiss-Webster mice (Taconic Farms; Germantown, NY). Animals were killed after 21 days and the hindquarter removed. The specimens were examined for the presence of calcification (usually, but not universally, indicative of the presence of calcified bone) radiographically using a Faxitron small part X-ray cabinet (Faxitron; Tucson, AZ) and the amount of deposited calcium quantified from implant areas that had been excised from the hindquarter by DEXA with a PIXImus2 instrument (Lunar; Madison, WI). Samples were then fixed in buffered formalin and transferred to 70% alcohol until computed tomography was undertaken.

MICRO-COMPUTERIZED TOMOGRAPHY (μCT)

Each excised implant mass was analyzed by high resolution micro-computed tomography (micro-CT) using a SkyScan 1172 scanner (SkyScan/Bruker; Kontich, Belgium) as previously described in detail [Johnson et al., 2011]. Virtual image slices were reconstructed using the cone-beam reconstruction software version 2.6 based on the Feldkamp algorithm (SkyScan/Bruker; Kontich, Belgium). Sample re-orientation and 2D visualization were performed using Data Viewer (SkyScan/Bruker; Kontich, Belgium). 3D visualization was performed using Dolphin Imaging version 11 (Dolphin Imaging & Management Solutions; Chatsworth, CA) which provided estimates of the total volume of the implant mass and "bone" (deposited calcium) volume within the mass, and the ratio (BV/TV) of these parameters.

HISTOLOGY

After CT analysis, the fixed specimens were decalcified by submersion in Cal-Ex (Thermo Fisher; Waltham, MA) for 48 hr and washed with running tap water. The specimens were carefully cut through the middle of the implant mass and then embedded in paraffin. Sections were cut in 5 μm and serial sections were deparaffinized in xylene, hydrated in graduated ethanols, and stained with hematoxylin and eosin (H&E) as well as Alcian Blue. Alcian Blue sections were stained with 1% Alcian Blue at pH 2.5 for 30 min, thoroughly rinsed with tap water and counterstained with nuclear Fast Red for 5 min.

IMMUNOHISTOCHEMISTRY

Immunohistochemical detection was performed using a standard protocol. In brief, serial sections were deparaffinized in xylene, hydrated in graduated ethanols, washed with PBS, blocked for endogenous peroxidase activity with 3% H_2O_2, and reacted with antibodies specific for osteocalcin (1:200; Santa Cruz Biotechnology, CA) or pSmad 1/5/8 (1:200; Cell Signaling, Danvers, MA) at 4°C overnight, placed in biotin conjugated species-specific secondary antibody (Vector Laboratories, Burlingame, CA), and stained using the ABC and AEC kits (Vector Laboratories). Slides were then counterstained with Hematoxylin (Sigma) and mounted with Faramount (Dako, Carpinteria, CA).

NUMERICAL ANALYSIS

Statistical analyses of numerical comparisons were conducted using InStat3.10 software (Graph Pad Software, Inc.; La Jolla, CA). Means of values for different treatments were compared by one-way ANOVA with subsequent Tukey–Kramer comparisons tests.

RESULTS

C-TERM c/t 14.5 HAS A HIGH AFFINITY FOR BMP-2/TGF-β PROTEINS

The results of surface plasmon resonance binding studies are shown in Table I. The affinity of C-term c/t 14.5 for BMP-2/TGF-β proteins

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TABLE I. Kinetic Analysis of the Interactions Between BMP-2, TGF-β1, TGF-β2, and Various Test Proteins and Peptides. K_D (M) Values are Shown in the Table Which Stands for "affinity." The Values for Full-Length spp24 are Ahown for Comparison and are Taken From Our Previous Paper [Brochmann et al., 2010]

	BMP-2	TGF-β1	TGF-β2
C-term c/t 18.1	9.72×10^{-5}	1.65×10^{-4}	3.64×10^{-4}
C-term c/t 14.5	1.77×10^{-7}	8.43×10^{-8}	2.62×10^{-7}
"scrambled" C-term c/t 18.1	5.04×10^{-4}	5.66×10^{-4}	5.68×10^{-4}
full-length spp24	1.77×10^{-8}	6.50×10^{-8}	1.94×10^{-7}

was much greater (about three orders of magnitude) than that of C-term c/t 18.1. The affinity of the C-term c/t 18.1 and scrambled C-term were not greatly different and were in the low and middle micromolar range.

C-TERM c/t 14.5 CAN EFFECTIVELY INHIBIT BMP-2 INDUCED BONE FORMATION

In the mouse hindquarter ectopic bone formation test for osteogenic activity, C-term c/t 14.5 did not demonstrate any such activity when implanted alone and effectively inhibited BMP-2 induced bone formation when the materials were co-implanted (Fig. 2A). This effect was confirmed by measurement of deposited calcium by DEXA which showed that the mineral content of implants containing BMP-2 + C-term c/t 14.5 was significantly lower than that of the BMP-2 only group (Fig. 2B). Histological examination found trabecular bone only in the BMP-2 only group and not in the sponge only, C-term c/t 14.5 only, and BMP-2 + C-term c/t 14.5 groups (Fig. 3). Positive Alcian Blue and osteocalcin staining were also seen in the BMP-2 only group indicating active bone formation and C-term c/t 14.5 effectively inhibited this effect, although slight Alcian Blue and osteocalcin staining could still be identified in association with fibrotic inflammation and metaplasia. Neither the sponge only group nor the C-term c/t 14.5 alone group showed any positive staining with either Alcian Blue or osteocalcin.

C-TERM c/t 14.5 INHIBITED ACTIVATION OF THE BMP-2 INDUCED SMAD PATHWAY

Immunohistochemical staining for phospho-Smad 1/5/8 was done for each group and positive staining, in the nuclei of some cells associated with trabecular bone, was seen only in the BMP-2 alone group indicating an inhibition of that pathway by the addition of C-term c/t 14.5 to BMP-2 (Fig. 4).

IN CONTRAST TO C-TERM c/t 14.5, ANOTHER C-TERMINAL FRAGMENT, C-TERM c/t 18.1, IMPROVED BONE QUALITY INDUCED BY BMP-2 AND ALSO INCREASE CALCIUM DEPOSITION WITHOUT TRABECULAR BONE FORMATION WHEN USED ALONE

On radiographic analysis, C-term c/t 18.1 produced a dramatically different effect from that of C-term c/t 14.5. Rather than inhibiting BMP-2 induced bone formation, it appeared to increase the amount of calcified bone induced by BMP-2 (Fig. 5). The amount of calcium deposited when C-term c/t 18.1 was co-implanted with BMP-2 was about half again the amount deposited in BMP-2 alone samples. Interestingly, C-term c/t 18.1 alone samples also contained about two-thirds as much calcium deposition as did the BMP-2 alone samples. Samples that contained the scrambled peptide, which was designed to control for non-specific charge effects, displayed only slightly more calcification than those in which only a sponge was implanted but significantly less calcification than those in which C-term c/t 18.1 was implanted.

Micro-CT images of these samples were acquired in order to evaluate the possible osteogenic effect of C-term c/t 18.1. The C-term c/t 18.1 alone group, however, induced a diffuse type of calcium deposition rather than trabecular bone (Fig. 6A and B). Because the calcium deposition induced by C-term c/t 18.1 was not bone per se, we then calculated the BV/TV value with a threshold which

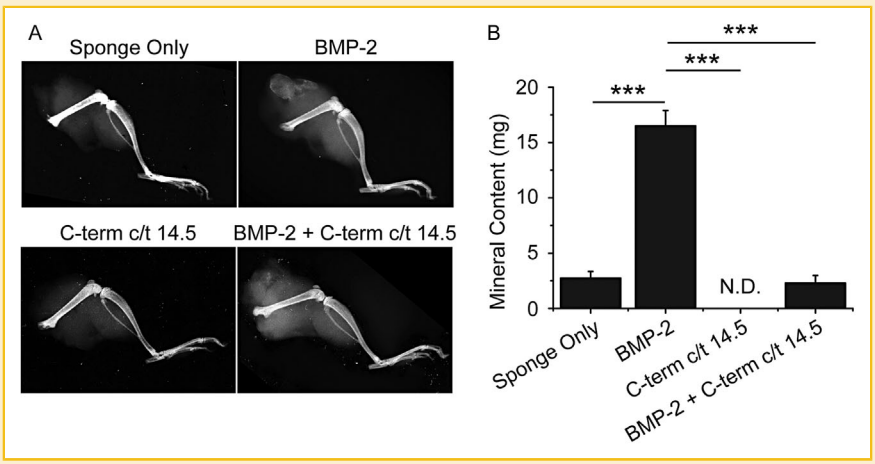

Fig. 2. Radiological examination of implants from the sponge only, BMP-2, C-term c/t 14.5 alone or BMP-2 + C-term c/t 14.5 groups. A: Radiographs of hindquarters of mice implanted with carrier sponge only, BMP-2, BMP-2 + C-term c/t 14.5, or C-term c/t 14.5 alone. Note the ectopic bone induced by BMP-2 and that C-term c/t 14.5 inhibited the osteogenic effect of BMP-2. B: Mineral content of implants of various test materials as assessed by DEXA. The mineral content induced by BMP-2 + C-term c/t 14.5 was significantly lower than that of BMP-2 only group. ***$P < 0.001$.

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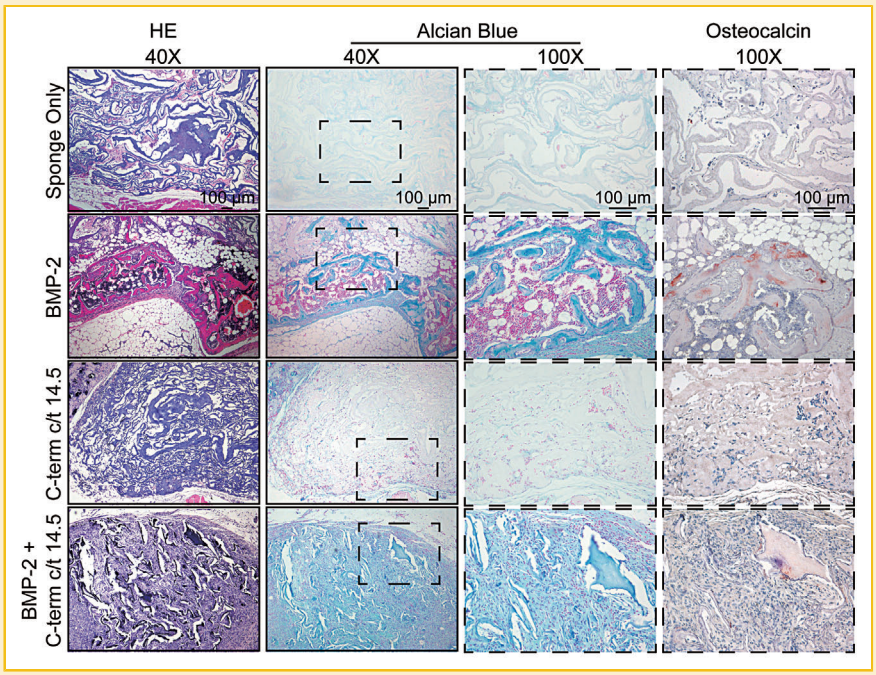

Fig. 3. Histological examination of implants from mice treated with sponge only, BMP-2, C-term c/t 14.5 alone, or BMP-2 + C-term c/t 14.5 stained with H&E, Alcian Blue, and for immunohistochemical detection of osteocalcin. Note that mature trabecular bone with bone marrow elements is seen in the BMP-2 group and that also positive Alcian Blue and osteocalcin staining, indicative of active bone formation, are present. Furthermore, in the group treated with C-term c/t 14.5 alone note that cells infiltrate the sponge with no positive staining of Alcian Blue or for osteocalcin. In the group treated with C-term c/t 14.5 + BMP-2, fibrotic inflammation and metaplasia is seen and is associated with slight Alcian Blue and osteocalcin staining.

eliminated the type of calcium deposition induced by C-term c/t 18.1 from the bone volume value. Compared to the BMP-2 + scrambled group, the BV/TV value is significantly higher for the BMP-2 + C-term c/t 18.1 group, confirming the finding of radiographic analyses that C-term c/t 18.1 improves the quality of bone induced by BMP-2 (Fig. 6C).

Histological examination of the same specimens is shown in . In the BMP-2 alone group, abundant new trabecular bone is present with osteoblastic lining cells, osteocytes, bone marrow, and remnant cartilage. These features of "full" bone were seen in all of the groups that received BMP-2 including the BMP-2 only group, the BMP-2 + scrambled peptide group, and the BMP-2 + C-term c/t 18.1 group. However, the BMP-2 and BMP-2 + scrambled peptide groups showed bone shelling with abundant internal adipocytes while more trabecular bone and fewer adipocytes were seen in the BMP-2 + C-term c/t 18.1 group. In the C-term c/t 18.1 alone group, remnants of the carrier sponge are apparent and there is a moderately fibrotic inflammatory response with foreign body multinucleated giant cells,

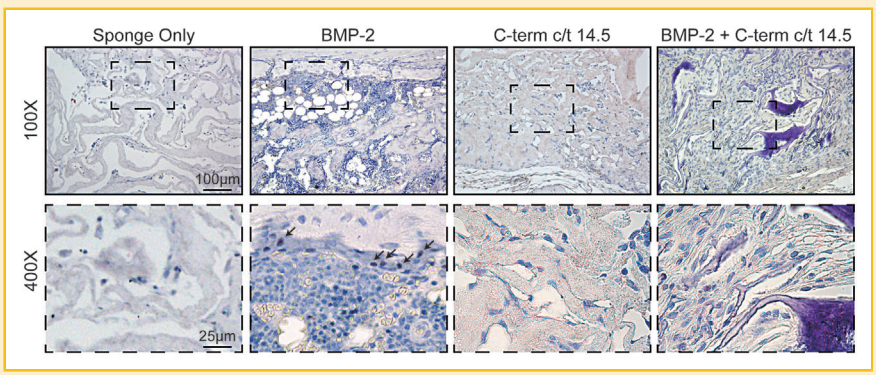

Fig. 4. Immunohistochemical staining for phospho-Smad 1/5/8 in specimens from the BMP-2, C-term c/t 14.5 alone, or BMP-2 + C-term c/t 14.5 treatment groups. Note that positive staining is only seen in the BMP-2 group and is localized to the nuclei of some cells around trabecular bone (see arrows).

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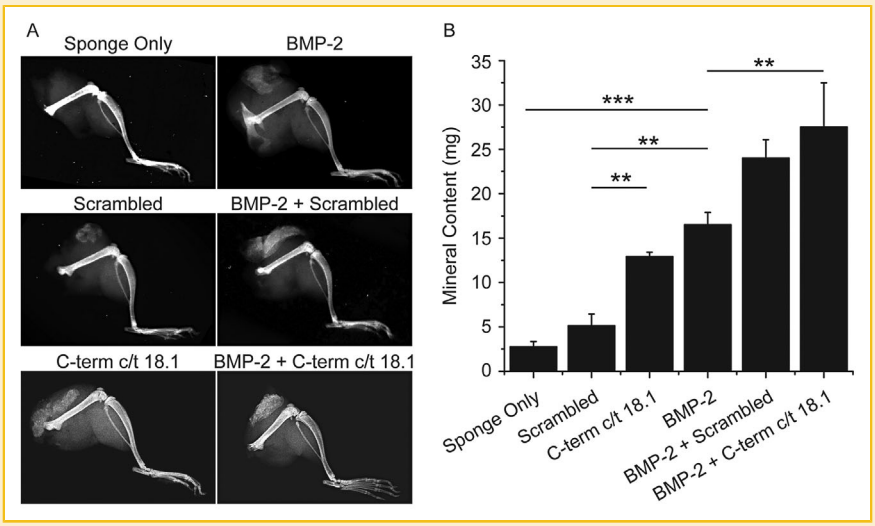

Fig. 5. Radiological examination of implants from the sponge only, BMP-2, scrambled peptide, BMP-2 + scrambled peptide, C-term c/t 18.1 alone and BMP-2 + C-term c/t 18.1 groups. A: Radiograph of hindquarters of mice implanted with sponge only, BMP-2, scrambled peptide, BMP-2 + scrambled peptide, C-term c/t 18.1 alone and BMP-2 + C-term c/t 18.1. Note especially the calcium deposition in the C-term 18.1 alone group; B: Mineral contents as assessed by DEXA. The mineral content of BMP-2 + C-term c/t 18.1 was significantly higher than BMP-2 only group ($P < 0.01$), whereas the mineral content of BMP-2 + scrambled peptide showed no significant difference with BMP-2 group ($P > 0.05$). $^{**}P < 0.01$, $^{***}P < 0.001$.

macrophages, and some lymphocytes present. The samples in which the implant contained the scrambled control peptide alone showed remnants of the collagen sponge only and in this regard was no different from controls in which only a sponge with no test protein was implanted. Furthermore, the BMP-2+ C-term c/t 18.1 group appeared to exhibit greater proteoglycan deposition, as manifested by Alcian Blue staining, than did either the BMP-2 alone group or the BMP-2 + scrambled peptide group, indicating more active bone formation. The BMP-2, BMP-2 + scrambled peptide, and the BMP-2 + C-term c/t 18.1 groups also showed strong osteocalcin staining, and the BMP-2 + C-term c/t 18.1 group again showed the strongest staining. Some degree of osteocalcin staining was also seen in the "bone-like structure" in the C-term c/t 18.1 group samples in contrast to the absence of such staining in the scrambled control peptide alone samples (Fig. 7).

C-TERM c/t 18.1 PROMOTED ACTIVATION OF THE SMAD PATHWAY

Immunohistochemical staining for phospho-Smad 1/5/8 was done for groups treated with sponge only, scrambled peptide only, C-term c/t 18.1 alone, BMP-2, BMP-2 + scrambled peptide, and BMP-2 + C-term

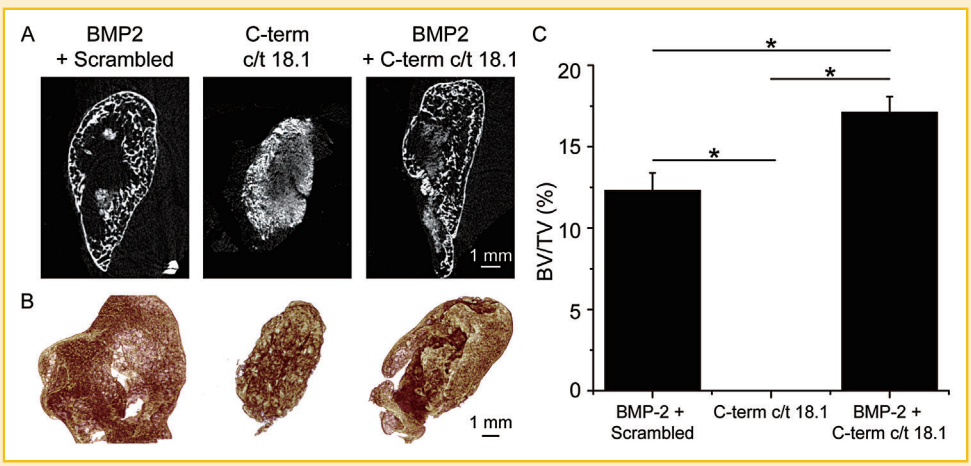

Figure 6. Micro-CT analysis of implants from BMP-2 + scrambled peptide, C-term c/t 18.1 alone and BMP-2 + C-term c/t 18.1 groups. A: μCT imaging and B: 3D reconstructions of specimens from BMP-2 + scrambled peptide and BMP-2 + C-term c/t 18.1 as well as C-term c/t 18.1 alone groups. Note the "egg shell" pattern of calcium in the BMP-2 + scrambled peptide whereas the BMP-2 + C-term c/t 18.1 group shows thicker bone "shell." C-term c/t 18.1 group shows a diffuse pattern of calcium deposition; C: Comparison of BV/TV for these three groups. $^{*}P < 0.05$.

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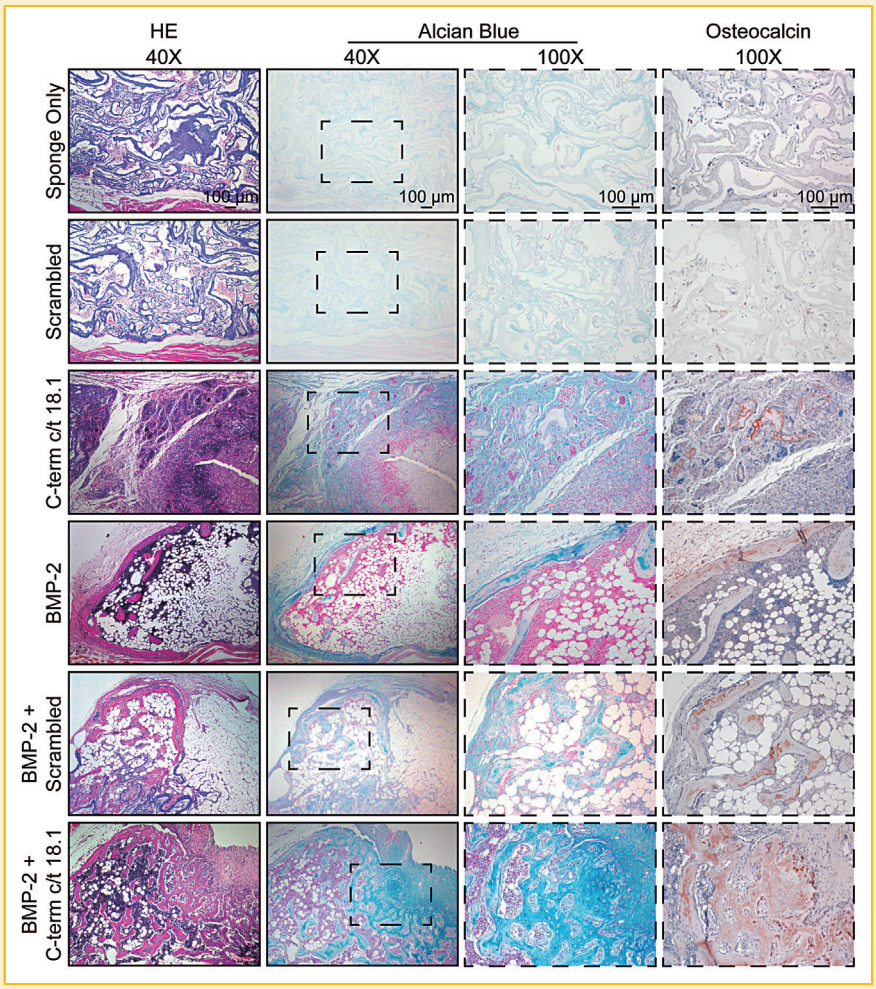

Fig. 7. Histological examination of implants from mice treated with sponge only, scrambled peptide only, C-term c/t 18.1 alone, BMP-2, BMP-2 + scrambled peptide, or BMP-2 + C-term c/t 18.1 stained with H&E, Alcian Blue, and for immunohistochemical detection of osteocalcin. Note that mature trabecular bone with bone marrow elements is seen in the BMP-2, BMP-2 + scrambled peptide, and BMP-2 + C-term c/t 18.1 groups, and that the BMP-2 and BMP-2 + scrambled peptide groups show bone shelling with abundant internal adipocytes. Note especially that more trabecular bone and fewer adipocytes are seen in the BMP-2 + C-term c/t 18.1 group and also that the structure that is similar to bone in the C-term c/t 18.1 group. Positive Alcian Blue staining is seen in the BMP-2, BMP-2 + scrambled peptide, and BMP-2 + C-term c/t 18.1 groups and the BMP-2 + C-term c/t 18.1 group shows the strongest staining, indicative of more active bone formation. Strong osteocalcin staining is also seen in the BMP-2, BMP-2 + scrambled peptide, BMP-2 + C-term c/t 18.1 groups, and the "bone-like structure" in C-term c/t 18.1 group.

c/t 18.1. Positive staining was seen in the nuclei of some cells around trabecular bone in the BMP-2 only and BMP-2 + scrambled peptide groups, and in accordance with the Alcian Blue staining and osteocalcin staining, more and stronger nuclear staining was seen in the BMP-2 + C-term c/t 18.1 group. No positive staining was found in the sponge only and scrambled peptide only groups. Interestingly, positive staining was also seen in the nuclei and cytoplasm of cells surrounding the "bone-like structure" in samples from the C-term c/t 18.1 group, indicating activation of the Smad signal pathway (Fig 8).

DISCUSSION

The activity of spp24 as a BMP binding protein was first brought to light by investigations into a protein fraction that was regarded to be the active component of Marshall Urist's "bone morphogenetic protein/non-collagenous protein" (BMP/NCP) [Behnam et al., 2005]. However, female transgenic mice that expressed this alleged "BMP" (full-length bovine spp24) were found to have diminished rather than increased bone mineral density (BMD) [Sintuu et al., 2008]. Sequence analysis of spp24 found a region in the cystatin domain of spp24 that was similar to the TRH-1 (TGF-β Receptor II Homology 1domain) within the cystatin domain of fetuin [Behnam et al., 2005]. The degree of similarity of these two domains to each other and to the TGF-β receptor is not great [Tian et al., 2013]. However, peptides generated from the TRH-1 domain sequences of both spp24 and fetuin bound to BMPs or TGF-β in a highly specific manner [Tian et al., 2013]. Subsequent studies demonstrated that the complete C-terminus of spp24 was required for greatest degree of inhibition of BMP-2 activity in the mouse hindquarter ectopic bone formation assay [Brochmann et al., 2010] which suggested that the C-terminal fragments that are produced when the truncated N-terminal

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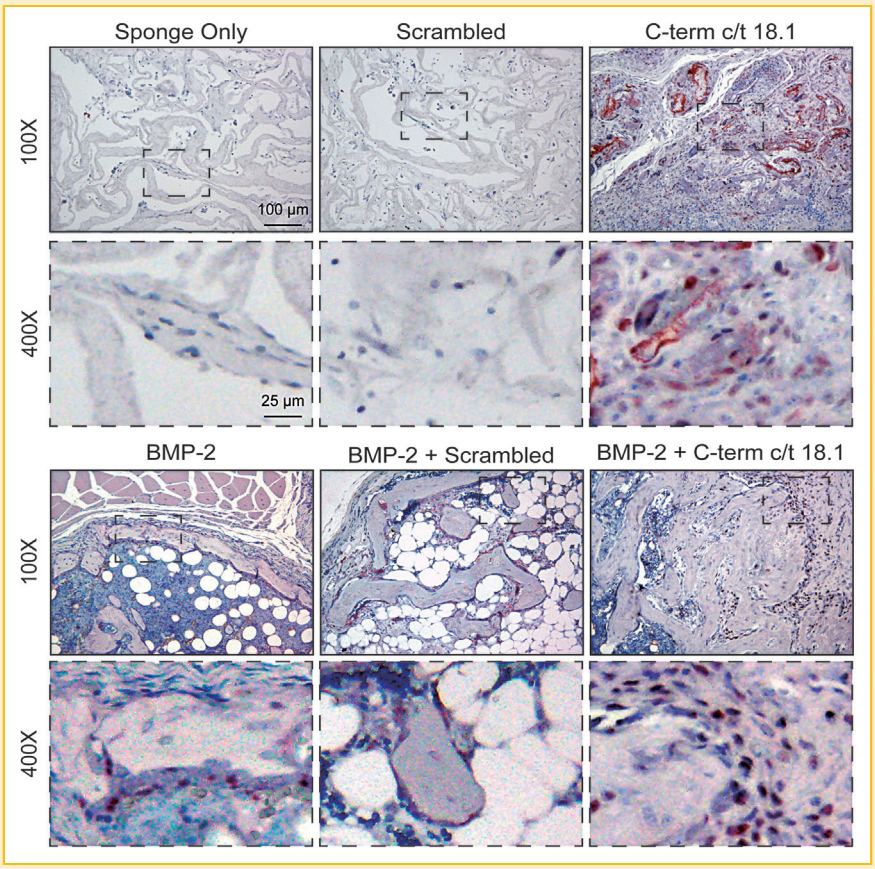

Fig. 8. Immunohistochemical staining for phospho-Smad 1/5/8 in implants from the groups treated with sponge only, scrambled peptide only, C-term c/t 18.1 alone, BMP-2, BMP-2 + scrambled peptide or BMP-2 + C-term c/t 18.1. No positive staining was found in the sponge only and scrambled peptide groups. Note that highly positive staining is seen in the nuclei and cytoplasm of cells surrounding the "bone-like structure" in C-term c/t 18.1 group. Positive staining is also seen in the nuclei of some cells around trabecular bone in the BMP-2 only and BMP-2 + scrambled peptide groups and more and stronger nuclear staining is seen in the BMP-2 + C-term c/t 18.1 group.

products are formed might have independent activities and that the C-terminus of the intact molecule cooperates in cytokine binding. In this report, data are presented that demonstrate the C-terminal fragment produced by the generation of spp14.5 (C-term c/t 14.5) binds to BMP-2 and TGF-β with similar affinity to full length spp24 whereas the C-terminal fragment produced by the cleavage that produces spp18.1 (C-term c/t 18.1) does not. This shorter fragment (C-term c/t 18.1) exhibited a very low affinity for BMP-2 and TGF-β (low μM range). Since full-length spp24 and the primary C-terminally truncated forms of spp24 bind BMPs, their biological effects in the skeletal environment have been assumed to relate to binding to and controlling the availability of BMPs and related cytokines. C-term c/t 14.5 bound to BMP-2 and inhibited the osteogenic effect of BMP-2 in a manner similar to that of full length spp24. In contrast, the shorter fragment, C-term c/t 18.1 did not bind BMP-2 with a high affinity and, therefore, is likely to have a totally different biological activity. When delivered together, C-term c/t 18.1 and BMP-2 showed a synergetic effect. Although the bone mass was not greatly increased, the quality of the new bone induced by BMP-2 was improved as reflected by an increased value of BV/TV in the micro-CT analyses. Histological evaluation showed that BMP-2 induced new bone shelling with abundant internal adipocytes whereas more trabecular bone and fewer adipocytes were seen in the samples from the BMP-2 + C-term c/t 18.1 treatment group. In addition, when used alone, C-term c/t 18.1 also manifested its own effect, forming "bone like structure." This material resembled bone radiologically but lacked trabecular structure when examined by micro-CT and by histological examination. The fact that osteocalcin staining could also be seen in the "bone-like structure" may indicate that the bone formation process may have been initiated but not completed.

Smad pathways are the main signal transduction pathways for the BMPs and Smads 1, 5, and 8 are key components of this pathway [Derynck and Zhang, 2003]. Activation of BMP receptors initiates phosphorylation of these downstream effector proteins, and we have previously reported that full length spp24 attenuates BMP-2-stimulated Smad 1/5 phosphorylation [Zhao et al., 2013]. In this study, we also tested the activation of phospho-Smad 1/5/8 to determine if the C-terminal fragments of spp24 affect the signal transduction pathways of BMP-2. Negative staining for phospho-Smad 1/5/8 revealed that C-term c/t 14.5 did inhibit activation of the downstream signal pathway of BMP-2 whereas more active staining

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was identified in the C-term c/t 18.1 + BMP-2 group than in the BMP-2 only group, demonstrating a synergetic effect of C-term c/t 18.1 with BMP-2. It remains unclear why the "bone-like structure" seen in the C-term c/t 18.1 only group showed highly positive staining yet did not form complete bone. Further study needs to be done to fully understand the exact mechanism of the biological effect of C-term c/t 18.1.

The exact functions of spp24 and the N-terminal and C-terminal fragments of spp24 in the biology of the bone environment remain incompletely defined but significant roles for them in regulating bone growth, remodeling, and repair is strongly suggested by studies reporting that the gene locus for spp24 (SPP2) is a significant determination of femoral neck BMD [Hsu et al., 2010]. The misidentification of spp24 as the active component of Urist's "BMP/NCP" can be attributed to co-purification. However, another investigator independently reported the N-terminal sequence of an "osteogenic" protein isolated from bovine bone as (FPVYDYSPARL-KEA) which compares very closely to the N-terminal sequence of what would later be called mature bovine spp24, the corresponding sequence of which is (FPVYDYDPASLKEA) [Sen et al., 1987]. One possible function for spp24-related proteins is to supply the "slow release" activity for BMPs that was first hypothesized by Wozney [Wozney et al., 1990]. A much more complex role for the spp24 proteins in directing the BMP/TGF-β economy of the bone matrix environment is suggested by data that showed different forms of spp24 had diverse effects in directing the periosteum to produce bone or cartilage in response to TGF-β2, a phenomenon that is likely related to effects on concentration and duration of exposure [Tian et al., 2013]. The effects of the C-terminal fragments on BMP-2 activity adds another level of complexity to the hypothesis that spp24-related proteins regulate the availability of BMPs and other related cytokines in the bone environment. Effects of spp24 proteins on cells in the bone environment that are related to properties other than growth factor binding are only beginning to be investigated.

The BMP binding properties of spp24 have been utilized in the design of several potential biotherapeutics. Materials can be engineered that utilize the same property, BMP binding, but within different contexts that provide for a net effect along a continuum from "slow release" (enhanced activity) to "sequestration" (inhibition of activity). Thus, a synthetic, cyclic 19 amino acid peptide based on the TRH-1 domain of bovine spp24, bone morphogenetic protein binding peptide (BBP) has been demonstrated to enhance bone healing in animal models of spinal fusion [Alanay et al., 2008; Taghavi et al., 2010] and long bone defect healing [Morishita et al., 2010]. The hypothesized mechanism of action for this material is "slow release" of BMP-2 or BMP-7 [Taghavi et al., 2010]. Initial studies have also indicated that spp24-derived materials can be used to reduce the inflammation associated with clinical applications of recombinant human BMP-2 and rhBMP-7 [Lee et al., 2011b]. On the other hand, full-length spp24 has been demonstrated to be effective in suppressing tumor growth in a model of skeletal tumors in which tumor growth is dependent upon BMP autocrine stimulation [Lee et al., 2011a]. How the unique properties of the C-terminal fragments of spp24 can be exploited in the design potential biotherapeutics remains to be determined. The longer C-term fragment (C-term c/t 14.5) did inhibit BMP activity. This material differs from N-terminal fragments that inhibit BMP activity in terms of charge and solubility and these properties may confer advantages in situations where suppression of tumor growth or inflammation is sought. It is recognized that large doses of BMP-2 usually induce significant amounts of bone "shelling" with associated adipocytes. This may compromise the quality of the bone formed and, therefore, the synergetic effect of the shorter fragment (C-term c/t 18.1) with BMP-2 might be utilized to improve the quality of BMP-2 induced bone in various clinical applications. The finding that C-term c/t 18.1 produced marked matrix calcium deposition needs to be studied further and it may find application in the enhancement of bone mineralization. In this regard, it is interesting to note that spp24 has been localized in matrix vesicles, structures critical for bone mineralization [Xiao et al., 2007].

Spp24 is a protein that is produced in the liver and transported to the bone matrix in a protective complex [Zhao et al., 2013]. Early studies suggested an "osteogenic" role for this protein but while this function has not been confirmed a number of other functions related to the growth factor binding properties of the parental protein and of proteolytic fragments have been suggested. The exploration for other biological properties for these proteins is an area of active research. Several potential biotherapeutics have been engineered from spp24 and the further definition of the properties of the various fragments increases the repertoire of materials available for testing in pre-clinical models.

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