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Biomechanical forces exert anabolic effects on osteoblasts by activation of SMAD 1/5/8 through type 1 BMP receptor

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Abstract

Osteoblasts are mechanosensitive cells, which respond to biomechanical stimuli to regulate the bone structure through anabolic and catabolic gene regulation. To examine the effects of mechanical forces on the osteogenic responses through the SMAD signaling in osteoblasts, the cells were cultured in well-characterized mechanoresponsive 3-D scaffolds and exposed to 10% dynamic compressive strain (*Cmp*) at 1 Hz. Subsequently, SMAD phosphorylation and osteogenic gene induction was examined. Osteoblasts cultured in 3-D scaffolds exhibited increased constitutive SMAD 1/5/8 phosphorylation, as compared to monolayers cultures. This SMAD 1/5/8 phosphorylation was further upregulated after 10, 30 and 60 min in response to *Cmp*, exhibiting a peak activation at 30 min. No significant changes in SMAD2 phosphorylation were observed, suggesting signals generated by *Cmp* may not activate the Transforming Growth Factor-β signaling cascade. Subsequently, biomechanical stimulation-induced SMAD 1/5/8 phosphorylation upregulated the expression of osteogenic genes such as Osteoprotegrin, Msx2 and Runx2. Dorsomorphin, a selective inhibitor of the bone morphogenetic protein (BMP) receptor type 1 (BMPR1), blocked *Cmp*-induced SMAD 1/5/8 phosphorylation, as well as Osteoprotegrin, Msx2 and Runx2 gene expression. Collectively, the present findings demonstrate that biomechanical stimulation of osteoblasts activates SMAD 1/5/8 in the BMP signaling pathway through BMPR1 and may enhance osteogenesis by upregulating SMAD-dependent osteogenic genes.

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Conflict

None of the authors have competing financial or non-financial interests in the work presented in this manuscript.

Keywords

Scaffold; mechanical signals; BMP; SMAD signaling; dorsomorphin

1. Introduction

Biomechanical stimuli are essential for the development and homeostasis of the skeletal system. Specifically, it is well documented that bone is a mechano-responsive tissue, which responds to mechanical forces through the induction of bone resorption and deposition [31,32]. In contrast, the lack of biomechanical forces leads to bone loss that requires an extended recovery time [1,30,37]. At the cellular level, osteoblasts embedded in bone, play an integral role in responding to biomechanically dynamic microenvironments to regulate the bone mass and architecture [7,15,34]. Several different forms of biomechanical stimuli take part in anabolic responses of osteoblasts. In 2-D cultures, tensile strain induces upregulation of genes such as alkaline phosphatase (*Alp*), osteocalcin (*Ocn*), runt-related transcription factor 2 (*Runx2*) and vascular endothelial growth factor (*Vegf*) [12,13,25]. Fluid shear has been shown to regulate the expression of Collagen type I (*Col1A1*) and *Vegf* [9,22,42]. Similarly, hydrostatic compression upregulates *Alp* and *Col1A1* in osteoblast monolayer-cultures [33]. Dynamic compressive strain in 3-D cultures is also shown to upregulate bone morphogenic protein 2 (*Bmp2*), osteopontin (*Opn*), *Alp*, *Col1A1* and osteocalcin (*Ocn*) [29]. However, the molecular mechanisms underlying these well-documented anabolic responses to mechanical stimuli in osteoblasts still remain elusive. Transforming growth factor beta (TGF-β) and bone morphogenic protein (BMP) pathways play an essential role in regulating osteogenesis as well as bone remodeling by modulating proliferation and differentiation of osteoblasts [5,36]. Traditionally, these pathways are believed to be activated by the binding of TGF-β or BMPs to ligand-specific membrane receptors, BMP receptor type 1 (BMPR1) and BMP receptor type 2 (BMPR2). The ligand/receptor binding initiates signal transduction through phosphorylation of various *Caenorrhabditis elegans protein SMA* (*mother against decapentaplegic MAD*; SMAD) proteins and their nuclear translocation. SMADs also function as transcription factors, controlling the expression of essential osteogenic genes involved in osteoblast proliferation (Msh homeobox 2, *Msx2*), matrix synthesis (*Runx2* and *Osterix* (*Osx*)), and inhibition of osteoclast differentiation (osteoprotegrin, *Opg*) [41]. Therefore, in this study we examined the SMAD signaling cascades that may mediate the biomechanical stimulation induced osteogenic gene induction.

2. Materials and methods

2.1. Scaffold synthesis

A 15 wt% solution of poly-ε-caprolacton (PCL) (M.W. 65000, Sigma-Aldrich, MO) dissolved in dichloromethane was electrospun as previously described [24]. Cylindrical scaffolds of 6 mm diameter and 3 mm height were treated with air plasma (Harrick Plasma, NY, USA) for 15 min immediately followed by collagen surface coating through incubating them in sterile 1 mg/ml collagen type I in 0.01 M hydrochloric acid overnight [4].

2.2. Cell culture

Calvarial osteoblasts were harvested from 3-day-old Sprague Dawley rats as previously described [29]. The procedure was approved by the IACUC at The Ohio State University. The cells were cultured in tissue culture medium (TCM); DMEM (Invitrogen Corp., CA, USA) containing 10% FBS (Atlanta Biologicals, GA, USA), 100 units penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml Amphotericin B and 2 mM L-glutamine (Invitrogen). A total of 2×10^5 cells in 50 μl TCM were seeded into each scaffold. The cells were allowed to attach for 2 h prior to adding additional culture medium. BMP and dorsomorphin concentration optimization studies were conducted on monolayer cultures by seeding 8×10^4 cells in 6-well tissue culture plates. Primary osteoblastic cells in second and third passages were used in all experiments.

2.3. Application of BMP-2 and dorsomorphin

Cells were grown in monolayers for 3 days. Twelve hours prior to the application of BMP-2 (Gen-Script, NJ, USA) and/or dorsomorphin (dissolved in DMSO, Sigma-Aldrich, MO, USA), TCM was replaced with medium containing 1% FBS. BMP-2 was added at a concentration of 50 ng/ml for 10, 30 or 60 min before extracting proteins from the cells. For the inhibitor studies, cells were preincubated for 30 min in dorsomorphin (0.5, 1, 5, 10, 20 or 40 μM) prior to the addition of BMP-2. Total cellular proteins were extracted 30 min after the addition of BMP-2.

2.4. Application of dynamic compression (Cmp)

Osteoblasts were cultured in 3-D scaffolds for 7 days. Subsequently, cell-scaffold constructs were subjected to 10% unconfined dynamic compression of saw-tooth profile, at 1 Hz for different durations, using a custom-dynamic compression device as described earlier [23]. In some experiments, dorsomorphin was added to the cell/scaffold constructs 30 min prior to the application of *Cmp*. All experiments were performed in an incubator at 37°C with 5% CO_2.

2.5. Western blot analysis

Whole-cells were extracted in RIPA buffer containing protease and phosphatase inhibitors (Santa Cruz Biotechnology, CA, USA). The protein concentration of cell lysates was measured using BCA protein assay (Pierce Biotechnology, IL, USA) and color intensity read at 540 nm in a Victor plate reader (PerkinElmer, MA, USA). Equal amounts of total proteins were resolved on SDS-10% PAGE and electrotransferred onto a nitrocellulose membrane (Bio-Rad, CA, USA). Membranes were probed with specific primary antibodies recognizing phospho-SMAD 1/5/8 (phospho-SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD8 (Ser426/428)), SMAD 5, phospho-SMAD 2 (phospho-SMAD 2 (Ser465/467)) and SMAD 2 (Cell Signaling, MA, USA). The same membranes were probed with anti-β-actin antibodies (Sigma-Aldrich, MO, USA) to normalize protein loading in each lane. An Odyssey Infrared Imaging System (LI-COR, NE, USA) was utilized to detect the antibody-specific bands using IRDye 800CW- or IRDye 680-conjugated secondary antibodies [23].

2.6. Immunofluorescence

Cell-scaffold constructs with or without compression for 30 min were fixed with 2% paraformaldehyde. The fixed constructs were horizontally sectioned in the middle of the scaffolds and the cells were permeabilized with 0.2% Triton-X for 30 min followed by staining with phospho-SMAD 1/5/8 primary antibody (Cell Signaling) and CY3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, PA, USA). To observe cell morphology, actin and nucleus were counterstained with fluorescein isothiocyanate (FITC) labeled phalloidin and 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, MO, USA), respectively. The stained samples were observed under an epifluorescence microscope (Axioplan2; Carl Zeiss, NY, USA).

2.7. Analysis of gene expression

Total RNA was extracted from cell-scaffolds constructs with the RNeasy Kit (Qiagen, CA, USA), and cDNA synthesized with 1 μg of RNA as described earlier [29]. Custom-designed (Primer Express, Applied Biosystems, CA, USA) primers (Table 1) were used to analyze the gene expression by real-time polymerase chain reaction (RT-PCR) (Bio-Rad, CA, USA). The data obtained were then analyzed by the comparative threshold cycle (CT) method [18].

2.8. Statistical analysis

All experiments were performed in triplicate. The statistical analysis was carried out using SPSS 15.0 (SPSS Inc., IL, USA). One-way analysis of variance (ANOVA) with Tukey's post hoc were performed to determine significance among the experimental groups. A p-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Dynamic compression leads to SMAD 1/5/8 but not SMAD 2 phosphorylation in osteoblasts

In the initial experiments we observed constitutive phosphorylation of SMAD 1/5/8 in osteoblasts grown in 3-D scaffolds. To eliminate the possibility of serum constituents activating osteoblasts, we next compared the SMAD 1/5/8 in osteoblasts grown in monolayers and 3-D constructs that were supplemented with same TCM containing 1% FBS, 12 h prior to examination of 1/5/8 SMAD phosphorylation. Western blot analysis showed an increased basal level of SMAD 1/5/8 phosphorylation in the cells cultured in 3-D scaffolds indicating constitutive activation of the bone morphogenetic protein (BMP) signaling pathway (Fig. 1A). No constitutive phosphorylation of SMAD 2 was observed (data not shown).

Previously, we reported that *Cmp* upregulates BMP-2 in osteoblasts [29]. Since BMP-2 is known to positively regulate its own expression through the SMAD signaling [19,35], we examined the effect of compressive forces on the phosphorylation of SMAD 1/5/8 (under BMP signaling pathway) and SMAD 2 (under TGF-β). Osteoblasts were subjected to *Cmp* for 10, 30 or 60 min and SMAD 1/5/8 phosphorylation of SMAD1 (Ser463/465)/SMAD5 (Ser463/465)/SMAD8 (Ser426/428) activation associated sites was examined by Western blot analysis. In parallel, total input of SMAD 5 and β-actin were assessed as controls for

equal input of proteins in each protein sample. The phosphorylation of SMAD 1/5/8 was increased in a time dependent manner as compared to untreated controls (Fig. 1B). The upregulation of SMAD 1/5/8 phosphorylation peaked at 30 min and was sustained up to 60 min under dynamic compression (Fig. 1B). On the other hand, total SMAD 5 was unchanged after subjecting the cells to various periods of compression regimens (Fig. 1B). Figure 1C confirmed the quantitative increase in the phosphorylation by digitization of the fluorescence intensity in bands shown in Fig. 1B. The increase in the relative fluorescence in compression-induced phosphorylation of SMAD 1/5/8 at 30 and 60 min was statistically significant as compared to uncompressed control. Importantly, there was no significant upregulation of SMAD 2 phosphorylation at Ser465/467 observed after 10, 30 and 60 min of *Cmp*. In these experiments, changes in the total SMAD 2 proteins were also not observed (Fig. 1D and E). To determine the functionality of the phosphorylated SMAD 1/5/8 as transcription factors, we next examined phospho-SMAD 1/5/8 localization in the cytoplasm and the nucleus by immunofluorescence. As shown in Fig. 2A phospho-SMAD 1/5/8 was observed both in the cytoplasm and nucleus in uncompressed control samples. After 30 min *Cmp* of the cell-scaffold constructs phospho-SMAD 1/5/8 were preferentially observed in the nucleus as opposed to control samples (Fig. 2B), demonstrating increased nuclear translocation of phospho-SMAD 1/5/8 after dynamic compression.

3.2. Dorsomorphin suppresses BMP-2 induced SMAD 1/5/8 phosphorylation

The upstream signal transduction event of SMAD 1/5/8 phosphorylation is BMPR1 activation. Therefore, in the next experiments, we determined whether biomechanical signals involve activation of BMPR1. For these studies dorsomorphin, an inhibitor of BMPR1 activity was used [46,47]. To determine the optimal stimulation time of BMP-2 and concentration of dorsomorphin, osteoblasts were first treated with 50 ng/ml BMP-2 for 10, 30 and 60 min in order to activate BMP-2 signaling. Similar to the changes in the phosphorylation by compressive forces, BMP-2 induced phosphorylation of SMAD 1/5/8, peaked at 30 min post-stimulation and it was maintained up to 60 min while there was no change in total SMAD 5 (Fig. 3A and B). Based on these experiments, osteoblasts were pre-incubated with dorsomorphin at various concentrations (0, 0.5, 1, 5, 10, 20 and 40 μM) for 30 min and BMP-2 added for the ensuing 30 min. The phosphorylation of SMAD 1/5/8 by BMP-2 (50 ng/ml) was increasingly suppressed by higher concentration of dorsomorphin (Fig. 3C). Approximately 80% suppression of SMAD 1/5/8 phosphorylation was observed at 5-μM dorsomorphin (Fig. 3C and D). BMP-2 or dorsomorphin did not affect the total amount of SMAD 5 protein over a period of 30 min (Fig. 3C).

3.3. Dorsomorphin inhibits SMAD phosphorylation induced by dynamic compressive strain

Next, to delineate whether biomechanical signals activate osteoblasts via activation of BMPR1, we examined SMAD 1/5/8 phosphorylation induced by *Cmp* in the absence or presence of dorsomorphin. The cell-scaffold constructs pre-incubated with dorsomorphin (5 μM) for 30 min, were subjected to *Cmp* for 30 min. Application of 5-μM dorsomorphin suppressed the *Cmp*-induced SMAD 1/5/8 phosphorylation by approximately 85% ($p < 0.01$) (Fig. 4A and B). These results suggested that SMAD 1/5/8 phosphorylation by dynamic compression may be mediated through the BMPR1.

3.4. Regulation of SMAD 1/5/8-dependent genes by biomechanical signals

Since SMAD 1/5/8 are shown to act as transcription factors, we next determined the SMAD 1/5/8 dependent gene expression. Cell-scaffold constructs were exposed to *Cmp* for 1 h with or without preincubation with dorsomorphin (5 μM). Subsequently, cells were rested for additional 1 h prior to RNA analysis. As shown in Fig. 5A and B, 10% of dynamic compressive strain significantly upregulated *Opg* and *Msx2* expression, as compared to control. Furthermore, dorsomorphin inhibited the *Cmp*-induced upregulation of *Opg* ($p < 0.01$) and *Msx2* ($p < 0.01$). In addition, the expression of *Runx2* was also significantly upregulated by *Cmp* ($p < 0.05$), and the presence of dorsomorphin inhibited the *Cmp*-induced upregulation of *Runx2* (Fig. 5C). However, the gene expression for *Osx2*, another transcription factor needed for ostegenesis was neither significantly upregulated by *Cmp* nor suppressed by dorsomorphin (Fig. 5D).

4. Discussion

In the present study, we analyzed the influence of dynamic compressive strain on the BMP signaling in osteoblasts. We previously demonstrated that 10% dynamic compressive strain resulted in osteoblast proliferation and differentiation by induction of osteogenic markers [29]. Similarly, a recent study showed that 5–20% strain during metaphyseal fracture healing induced bone formation, whereas strain lower 5% resulted in significantly less bone formation and strain higher 20% led to fibrocartilage layers [6]. Another study demonstrated that an intramedullary bone pressure reaches up to 45 ± 9 mmHg during muscle stimulation that could cause significant matrix deformation [28].

We observed that application of dynamic compressive strain induces phosphorylation of SMAD 1/5/8 in osteoblasts leading to the induction of osteogenic genes. On the other hand, SMAD 2 phosphorylation required for the activation of TGF-β signaling cascade was not observed during the initial dynamic compression-induced signaling. This suggests that dynamic compressive forces may use the BMP signaling cascade for the activation of osteogenic genes in osteoblasts. Interestingly, the activation of SMAD 2 by TGF-β isoforms is shown to suppress osteoblastic differentiation that may suggest an inhibitory effect of the TGF-β signaling pathway on osteogenesis [8,20,39]. These findings support our observations that selective SMAD 1/5/8 but not SMAD 2 activation may be associated with the osteogenic potential of the biomechanical signals. We have observed that phosphorylation of SMAD 1/5/8 led to upregulation of *Opg*, *Msx2* and *Runx2*. All of these genes are osteogenic. Osteoprotegerin inhibits osteoclast differentiation and thereby regulates bone mass [38]. In this respect, compressive strain acts similar to BMP-2, which is shown to induce OPG expression in osteoblasts [10,44]. On the other hand, MSX2 is involved in intramembranous ossification and supports osteoblastic differentiation. MSX2 is also shown to be directly regulated by BMP-2 through SMAD activation [2,11]. In addition, biomechanical signals regulate expression of Runx2, a transcription factor upregulated by BMP-2 that is essential for osteoblast differentiation [14,26,27,40]. On the contrary, Osterix, also a transcription factor that promotes osteoblast proliferation and bone deposition in response to BMP-2 [43], was not significantly regulated by compressive strain. These findings suggest that the signals generated by biomechanical signals may not activate gene

expression identical to BMP-2, rather only some of the genes that are activated by BMP-2 [3]. Since SMAD activation could occur through BMP independent pathways [16,45] we next examined whether BMP receptors are involved in the mechanoactivation of SMAD by utilizing dorsomorphin, a selective inhibitor of the BMPR1 (activin receptor-like kinase-2, ALK2) [46,47]. Interestingly, the inhibitor not only abolished the biomechanically induced SMAD 1/5/8 phosphorylation, but also suppressed subsequent gene expression of the mechano-responsive genes. Furthermore, the concentrations of dorsomorphin that inhibited BMP-2 mediated SMAD 1/5/8 phosphorylation were sufficient to inhibit biomechanical signal activated SMAD 1/5/8 signaling. Considering the rapid SMAD 1/5/8 phosphorylation by mechanostimulation, BMPR1 could be a putative mechanoreceptor. Another interesting finding in this study is that, *Runx2*, *Msx2* and *Osx*, transcription factors are also regulated by SMADs under the BMP signaling pathway [11,27,43]. However, compressive forces activate these genes via SMAD 1/5/8 signaling. In osteoblasts, mechanical signals are also shown to activate other pathways such as estrogen receptor signaling pathway, IGF-I signaling pathway, and Wnt signaling pathway in addition to the BMP signaling pathway [17]. The possibility that genes activated by SMAD 1/5/8 mediated mechanotransduction are also indirectly activated by other signaling pathways, cannot be excluded.

In summary, the present findings collectively suggest that the BMP signaling is an important mechanoresponsive pathway in regulating the osteogenic and proliferative activities of osteoblasts. In this pathway, BMPR1 possibly serves as a mechanosensory protein and activates the SMAD 1/5/8 signaling cascade. Activation of SMAD 1/5/8 leads to upregulation of the osteogenic gene expression such as *Opg*, *Msx2* and *Runx2*. Further research projects focusing on the molecular events that are involved in osteogenesis via mechanoactivation are necessary to utilize full potential of these signals to repair and remodel bone.

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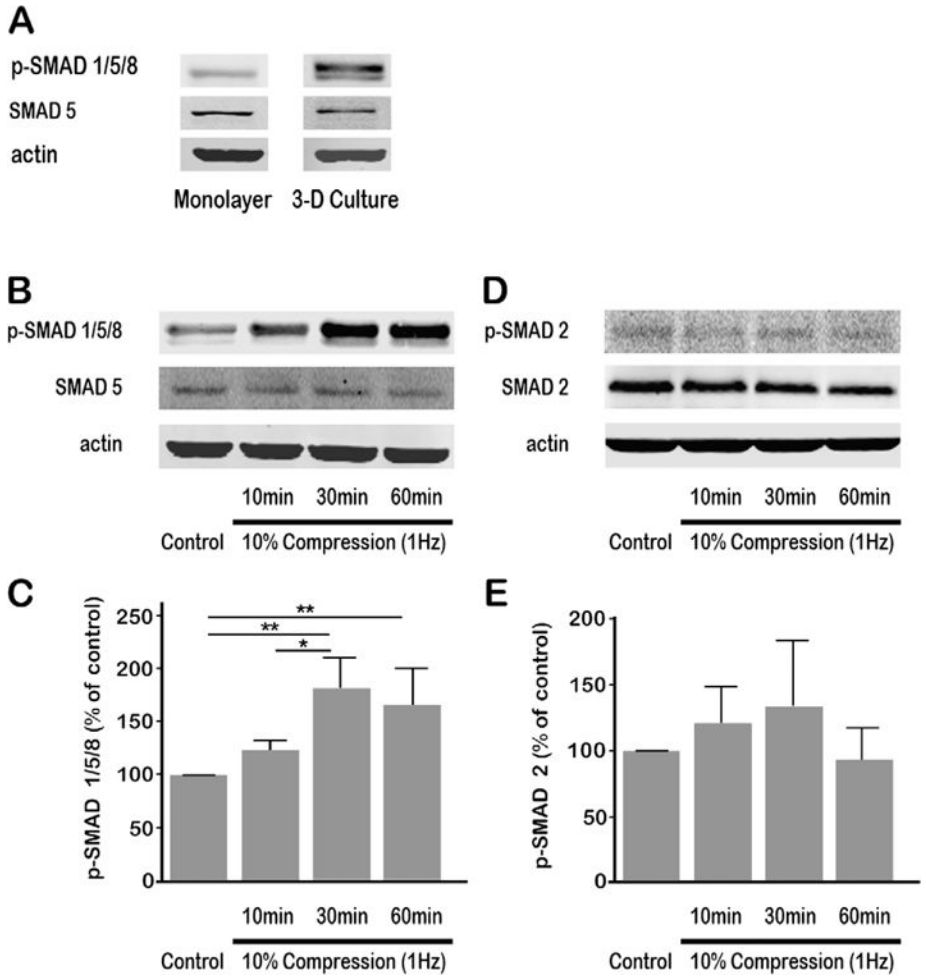

Fig. 1.

(A) Constitutive phosphorylation level of SMAD 1/5/8 in osteoblasts grown as monolayers or in 3-D scaffolds. (B) SMAD 1/5/8 phosphorylation in osteoblasts in response to dynamic compression for 10, 30 and 60 min. SMAD 5 and β-actin show equal input of proteins in each lane. (C) Digitization of phospho-SMAD 1/5/8 bands in (B) showing statistically significant upregulation of SMAD 1/5/8 phosphorylation. (D) SMAD 2 phosphorylation following exposure to dynamic strain for 10, 30 and 60 min. Equal protein input is shown by levels of total SMAD 2 and actin in all lanes. (E) Digitization of bands in (D) showing lack of SMAD 2 phosphorylation. Bars represent mean ± SEM of 3 separate experiments; *$p <$ 0.05 and **$p < 0.01$.

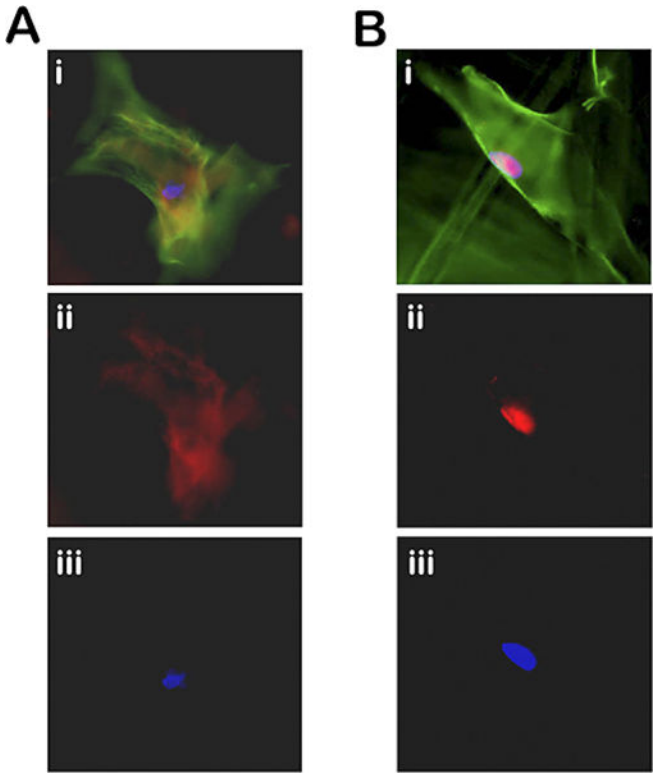

Fig. 2.

Immunofluorescence analysis of phospho-SMAD 1/5/8 in control scaffolds and cell-scaffold constructs after 30 min compression. (A) (i) In untreated samples phospho-SMAD 1/5/8 was observed in the cytoplasm and in the nucleus; (ii) phospho-SMAD 1/5/8 staining; (iii) DAPI staining. (B) 30 min of compression-induced nuclear translocation of phospho-SMAD 1/5/8 (i). Phospho-SMAD 1/5/8 was observed mainly inside the nucleus; (ii) phospho-SMAD 1/5/8 staining; (iii) DAPI staining. (Colors are visible in the online version of the article; http://dx.doi.org/10.3233/BIR-2011-0580.)

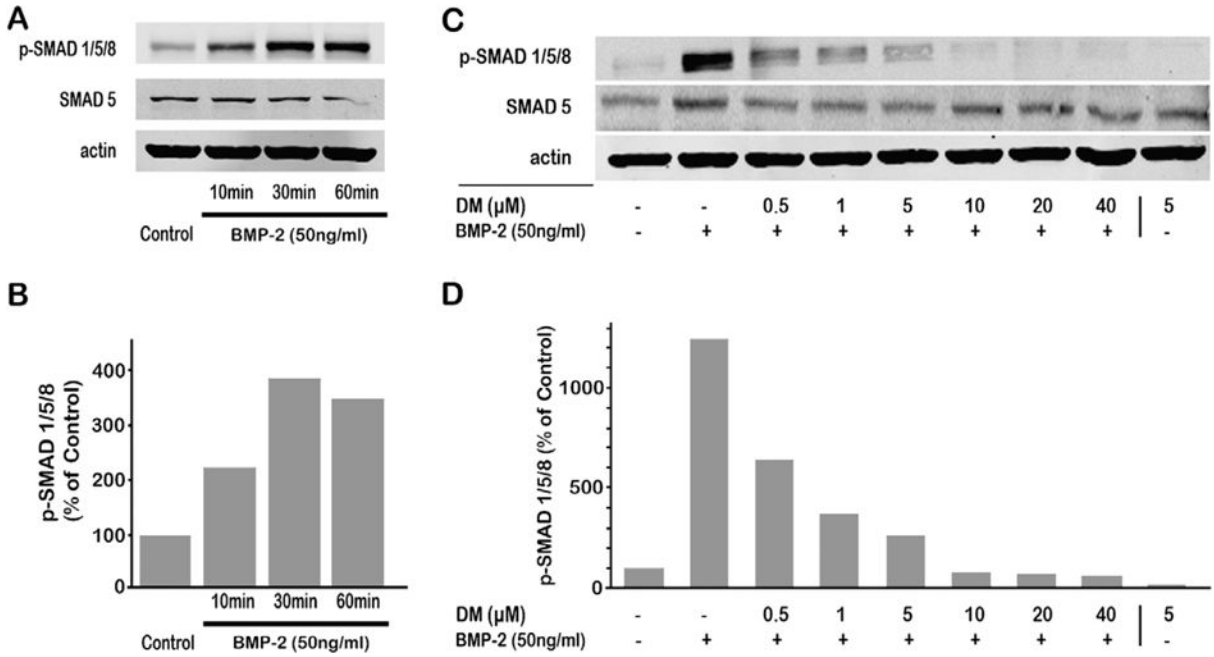

Fig. 3.

(A) SMAD 1/5/8 phosphorylation in osteoblasts in response to BMP-2 (50 ng/ml) at 10, 30 and 60 min. SMAD 5 and *β*-actin bands showing equal protein levels in all lanes. (B) Digitization of bands in (A). The results represent one of two separate experiments. (C) Inhibition of SMAD 1/5/8 phosphorylation by dorsomorphin in a dose dependent manner. Osteoblasts pretreated with dorsomorphin for 30 min were exposed to BMP-2 (50 ng/ml) for the ensuing 30 min, and SMAD 1/5/8 phoshorylation analyzed as described in Section 2. Five μM dorsomorphin suppressed BMP-2-induced SMAD 1/5/8 phosphorylation by more than 80%. Last lane shows that dorsomorphin also abolished constitutive SMAD 1/5/8 phosphorylation in the osteoblasts. No changes in protein levels were observed in cells treated with BMP-2 and/or dorsomorphin. (D) Digitization of bands in (C) showing a dose dependent inhibition of phospho-SMAD 1/5/8 by dorsomorphin. The results represent one of two separate experiments.

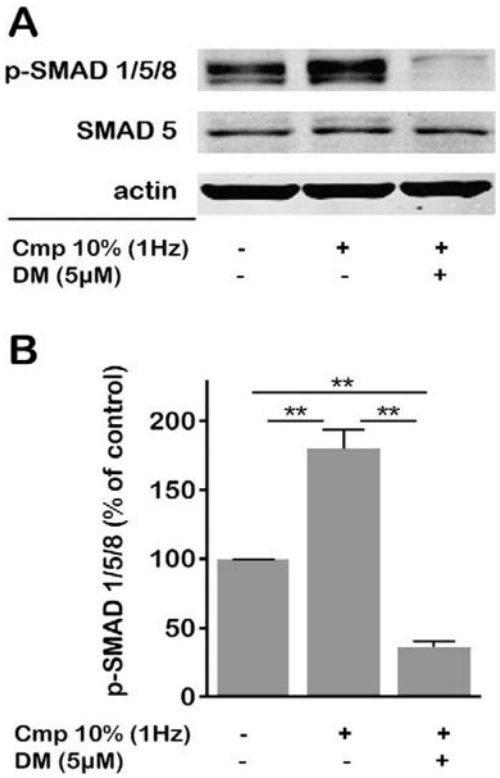

Fig. 4.

(A) Western blot analysis shows upregulation of SMAD 1/5/8 phosphorylation by dynamic compression (*Cmp*) after 30 min, and its inhibition by 5 μM dorsomorphin. (B) Digitization of bands in (A) showing that dorsomorphin blocked *Cmp*-induced SMAD 1/5/8 phosphorylation significantly. Bars represent mean ± SEM of 3 separate experiments; *$p <$ 0.05 and **$p < 0.01$.

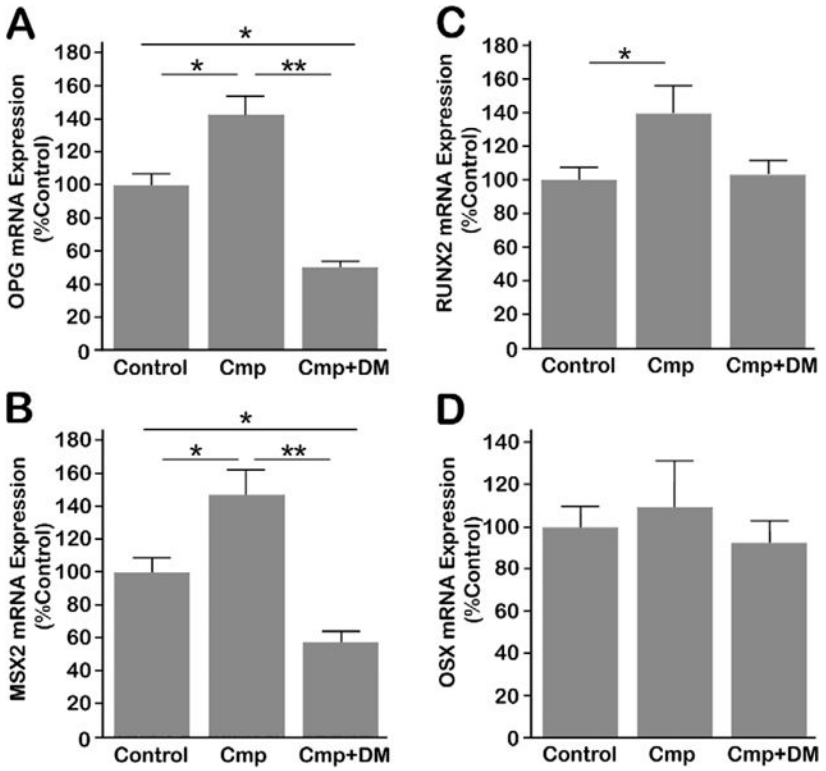

Fig. 5.

(A–D) Osteoblasts were exposed to 10% compression at 1 Hz for 1 h followed by 1 h of rest in the absence or presence of dorsomorphin. Subsequently expression of mRNA for (A) *Opg*, (B) *Msx2*, (C) *Runx2* and (D) *Osx*, examined by RT-PCR. Bars represent mean ± SEM ($n = 3$); *$p < 0.05$ and **$p < 0.01$.

Table 1

Primer sequences for RT-PCR

	Sense (5′–3′)	Antisense (5′–3′)	Length (bp)	GenBank accession
MSX2	TGA AAA TGG CTG CCA AAC CT	CTT GCA AGC GGC ATC CAT A	83	NM_012982
OSX	CTG CAA CTG GCT TTT CTG TGG	CAC TTG AGC AAA CAT CAG CGC	148	AY177399
OPG	GTG CGA AGA GGC ATT CTT CAG	TGT TCT GGT GGA CAG TTT GCC	81	NM_012870
RUNX2	TCA CAA ATC CTC CCC AAG TGG	GTG ATT TAG GGC GCA TTC CTC A	151	NM_053470
RPS18	GCG GCG GAA AAT AGC CTT CG	CAG CAC ACC AAG ACC ACT GGC C	356	NM_213557