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# Development and optimization of a cell-based assay for the selection of synthetic compounds that potentiate bone morphogenetic protein-2 activity<sup>†‡</sup>

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The requirement of large amounts of the recombinant human bone morphogenetic protein-2 (BMP-2) produces a huge translational barrier for its routine clinical use due to high cost. This leads to an urgent need to develop alternative methods to lower costs and/or increase efficacies for using BMP-2. In this study, we describe the development and optimization of a cell-based assay that is sensitive, reproducible, and reliable in identifying reagents that potentiate the effects of BMP-2 in inducing transdifferentiation of C2C12 myoblasts into the osteoblastic phenotype. The assay is based on a BMP-responsive Smad1-driven luciferase reporter gene. LIM mineralization protein-1 (LMP-1) is a novel intracellular LIM domain protein that has been shown by our group to enhance cellular responsiveness to BMP-2. Our previous report elucidated that the binding of LMP-1 with the WW2 domain in Smad ubiquitin regulatory factor-1 (Smurf1) rescues the osteogenic Smads from degradation. Here, using the optimized cell-based assay, we first evaluated the activity of the recombinantly prepared proteins, LMP-1, and its mutant (LMP-1ΔSmurf1) that lacks the Smurf1-WW2 domain-binding motif. Both the wild type and the mutant proteins were engineered to contain an 11-amino acid HIV-TAT protein derived membrane transduction domain to aid the cellular delivery of recombinant proteins. The cell-based reporter assay confirmed that LMP-1 potentiates the BMP-induced stimulation of C2C12 cells towards the osteoblastic phenotype. The potentiating effect of LMP-1 was significantly reduced when a specific-motif known to interact with Smurf1 was mutated. We validated the results obtained in the reporter assay by also monitoring the expression of mRNA for osteocalcin and alkaline phosphatase (ALP) which is widely accepted osteoblast differentiation marker genes. Finally, we provide further confirmation of our results by measuring the activity of alkaline phosphatase in support of the accuracy and reliability of our cell-based assay. Direct delivery of synthesized protein can be limited by high cost, instability or inadequate post-translational modifications. Thus, there would be a clear benefit for a low cost, cell penetrable chemical compound. We successfully used our gene expression-based assay to choose an active compound from a select group of compounds that were identified by computational screenings as the most likely candidates for mimicking the function of LMP-1. Among them, we selected SVAK-3, a compound that showed a dose-dependent potentiation of BMP-2 activity in inducing osteoblastic differentiation of C2C12 cells. We show that either the full length LMP-1 protein or its potential mimetic compound consistently exhibit similar potentiation of BMP-2 activity even when multiple markers of the osteoblastic phenotype were parallelly monitored. Published in 2009 by John Wiley & Sons, Ltd.

KEY WORDS — BMP-2; LMP-1; Smad; Smurf1; potentiation

ABBREVIATIONS — ALP, alkaline phosphatase; BMP, bone morphogenetic protein; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; LMP, LIM, mineralization protein; RT-PCR, Reverse transcriptase-polymerase chain reaction; RUL, Relative Units of Luciferase; Smurf1, Smad Ubiquitin Regulatory Factor-1

## INTRODUCTION

Bone morphogenetic proteins (BMPs) belong to the TGF $\beta$  superfamily and are capable of initiating the osteoblast differentiation cascade and inducing ectopic bone formation.<sup>1–6</sup>

BMP-2 has been studied most extensively *in vitro* and *in vivo*. Recombinant human BMP-2 was approved by the FDA in 2002 and is being used clinically as a bone graft substitute to achieve solid bony fusion and reduce the morbidity of iliac bone harvest in patients who require spine fusion surgery due to spinal instability.<sup>7</sup> However, the requirement for a 15 000-fold higher concentration in humans (1.5 mg ml<sup>-1</sup>) than in cell culture (100 ng ml<sup>-1</sup>) creates a huge translational barrier for routine clinical use due to high cost.<sup>8–12</sup> The traditional biochemical screening approaches involving the use of *in vitro* binding assays are not a true

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representation of interactions that occur in living cells. In this study, we describe a new gene expression-based and normal cell monitoring approach that selects reagents based on their real effects on cell physiology.

LIM mineralization protein-1 (LMP-1) is a novel intracellular LIM domain protein identified by our group several years ago.<sup>13,14</sup> LMP-1 can enhance BMP-2 activity by increasing the cellular responsiveness to BMP-2. We recently described the interaction between LMP-1 and Smad Ubiquitin Regulatory Factor-1 (Smurf1) in human mesenchymal stem cells.<sup>15</sup> The ubiquitin-mediated proteasomal pathway is the major intracellular mechanism for degradation of many short-lived regulatory proteins.<sup>16</sup> Smurf1 is a member of the Hect family of E3 ligases and has been reported to interact with Smad1, Smad5, Smad6, Smad7, Runx2, Tumor Growth Factor- $\beta$ 1 (TGF $\beta$ 1), and BMP receptors.<sup>17–21</sup> These interactions result in ubiquitination of the targeted protein followed by subsequent degradation by proteasomes. Our previous report suggested that the interaction of Smurf1 with LMP-1 prevents the ubiquitination of Smad1 and 5 which are the key intracellular messengers in the BMP signaling pathway. Further, we reported that the interaction of LMP-1 or Smads with Smurf1 was based on the presence of a unique motif in LMP-1 that binds the WW2 domain in Smurf1.<sup>22,23</sup> The ability of LMP-1 to block Smurf1 from binding to Smad1/5 results in protection of Smads from degradation which leads to an increase in the cellular responsiveness to BMP-2.

BMP signaling involves a complex interaction of regulatory proteins. Upon binding of BMP ligand to its specific cell surface receptor, receptor-associated intracellular signaling proteins Smads1/5 are phosphorylated. The activated Smads1/5 associate with a common component of both the BMP and TGF $\beta$  signaling pathways, Smad4. The oligomerized Smad complex enters the nucleus to induce BMP-responsive genes such as alkaline phosphatase and osteocalcin in concert with other transcription factors. LMP-1 specifically interacts with Smurf1 and disrupts association of Smad1/5 with Smurf1 resulting in decreased ubiquitination and decreased proteasomal degradation of Smad1/5 leading to increased cellular responsiveness to BMP-2. (Figure 1).

Our earlier published data<sup>15,22</sup> demonstrated that: (1) LMP-1 and Smurf1 exhibit binding affinity for each other both in cell extracts and in slot-blot binding assays performed with purified recombinant proteins; (2) LMP-1 successfully blocks the binding of osteogenic Smads1/5 to Smurf1; and (3) LMP-1 inhibits ubiquitination of Smad1/5 by Smurf1 in a concentration dependent manner. Physical binding of target molecules to Smurf1 is considered a requirement for the transfer of ubiquitin moieties to one or more lysine residues in the target protein thus targeting them for degradation. Our overall goal is to translate our knowledge of the LMP-1 and Smurf1 interaction interface into therapeutic applications for clinical bone-induction. Compounds that physically block osteogenic Smads from interacting with Smurf1 will be good candidates for development into drugs to promote BMP-2 activity.

## LMP-1 Rescues Smads from Smurf1-Mediated Proteasomal Degradation

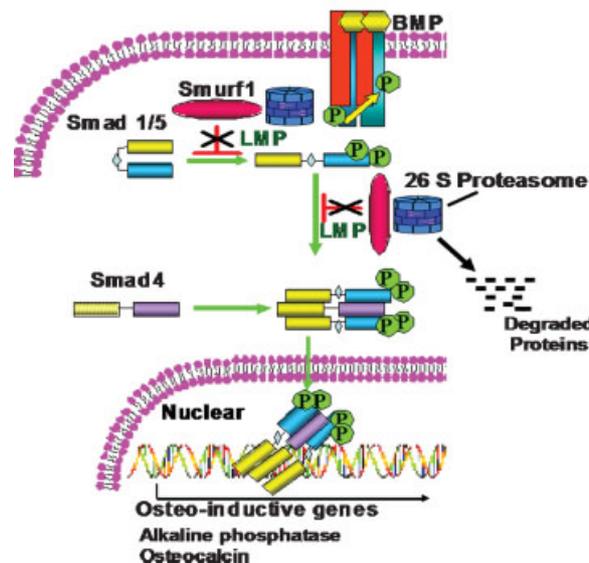


Figure 1. LMP-1 regulates cellular responsiveness to BMP. Upon binding of BMP ligand to its specific cell surface receptor, intracellular signaling proteins Smad1/5 are phosphorylated. The activated Smad1/5 associate with Smad4. The oligomerized Smad complex then enters the nucleus to induce BMP-responsive genes in concert with other transcription factors. LMP-1 competitively binds to Smurf1 and rescues Smads1/5 from being targeted for Smurf1-dependent ubiquitin-mediated proteasomal degradation. Consequently, the rescued Smads1/5 lead to potentiation of the BMP-pathway by enhancing the expression of BMP-induced genes such as alkaline phosphatase and osteocalcin

In this study, we focused on developing and optimizing a cell-based method to use as a tool to identify reagents that potentiate BMP-2 activity. Using this assay we confirmed that the Smurf1-interacting motif was necessary for the optimal function of LMP-1 in potentiating BMP-2 activity by applying the recombinantly prepared LMP-1 wild type protein and LMP-1 $\Delta$ Smurf1 mutant protein that lacks the WW2 domain-binding motif. Both recombinant proteins were engineered to contain an 11-amino acid HIV-derived membrane transduction domain to facilitate their cellular entry when applied extracellularly. Finally, as proof of concept, we selected a small drug-like compound from a set of lead compounds that were previously selected based on computational homology modeling, DOCKING, and *in silico* screenings. The BMP-potentiating activities of both LMP-1 and the LMP mimetic compound were further evaluated by monitoring several markers of the osteoblastic phenotype corresponding to various time points during phenotype differentiation of C2C12 cells towards the osteoblast.

## MATERIALS AND METHODS

### Cell culture

Mouse C2C12 cells and Dulbecco's modified Eagle's medium (DMEM) were purchased from ATCC (Manassas,

VA). Fetal bovine serum (FBS) was purchased from HyClone Laboratories, Inc. (Logan, UT). The C2C12 cells at passages 5–10 were subcultured in T-75 cm<sup>2</sup> flasks in DMEM supplemented with 10% FBS at 37°C in 5% CO<sub>2</sub> with humidification. When the flasks reached 80% confluence, the cells were trypsinized and seeded in triplicate at 200 000 cells per well in a six-well plate for quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Alkaline phosphatase (ALP) assays or at 50 000 cells per well in a 12-well plate for the Dual-Luciferase reporter assay.

*Preparation of a mutant of LMP-1 fusion protein that lacks the WW2 domain-binding motif (LMP-1ΔSmurf1)*

The detailed methods for purification of the TAT-LMP-1 fusion proteins were previously described by us and we have already confirmed in those studies that the protein enters into cells and executes its function.<sup>24</sup> LMP-1ΔSmurf1 was generated using the following primers: forward primer, 5'-GGC CCG GCC CTT TGG GGC GGC AGC AGC AGC TGA CAG CGC CCC GCA AC-3'; reverse primer, 5'-GTT GCG GGG CGC TGT CAG CTG CTG CTG CCG CCC CAA AGG GCC GGG CC-3'. Mutagenesis was performed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

*RNA extraction and reverse transcription*

The C2C12 cells were plated at a density of 200 000 cells per well in six-well plates and grown overnight. On day 2, culture media was replaced with DMEM containing 2% FBS and the cells were treated with LMP-1 or LMP-1ΔSmurf1 or various concentrations of LMP-1 mimetic compound (diluted from 10 mg ml<sup>-1</sup> stock solutions prepared in DMSO) for 24 h. In controls, for compound experiments a DMSO solvent concentration of 0.01% (v/v) was used. On day 3, media was replaced with fresh DMEM containing 2% FBS and the cells were treated with BMP-2 for 24 h.

Total RNA was harvested using the RNeasy Mini Kit according to the manufacturer's instruction (Qiagen, Valencia, CA). The harvested RNA was digested with RNase-free DNase I (Qiagen, Valencia, CA) to remove DNA contamination. The concentration of the isolated RNA was determined by measuring the absorbance at 260 nm wavelength with a spectrophotometer. The ratio of A<sub>260</sub>/A<sub>280</sub> was between 1.6 and 1.8. Reverse transcription was carried out to synthesize cDNA in a 100 μl volume with 2 μg of total RNA, 10 × RT buffer, 5.5 mM MgCl<sub>2</sub>, 2 mM dNTP mixture, 0.25 μM oligo d(T), 0.25 μM random primer, 40 U RNase inhibitor, and 125 U murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) for 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C.

*Quantitative real-time RT-PCR*

Quantitative real-time RT-PCR was performed to determine the mRNA expression level of ALP and osteocalcin. The sequences of the primers were follows: ALP (forward,

5'-TCA GGG CAA TGA GGT CAC ATC-3'; reverse, 5'-CAC AAT GCC CAC GGA CTT C-3'), osteocalcin (forward, 5'-CGG CCC TGA GTC TGA CAA AG-3'; reverse, 5'-CTC GTC ACA AGC AGG GTC AA-3'). Twenty-five microliters of reaction volume included 5 μl of cDNA, 0.5 μl of 10 μM of each primer, and 12.5 μl of 2 × SYBR green master mix (Applied Biosystems). Real-time PCR was performed with the following three-step protocol: step 1, 50°C for 2 min; step 2, 95°C for 10 min; step 3, 40 cycles of 95°C for 15 s and 62°C for 1 min using the 7500 real-time PCR System (Applied Biosystems, Foster City, CA). To confirm the amplification specificity, the PCR products were subjected to a dissociation curve analysis. The threshold cycles (Ct) of each reaction were normalized to those obtained for 18S mRNA using <sup>-ΔΔ</sup>Ct method. All PCR reactions were performed in duplicates.

*Alkaline phosphatase (ALP) assay*

The C2C12 cells were plated at 200 000 cells per well in six-well plates and grown overnight. On day 2, culture media was replaced with DMEM containing 2% FBS and the cells were treated with 25 nM LMP-1 or LMP-1ΔSmurf1 or various concentrations of the LMP-1 mimetic compound for 24 h. On day 3, media was replaced with fresh DMEM containing 2% FBS and the cells were treated with 50 ng ml<sup>-1</sup> of BMP-2 for 72 h. The cells were washed with phosphate-buffered saline (PBS) and lysed by addition of lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM MgCl<sub>2</sub>, and 0.5% Triton X-100). The cell lysates were centrifuged for 5 min at 13 000 g. The supernatant was removed and the aliquots were assayed for ALP activity and protein amount. The ALP activity was measured using an ALP assay kit (Sigma-Aldrich, St. Louis, MO) in triplicates in microtiter plates. The protein amount was determined with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard. The ALP activity (nmoles of *p*-nitrophenol per ml) was normalized to the protein amount (nmoles of *p*-nitrophenol per μg).

*Dual-luciferase reporter assay*

The BMP-specific Smad1-driven 9 × GCCG (a consensus binding sequence for Smad1) reporter plasmid was kindly provided by Dr Miyazono (The Institute of Japanese Foundation for Cancer Research, Tokyo). The C2C12 were trypsinized and seeded in triplicates at 50 000 cells per well in 12-well plates on day 1. On day 2, the cells were cotransfected with the 9 × GCCG-reporter construct and the renilla-luciferase control vector using SuperFect (Qiagen, Valencia, CA) for 24 h. A total of 1 μg of plasmid was used for cotransfection in each well and the concentration of renilla-luciferase vector was 1/15 of 9 × GCCG-reporter plasmid. On day 3, media was replaced with DMEM containing 2% FBS and the cells were treated with 100 nM LMP-1 or LMP-1ΔSmurf1 or various concentrations of the LMP-1 mimetic compound. On day 4, the cells were treated with BMP-2. On day 5, the luciferase activities were

measured in 20  $\mu\text{l}$  of cell-lysate using the dual-luciferase assay system (Promega, Madison, WI) with a luminometer (LumiCount; Packard Bioscience, Meriden, CT) following the manufacturer's instructions. The luciferase activity was expressed as relative units of luciferase (RUL; a ratio of firefly luciferase and renilla luciferase activity).

### Statistics and calculations

Results are presented as the mean of three determinations ( $n$ ) with error bars representing the standard error of the mean (SEM). Real time RT-PCR was performed on duplicate samples. Experimental results that are visually represented are from consistent experiments where one representative experimental result is shown. Statistical significance ( $p < 0.05$ ) was calculated using a one-way analysis of variance (ANOVA) and a Holm-Sidak post hoc test. These results were also confirmed by Bonferroni post hoc test (equal variances assumed) or Dunnett's T3 post hoc test (equal variances not assumed) using SigmaStat, version 3.1 (Systat Software, Inc., Point Richmond, CA) to compare various treatments in multi-group analysis. Statistical probability of  $p < 0.05$  was considered significant and is denoted as (\*) and/or (#) in figures.

## RESULTS

### Optimization of a cell-based method to monitor BMP-2 induced responses

To develop a sensitive and reliable cell-based assay, we utilized a Smad1-specific luciferase reporter plasmid which contains a multimerized -GCCG- motif (nine copies) to monitor transcriptional activity driven by activated Smad1 in C2C12 cells. The BMP-specific Smad1-driven  $9 \times \text{GCCG}$  reporter construct is widely used to assay the BMP activity in many cell types at a typical concentration of 50–100  $\text{ng ml}^{-1}$  of BMP-2. To select a sub-optimal dose of BMP-2 for studying the potentiating effect of LMP-1 or its mimetic compound, we performed the reporter assay with lower BMP-2 concentrations ranging from 0.25 to 75  $\text{ng ml}^{-1}$ . A dose response was established with a concentration range of BMP-2 required for activating the reporter assay (Figure 2). The results from this experiment allowed us to select a sub-optimal dose of BMP-2 to assess the potentiating effects of wild type LMP-1, LMP-1 $\Delta\text{Smurf1}$  mutant, or LMP-1 mimetic compounds in the subsequent experiments.

### Smurf1-interacting motif in LMP-1 is required for potentiation of BMP-2 induced reporter activity

We prepared recombinant proteins of LMP-1 wild type and a mutant form of LMP-1 (LMP-1 $\Delta\text{Smurf1}$ ) that fails to bind Smurf1. We then compared the induction of luciferase activity by 5  $\text{ng ml}^{-1}$  of BMP-2 when the C2C12 cells transfected with reporter plasmid were treated with or without 25 nM LMP-1 or LMP-1 $\Delta\text{Smurf1}$ . We observed that the BMP-2 induced luciferase activity was enhanced 2.7-

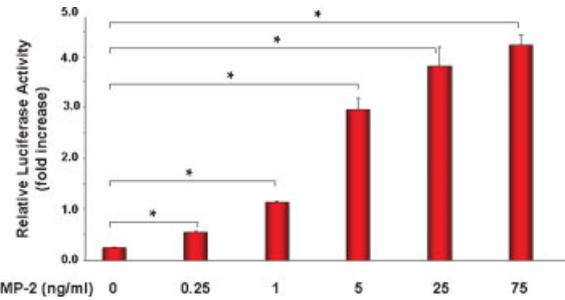


Figure 2. Determination of BMP-2 activity in a cell-based reporter assay. A dose-dependent induction of luciferase activity by BMP-2 is shown in C2C12 cells transfected with the BMP-specific and Smad1-driven  $9 \times \text{GCCG}$  reporter plasmid for 24 h. Luciferase activities were determined in triplicates. \* Denotes statistical significance, determined as described in methods, between the indicated treatments ( $p < 0.001$ )

fold by LMP-1 ( $p < 0.001$ ) when compared to BMP-2 alone and that treatment with LMP-1 $\Delta\text{Smurf1}$  resulted in a 45% lower BMP-2 induction of luciferase activity ( $p < 0.001$ ) compared with wild type LMP-1 treatment (Figure 3).

### LMP-1 enhancement of BMP-2-induced alkaline phosphatase and osteocalcin mRNA levels requires interaction with Smurf1

ALP and osteocalcin are widely accepted osteoblast differentiation markers. We compared the activities of LMP-1 and LMP-1 $\Delta\text{Smurf1}$  proteins in enhancing the BMP-2-induced expression of these markers in C2C12 cells. The cells were treated with LMP-1 or LMP-1 $\Delta\text{Smurf1}$  for 24 h followed by treatment with BMP-2 for 24 h. We observed that the BMP-2 induced elevation of the ALP mRNA level was enhanced 2.2-fold ( $p < 0.001$ ) by 25 nM

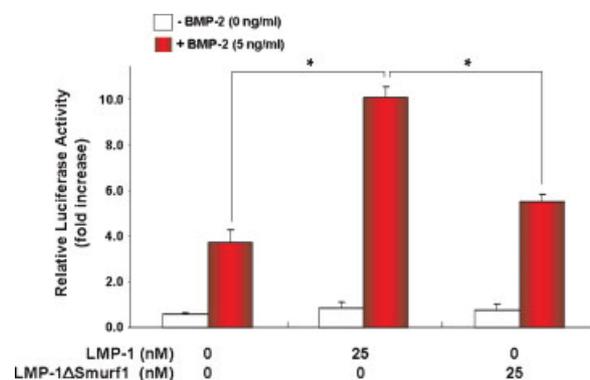


Figure 3. The Smurf1-interacting motif in LMP-1 is required for potentiation of BMP-2 induced reporter activity. A comparison of the effect of wild type LMP-1 protein and the mutant LMP-1 $\Delta\text{Smurf1}$  protein on BMP-2 induced  $9 \times \text{GCCG}$  reporter activity is shown. The LMP-1 wild type protein enhanced the BMP-2 induced luciferase activity by 2.7-fold. LMP-1 $\Delta\text{Smurf1}$  mutant protein treatment resulted in a 45% loss of the activity observed with the wild type LMP-1 protein. Luciferase activities were determined in triplicates. Statistical significance among different treatment groups was ( $p < 0.001$ ) obtained as detailed in methods

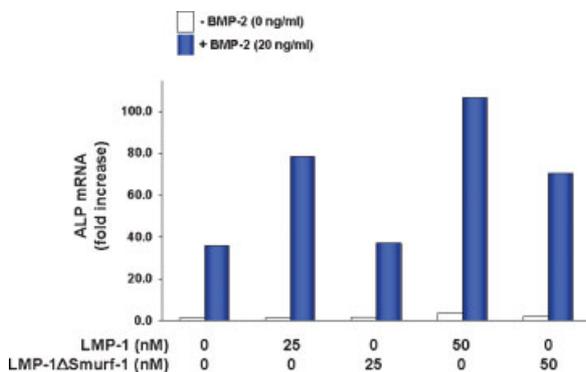


Figure 4. The Smurf1-interacting motif in LMP-1 is required to enhance BMP-2 induced expression of ALP mRNA. Comparison of the effect of wild type LMP-1 protein and the mutant LMP-1ΔSmurf1 protein on the potentiation of the BMP-2-induced increase in the ALP mRNA level is shown at 25 and 50 nM concentration of proteins. The BMP-2 induced increase in the ALP mRNA level was 2.2-fold and 3.0-fold by 25 nM and 50 nM LMP-1, respectively. These increases were reduced by 53% and 34% when the cells were treated with LMP-1ΔSmurf1 mutant, respectively. Quantitation of mRNA was based on duplicate determinations

LMP-1 when compared to BMP-2 alone control and treatment with 25 nM LMP-1ΔSmurf1 resulted in a 53% lower ( $p < 0.001$ ) induction of ALP mRNA expression compared with wild type LMP-1 treatment (Figure 4).

Next we measured BMP-2 induction of the osteocalcin mRNA level in the presence or absence of 25 or 50 nM LMP-1 or LMP-1ΔSmurf1 proteins. BMP-2 alone showed a 10.6-fold increase in osteocalcin mRNA over the no treatment control. The LMP-1 (25 nM) treatment caused a 1.7-fold increase ( $p < 0.001$ ) compared to BMP-2 alone. Treatment with 25 nM LMP-1ΔSmurf1 protein resulted in a 30% lower ( $p < 0.001$ ) BMP-2 induction of osteocalcin mRNA expression compared with the wild type LMP-1. Similar observations were made in experiments performed with 50 nM LMP-1 or LMP-1ΔSmurf1 proteins. When 50 nM LMP-1 was used, we observed a 2.6-fold ( $p < 0.001$ ) enhancement of the BMP-2-induced osteocalcin mRNA level as compared to BMP-2 alone. When 50 nM LMP-1ΔSmurf1 was used, we observed a 24% lower induction of the osteocalcin mRNA level compared with 50 nM wild type LMP-1 ( $p < 0.001$ ) (Figure 5). These observations confirmed that the interaction of LMP-1 with Smurf1 was indeed involved in the potentiation of BMP-2 activity by LMP-1.

#### The Smurf1-interacting motif in LMP-1 is required for enhancement of BMP-2 induced alkaline phosphatase activity

ALP is a highly active and stable enzyme, making a direct and sensitive assay of this enzyme activity feasible. To further confirm the results obtained in Figure 5, we determined the effect of LMP-1 and LMP-1ΔSmurf1 proteins on BMP-2 induced ALP enzyme activity in the

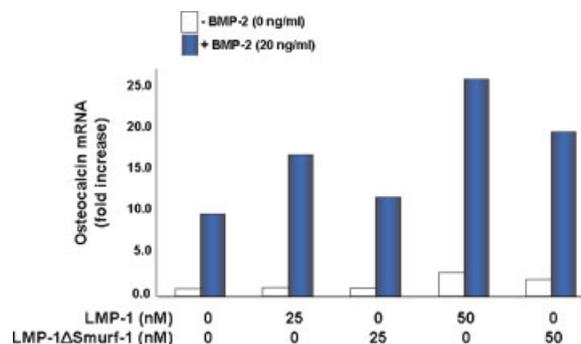


Figure 5. The Smurf1-interacting motif in LMP-1 is required for enhancing BMP-2 induced expression of osteocalcin mRNA. Comparison of the effect of the wild type LMP-1 protein and the LMP-1ΔSmurf1 protein on enhancement of BMP-2 induced osteocalcin mRNA expression is shown at 25 and 50 nM concentration of proteins, respectively. BMP-2 induced osteocalcin mRNA expression was enhanced 1.7-fold and 2.6-fold by 25 nM and 50 nM LMP-1, respectively. These increases were reduced by 30% and 24% when the cells were treated with 25 and 50 nM LMP-1ΔSmurf1 mutant, respectively. Quantitation of mRNA was based on duplicate determinations

C2C12 cells. Cells were treated with 100 nM LMP-1 or LMP-1ΔSmurf1 protein for 24 h followed by a treatment with BMP-2 (50 ng ml<sup>-1</sup>) for 72 h. Similar to the results obtained in the reporter assays and real-time RT-PCR, the C2C12 cells caused a significant increase in BMP-2 induced ALP activity by LMP-1 protein (4.7-fold,  $p < 0.001$ ) when compared to BMP-2 alone. The treatment with LMP-1ΔSmurf1 protein resulted in a 42% lower ( $p < 0.001$ ) induction of ALP activity compared to BMP alone or no treatment controls which showed little or no activity (Figure 6).

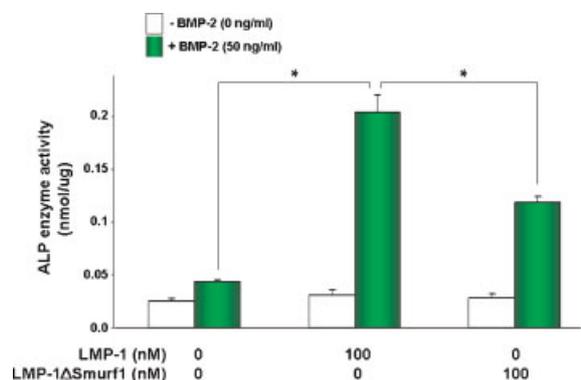


Figure 6. The Smurf1-interacting motif in LMP-1 is required for enhancing BMP-2 induced ALP enzyme activity. Comparison of the effect of the wild type LMP-1 protein and the LMP-1ΔSmurf1 mutant protein on the enhancement of BMP-2 induced ALP enzyme activity is shown. BMP-2 induced ALP activity was enhanced 4.7-fold by 100 nM LMP-1. This enhancement was reduced by 42% when cells were treated with 100 nM LMP-1ΔSmurf1 mutant instead of wild type LMP-1. Data points were determined in triplicates. Statistical significance among different treatment groups was ( $p < 0.001$ ) obtained as detailed in methods

*SVAK-3, an LMP-1 mimetic compound, potentiates BMP-2 induced Smad1-driven luciferase reporter activity*

In an effort to identify a novel small drug-like molecule that could disrupt Smurf1–Smad1/5 interaction in a way similar to LMP-1 function we used the computer simulation techniques of molecular docking to virtually screen compounds from a library enriched for FDA-approved drugs from the National Cancer Institute and a chemical database MDL Available Chemicals Directory (ACD). Compounds were ranked according to their relative binding energy, favorable shape complementarity, and potential to form hydrogen bonds within the modeled Smurf1 WW2 domain hydrophobic pocket.<sup>22,23</sup> For each docked compound, the best-scoring complexes were ranked, clustered, and re-ranked using calculations which included more accurate estimates of desolvation. Finally, a diversity selection, followed by visual inspection using 3D stereographics, of putative compounds was employed as filters for discovery of potential “lead” compounds. A representative sample of the most favorable compounds (54 compounds) was tested experimentally for their ability to potentiate BMP-2 activity in our cell-based luciferase reporter assay. Out of these candidate lead compounds, we selected SVAK-3 for the current studies as a promising LMP-1 mimetic synthetic compound. Although SVAK-5, 6, and 25 showed relatively more enhancement of BMP-2 induced luciferase activity than SVAK-3, we focused on additional characterization of SVAK-3 activity because its parent compound and the related chemical derivatives were pharmacologically more defined (Figure 7). Similar to LMP-1 protein alone treatments, the compounds by themselves showed no

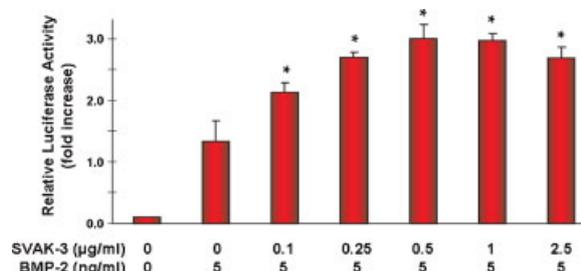


Figure 8. SVAK-3 potentiates BMP-2 responsiveness in the Smad1-driven reporter assay. A 2.3-fold enhancement of luciferase activity was observed at a compound concentration of  $0.5 \mu\text{g ml}^{-1}$  when compared to BMP-2 alone ( $5 \text{ ng ml}^{-1}$ ). Data points were determined in triplicates. Statistical significance from the BMP-2 control group was ( $p < 0.001$ ) obtained as detailed in Methods section and is denoted by (\*)

enhancement of reporter activity in the absence of BMP-2. The solvent dimethylsulfoxide (DMSO) controls showed only basal activity similar to no treatment controls. The DMSO solvent concentration of 0.01% (v/v) was not toxic to cells as determined by cell number, total protein amount, and cell phenotype consistent with the literature (data not shown).

Next, we determined the effectiveness of compound SVAK-3 on potentiation of BMP-2 activity over the concentration range from  $0.1$  to  $2.5 \mu\text{g ml}^{-1}$  while keeping the BMP-2 concentration at  $5 \text{ ng ml}^{-1}$  in the luciferase reporter assay as shown in Figure 8. SVAK-3 showed a dose-dependent effect in enhancing the luciferase activity with an optimum enhancement of 2.3-fold ( $p < 0.001$ ) observed at a concentration of  $0.5 \mu\text{g ml}^{-1}$  when compared to BMP-2 alone.

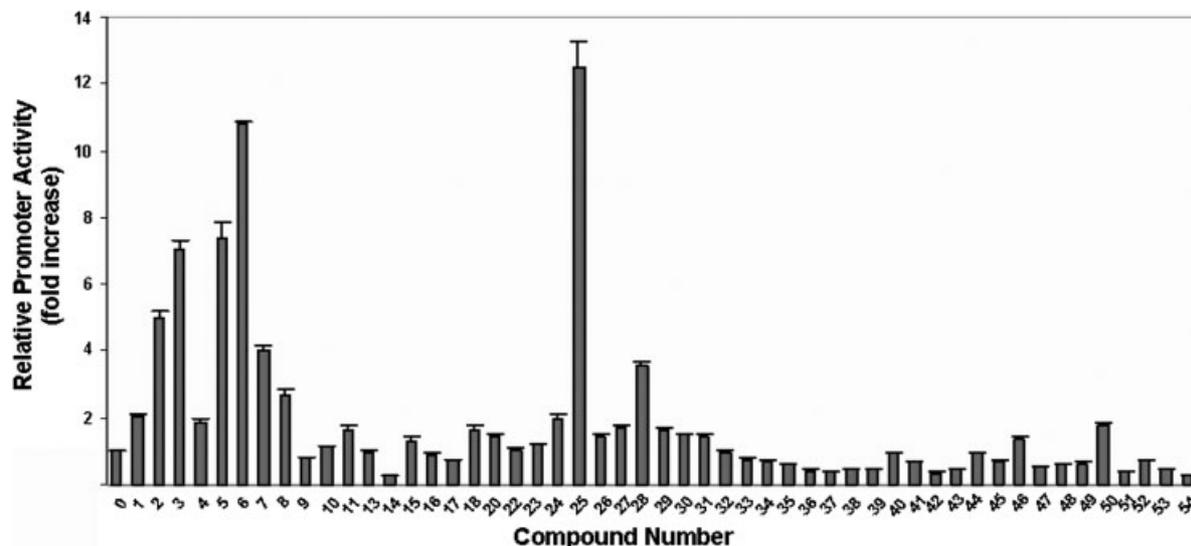


Figure 7. Determination of the efficacy of various mimetic compounds to enhance BMP-induced luciferase activity. Relative activities of a representative set of candidate LMP-1 mimetic compounds in the luciferase reporter assay are shown. Compounds were tested at a concentration of  $1.0 \mu\text{g ml}^{-1}$  while BMP-2 was used at  $0.25 \text{ ng ml}^{-1}$ . Data points were determined in triplicate. Statistical significance among different treatment groups was ( $p < 0.001$ ) obtained as detailed in methods. In no compound controls, cells were treated with DMSO (0.01%) alone. Compounds showed no activity in the absence of BMP-2 at the concentration ( $1.0 \mu\text{g ml}^{-1}$ ) tested. The same compounds that were identified as being active were also determined to be active at compound concentrations of  $0.1$  and  $10 \mu\text{g ml}^{-1}$  (data not shown)

### BMP-2 induced alkaline phosphatase and osteocalcin mRNA levels are enhanced by the compound SVAK-3

In Figure 4, we determined the potentiating effect of LMP-1 on BMP-2 activity by determining the enhancement of the BMP-induced alkaline phosphatase mRNA level by LMP-1. Here, we tested whether the LMP-1 mimetic compound, SVAK-3, that enhanced BMP-induced reporter activity would also exhibit potentiating activity on BMP-2-induced marker gene expression. We determined the effectiveness of compound SVAK-3 over the concentration range from 0.125 to 1.0  $\mu\text{g ml}^{-1}$  while keeping the BMP-2 concentration constant at 20  $\text{ng ml}^{-1}$  by determining alkaline phosphatase mRNA levels as shown in Figure 9. SVAK-3 showed a dose-dependent increase in the BMP-induced alkaline phosphatase mRNA level with the maximal 3.4-fold increase ( $p < 0.001$ ) compared to BMP-2 alone observed at a compound concentration of 0.25  $\mu\text{g ml}^{-1}$ . At higher concentrations of compound this activity was partly diminished probably due to undesired/toxic effects in cells.

In Figure 5, we showed the potentiating effect of LMP-1 on BMP-2 activity by determining the enhancement of the BMP-induced osteocalcin mRNA level by LMP-1. Here, we tested whether the LMP-1 mimetic compound, SVAK-3, that enhanced BMP-induced reporter activity and ALP mRNA expression would also exhibit potentiating activity by increasing BMP-induced osteocalcin gene expression. Such an observation would strengthen its biological role in promoting the osteoblastic phenotype. We determined the effectiveness of compound SVAK-3 over the concentration range from 0.125 to 1.0  $\mu\text{g ml}^{-1}$  while keeping the BMP-2 concentration constant at 20  $\text{ng ml}^{-1}$  as shown in Figure 10. SVAK-3 caused a dose-dependent increase in the BMP-induced osteocalcin mRNA level with maximal 2.6-fold increase ( $p < 0.001$ ) compared to BMP-2 alone observed at a compound concentration of 0.25  $\mu\text{g ml}^{-1}$ .

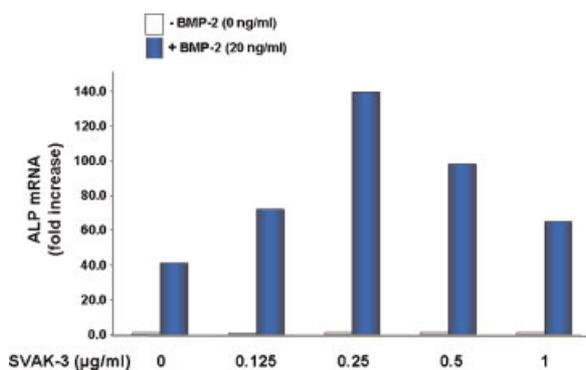


Figure 9. SVAK-3 enhances the BMP-induced increase of the ALP mRNA level in C2C12 cells. The compound dose-dependently enhanced the BMP-2 induced ALP mRNA level. The peak 3.4-fold enhancement of ALP mRNA level was observed at a SVAK-3 compound concentration of 0.25  $\mu\text{g ml}^{-1}$ . LMP-1 alone or compound alone controls showed no significant effect. Quantitation of mRNA was based on duplicate determinations

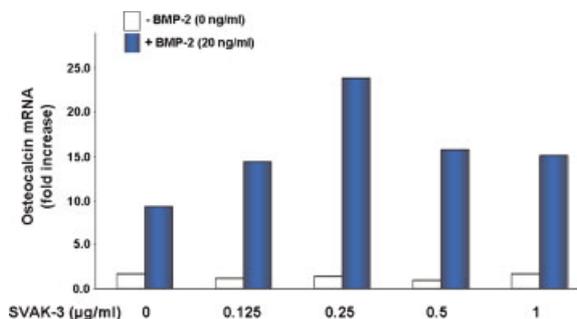


Figure 10. SVAK-3 enhances the BMP-induced osteocalcin mRNA level in C2C12 cells. The peak 2.6-fold enhancement of the osteocalcin mRNA level was observed at a SVAK-3 compound concentration of 0.25  $\mu\text{g ml}^{-1}$ . LMP-1 alone or compound alone showed no significant effect. Quantitation of mRNA was based on duplicate determinations

### BMP-2 induced ALP enzyme activity is enhanced by the compound SVAK-3

We used SVAK-3 in the concentration range of 0.125–1  $\mu\text{g ml}^{-1}$  while keeping the BMP-2 concentration constant at 100  $\text{ng ml}^{-1}$  to evaluate its enhancement of the BMP-induced alkaline phosphatase activity. The ALP enzyme assay demonstrated that the compound dose-dependently enhanced the BMP-2 induced ALP activity. The peak activity of 3.6-fold increase ( $p < 0.001$ ) was observed at a compound concentration of 0.25  $\mu\text{g ml}^{-1}$  when compared to BMP-2 alone, further confirming the earlier observations (Figure 11). We also used 100 nM LMP-1 protein as a positive control and observed an enhancement of ALP activity of 2.8-fold ( $p < 0.001$ ) when compared to BMP-2 (50  $\text{ng ml}^{-1}$ ) alone in these experiments. These results

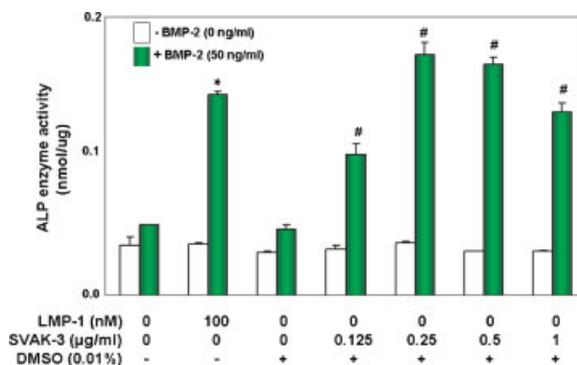


Figure 11. Enhancement of BMP-2 induced ALP enzyme activity by LMP-1 and SVAK-3. The compound dose-dependently enhanced the BMP-2 (100  $\text{ng ml}^{-1}$ )-induced ALP activity at 100  $\text{ng ml}^{-1}$  of BMP-2. The peak 3.6-fold enhancement of ALP activity was observed at a SVAK-3 compound concentration of 0.25  $\mu\text{g ml}^{-1}$ . Similarly, 100 nM LMP-1 protein treatment, when used as a positive control, showed a 2.8-fold enhancement of ALP activity. Data points were determined in triplicates. \* Denotes significant difference from BMP alone control. # Denotes significant difference from BMP alone control in the presence of equal amount of DMSO (0.01%) as in compound treatments. Statistical significance among different treatment groups was ( $p < 0.001$ ) obtained as detailed in Methods section

support our hypothesis that SVAK-3 is potentially a low-cost substitute for a recombinantly prepared LMP-1 protein in enhancing BMP-induced osteoblastic responses in cells.

## DISCUSSION

LMP-1 is a novel intracellular LIM domain protein shown to induce bone formation *in vitro* and *in vivo*. An interesting function of LMP-1 is its ability to synergize with BMP-2. We previously reported that LMP-1 binds to Smurf1, which is capable of degrading its natural targets, the osteogenic Smad1/5. The interaction between LMP-1 and Smurf1 prevents ubiquitination of these Smads and their degradation, increasing the cellular responsiveness to BMP-2. By comparing the LMP-1 wildtype and the mutant protein that lacked the Smurf1-binding motif, we confirmed that the interaction of LMP-1 with Smurf1 via its WW2-domain interacting site was necessary for the synergistic effect of LMP-1 with BMP-2. The reporter assay using BMP-specific Smad1-driven  $9 \times$  GCCG reporter plasmid, real-time RT-PCR for ALP and osteocalcin mRNA expression, and the ALP enzyme assay all showed that LMP-1 potentiated BMP-2 activity via binding to Smurf1.

Small-molecule compounds with potential BMP-potentiating activity will provide a pharmacological approach to help further extend the therapeutic use of BMPs. Any identification of a low cost compound that potentiates a BMP-induced cellular response will broaden the use of BMP-2 towards a more efficient bone regeneration application. Therefore, we extended the use of our cell-based assay using the Smad1-specific luciferase reporter to test potentially active candidates among the LMP-1-based synthetic compounds that were previously selected as the most likely "leads" after extensive chemo-informatic analyses of the Smurf1 WW2 domain and its interacting site in LMP-1. We selected SVAK-3 as one of such active "lead" candidates.

To test the validity of the cell-based reporter assay further, we examined the potentiating activity of SVAK-3 on BMP-2-induced cellular responses. We performed real-time RT-PCR for ALP and osteocalcin mRNA expression and the ALP enzyme assay in C2C12 cells. These determinations also demonstrated a dose-dependent activity of SVAK-3 in potentiating BMP-2 induced signaling. From these results we conclude that the activity of LMP-1 or SVAK-3 was not dependent on any particular assay and similar BMP-potentiating effects were observed both in reporter plasmid-transfected and non-transfected cells.

Two procedural details were critical to the success of this assay. We used a much lower concentration of BMP-2 ( $0.25\text{--}5\text{ ng ml}^{-1}$ ) than typical concentrations ( $50\text{--}100\text{ ng ml}^{-1}$ ) to improve the sensitivity of detecting enhancement of BMP-2 activity.<sup>25</sup> In addition, we increased the transfection time from 2 to 24 h which substantially improved the reliability, consistency, and reproducibility of the response. We showed that the specificity of the assay was well maintained despite monitoring multiple cellular outputs at various time points. This is a generic approach that can, in principle, be applied

to the identification of any reagent that influences cellular responsiveness to BMPs.

The compound SVAK-3 elevated the BMP-2-induced response significantly even at a dose of  $0.25\text{ }\mu\text{g ml}^{-1}$ , a non-toxic concentration based on cell number, total protein and cellular phenotype. The effective dose of compound remained consistent ( $0.25\text{ }\mu\text{g ml}^{-1}$ ) among multiple assays. This suggested that the biological markers in C2C12 cells that we chose to investigate are tightly controlled by the same BMP-signaling pathway. All these studies suggest that the compound, SVAK-3 or a potentially more efficacious derivative, may be useful in potentiating the BMP-2 responsiveness of cells. The combined use of such a compound with BMP-2 may reduce the high doses of BMP-2 protein currently required for its effective clinical use. The abundance and low cost of SVAK-3 makes it a highly desirable drug-candidate. Thus, SVAK-3 may be a useful pharmacological tool that induces BMP-mediated osteoblastic progression.

In summary, we established the  $9 \times$  GCCG reporter transfected C2C12 cells as a sensitive cell-based sensor of BMP-2 osteogenic effects. This system allowed us to monitor successfully the complex process of BMP-induced transdifferentiation of C2C12 cells towards the osteoblastic phenotype by determining various cellular markers at different time points in living cells. To our knowledge, this is the first report describing the effectiveness of an intact recombinant protein along with its mimetic compound in potentiating BMP-2 signaling. Also the identification and activity determination of SVAK-3 through novel approaches: chemo-informatics, virtual screenings, and cell-based biochemical evaluations lay promising ground for further applications in bone biology. Future experiments will evaluate enhancement of BMP-2-induced bone formation by SVAK-3 or its more efficacious derivatives in animal model.

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## REFERENCES

1. Cheng H, Jiang W, Phillips FM, *et al.* Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg Am* 2003; **85-A**: 1544–1552.
2. Cheng SL, Shao JS, Charlton-Kachigian N, Loewy AP, Towler DA. MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors. *J Biol Chem* 2003; **278**: 45969–45977.
3. Ying SX, Hussain ZJ, Zhang YE. Smurf1 facilitates myogenic differentiation and antagonizes the bone morphogenetic protein-2-induced

- osteoblast conversion by targeting Smad5 for degradation. *J Biol Chem* 2003; **278**: 39029–39036.
4. Peng Y, Kang Q, Cheng H, *et al.* Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signaling. *J Cell Biochem* 2003; **90**: 1149–1165.
  5. von Bubnoff A, Cho KW. Intracellular BMP signaling regulation in vertebrates: pathway or network? *Dev Biol* 2001; **239**: 1–14.
  6. Morone MA, Boden SD, Hair G, *et al.* Gene expression during autograft lumbar spine fusion and the effect of bone morphogenetic protein 2. *Clin Orthop Relat Res* 1998; **351**: 252–265.
  7. Boden SD, Zdeblick TA, Sandhu HS, Heim SE. The use of rhBMP-2 in interbody fusion cages. Definitive evidence of osteoinduction in humans: a preliminary report. *Spine* 2000; **25**: 376–381.
  8. Polly DW Jr, Ackerman SJ, Shaffrey CI, *et al.* A cost analysis of bone morphogenetic protein versus autogenous iliac crest bone graft in single-level anterior lumbar fusion. *Orthopedics* 2003; **26**: 1027–1037.
  9. Boden SD, Kang J, Sandhu H, Heller JG. Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial: 2002 Volvo Award in clinical studies. *Spine* 2002; **27**: 2662–2673.
  10. Boden SD, Schimandle JH, Hutton WC. The use of an osteoinductive growth factor for lumbar spinal fusion. Part I: biology of spinal fusion. *Spine* 1995; **20**: 2633–2644.
  11. Kusumoto K, Bessho K, Fujimura K, *et al.* Osteoinduction by recombinant human bone morphogenetic protein-2 in muscles of non-human primates. *J Int Med Res* 2002; **30**: 251–259.
  12. Ackerman SJ, Mafilios MS, Polly DW Jr. Economic evaluation of bone morphogenetic protein versus autogenous iliac crest bone graft in single-level anterior lumbar fusion: an evidence-based modeling approach. *Spine* 2002; **27**: S94–99.
  13. Boden SD, Liu Y, Hair GA, *et al.* LMP-1, a LIM-domain protein, mediates BMP-6 effects on bone formation. *Endocrinology* 1998; **139**: 5125–5134.
  14. Liu Y, Hair GA, Boden SD, Viggswarapu M, Titus L. Overexpressed LIM mineralization proteins do not require LIM domains to induce bone. *J Bone Miner Res* 2002; **17**: 406–414.
  15. Sangadala S, Boden SD, Viggswarapu M, Liu Y, Titus L. LIM mineralization protein-1 potentiates bone morphogenetic protein responsiveness via a novel interaction with Smurf1 resulting in decreased ubiquitination of Smads. *J Biol Chem* 2006; **281**: 17212–17219.
  16. Goldberg AL, Elledge SJ, Harper JW. The cellular chamber of doom. *Sci Am* 2001; **284**: 68–73.
  17. Zhu H, Kavsak P, Abdollah S, Wrana JL, Thomsen GH. A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* 1999; **400**: 687–693.
  18. Zhao M, Qiao M, Harris SE, Oyajobi BO, Mundy GR, Chen D. Smurf1 inhibits osteoblast differentiation and bone formation *in vitro* and *in vivo*. *J Biol Chem* 2004; **279**: 12854–12859.
  19. Zhao M, Qiao M, Oyajobi BO, Mundy GR, Chen D. E3 ubiquitin ligase Smurf1 mediates core-binding factor alpha1/Runx2 degradation and plays a specific role in osteoblast differentiation. *J Biol Chem* 2003; **278**: 27939–27944.
  20. Ebisawa T, Fukuchi M, Murakami G, *et al.* Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 2001; **276**: 12477–12480.
  21. Izzi L, Attisano L. Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. *Oncogene* 2004; **23**: 2071–2078.
  22. Sangadala S, Boden SD, Metpally RP, Reddy BV. Modeling and analysis of molecular interaction between Smurf1-WW2 domain and various isoforms of LIM mineralization protein. *Proteins* 2007; **68**: 690–701.
  23. Sangadala S, Metpally RP, Reddy BV. Molecular interaction between Smurf1 WW2 domain and PPXY motifs of Smad1, Smad5, and Smad6—modeling and analysis. *J Biomol Struct Dyn* 2007; **25**: 11–23.
  24. Sangadala S, Okada M, Liu Y, Viggswarapu M, Titus L, Boden SD. Engineering, Cloning and Functional Characterization of Recombinant LIM Mineralization Protein-1 Containing an N-terminal HIV-derived Membrane Transduction Domain. *Protein Expr Purif* 2009; **65**: 165–173.
  25. Sowa H, Kaji H, Hendy GN, *et al.* Menin is required for bone morphogenetic protein-2 and transforming growth factor beta-regulated osteoblastic differentiation through interaction with Smads and Runx2. *J Biol Chem* 2004; **279**: 40267–40275.