Involvement of BMPs/Smad Signaling Pathway in Mechanical Response in Osteoblasts

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Key Words

Osteoblasts • Mechanical strain • Bone morphogenetic proteins • Smad • Smurf1

Abstract

Background/Aims: Mechanical strain plays an important role in osteoblasts differentiation and bone formation but the underlying mechanism remains unclear. The aim of this study was to determine whether Bone Morphogenetic Proteins (BMPs)/Smad signaling pathway is involved in mechanical response in osteoblasts. **Methods:** MC3T3-E1 cells were exposed to mechanical strain via a four-point bending system. mRNA levels and protein levels of BMP-2, BMP-4, Smad1, Smad5, Smurf1, and Smurf2 were assessed using RT-PCR and immunoblotting. Protein levels of BMP-2 and BMP-4 in the culture medium were also determined using Enzyme-linked Immunosorbent Assay (ELISA). Pretreatment with Noggin and transfection with Smad4 siRNA were carried out to block the BMPs/Smad signaling pathway and MG132 was used to inhibit the proteasome pathway. **Results:** We found that mechanical strain enhanced alkaline phosphatase (ALP) expression and activated BMPs/Smad signaling pathway.

Mechanical strain induced expression of ALP was attenuated by Noggin and by Smad4 siRNA. The protein levels of Smad1 and Smad5, but not their mRNA levels, were up-regulated by mechanical strain. This finding could be explained by the down-regulation of Smurf1. The protein degradation of Smad might be inhibited by mechanical strain through down-regulation of Smurf1 expression. The addition of MG132 further enhanced the mechanical strain induced activation of Smad proteins and the increased expression of ALP. **Conclusions:** Mechanical strain might promote osteoblasts differentiation through BMPs/Smad signaling pathway. The strain causes a drop in Smurf1 levels, leading to accumulation of Smad proteins and, subsequently, to enhanced BMPs/Smad signaling.

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Introduction

Mechanical strain plays an important role in bone homeostasis. Application of mechanical strain promotes bone formation, whereas lack of strain leads to bone loss

[1]. Osteoblasts, originating from mesenchymal stem cells (MSCs), are not only the bone-forming and bone-remodeling cells, but also are involved in mechanical signal transduction [2]. Osteoblasts and their precursor cells are sensitive to mechanical strain. However, knowledge of the molecular events involved in mechanical signal transduction in osteoblasts is still limited.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)- β superfamily; they are potent osteoblastic differentiation factors playing a pivotal regulatory role in bone formation [3, 4]. The intracellular signaling of BMPs is mediated primarily by Smad proteins. BMPs act through a complex serine-threonine receptor mechanism to activate the signal transduction molecules, Smad proteins. The activated Smad proteins then move into the nucleus to modulate the expression of osteoblasts related genes [5, 6]. Many studies have demonstrated that mechanical strain can influence the expression of BMPs [7-10], indicating the potent regulatory role of BMPs/Smad signaling pathway in mechanical signal transduction in osteoblasts.

As we know, the amount of signaling proteins is regulated at both transcriptional and post-translational levels. Ubiquitin-mediated proteasome proteolytic pathway is an important post-translation regulatory mechanism; this pathway requires three enzymes that participate in a cascade of ubiquitin transfer reactions [11]. Smurf1 is a member of the Hect domain family of E3 ubiquitin ligases and it has been shown to interact with Smad1, Smad5 and Runx2, triggering their ubiquitination and degradation [12-15]. Thus, Smurf1 is a negative regulator of BMPs signaling. The role of Smurf1 *in vivo* has been studied in Smurf1 transgenic mice and Smurf1 deficient mice. The bone formation rates and the osteoblast differentiation of Smurf1 transgenic mice are significantly suppressed [16]; in contrast, Smurf1-deficient mice exhibit an age-dependent increase in bone mass [17]. These data indicate that Smurf1 is an important inhibitor of the osteoblast differentiation and bone formation processes. However, it has never been elucidated whether Smurf1 mediated ubiquitin-proteasome pathway are involved in mechanical signal transduction in osteoblasts.

Considering the important role of BMPs/Smad signaling pathway in osteoblasts differentiation and bone formation, we hypothesized that this signaling pathway is involved in mechanical response in osteoblasts. The purpose of this study was to test this hypothesis by investigating: (1) the effect of mechanical strain on osteoblasts differentiation and BMPs/Smad pathway; (2)

the involvement of BMPs/Smad signaling pathway and Smurf1 mediated ubiquitin-proteasome pathway in mechanical response in osteoblasts.

Materials and Methods

Materials

BMP-2, BMP-4 ELISA kit and Noggin were purchased from R&D System (Minneapolis, MN, USA). MG132 was obtained from Sigma-Aldrich (St. Louis, MO) and prepared as 10mmol/L stock solutions in DMSO. BMP-2, BMP-4, Smad1/5, Smad4, Smurf1 and Smurf2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and phospho-Smad1/5 antibody from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

MC3T3-E1 cells, a mouse monoclonal pre-osteoblastic cell line that has been shown to differentiate into osteoblasts and osteocytes [18, 19], were maintained in alpha minimal essential medium (α -MEM; Invitrogen, San Diego, CA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. The pharmacological agents used to evaluate the potential role of various signaling pathways in mechanical response were purified Noggin (50ng/ml), the proteasome inhibitor MG132 (10 μ M) or DMSO (0.1%) as a solvent control. They were added to cell culture 2 hours prior to mechanical strain application and remained in the culture media throughout the experiment.

Application of mechanical strain to cultured cells

Uniaxial and homogeneous mechanical strain was generated by a specially designed four-point bending device described previously [20]. Cells were seeded at the density of 10⁴/cm² in the cell culture dishes and cultivated until they reached 80% confluence. The medium was then changed to FBS-free medium and the cell cultures were subjected to mechanical strain of 2000 microstrain (μ ϵ) at 0.5 Hz for the indicated time. Unstrained (control) cultures were incubated under the same conditions for the maximum period of mechanical strain application.

RNAi against Smad4

siRNA targeting mouse Smad4 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MC3T3-E1 cells (60%-70% confluence) were transfected with Smad4 siRNA (siSd4) or control siRNA (siCtl) using Lipofectamine2000 (Invitrogen, San Diego, CA, USA), according to the manufacturer's recommendations. Mechanical strain or pharmacological treatment was applied 48 hours after transfection.

Alkaline phosphatase (ALP) activity analysis

After mechanical loading, cells were harvested and lysed by brief sonication in the lysis buffer (10 mmol/L HEPES, 250 mmol/L sucrose, 5 mmol/L Tris-HCl, 0.1% TritonX-100, pH 7.5). ALP activity in the cellular fraction was measured by a

Gene	Primer sequence	Accession number	Expected size (bp)	Annealing temperature (°C)
ALP	5'-CGGGACTGGTACTCGGATAA -3' 5'-ATTCCACGTCGGTTCTGTTC -3'	AB473959.1	156	58
BMP-2	5'-AGCTGCAAGAGACACCCCTTT -3' 5'-CATGCCTTAGGGATTTTGA -3'	NM_007553.2	196	58
BMP-4	5'-ATTGGCTCCCAAGAATCATGG -3' 5'-CGTGATGGAACTCCTCACAGT -3'	NM_007554.2	426	56
Smad1	5'-CTCACATGCCACTGAACGCC -3' 5'-TCCGCATACACCTCTCCTCC -3'	NM_008539.3	546	56
Smad5	5'-TTTGCAGAGCCATCACGAGC -3' 5'-TTTCCAATATGCCGCTAGT -3'	AF063006.1	704	57
Smurf1	5'-CTACCAGCGTTTGGATCTAT -3' 5'-TGTCTCGGGTCTGTAAACT -3'	BC029097.1	89	55
Smurf2	5'-ATGAGCAGGACACACTTACA -3' 5'-CACCAGTCTGAGTATGTAAGA -3'	NM_025481.2	102	58
Tubulin	5'-CAACGTCAAGACGGCCGTGTG -3' 5'-GACAGAGGCAAACGTGAGCACC -3'	NM_009450.2	394	58

Table. 1. Sequences of primer used in this study

Fig. 1. Effect of mechanical strain on mRNA expression of ALP and ALP activity. MC3T3-E1 cells were subjected to mechanical strain for 2 to 24 hours. mRNA levels of ALP (A) and ALP activity (B) significantly increased in the strained cells. Results are shown as mean $\pm$ S.E. of data from three independent experiments. * P <0.05 versus Control (0h).

fluorometric detection kit (Nanjing Jiancheng Biotechnology Co. Ltd, Nanjing, China). ALP activity of each sample was normalized by protein concentration.

RNA isolation and RT-PCR

To investigate the influence of mechanical strain on gene expression, reverse transcription (RT)-polymerase chain reaction (PCR) was performed, as previously described [21]. Briefly, total RNA was extracted from MC3T3-E1 cells with Trizol reagent (Invitrogen, San Diego, CA, USA). A total of 2 μ g RNA was reverse transcribed using the Superscript III Reverse Transcriptase Kit (Invitrogen, San Diego, CA, USA), then the cDNA mixture was subjected to PCR amplification. The conditions for PCR analysis were established so that PCR was stopped in the linear range and the reaction products could be accurately quantified and compared [22]. The primers used in this study are shown in Table 1. The electrophoresis of amplification products was performed on 1% agarose gels. Three independent experiments were carried out to study relative mRNA levels.

Western blot analysis

After mechanical strain, whole-cell or nuclear lysates were obtained as described below. Total cell lysates were prepared in RIPA lysis buffer containing protease and phosphatase inhibitors. Nuclear lysates were prepared according to Ziros et al [23]. Protein concentration was determined according to Bradford's method. Equal amounts of proteins were then separated by SDS-PAGE and electrotransferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes blocked with 5% skim milk were incubated overnight with the primary antibody. After washing, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase. The immunoreactive bands were visualized using enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Optical density of the protein bands was determined with Gel Doc 2000 (Bio-Rad, CA, USA). The expression of tubulin was used as a loading control and data were normalized against those of corresponding tubulin. Results were expressed as relative to control.

Fig. 2. Effect of mechanical strain on BMPs/Smad signaling pathway. MC3T3-E1 cells were mechanically strained for 2 to 24 hours. mRNA levels (A) and the levels of BMP-2 and BMP-4 proteins (B) increased significantly after strain. (C) Mechanical strain had no effect on mRNA levels of Smad1 and Smad5. (D) Protein levels of Smad1 and Smad5 were increased in MC3T3-E1 cells mechanically strained for 4 to 12 hours. p-Smad1 and p-Smad5 accumulated in whole cell (E) or nucleus (F). Results are shown as mean $\pm$ S.E. of data from three independent experiments. * $P < 0.05$ versus Control (0h).

Fig. 3. Pretreatment with Noggin attenuated the effect of mechanical strain. MC3T3-E1 cells were pretreated with purified Noggin (50ng/ml) for 2 hours and then subjected to the strain for 8 hours. (A) Pretreatment with Noggin attenuated the effect of the strain on Smad proteins activation. (B) Noggin reduced strain induced increase in mRNA levels of ALP and in ALP activity. * $P < 0.05$ versus Control or between the indicated groups. Results represent three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) for BMP-2 and BMP-4

BMP-2 and BMP-4 ELISA kits were used to detect BMP-2 and BMP-4 levels in the culture medium. The culture medium was collected after mechanical strain, the samples were placed into microtiter plates coated with BMP-2 or BMP-4 antibody and incubated for 2 hours at room temperature. After washing, horseradish peroxidase conjugated streptavidin was added to the plates to catalyze the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution. The absorbance was measured at 450 nm. The results are presented

as the percentage of activity change, compared to the control.

Statistical analysis

All results are shown as mean $\pm$ S.E. of data from three separate experiments with triplicate samples. The data were tested for normal distribution using Shapiro-Wild test. Differences between the groups were analyzed for statistical significance using one-way ANOVA followed by Duncan's multiple range test. Statistical analysis was performed using SPSS13.0 software. P values < 0.05 were considered statistically significant.

Fig. 4. Transfection with Smad4 siRNA reduced the effect of mechanical strain. MC3T3-E1 cells were transfected with Smad4 siRNA (siSd4) or control siRNA (siCtl); 48 hours after transfection the cells were subjected to mechanical strain for 8 hours. (A) Smad4 mRNA expression and protein levels were reduced. (B) Mechanical strain induced accumulation of p-Smad1 and p-Smad5 in nucleus was reduced after transfection with Smad4 siRNA. (C) Knockdown of Smad4 decreased the effect of mechanical strain on ALP mRNA expression and ALP activity. Results are shown as mean $\pm$ S.E. of data from three independent experiments. * $P < 0.05$, # $P < 0.01$ versus Control or between the indicated groups.

Results

Mechanical strain induces ALP expression

ALP is an early differentiation marker for the osteoblast [24]. To see if mechanical strain modulates osteoblasts differentiation, MC3T3-E1 cells were subjected to mechanical strain for 2 to 24 hours. As shown in Fig. 1A, mRNA levels of ALP increased significantly after 4 hours of strain and returned to basal level at 24 hours. The maximum expression was detected 8 hours after strain initiation. We also wanted to determine whether such up-regulation of ALP mRNA levels would be followed by an increase in ALP activity. We found that ALP activity was also enhanced by mechanical strain, following the time dependent changes of its mRNA levels (Fig. 1B).

BMPs/Smad signaling pathway is activated by mechanical strain

ALP is the target gene of BMPs/Smad signaling pathway [24]. To confirm whether mechanical strain induced expression of ALP was mediated by this pathway, first we examined the effect of mechanical strain on BMPs/Smad signaling pathway. We observed

that mRNA levels of BMP-2 and BMP-4 were significantly increased in cells strained for 4 to 12 hours (Fig. 2A). The increased protein levels of these two molecules, both in cells and in the culture medium, were confirmed by western blot and ELISA analysis (Fig. 2B). Though mechanical strain had no significant effect on mRNA levels of Smad1 and Smad5 (Fig. 2C), the protein levels of these two proteins were significantly increased in cells strained for 4 to 12 hours (Fig. 2D). In addition, a significant increase in activated Smad1 (p-Smad1) and Smad5 (p-Smad5), both in whole cell (Fig. 2E) and in nucleus (Fig. 2F), was observed 4 hours after mechanical strain initiation; this increase peaked at 8h and returned to basal level at 24h. These results suggest that BMPs/Smad signaling pathway is activated by mechanical strain.

Involvement of BMPs/Smad signaling pathway in mechanical strain induced osteoblasts differentiation

Pretreatment with Noggin or transfection with Smad4 siRNA has been reported to inhibit the BMPs/Smad signaling [25, 26]. To confirm further the involvement of BMPs/Smad signaling pathway in

Fig. 5. Effect of mechanical strain on Smurf expression. MC3T3-E1 cells were mechanically strained for 2 to 24 hours. (A) mRNA levels of Smurf1 were down-regulated in MC3T3-E1 cells strained for 4 to 12 hours, mRNA levels of Smurf2 were not affected. (B) Mechanical strain down-regulated protein levels of Smurf1 but had no effect on Smurf2. Results are shown as mean $\pm$ S.E. of data from three independent experiments, * $P < 0.05$ versus Control (0h).

mechanical response, MC3T3-E1 cells were pretreated with Noggin or transfected with Smad4 siRNA (siSd4) before application of mechanical strain, then subjected to strain for 8 hours. Mechanical strain induced phosphorylation of Smad proteins was attenuated by Noggin (Fig. 3A) and p-Smad accumulation in the nucleus was also decreased by Smad4 siRNA (Fig. 4B). These events were followed by the down-regulation of mRNA levels of ALP as well as ALP activity (Fig. 3B, 4C). Our results clearly demonstrate that BMPs/Smad signaling pathway plays an important role in regulating osteoblasts differentiation.

Smurf1, but not Smurf2, is down-regulated by mechanical strain

The results described above show that the protein levels of Smad1 and Smad5 were not consistent with their mRNA levels in mechanically strained MC3T3-E1 cells. Protein levels of Smad1 and Smad5 were up-regulated by mechanical strain, whereas their mRNA expression levels were not affected. This implies that Smad protein degradation might have been suppressed by mechanical strain. It has been reported that Smurf mediates the degradation of Smad proteins in a proteasome dependent manner [13, 14]. We demonstrated that both mRNA levels (Fig. 5A) and protein levels (Fig. 5B) of Smurf1 were significantly decreased at 4 hours and returned to basal level 24 hours after strain initiation, with the minimum expression at 8 hours. However, neither mRNA levels (Fig. 5A) nor protein levels (Fig. 5B) of Smurf2 were affected by mechanical strain at any time during the treatment. These results indicate that the expression of Smurf1 is down-regulated by mechanical strain, suggesting

a potential regulatory role of Smurf1 in osteoblasts' strain response.

Pretreatment with MG132 augments the effect of mechanical strain on Smad activation and ALP expression

The protein degradation of Smad mediated by Smurf1 is dependent on the proteasome pathway. To study the role of this pathway in osteoblasts' strain response, we pretreated MC3T3-E1 cells with a proteasome inhibitor MG132 (10 μ M) or DMSO (0.1%, solvent control) 2 hours before application of mechanical strain, then subjected the cells to mechanical strain for 8 hours. Compared to unstrained (control) cells or cells only mechanically strained, protein levels of Smad and phospho-Smad were both significantly increased (Fig. 6A) in pretreated cells. This was followed by a significant increase in ALP mRNA levels (Fig. 6B) and in ALP activity (Fig. 6C). Thus, our results indicate that the proteasome pathway affects the mechanical strain response in MC3T3-E1 cells by regulating Smad protein degradation.

Discussion

During evolution, bone has optimized its load-bearing role by adapting its architecture and function to mechanical forces. Removal of mechanical loading results in declined bone mass while a suitable dynamic mechanical strain promotes bone formation [27]. However, bones are sensitive to dynamic but not static strain, a static load has no effect on bone remodeling,

Fig. 6. Pretreatment with MG132 augmented the effect of mechanical strain. MC3T3-E1 cells were pretreated with a proteasome inhibitor MG132 (10 μ M) or DMSO (0.1%, solvent control) for 2 hours and then subjected to the strain for 8 hours. (A) Mechanical strain induced increase in protein levels of Smad and p-Smad was further augmented by MG132. (B and C) MG132 increased the effect of mechanical strain on ALP mRNA expression and ALP activity. * $P < 0.05$, # $P < 0.01$ versus Control or between the indicated groups. Results represent three independent experiments.

whereas a similar dynamic load is associated with the increased bone mass [28]. A single period of dynamic strain not only induces the periosteal surface to transform directly from quiescence to active bone formation [29], but also modulates the bone loss caused by calcium insufficiency [30]. Therefore, dynamic mechanical loading is a fundamental physiological factor for regulating structure and function of bones [31]. The ability of bone tissues to adapt to the mechanical environment depends on the bone cells [32]. Osteoblasts are located on the surface of bones and are bone-forming cells; thus, they can be activated by dynamic mechanical strain *in vivo*. ALP is the differentiation marker of osteoblasts and its expression was enhanced in mechanical strained cells in our study, thus, osteoblasts are also sensitive to mechanical stimulus *in vitro*. Osteocytes are derived from osteoblasts and compose over 90–95% of bone cells; they are dispersed throughout the mineralized matrix. It has been confirmed that osteocytes are mechanosensory cells in bones and critical for bone functional adaptation through their extensive communication network [33]. Gap junctions and the ERK signaling pathway are essential components in the osteoblastic response to osteocytes

communicated mechanical signals [34, 35]. Connexin43 is the most abundant gap junction proteins, mechanical stimulation can induce the translocation of connexin43 to the membrane surface and open connexin43 hemichannels in osteocytes and further increase the release of prostaglandin [36]. Furthermore, mechanical stimulation also prevents osteocytes apoptosis by activation of integrin/cytoskeleton/Src/ERK signaling pathway [37] and by releasing nitric oxide [38].

Mechanical stimulation parameters like the strain kind, magnitude, frequency and time course strongly affect cellular reaction. In our experiments, MC3T3-E1 cells were grown in a flexible cell culture system and were subjected to mechanical strain of 2000 μ ε. The cell culture system and the four-point bending system have been described in Tang's study; it can supply uniaxial and homogeneous mechanical strain to the cultured cells [20]. The strain magnitude of 2000 μ ε has been shown to induce osteogenic differentiation of MSCs [39]; our previous research also demonstrates that application of mechanical strain of 2000 μ ε can induce the differentiation of MC3T3-E1 cells (unpublished data). The loading time is another important parameter of mechanical strain, determining

the response of osteoblasts. A strain of moderate duration can induce the differentiation of osteoblasts, while too short or too long exposure might have no effect [40]. In this study, MC3T3-E1 cells were mechanically strained for 2-24 hours but the enhanced ALP expression and Smad proteins' activation were only detected in cells strained for 4-12 hours. Hence, 4-12 hours of strain might be the appropriate time frame for inducing the differentiation of MC3T3-E1 cells.

BMPs play an important role in the regulation of bone formation and remodeling; the action of BMPs is mediated by their receptors and downstream Smad proteins [5, 6]. The role of BMPs/Smad signaling pathway in distraction osteogenesis and fracture nonunions has been reported in several studies [41-44]. However, it is not very clear whether this pathway is involved in osteoblasts' mechanical response. Here, we show that the level of BMP-2 and BMP-4 was increased both in cells and in the culture medium after mechanical strain. Thus, mechanical strain can induce the expression and secretion of BMPs. It has been demonstrated that NF- κ B pathway is involved in pressure-induced BMP-2 expression in endothelial cells [45]. Therefore, mechanical strain might up-regulate BMPs expression through NF- κ B pathway in MC3T3-E1 cells-this merits further investigation. Moreover, we observed an interesting phenomenon: though BMP-2 and BMP-4 amounts in the culture medium were increased 4-12 hours after strain initiation, these elevated levels were not maintained and returned to basal level at 24h. Thus, BMPs secreted to the medium after mechanical strain are not stable. It has been reported that the degradation of BMPs is mediated by BMPase [46]; we could assume that BMPase is synthesized and secreted by MC3T3-E1 cells in the late stage of mechanical strain (12-24h) to initiate the degradation of BMPs. This might contribute to the decrease in BMPs levels in the culture medium. However, further research is needed to confirm this hypothesis. Furthermore, we demonstrate that the phosphorylation and nuclear translocation of Smad1 and Smad5 are enhanced simultaneously. Throughout the duration of mechanical strain the cells were cultured in the FBS-free medium, thus we can exclude the effect of exogenous BMPs contained in FBS on the activation of Smad proteins. Knowing that BMP-2 and BMP-4 binding to their receptors can activate Smad proteins, we could attribute the enhanced activation of Smad proteins to the increased BMPs secretion from the mechanically strained cells. To confirm the involvement of BMPs/Smad signaling pathway in mechanical strain response in osteoblasts, the

Fig. 7. A scheme for signaling pathway involved in mechanical response in osteoblasts.

BMPs antagonist Noggin and Smad4 siRNA were employed. We show that treatment with Noggin abolishes the activation of Smad proteins caused by mechanical strain, and knockdown of Smad4 blocks the nuclear translocation of p-Smad proteins. The mRNA levels and the activity of ALP, up-regulated by mechanical strain, were simultaneously attenuated. Our results clearly demonstrate that BMPs/Smad signaling pathway plays an important role in mechanical signal transduction in osteoblasts.

We also observed that mechanical strain did not affect Smad1 and Smad5 mRNA expression. However, protein levels of Smad1 and Smad5 were elevated by mechanical strain. The transcription of these two proteins' genes was not affected by mechanical strain, but we believe that the protein degradation of Smad1 and Smad5 might have been suppressed. The degradation of Smad proteins is mediated by Smurf in an ubiquitin-proteasome dependent manner [13, 14, 47, 48]. Our results show that the expression of Smurf1, but not of Smurf2, is suppressed by mechanical strain. We show here, for the first time, that environmental effects can alter Smurf1 expression. Mechanical strain treatment can suppress the proteasome pathway mediated protein degradation of Smad1 and Smad5 by down-regulating Smurf1 expression. To block the proteasome pathway, we treated MC3T3-E1 cells with MG132. Mechanical strain induced increase in the levels of Smad and p-Smad proteins, as well as augmented ALP expression, were enhanced further in the presence of MG132. However, MG132 has multiple effects on BMPs/Smad pathway in mechanically strained cells. Firstly, MG132 could inhibit the proteasome pathway

mediated degradation of Smad proteins, and so contribute to the elevated Smad protein levels and enhance the activation of Smad proteins. Secondly, it has been shown that the expression of BMP-2 could be up-regulated by MG132 [49, 50]. Thus, MG132, together with mechanical strain, might up-regulate BMP-2 expression, and this can lead to the increase in Smad activation and ALP expression.

In conclusion, our study demonstrates that mechanical strain can be involved in osteoblasts differentiation through BMPs/Smad signaling pathway. The diminished Smurf1 levels lead to an accumulation of Smad proteins and to subsequently enhanced BMPs/Smad signaling, induced by mechanical strain (Fig. 7).

Abbreviations

BMPs (Bone morphogenetic proteins); ALP (alkaline phosphatase); FBS (Fetal bovine serum); MEM (Minimum essential medium); ELISA (Enzyme-linked immunosorbent assay); RT-PCR (Reverse transcription-polymerase chain reaction).

Acknowledgements

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