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Spp24 Derivatives Stimulate a G_i-Protein Coupled Receptor-Erk1/2 Signaling Pathway and Modulate Gene Expressions in W-20-17 Cells

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ABSTRACT

Secreted phosphoprotein 24 kDa (Spp24) is an apatite- and BMP/TGF-β cytokine-binding phosphoprotein found in serum and many tissues, including bone. N-terminally intact degradation products ranging in size from 14 kDa to 23 kDa have been found in bone. The cleavage sites in Spp24 that produce these short forms have not been definitively identified, and the biological activities and mechanisms of action of Spp24 and its degradation products have not been fully elucidated. We found that the C-terminus of Spp24 is labile to proteolysis by furin, kallikrein, lactoferrin, and trypsin, indicating that both extracellular and intracellular proteolytic events could account for the generation of biologically-active Spp18, Spp16, and Spp14. We determined the effects of these truncation products on kinase-mediated signal transduction, gene expression, and osteoblastic differentiation in W-20–17 bone marrow stromal cells cultured in basal or pro-osteogenic media. After culturing for five days, all forms inhibited BMP-2-stimulated osteoblastic differentiation, assessed as induction of alkaline phosphatase activity, in basal, but not pro-osteogenic media. After 10 days, they also inhibited BMP-2-stimulated mineral deposition in pro-osteogenic media. Spp24 had no effect on Erk1/2 phosphorylation, but Spp18 stimulated short-term Erk1/2, MEK 1/2, and p38 phosphorylation. Pertussis toxin and a MEK1/2 inhibitor ablated Spp18-stimulated Erk 1/2 phosphorylation, indicating a role for G_i proteins and MEK1/2 in the Spp18-stimulated Erk1/2 phosphorylation cascade. Truncation products, but not full-length Spp24, stimulated RUNX2, ATF4, and CSF1 transcription. This suggests that Spp24 truncation products have effects on osteoblastic differentiation mediated by kinase pathways that are independent of exogenous BMP/TGF-β cytokines. J. Cell. Biochem. 116: 767–777, 2015.

Spp24 (secreted phosphoprotein 24 kDa, sometimes referred to as Spp2 in deference to the gene SPP2) is an apatite- and cytokine-binding bone matrix protein [Behnam et al., 2005; Zhou, 2007] that is produced primarily in the liver [Agarwal et al., 1995; Hu et al., 1995] and found exclusively in vertebrates [Bennett et al., 2004]. Spp24 was originally cloned from dwarf chickens [Agarwal et al., 1995] and bovine cortical bone [Hu et al., 1995]. It was later identified as the precursor of a "pro-osteogenic" 18.5 kDa protein that was isolated from demineralized bone powder during the search for bone morphogenetic proteins [Behnam et al., 2005]. Spp24 is also found in the periosteum [Hu et al., 1995], cartilage [Meng et al., 2005], calvaria [van den Bos et al., 2008], and kidneys [Agarwal et al., 1995; Hu et al., 1995]. Like all secretory proteins, Spp24 contains a short N-terminal signal peptide that is cleaved by signal peptidase [Hu et al., 1995]. Mature secretory or full-length (FL) Spp24 consists of three major domains including the N-terminal cystatin- (cysteine protease inhibitor) or cathelicidin-like domain, a variable polyserine domain that is heavily phosphorylated, and an arginine-rich carboxy (C)-terminal domain (Figs. 1 A and B) [Hu et al., 1995; Bennett et al., 2004]. In spite its similarity to cystatins [Hu et al., 1995; Bennett et al., 2004], Spp24 lacks the required protease inhibitory domain [Kordis and Turk, 2009] and does not inhibit cathepsins B and K [van den Bos et al., 2008]. Unfortunately, the concept that Spp24 is a cathepsin inhibitor that regulates some aspect of bone turnover [Hu et al., 1995; Bennett et al., 2004] has persisted in the databases.

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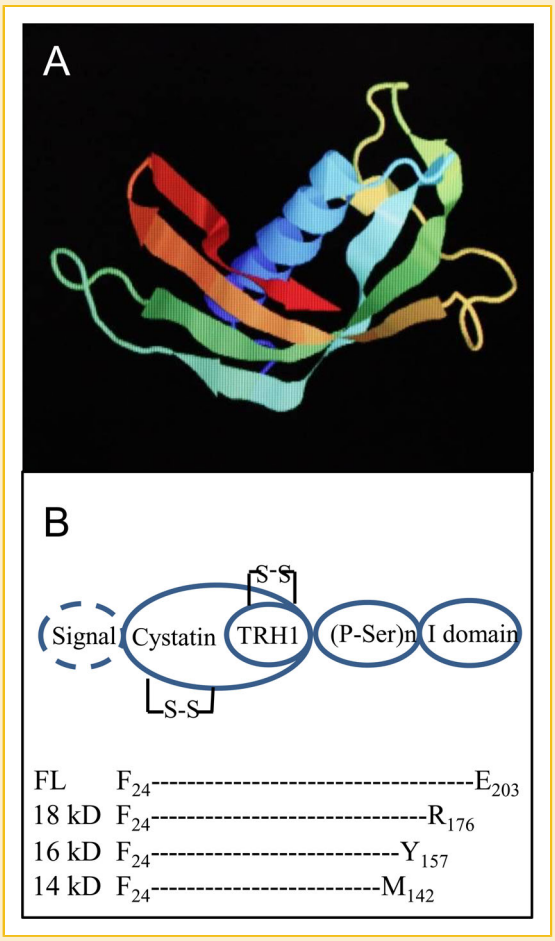

Fig. 1. Bovine Spp24 modeling image and structural organization. A: Phyre[2]-generated 3D image (http://www.sbg.bio.ic.ac.uk/phyre2) of FL Spp24. The rainbow color spectrum proceeds from N (red) to C (blue) termini. B: Schematic representation of the domain organization of Spp24. The signal peptide (dotted circle) is removed by signal peptidase. The cystatin-like domain contains two pairs of disulfide bonds and the TRH1 (TGF-β receptor II homology domain 1) subdomain. It is followed by a polyphosphoserine domain and an arginine-rich domain. The *E. coli* expressed bovine FL-Spp24, Spp18, Spp16, and Spp14 used in this study, representing amino acid residues F_{24} to E_{203}, F_{24} to R_{176}, F_{24} to Y_{157}, and F_{24} to F_{143}, respectively, did not contain phosphorylated serine residues.

The biological function of Spp24 was completely unknown until a key element of its similarity to fetuin, another member of the cystatin family of proteins, was recognized [Behnam et al., 2005; Brochmann et al., 2009]. Both Spp24 and fetuin contain a short transforming growth factor-β type II receptor homology-1 domain (TRH1) subdomain within their cystatin-like domains [Demetriou et al., 1996]. The TRH1 subdomain contains an 18- or 19-amino acid residue β-pleated sheet/turn/β-pleated sheet (BTB) motif that is cross-linked at its amino (N)- and carboxy (C)-termini by a disulfide bond [Demetriou et al., 1996]. Spp24 and cyclic BMP binding peptide (cBBP: the synthetic cyclic peptide corresponding to the TRH1 domain of Spp24) bind cytokines of the TGF-β/BMP superfamily with high affinity and specificity [Behnam et al., 2005; Taghavi et al., 2010; Tian et al., 2013]. Cyclic BBP enhances BMP-2 [Behnam et al., 2005; Sun et al., 2010] and -7-stimulated bone formation [Taghavi et al., 2010], spinal fusion [Alanay et al., 2008], and femoral bone defect healing [Morishita et al., 2010], while FL-Spp24 inhibits ectopic bone formation [Sintuu et al., 2008] and spinal fusion [Sintuu et al., 2011]. Cyclic BBP also reduces the inflammatory response to recombinant human (rh)BMP-2 and -7 in soft tissue, while FL-Spp24 inhibits the growth of BMP-dependent human A549 lung cancer cells [Lee et al., 2011a, 2011b]. Over-expression of FL-Spp24 under the control of the estrogen-regulated osteocalcin promoter resulted in reduced femoral and vertebral bone mineral density in female 3- and 8-month old transgenic mice [Sintuu et al., 2008]. These findings support a mechanism of action of Spp24 wherein it acts as a pseudoreceptor for BMP/TGF-β cytokines and competes with the cellular receptors for binding [Brochmann et al., 2009]. This is supported by recent observations that Spp24 attenuates downstream post-receptor BMP-2-stimulated Smad 1/5 phosphorylation, alkaline phosphatase induction, and mineralization in W-20–17 bone marrow stem cells [Zhao et al., 2013b].

FL-Spp24 over-expressed in *E. coli* is both highly insoluble and exquisitely susceptible to proteolysis of the C-terminal Arg-rich domain *in vitro* [Murray et al., 2007]. Furthermore, Spp24 truncation products ranging in size from 14 kDa to 23 kDa have been detected in bone extracts [Urist et al., 1987; Hu et al., 1995; Behnam et al., 2005; Sen et al., 1987]. In contrast, the native protein is secreted in soluble form by the liver in a stable protective complex with α_2-macroglobulins (Spp24∼A2M) and the protease inhibitor anti-thrombin III (serpin C1) [Zhao et al., 2013b]. Many of the C-terminal degradation products of Spp24, such as Spp18, Spp16, and Spp14 retain the TRH1 cytokine binding domain [Murray et al., 2007], but do not inhibit bone formation as much as FL-Spp24 [Brochmann et al., 2010; Sintuu et al., 2011]. Therefore, it is likely that the C-terminus of Spp24 contains additional domains that are required for its full inhibition of BMP/TGF-β cytokine activity [Tian et al., 2013]. This is currently an area of active research. For example, the sizes of the C-terminal 18 kD, 16 kD, and 14 kD degradation products (Fig. 1) were estimated by mass spectroscopy of spots excised from 2D gels [Murray et al., 2007], but the actual structures of the proteins have not been verified by isolating and sequencing them. In addition, the protease(s) that cleave Spp24 and the location of the precise cleavage sites are unknown. The presence of large numbers of Arg residues [Hu et al., 1995; Bennett et al., 2004] and multiple cleavage sites in the C-terminus [Murray et al., 2007] suggest that FL-Spp24 could be cleaved by the many different proteases, such as lactoferrin, trypsin, furin, or kallikrein, which favor Arg residues at the P1 position of the substrate [Rawlings et al., 2014]. Furthermore, the biological functions and mechanisms of action of the C-terminal truncation products of Spp24 have not been determined. Since at least two of the truncation products of Spp24 (e.g., 18.5 kD and 23 kD) appear to be pro-osteogenic in some manner [Sen et al., 1987; Urist et al., 1987], we hypothesized that these truncation products are biologically active in mineralized tissue and may mediate their effects by stimulating the kinase-dependent pathways that regulate gene transcription in (pro) osteoblastic cells. The mitogen-activated protein kinase/extracellular signal-regulated kinases 1 and 2 (MAPK/Erk1/2) signaling pathway is essential for the differentiation of mesenchymal cell types, such as the MC-3T3 cell line, towards the osteoblastic phenotype [Yu et al., 2010],

particularly in response to BMP-2 activation [Jun et al., 2010]. MAPK/Erk phosphorylation enhances the transcription of Runt related transcription factor 2 (RUNX2) and activating transcription factor 4 (ATF4), thus inducing osteoblastogenesis [Franceschi et al., 2009]. Therefore, an examination of the effects of C-terminal truncation products of Spp24 on both osteoblast differentiation and intracellular signal transduction by MAPK/Erk may provide valuable insights into the functions of these proteins.

To summarize, our major objective was to determine the biological effects of C-terminal truncation products of Spp24 on osteoblast differentiation and to determine their effects on the MAPK/Erk signal transduction pathway. Our secondary objective was to identify specific protease cleavage sites in the Arg-rich C-terminus of Spp24 and to determine how they might give rise to the many C-terminal truncation products of FL-Spp24. We successfully demonstrated that C-terminal truncation products of Spp24 affect osteoblastic differentiation and gene transcription independently of BMP/TGF-β cytokines and that the C-terminus contains a variety of protease-sensitive sites that could be cleaved to give rise to the N-terminal proteins identified in bone extracts.

MATERIALS AND METHODS

PROTEASE DIGESTIONS OF HUMAN SPP24 C-TERMINAL SPP151–211

The effects of specific proteases on the cleavage of the labile C-terminal domain of human Spp24 were determined under defined conditions in vitro. A fusion protein containing thioredoxin and the C-terminal amino acids 151–211 of human Spp24 was constructed using the pET32 vector (Novagene, Gibbstown, NJ) as described previously [Murray et al., 2007]. The fusion protein was overexpressed in BL21 (DE3) bacterial cells and purified from inclusion bodies by IMAC chromatography [Murray et al., 2007]. The purified protein was diluted in 10 mM Tris-HCl, pH 6.8 containing 1 mM $CaCl_2$ and 1 mM β-mercaptoethanol to a final concentration of 200 μg/ml. Aliquots of 200 μl were divided and mixed with 1 μl of vehicle or 1 μl of 2000 U/ml recombinant furin (P8077S: New England Biolabs, Ipswich, MA); 1 μl of 250 μg/ml kallikrein (16–16-110112: Athens Research, Athens, GA); 1 μl of 1 μg/ml lactoferrin (16–14-120103: Athens Research); or 1 μl of 250 μg/ml trypsin (Boehringer-Manheim, Petersburg, VA). The digestion reactions were conducted at 30 °C. Samples (50 μl) were removed at 1, 3, 5, and 18 h and boiled for 5 min prior to analysis. Samples were passed through Supel-Tip C18 pipette tips (Sigma-Aldrich, St. Louis, MO) to remove salt and buffer. Bound peptides were washed with 0.1% trifluoroacetic acid (TFA), followed by elution with 10 μl of 70% acetylnitrile/0.1%TFA. Aliquots (1 μl) of eluted samples were mixed with α-cyano-4-hydroxinnamic acid matrix and subjected to MALDI-ToF analysis as described previously [Zhao et al., 1997]. Peptide assignment was carried out by using FindPept at ExPASy (http://web.expasy.org/findpept/).

CELL CULTURE

The W-20–17 mouse bone marrow stromal cell line was obtained from the American Type Culture Collection (Manassas, VA) and cultured in basal culture media (BCM: Dulbecco's modified Eagle's medium [DMEM] plus 0.15% $NaHCO_3$, 4 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin) supplemented with 10% fetal calf serum (FCS) (BCM/10% FCS). Routine culture was conducted at 37 °C in a humid atmosphere of 95% air and 5% CO_2. Cells were sub-cultured at about 80% confluence and only low passage-number (< 10) cells were used for experiments. Cells were seeded in 6-well (80,000 cells/well) or 24-well (20,000 cells/well) plates (Laguna Scientific, Laguna Niguel, CA) for Western blotting and qPCR or alkaline phosphatase and mineral staining, respectively. After plating and overnight culture in BCM/10% FCS, cells were cultured for another day in BCM plus 1% FCS (BCM/1% FCS) to adapt them to low-serum conditions. Cells were treated with BCM supplemented with 0.2% FCS (BCM/0.2% FCS) and vehicle, 100 nM FL-Spp24 or one of its C-terminally truncated short forms (Spp18 kD, Spp16 kD, or Spp14 kD) [Murray et al., 2007], and incubated for up to 60 min at 37 °C. In some studies, various inhibitors were added to the medium two hours prior to the addition of the Spp24-related materials. All inhibitors were purchased from Calbiochem (La Jolla, CA). The inhibitors included 100 ng/ml pertussis toxin (PTX, catalyzes ADP-ribosylation of the α_i subunits of G proteins) or 25 μM PD98059 (inhibits MEK1/2 phosphorylation).

For studies of alkaline phosphatase, low-serum adapted cells were then cultured in BCM supplemented with 0.5% FCS (BCM/0.5% FCS) or pro-osteogenic basal culture medium supplemented with 0.5% FCS, 50 μg/ml ascorbic acid, 7 mg/ml β-glycerol phosphate, and 10 nM dexamethasone (OBCM/0.5% FCS) for an additional 5 days plus vehicle, 100 ng/ml rhBMP-2, 100 ng/ml of the recombinant bovine Spp derivatives, or 100 ng/ml rhBMP-2 plus 100 ng/ml Spp derivatives [Murray et al., 2007].

For studies of mineralization, low-serum adapted cells were cultured in αMEM/2% FCS or pro-osteogenic OαMEM/2% FCS for an additional 10 days in the presence of vehicle, 500 ng/ml rhBMP-2, 5000 ng/ml of each Spp derivative only, or rhBMP-2 plus 1250 to 5000 ng/ml of each Spp derivative. Cells were cultured under reduced serum conditions (2% FCS) to minimize the potentially confounding effects of the Spp24∼A2M complex found in normal serum [Zhao et al., 2013b].

SDS-PAGE AND WESTERN BLOTTING

Cultured cells were lysed directly with 0.3 ml Laemmli buffer [Laemmli, 1970]. All samples were heated for 5 min in a 100 °C water bath prior to loading (5–30 μl), separated by 4–20% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF), and probed as described previously [Zhao et al., 2013b]. Primary antibodies directed against phosphorylated antigens were from Cell Signaling (Danvers, MA). All other primary antibodies were from Santa Cruz Biotechnology (Dallas, TX). Each PVDF membrane was first probed with anti-phospho-molecule antibodies, stripped with Restore™ Western Blot Stripping Buffer (Pierce, Rockford, IL), and re-probed with anti-regular protein antibodies. Primary and secondary antibodies and anti-rabbit or mouse IgG-horseradish peroxidase were used at 1: 5000 dilutions.

QUANTITATIVE GENE EXPRESSION ANALYSIS BY QPCR

For gene expression assays, cells were cultured for a further 24 h after the addition of the Spp derivatives (100 nM FL-Spp24, Spp18,

TABLE I. Primers Used for Quantitative PCR Amplification of Mouse Genes

Gene symbol	GenBank ID #	Forward sequence (5' to 3')	Reverse sequence (5' to 3')
ATF4	NM_009716.2	ATAAAGGGCGGGTTTAGGGC	GTGAAGAGCGCCATGGCTTA
CSF1	NM_007778.4	AGGCTCCAGGAACTCTCCAA	TGGTCACCACATCTCGGCTA
RUNX2	NM_001146038	GCGGTGCAAACTTTCTCCAG	CTGCTTGCAGCCTTAAATATTCCTG
18S rRNA	NR_003286	CGGGTCATAAGCTTGCGTT	CCGCAGGTTCACCTACGG

Spp16, or Spp14) in BCM/10% FCS. Cells were harvested by direct lysis without washing using 0.3 ml Buffer RLT (RNeasy mini kit; Qiagen, Valencia, CA) for total RNA extraction according to the manufacturer's protocols. The expression of osteoblastic differentiation gene markers was quantitatively analyzed by qPCR following a separate reverse transcription reaction as described previously [Zhao et al., 2013a]. Primers for target genes were designed using Primer-Blast (NCBI, www.ncbi.nlm.nih.gov), and are listed in Table I. The relative expression of genes was calculated as fold changes using 18 S rRNA as a reference marker by the method of $2^{-\Delta\Delta Ct}$ (increases) or $-2^{\Delta\Delta Ct}$ (decreases) adopted from previously described methods [Livak and Schmittgen, 2001]. Untreated controls were set at 1 (2°) or -1 (-2°) and only fold changes of >2 or <-2 were considered as meaningful for the purpose of further pursuit and analysis.

ALKALINE PHOSPHATASE AND MINERALIZATION STAINING

At the end of the culture period in vehicle, BMP-2 (100 ng/ml), the Spp derivative indicated (100 ng/ml), or BMP-2 (100 ng/ml) plus the derivative (100 ng/ml), the cells in 24-well plates were fixed with 10% formaldehyde in phosphate-buffered saline for 15 min and stained for alkaline phosphatase using a commercial kit (Sigma–Aldrich, St. Louis, MO; #86R-1KT). Mineralization was detected in cells treated with vehicle or 500 ng/ml rhBMP-2, plus or minus 1250 to 5000 ng/ml Spp24 by staining with Alizarin red [Zhao et al., 2013b]. The color images were photographed and scanned for subsequent image analysis.

STATISTICAL ANALYSIS AND REPLICATION OF DATA

Numerical data are expressed as the mean ± S.E.M. (n = 3). Data were analyzed by Student's *t*-test using GraphPad Instat (version 3.0, San Diego, CA). All figures and numerical data are representative examples of experiments that were independently repeated multiple (>3) times with similar results.

RESULTS

IDENTIFICATION OF PROTEASE CLEAVAGE SITES IN THE C-TERMINUS OF SPP24

In order to identify candidate protease(s) involved in the cleavage of FL-Spp24 to smaller forms, such as Spp18, the C-terminal domain of human Spp24 (amino acid residues 151–211) was overexpressed as a fusion protein with thioredoxin in *E. coli*, purified to homogeneity, and treated in vitro with highly purified proteases. Based on the high number of Arg residues in the labile C-terminal domain and what is known about the identity of protease substrates, we chose to examine proteolysis by furin, kallikrein, lactoferrin, and trypsin (all of which have a preference for Arg at the P1 site) [Seidah, 2011; Constam, 2014]. The cleavage products were separated and identified by MALDI-ToF and computerized data reduction (Fig. 2A and Table II). One peptide contains a methionine (M_{154}) that is C-terminal to an aspartic acid (D_{153}) and a tryptophan (W_{160}) in addition to being surrounded by multiple arginines, both of which are oxidized or one of which is doubly oxidized (+32 Da), a common phenomenon in MALDI-ToF (Zhao et al., 1997; Guan et al., 2003). A loss of 64 Da represents a signature neutral ejection of a methane sulfenic acid (CH3-S-OH) from the oxidized methionine under collision induced dissociation which is the basis of MALDI-ToF [Guan et al., 2003]. Multiple C-terminal cleavage sites were identified, with the greatest lability observed within a few residues of the site that would give rise to Spp16 and Spp18 proteins (Fig. 2B). Cleavage products of the arginine-rich region (R_{187} to E_{211}) were not seen due possibly to the extensive digestion by all four arginine-specific proteases and the detection limit or high noises around m/z 1500 range in MALDI-ToF.

ALL C-TERMINALLY TRUNCATED FORMS OF SPP24 ATTENUATE BMP-2 INDUCED ALKALINE PHOSPHATASE ACTIVITY AND MINERALIZATION

Culturing W-20–17 cells in BCM/0.5% FCS or in OBCM/0.5% FCS for 5 days did not induce alkaline phosphatase activity, while addition of BMP-2 (100 ng/ml) did, as would be predicted (Fig. 3, Panel A) [Zhao et al., 2013b; Thies et al., 1992]. In the absence of BMP-2, addition of Spp24, -18, -16, or -14 had no effect on alkaline phosphatase activity in BCM/0.5% FCS (non-osteogenic media) or in OBCM/0.5% FCS (pro-osteogenic media) (Fig. 3, Panel A). However, FL-Spp24, Spp18, Spp16, and Spp14 inhibited BMP-induced stimulation of alkaline phosphatase in BCM/0.5% FCS (non-osteogenic media) (Fig. 3, Panel A), but the inhibitory effects of the Spp proteins on BMP-2-stimulated induction of alkaline phosphatase were not observed when the cells were cultured in OBCM/0.5% FCS (pro-osteogenic media) (Fig. 3, Panel A).

When W-20–17 cells were cultured in osteogenic (O) αMEM/2% FCS for 10 days in the absence of BMP, no mineralization was observed (well a1, Fig. 3B). Likewise, addition of 5,000 ng/ml of FL-Spp24, Spp18, Spp16, Spp14, or cBBP (wells a2 to a6) did not induce mineral deposition in the absence of BMP-2 (Fig. 3B). Very high concentrations of rhBMP-2 (500 ng/ml) induced mineralization in OαMEM/2% FCS (wells b1, c1, and d1), and addition of Spp24 derivatives (1250–5000 ng/ml, wells b2 to d6) ranging in size from FL- to cBBP along with BMP-2 inhibited BMP-2-stimulated mineralization (Fig. 3B).

SPP18, BUT NOT FL-SPP24, STIMULATES ERK1/2 PHOSPHORYLATION

The effects of both FL-Spp24 and Spp18 on osteoblastic differentiation pathways were investigated in W-20–17 cells in the

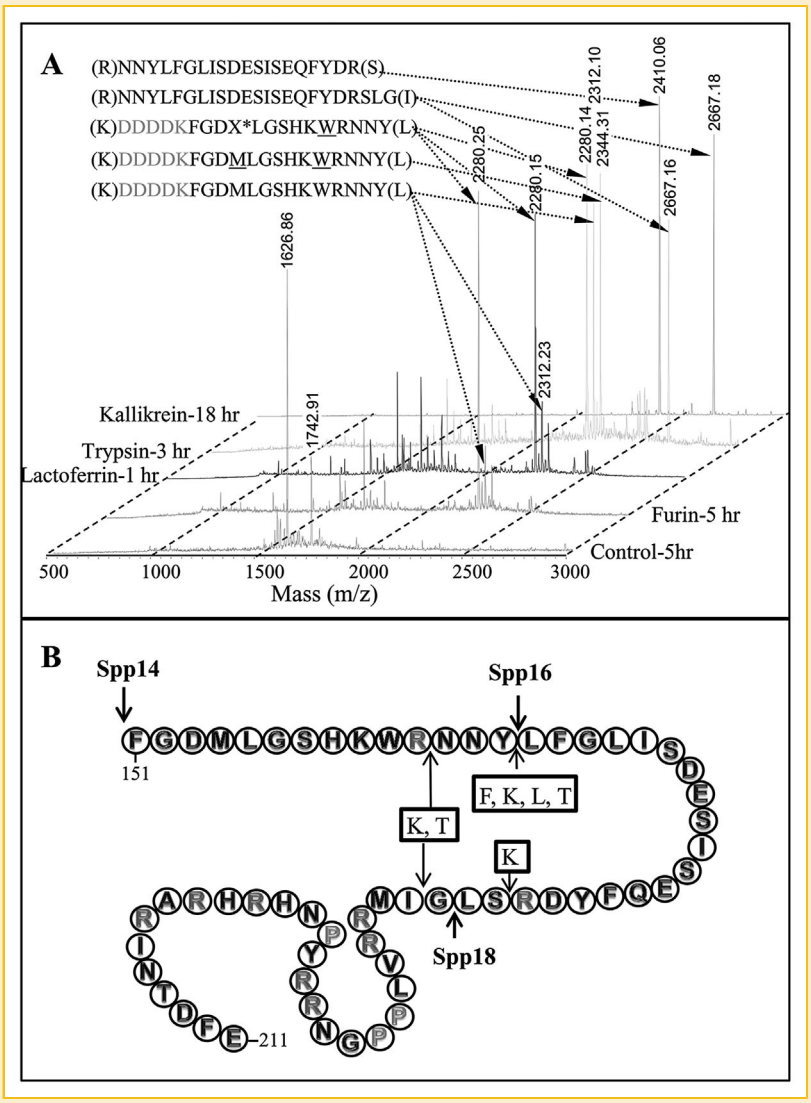

Fig. 2. Degradation products of the thioredoxin-human Spp24 C-terminal domain fusion protein. Mass spectrograms of peptides produced after the digestion of the C-terminal fragment of human Spp24 with highly purified proteases for 1–18 h, as indicated. Prominent peaks are assigned peptides as indicated by arrows. Amino acids shown in grey are part of a junction between thioredoxin and Spp151–211, and oxidized amino acids are underlined (Panel A). Panel B is a schematic illustration of cleavage sites in the human Spp24 C-terminus. Arrows indicate the sites cleaved by furin (F), kallikrein (K), lactoferrin (L), and/or trypsin (T). Conserved Arg residues are highlighted in red, and proline residues are shown in green. Bold arrows indicate known cleavage sites for Spp18, Spp16, and Spp14.

absence of any exogenous TGF-β/BMP cytokines. Phosphorylation of Erk1/2 was examined first, and the results are shown in Figure 4. Under basal conditions as well as with additional FL-Spp24 (12.5 to 100 nM), no effect on Erk1/2 phosphorylation was observed after an incubation of 30 min. In contrast, Spp18 stimulated phosphorylation of Erk1/2 in a concentration-dependent manner, from 25 nM to 100 nM (Fig. 4A). The time course for Spp18-stimulated induction of Erk1/2 phosphorylation was subsequently studied (Fig. 4B). At a concentration of 100 nM, Spp18 stimulated Erk1/2 phosphorylation, with maximum signal intensity observed around 15 min after addition. The total level of Erk2 was not affected by addition of Spp18 (Fig. 4B). Spp16 and Spp14 also caused Erk1/2 phosphorylation (data not shown). The film exposure time was longer in Figure 4A than in Figure 4B in order to track all proteins.

THE SPP18 SIGNAL TRANSDUCTION PATHWAY IN W-20–17 CELLS INVOLVES MEK1/2 PHOSPHORYLATION

The effects of Spp18 on the magnitude and time course of phosphorylation of other members of the MAPK cascade were also determined. The phosphorylation of MEK1/2 (a kinase immediately upstream from Erk1/2) was examined first. Basal levels of p-MEK1/2 were very low (Fig. 4B, Panel 2). Addition of 100 nM Spp18 stimulated MEK1/2 phosphorylation above basal levels. MEK1/2 phosphorylation peaked at about 10 min after treatment with

TABLE II. Cleavage of the C-Terminus of Human Spp24 by Various Proteases. Peptides Generated by Digestion of Thioredoxin Fused Spp24 C-Terminal Spp151–211 With Various Proteases and Analyzed by MALDI-TOF. Amino Acids in Grey Color Belong to the Thioredoxin Part. Amino Acids With an Underline Represent Oxidation Modification (+16 Da), and X* Denotes Neutral Loss of Methane Sulfenic Acid (CH3-S-OH, 64 Da) From Methionine Oxide by Collision-Induced Dissociation

	Mass			
Peptides	$[M+H]^+$ obs	$[M+H]^+$ cal	Intensity (A.U.)	Digestion (H)
Furin				
(K)DDDDKFGDX*LGSHKWRNNY(L)	2280.23	2280.00	0.95	5
(K)DDDDKFGDMLGSHKWRNNY(L)	2312.20	2312.26	0.37	5
Kallikrein				
(K)DDDDKFGDX*LGSHKWRNNY(L)	2280.25	2280.00	1.00	5
(K)DDDDKFGDMLGSHKWRNNY(L)	2312.28	2312.26	0.20	5
(R)NNYLFGLISDESISEQFYDR(S)	2410.06	2410.02	3.20	18
(R)NNYLFGLISDESISEQFYDRSLG(I)	2667.18	2667.26	3.18	18
Lactoferrin				
(K)DDDDKFGDX*LGSHKWRNNY(L)	2280.15	2280.00	1.70	1
(K)DDDDKFGDMLGSHKWRNNY(L)	2312.23	2312.26	0.45	1
Trypsin				
(K)DDDDKFGDX*LGSHKWRNNY(L)	2280.19	2280.00	2200	3
(K)DDDDKFGDMLGSHKWRNNY(L)	2312.10	2312.26	1900	3
(K)DDDDKFGDMLGSHKWRNNY(L)	2344.31	2344.99	2100	3
(R)NNYLFGLISDESISEQFYDRSLG(I)	2667.16	2667.26	1750	3

100 nM Spp18 (Fig. 4B, Panel 2), thus slightly preceding peak Erk1/2 phosphorylation (Fig. 4B, Panel 1).

SPP18 STIMULATES β-ARRESTIN 1 PHOSPHORYLATION, BUT OVER A LATER, LONGER TIME COURSE THAN MEK1/2 AND ERK1/2

β-arrestin 1 (βArr1) functions as a scaffold to facilitate clathrin-mediated endocytosis of G-protein-coupled receptor (GPCR) and to negatively regulate Erk signaling [Lin et al., 1999]. βArr1/S412 phosphorylation was not immediately enhanced in W-20–17 cells treated with 100 nM Spp18 (Fig. 4B, Panel 3). β-Arr1/S412 phosphorylation began to increase 10 min after treatment and gradually reached its plateau at around 60 min. Thus, peak β-Arr1/S412 phosphorylation occurred after peak phosphorylation of MEK1/2 and Erk1/2.

OTHER SIGNAL MOLECULES INVOLVED IN THE SPP18 ACTIVATION

In addition to the findings presented above, we found that treatment of W-20–17 cells with Spp18 was associated with short-term (5 to 10 min) increased phosphorylation of p38. The magnitude of the change was slight when compared to their basal levels (Fig. 4B, Panel 4).

SPP18 STIMULATES ERK1/2 PHOSPHORYLATION THROUGH A G_i-PROTEIN COUPLED RECEPTOR

In order to further elucidate the signal transduction pathways stimulated by Spp18, W-20–17 cells were pre-treated with two inhibitors for proteins or receptors upstream of Erk1/2: PD98059 which inhibits MEK1/2 phosphorylation and PTX that ADP-ribosylates G_i proteins and prevents them from interacting with GPCR in the plasma membrane. In this experiment, and in the absence of inhibitors, Spp18-stimulated Erk1/2 phosphorylation was observed 30 min after its addition (Fig. 5A, Lane 2). Spp18-stimulated Erk1/2 phosphorylation was abolished when PTX and PD98059 were added (Fig. 5A, Lanes 3 to 6). These results demonstrated that Spp18 mediates a GPCR-MEK1/2-Erk1/2 phosphorylation pathway.

BMP2 and TGFβ1 can also stimulate Erk1/2 phosphorylation as non-canonical pathways, in addition to its canonical pathway of stimulating Smad1/5 or Smad2, respectively (Fig. 5B). However, Spp18 stimulated Erk1/2 phosphorylation affects neither Smad1/5 nor Smad2 phosphorylation (Fig. 5B), suggesting that the putative GPCR-Erk1/2 phosphorylation pathway is independent of receptor (s) for BMP2 or TGFβ1.

TRUNCATED FORMS OF SPP24 BUT NOT FULL-LENGTH SPP24 STIMULATE GENE EXPRESSIONS OF TRANSCRIPTION FACTORS IMPORTANT IN OSTEOGENIC DIFFERENTIATION

In order to examine the effects of the truncated forms of Spp24 on osteoblastogenesis, W-20–17 cells were cultured in BCM/10% FCS after a single treatment with 100 nM FL-Spp24, Spp18, Spp16 or Spp14, and the expression of two transcription factors critical for osteoblast differentiation (RUNX2 and ATF4) and a cytokine that stimulates osteoclast recruitment and development (CSF1) were measured 24 h after the initial treatment. The results of these studies are presented in Figure 6. In each case, Spp18, Spp16, and Spp14 significantly increased the expression of these genes, but FL-Spp24 did not. Spp24 and its derivatives did not significantly alter the expression of other genes, including amphiregulin, osteoprotegerin, and chemokine (C-C) ligand 2 (data not shown).

DISCUSSION

To date, the protease that cleaves the C-terminus of Spp24 has not been identified, but all four of the proteases tested in this study (furin, kallikrein, lactoferrin, and trypsin) do cleave FL-Spp24 at C-

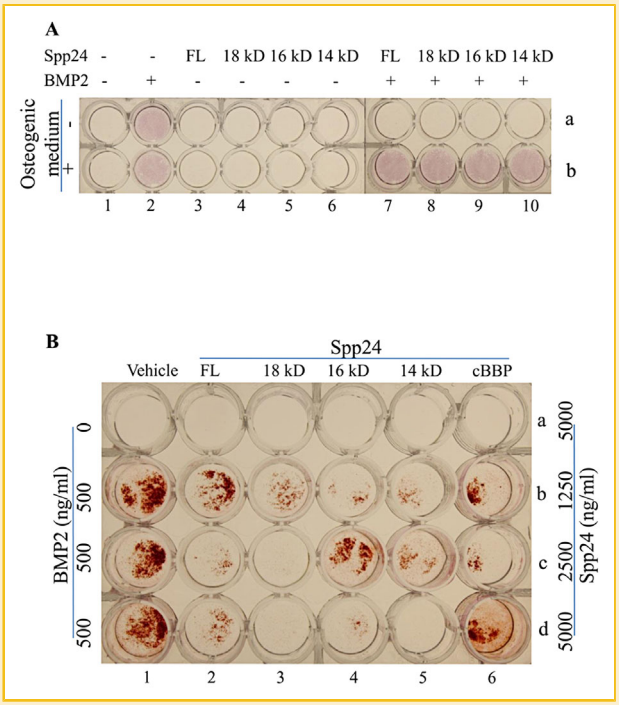

Fig. 3. Effects of Spp-derivatives on alkaline phosphatase activity and mineralization in W-20–17 cells. Panel A. Alkaline phosphatase active in W-20–17 cells treated with or without BMP-2, Spp24 or its C-terminal truncation products or cBBP (cyclic BMP binding peptide) (all at 100 ng/ml) and cultured in basal culture media (BCM)/0.5% fetal calf serum plus or minus pro-osteogenic agents (ascorbate, β-glycerol phosphate, and dexamethasone) for 5 days. Panel B. Mineralization (assessed as Alizarin red staining) in W-20–17 cells cultured for 10 days in osteogenic media (αMEM plus osteogenic agents)/2% fetal calf serum in vehicle treated (well a1), 500 ng/ml rhBMP2-treated (wells b1, c1, and d1) or 500 ng/ml rhBMP2 plus 1250 to 5000 ng/ml Spp24 or its derivatives (wells b2 to d6) treated conditions.

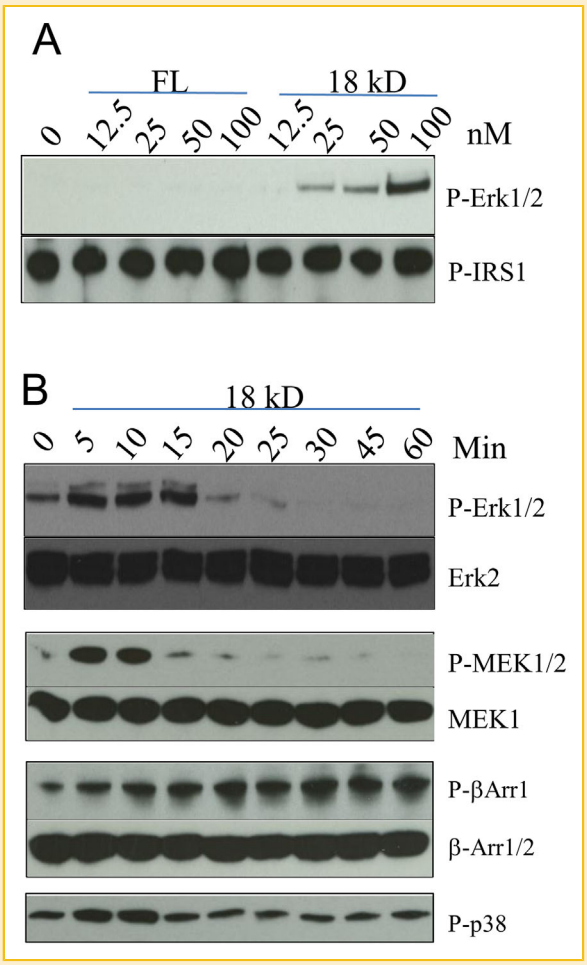

Fig. 4. Spp18, but not Spp24, stimulates phosphorylation of Erk1/2, MEK1/2, β-arrestin1, and p38 in W-20–17 cells. Panel A. Dose-response curve for ERK1/2 phosphorylation by up to 100 nM FL Spp24 or Spp18 after a 30 min incubation and lysis with Laemmli buffer. An insulin receptor substrate-1 phosphorylated at serine 612 (P-IRS1) serves as a loading control. Panel B: Time-course of phosphorylation of ERK1/2, MEK1/2, β-arrestin1, and p38 by 100 nM Spp18 treatments. Cells were lysed directly by Laemmli buffer, probed first with antibodies against phosphorylated signal molecules and then re-probed with antibodies against respective unphosphorylated signal molecules after stripping the PVDF membranes. Note that the film exposure time for P-Erk1/2 is short relative to Panel A.

terminal locations that would give rise to proteins of approximately 14 kD, 16 kD, and 18 kD. Potential cleavage by furin was of particular interest because furin or furin-like proprotein convertases are involved in activation of proteins important in bone metabolism, including pro-BMP1 and TGF-β precursors [Leighton and Kadler, 2003; Constam, 2014]. The C-terminal sequence of Spp24 is theoretically labile to proteolysis by so many enzymes [Rawlings et al., 2014] that a definitive identification of the protease that cleaves it may remain difficult. Given the affinity of Spp24 for apatite [Hu et al., 1995; Behnam et al., 2005; Zhou, 2007], it is possible that matrix turnover liberates bound Spp derivatives, which have a short-term local pro-osteogenic effect mediated by a GPCR-MEK1/2-Erk1/2 kinase pathway. This could account, in part, for the more rapid progression of osteogenesis, as opposed to chondrogenesis [Franceschi et al., 2009], which is observed in vivo in response to combined BMP-2 and Spp14 treatment [Tian et al., 2013].

Previous studies have clearly demonstrated that FL-Spp24 binds BMP/TGF-β cytokines with high affinity and specificity [Behnam et al., 2005; Taghavi et al., 2010; Tian et al., 2013] and attenuates BMP-2-stimulated signal transduction and osteoblastic differentiation, assessed as inhibition of post-receptor Smad 1/5 phosphorylation, alkaline phosphatase activity, and mineral deposition [Zhao et al., 2013b]. However, the biological functions and mechanisms of action of the C-terminal truncation products of Spp24, notably Spp18, 16, and 14, have not been elucidated in detail, despite their abundance in demineralized bone matrix (DBM) extracts [Sen et al., 1987; Urist et al., 1987; Behnam et al., 2005] and their cytokine-binding properties [Tian et al., 2013]. Furthermore, the observations that the C-terminus of Spp24 is required for full inhibition of BMP-stimulated ectopic bone formation [Sintuu et al., 2008] and spinal fusion [Sintuu et al., 2011] were difficult to

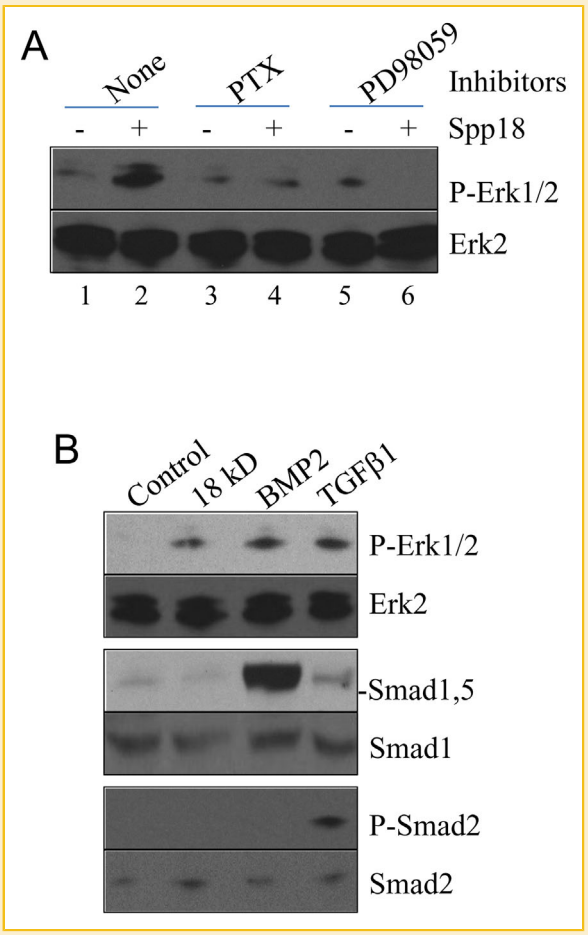

Fig. 5. Spp18 stimulates ERK1/2 phosphorylation via a GPCR but not receptors for BMP2 or TGFβ1. Panel A: W-20–17 cells were cultured in the absence (lanes 1–2) or presence of 100 ng/ml PTX (G_i protein inhibitor, lanes 3–4) or 25 μM PD98059 (MEK1/2 inhibitor, lanes 5–6) for 1.5 h, and continued in culture for 30 min in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of additional 100 nM Spp18. Cells were lysed in Laemmli buffer and phosphorylated and non-phosphorylated Erk1/2 and βArr1 levels were analyzed by Western blots; Panel B: W-20–17 cells were treated by control (equivalent volume of water solvent for agonists), 100 nM Spp18, 500 ng/ml BMP2, or 10 ng/ml TGFβ1 and incubated for 30 min prior to lysis as in Panel A. Phosphorylated and total Erk1/2, Smad1/5, and Smad2 for respective agonists were analyzed by Western blots.

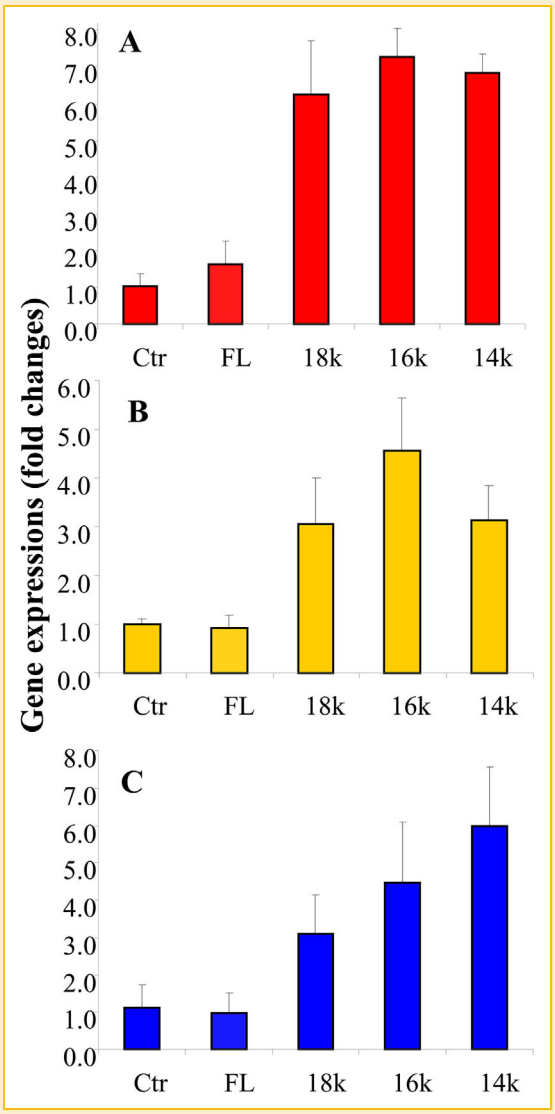

Fig. 6. Effects of treatment with FL-Spp24 and its truncated forms on gene expression in W-20–17 cells. Cells were cultured with the designated Spp proteins at 100 nM and assayed after 24 h. Gene expression levels are quantitated by qPCR and are shown as fold changes versus control for RUNX2 (A), ATF4 (B), and CSF1 (C). No statistical difference between FL-Spp24 and control were observed, while all Spp18, Spp16, and Spp14 showed statistical differences in comparison to control or FL-Spp24 ($P < 0.01$).

reconcile with the reports that 23 kD and 18.5 kD fragments of Spp24 are pro-osteogenic [Behnam et al., 2005; Sen et al., 1987; Urist et al., 1987] and that FL-Spp24 inhibits TGF-β2-stimulated bone formation in vivo, while Spp14 enhances it [Tian et al., 2013].

In the studies reported here, we tested the earlier hypothesis that proteolysis of Spp24 gives rise to bioactive proteins, just as proteolysis of other proteins with cystatin-like domains gives rise to antibiotic and vasoactive peptides such as Bac5 and bradykinin [Hu et al., 1995]. It should be noted, however, that cleavage of cystatin-like domains in other proteins usually gives rise to bioactive C-terminal peptides [Hu et al., 1995], while cleavage of Spp24 gives rise to stable, biologically-active N-terminal proteins and an unstable C-terminus that is rapidly degraded in vitro [Murray et al., 2007]. The possible functions of C-terminal fragments are also the subject of research. The hypothesis was tested in vitro in the W-20, clone 17, bone marrow stromal cell line, which is the standard cell line in which to evaluate the biological activity of recombinant BMP-2 [Thies et al., 1992]. BMP-2 induces ALP and osteocalcin expression in W-20–17 cells and sensitizes them to PTH [Thies et al., 1992]. However, low-dose BMP-2 (<100 ng/ml) does not stimulate mineralization of W-20–17 cells in media containing ascorbate and β-glycerophosphate [Thies et al., 1992]. While it was generally assumed that W-20–17 cells do not undergo mineralization [Bahamonde and Lyons, 2001], we have shown that high doses of

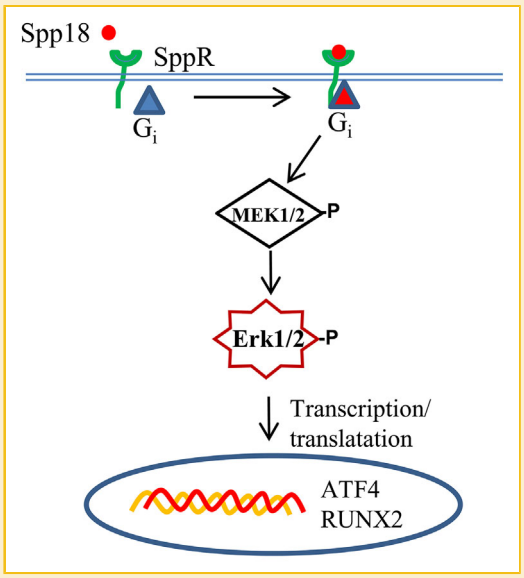

Fig. 7. Proposed model of cytokine-independent Spp18 signaling in W-20-17 cells. Spp18 is delivered to a GPCR and stimulates phosphorylation of MEK1/2 and ERK1/2. Short-term down-stream activation of ATF4, RUNX2 and CSF-1 transcription then occurs.

BMP-2 (500 ng/ml) stimulate mineral deposition in W-20–17 cultures [Zhao et al., 2013b]. When we examined the effects of FL-Spp24 and its C-terminal truncation products on osteoblastic differentiation of W-20–17 cells cultured under low serum conditions using the preferred basal control media and compared the results to those obtained using the same media supplemented with three pro-osteogenic additives (OBCM: basal control media plus dexamethasone, ascorbate, and β-glycerophosphate) after 5 days in culture, we observed that: (1) Spp24 and its derivatives have no independent effects on alkaline phosphatase (ALP) activity in the absence of BMP-2; (2) they inhibit BMP-stimulated ALP activity when cultured in the absence of the three pro-osteogenic agents; and (3) the inhibition of BMP-stimulated ALP activity by Spp24 and its derivatives is over-ridden upon addition of the pro-osteogenic additives dexamethasone, glycerophosphate, and ascorbate. As expected for negative and positive control cultures, ALP staining was low in the absence of BMP-2, even in osteogenic media (negative control) and increased following addition of BMP-2 (100 ng/ml) (positive control) [Zhao et al., 2013b Thies et al., 1992]. Similar, but less emphatic, results were observed when high-dose BMP was used to induce mineralization in osteogenic media. In the absence of very high levels (>500 ng/ml) of rhBMP-2, W-20–17 cells do not undergo mineralization, as previously reported [Thies et al., 1992]. Our present study demonstrates that the effects of Spp24 and its derivatives on BMP-stimulated osteogenesis in 5- to 10-day cultures vary depending upon the culture conditions and are most clearly observed in subconfluent cultures that are not driven to undergo osteoblastogenesis by addition of ascorbate, β-glycerol phosphate, and dexamethasone. Ascorbic acid induces osteoblastic gene expression in parallel with increased Erk phosphorylation and RUNX2 transcription, and acts synergistically with BMP2/7 [Ge et al., 2012]. Dexamethasone enhances the transcriptional activity of RUNX2 [Mikami et al., 2007]. Acting in concert, these three media additives are sufficient to overcome the inhibitory effects of Spp24 and its derivatives on BMP-2-stimulated alkaline phosphatase activity and may attenuate its effects on mineralization. Thus, we demonstrated that C-terminal truncation products of Spp24 have measurable short-term effects on Erk1/2 phosphorylation and RUNX2 transcription, as well as long-term BMP-2-stimulated osteoblastic differentiation.

Previous investigators have established that MAPK/Erk pathway plays a central role in the differentiation of the osteoblast [Ge et al., 2007; Franceschi et al., 2009]. Others have shown that phospho-Erk also co-localizes with RUNX2 in the nuclei of differentiating osteoblasts, with a portion of the nuclear phospho-Erk directly binding to the promoter regions of osteoblast-related genes [Mikami et al., 2007]. In this report, we demonstrate for the first time that a C-terminally truncated form of Spp24, but not FL-Spp24, contributes to the regulation of osteoblast differentiation and gene expression independently of exogenous BMP/TGF-β cytokines by an MAPK/Erk-dependent pathway mechanism. The physical and biological properties of Spp24 and Spp18 (e.g., solubility, binding affinity for BMP-2 and TGF-β cytokines, and effects on bone formation) can differ significantly [Brochmann et al., 2010; Taghavi et al., 2010; Sintuu et al., 2011]. For example, Spp24 inhibits spinal fusion in rodents, while Spp18 does not [Sintuu et al., 2011], and the C-terminus of Spp24 is required for full inhibition of BMP-2-stimulated ectopic bone formation [Sintuu et al., 2008]. Furthermore, an 18.5 kD C-terminal degradation product of Spp24 has been reported to have osteogenic effects [Urist et al., 1987]. The differences in the effects of Spp24 and Spp18 on Erk phosphorylation support the hypothesis that cleavage of Spp24 at a labile site C-terminal to the cystatin domain may generate biologically-active peptides [Hu et al., 1995].

These observations contribute to our understanding of the mechanisms by which Spp24 degradation products may mediate their effects. Under basal conditions, very low levels of Erk1/2 phosphorylation are observed, relative to the total amount of Erk1/2 detected, suggesting that the effects of Spp18 are specific to the protein, not generalized nonspecific effects due to media composition or culture conditions. We propose, based on our observations related to Spp18-stimulated phosphorylation, that Spp18 binds to a GPCR, which stimulates a signal cascade pathway involving phosphorylation of down-stream kinases, including MEK1/2, Erk1/2, and p38. The net result of this signal transduction pathway is enhanced short-term transcription of proteins, such as RUNX2 and ATF4, important in osteoblast development, as well as the coupling factor, CSF1. It appears that shorter C-terminal truncation products (Spp16 and Spp14) also up-regulate the transcription of these proteins and may account for previous observations that degradation products of Spp24 are present in pro-osteogenic preparations isolated from bone matrix [Behnam et al., 2005; Sen et al., 1987; Urist et al., 1987]. A simplified model of the proposed pathway is shown in Figure 7. We also observed that C-terminal Spp degradation products have no effect on SMAD phosphorylation, which further supports our hypothesis in that the effects on the

MAPK pathway are specific, rather than a general, nonspecific effect on BMP pathways. Degradation of phosphorylated intermediates or Spp proteins by the ubiquitin-proteasome pathway may account for the short period of action observed for Spp24 derivatives.

Although the significance of βArr1 phosphorylation in the Spp18 mediated GPCR-Erk1/2 signaling is not fully understood, it may be involved in the processing and delivery of Spp18 from its native precursor in the form of Spp24∼A2M complex [Zhao et al., 2013b]. Insulin-like growth factor-1 (IGF1) receptor endocytosis is facilitated by βArr1, which is necessary for the IGF1-stimulated GPCR-MAPK pathway [Dalle et al., 2002]. A receptor (low density lipoprotein receptor-related protein 1 or LRP1)-mediated endocytosis of the complex could facilitate the delivery, processing, and exposure of Spp18 to its GPCR for signaling, and βArr1 may play an important role in the process. It is not surprising that both Spp24 and IGF1 shared a similar GPCR-MAPK signaling pathway: they are "twins" in at least two aspects: secretion of both IGF1 and Spp24 from the liver are regulated by the pituitary-secreted growth hormone [Agarwal et al., 1995], and both function as important molecules in bone metabolism [Sintuu et al., 2008, 2011; Sheng et al., 2014]. However, the Spp18-mediated GPCR-Er1/2 pathway does not involve phosphorylation of the IGF1 receptor (data not shown).

Genetic studies have indicated a critical role for Spp24, which is to say the SPP2 gene locus, in bone mineral density and quality [Wilson et al., 2004; Hsu et al., 2010]. These studies did not distinguish a positive correlation from a negative correlation in terms of Spp24 activity. It remains unclear whether increased Spp24 activity results in increased or decreased bone quality or density. In this regard, the observation that FL-Spp24 expressed under control of the osteocalcin promoter produced female transgenic animals with reduced bone mineral density is relevant [Sintuu et al., 2008]. Spp24 could affect bone quality by a number of mechanisms and may, in fact, have more than one activity. The hypothesized control, through specific binding, of the relative availability of TGF-βs and BMPs in the bone environment remains feasible. The studies described in this report provide an entirely different hypothesis according to which Spp24 is the precursor of growth factor agonists that are anabolic with respect to bone. In any case, the elucidation of the natural function(s) of Spp24 and its products will improve our understanding of bone development, growth, turnover, and repair and will also allow for the rational development of more potential therapeutics based on this important but poorly understood protein.

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