ARTICLE
Journal of Cellular Biochemistry 106:7–15 (2009)

Journal of Cellular Biochemistry

Ultrasound Increased BMP-2 Expression Via PI3K, Akt, c-Fos/c-Jun, and AP-1 Pathways in Cultured Osteoblasts

Chun-Han Hou,[1,2] Sheng-Mou Hou,[3*] and Chih-Hsin Tang[4**]

[1]*Institute of Biomedical Engineering, National Taiwan University, Taipei, Taiwan*
[2]*Department of Orthopedic Surgery, National Taiwan University Hospital, Yun-Lin Branch, Yun-Lin County, Taiwan*
[3]*Department of Orthopedic Surgery, National Taiwan University Hospital, Taipei, Taiwan*
[4]*Department of Pharmacology, College of Medicine, China Medical University, Taichung, Taiwan*

ABSTRACT

It has been shown that ultrasound (US) stimulation accelerates fracture healing in the animal models and in clinical studies. Bone morphogenetic protein (BMP) is a crucial mediator in bone formation during fracture healing. Here we found that US stimulation increased BMP-2 expression but not other BMPs. US induced BMP-2 transcription is mediated by AP-1 element but not estrogen receptor response element and GC-rich Sp1 response element. Pretreatment of osteoblasts with phosphatidylinositol 3-kinase (PI3K) inhibitor (Ly294002) and Akt inhibitor inhibited the potentiating action of US; these results were further substantiated by transfecting with the dominant negative mutants of p85 and Akt. US stimulation increased the phosphorylation of p85 subunit of PI3K and serine 473 of Akt. Transfection of osteoblasts with c-Fos and c-Jun antisense oligonucleotide also reduced US-increased BMP-2 expression. US-increased the binding of c-Fos and c-Jun to the AP-1 element on the BMP-2 promoter and the enhancement of AP-1 luciferase activity was inhibited by Ly294002 and Akt inhibitor. Our results suggest that US increased BMP-2 expression in osteoblasts via the PI3K, Akt, c-Fos/c-Jun, and AP-1 signaling pathway. J. Cell. Biochem. 106: 7–15, 2009.

KEY WORDS: ULTRASOUND; AP-1; BMP-2; OSTEOBLASTS; Akt

Fracture healing is a complex physiologic process by the coordinated participation of several cell types. Among all the means to influence fracture healing, ultrasound (US) distinguishes itself by being non-invasive and easy to apply. Low intensity levels are used to accelerate fracture healing and are considered neither thermal nor destructive. It has been shown that low-intensity US accelerates fracture healing in animal models [Duarte, 1983; Wang et al., 1994] and clinical studies [Heckman et al., 1994; Cook et al., 1997].

Bone is a dynamic tissue that remodels in response to mechanical loads from the external environment [Rubin and Lanyon, 1985; Turner et al., 1994]. Whereas the augmentation of fracture healing by US is well documented, the underlying mechanism of the mechanotransduction pathway involved in cellular responses to US is largely unknown. It has been demonstrated that exposure to low-intensity US pulses increases the expression of bone formation genes including: cyclooxygenase-2 (COX-2) and inducible nitric-oxide synthase (iNOS), which are required for mechanically induced bone formation [Reher et al., 2002; Tang et al., 2006]. However, the mechanisms involved in osteoblasts to detect US stress and transduce the signal across the membrane for activating signaling pathways in bone metabolism, such as the induction of COX-2 and iNOS, remain poorly understood.

Bone morphogenetic proteins (BMPs), with more than 20 members, belong to the TGF-β superfamily and were originally identified by their unique ability to induce ectopic cartilage and bone formation in vivo [Urist, 1965; Reddi, 1994]. BMPs play important roles in bone formation and bone cell differentiation by stimulating alkaline phosphatase activity and synthesis of proteoglycan, collagen, fibronectin, and osteocalcin [Reddi, 1994; Sakou

Grant sponsor: Department of Health, Executive Yuan, Taiwan; Grant number: DOH97-PA-1001; Grant sponsor: National Taiwan University Hospital, Yun-Lin Branch; Grant numbers: NTUHYL97.S0012, NTUHYL97.S009, NTUHYL 97.N004.

*Correspondence to: Sheng-Mou Hou, Department of Orthopedic Surgery, National Taiwan University Hospital, Taipei, Taiwan. E-mail: shengmou@ntuh.gov.tw

**Correspondence to: Chih-Hsin Tang, Department of Pharmacology, College of Medicine, China Medical University, No. 91, Hsueh-Shih Road, Taichung, Taiwan. E-mail: chtang@mail.cmu.edu.tw

Received 30 May 2008; Accepted 26 August 2008 • DOI 10.1002/jcb.21934 • 2008 Wiley-Liss, Inc.
Published online 13 November 2008 in Wiley InterScience (www.interscience.wiley.com).

et al., 1999]. It has been shown that BMP-2 and BMP-4 are synthesized by osteoblasts [Centrella et al., 1994]. Among BMP family members, BMP-2 has been extensively studied and demonstrated to play a crucial role in inducing osteoblast differentiation and bone formation during embryonic skeletal development and postnatal bone remodeling [Wozney et al., 1998; Zhao et al., 2002]. After specific receptors binding, BMP-related effects are mediated by different signaling pathways including the Ras/MAPK system, different Smad proteins, Ca^{2+}, cAMP, the Runx/Cbfa1 pathway, and the Wnt/b-catenin system [Lian et al., 2006; Sapkota et al., 2007]. Especially, BMP-2, -6, -7, and -9 are potent to induce osteoblast differentiation and produce a distinct set of molecular fingerprints during osteogenic differentiation [Luu et al., 2007].

Intracellular signals that promote osteoblast differentiation, including those mediated by bioactive radicals such us nitric oxide, prostaglandin, and calcium, may occur in response to cellular homeostatic disturbance induced by US [Reher et al., 2002]. It has been reported that BMP may play an important role in the maturation of osteoblast during fracture healing [Szczesny, 2002]. However, the signaling pathway for US stimulation on BMP-2 expression and bone formation are mostly unknown. Here we found that US stimulation increased BMP-2 expression in osteoblasts in a phosphatidylinositol 3-kinase (PI3K), Akt, c-Fos/c-Jun, and AP-1 dependent pathway.

EXPERIMENTAL PROCEDURES

MATERIALS

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for p85, phosphotyrosine residues (PY20), and phosphor-Akt (Ser473), estrogen receptor α, SP-1, Akt, c-Fos, c-Jun, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-BMP-2 antibody and BMP-2 ELISA kit were purchased from R&D Systems (Minneapolis, MN). Ly294002 and Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((*R*)-2-*O*-methyl-3-*O*-octadecyl-carbonate)) were purchased from Calbiochem (San Diego, CA). The AP-1 luciferase plasmid was purchased from Stratagene (La Jolla, CA). The BMP-2 luciferase plasmid (p-BMP-2-Luc; −2,712 to +165) was gift from Dr. Ming Zhao (Vanderbilt University, TN). The p85 (Δp85; deletion of 35 aa from residues 479–513 of p85) and Akt (Akt K179A) dominant negative mutants were gifts from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, MA). All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURES

The murine primary osteoblastic cells were prepared by the method described previously [Tang et al., 2007a]. The calvaria of fetal murine were dissected from fetal murine, divided into small pieces, and then treated with 0.1% type I collagenase solution for 10 min at 37°C. The next two 20-min sequential collagenase digestions were then pooled and filtered through 70-μm nylon filters (Falcon). The cells were grown on the plastic cell culture dishes in 95% air, 5% CO_2 with α-MEM that was supplemented with 20 mM HEPES and 10% heat-inactivated fetal calf serum, 2 mM-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The characteristics of osteoblasts were confirmed by morphology and the expression of alkaline phosphatase.

ULTRASOUND TREATMENT

Cells (3×10^5 cells/well, six-well plates) were cultured for 24 h and subjected to US treatment. A UV-sterilized unfocused circular transducer (Exogen®; Smith & Nephew, Inc., Memphis, TN) was immersed vertically into each culture well and placed to just contact the surface of the culture medium. The transducer generated a low intensity pulsed ultrasound signal that has been clinically proven to enhance fracture healing [Heckman et al., 1994]. The driving signal consists of a 1.5 MHz sinusoidal ultrasound carrier wave, amplitude modulated with a 1 kHz pulse, and a pulse width of 200 μs (20% duty cycle). The I_{SATA} (Spatial Average Time Average Intensity) was 30 mW/cm^2 with a temporal average power of 117 mW and an effective radiating area (ERA) of 3.88 cm^2. Exposure time was 20 min for all cultures, which is the FDA approved treatment time for bone healing. The distance between the transducer and the cells was approximately 5 mm. Control samples were prepared in the same manner without US exposure. Cells were harvested at 10, 15, 30, and 60 min after US stimulation. For confirmation of the downstream and signaling pathways after US treatment, osteoblasts were pretreated with various inhibitors (DMSO as vehicle) for 30 min before US stimulation was added [Tang et al., 2007b].

WESTERN BLOT ANALYSIS

The cellular lysates were prepared as described previously [Tang et al., 2007a,b]. Proteins were resolved on SDS–PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-mouse antibodies against c-Fos, c-Jun, Akt, or p-Akt (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

mRNA ANALYSIS BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

The method of RT-PCR was prepared as described previously [Tang et al., 2007b]. Total RNA was extracted from osteoblasts using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg of total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified for 33 cycles using two oligonucleotide primers: c-Fos: GAATAACATGGCTGTGCAGC-CAAATGCCGCAA and CGTCAGATCAAGGGAAGCCACAGACATCT c-Jun: GGAAACGACCTTCTATGACGATGCCCTCAA and GAACCCCT-CCTGCTCATCTGTCACGTTCTT.

The primer sequences for BMPs were designed as described previously [van der Horst et al., 2002; Su et al., 2007].

Each PCR cycle was carried out for 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C.

PCR products were then separated electrophoretically in a 2% agarose DNA gel and stained with ethidium bromide.

QUANTITATIVE REAL TIME PCR

The quantitative real time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, CA). One hundred nanograms of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (GAPDH was used as internal control) (Applied Biosystems). qPCR assays were carried out in triplicate on an ABI Prism 7900 sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

OLIGONUCLEOTIDE (ODN) TRANSFECTION

Cells were cultured to confluence; the complete medium was replaced with Opti-MEM (Invitrogen) containing the antisense phosphorothioate oligonucleotides (5 μg/ml) that had been preincubated with 10 μg/ml Lipofectamine 2000 (Invitrogen) for 30 min. The cells were washed after 24 h of incubation at 37°C and washed before the addition of medium containing All antisense ODNs were synthesized and highpressure liquid chromatography-purified by MDBio, Inc. The sequences used are as follows: c-Fos antisense (AS)-ODN, GCGTTGAAGCCCGAGAA and missense (MS)-ODN, GCATTGACGCCAGAGCA; and c-Jun AS-ODN, CGTTTCCATCTTTGCAGT and MS-ODN, ACTGCAAAGATG-GAAACG [Chiu et al., 2007].

SYNTHESIS OF AP-1 DECOY ODN

We used a phosphorothioate double-stranded decoy ODN carrying the AP-1 decoy ODN sequence was 5′-TGTCTGACTCATGTC-3′/3′-ACAGACTGAGTACAG-5′. The mutated (scrambled) form 5′-TTGCCGTACCTGACTTAGCC-3′/3′-AACGGCATGGACTGAATCGG-5′ was used as a control. ODN (5 μM) was mixed with Lipofectamine 2000 (10 μg/ml) for 25 min at room temperature, and the mixture was added to cells in serum-free medium. After 24 h of transient transfection, the cells were used for the following experiments.

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously [Chiu et al., 2007]. DNA immunoprecipitated by anti-c-Fos or anti-c-Jun antibody was purified. The DNA was then extracted with phenol-chloroform. The purified DNA pellet was subjected to PCR. PCR products were then resolved by 1.5% agarose

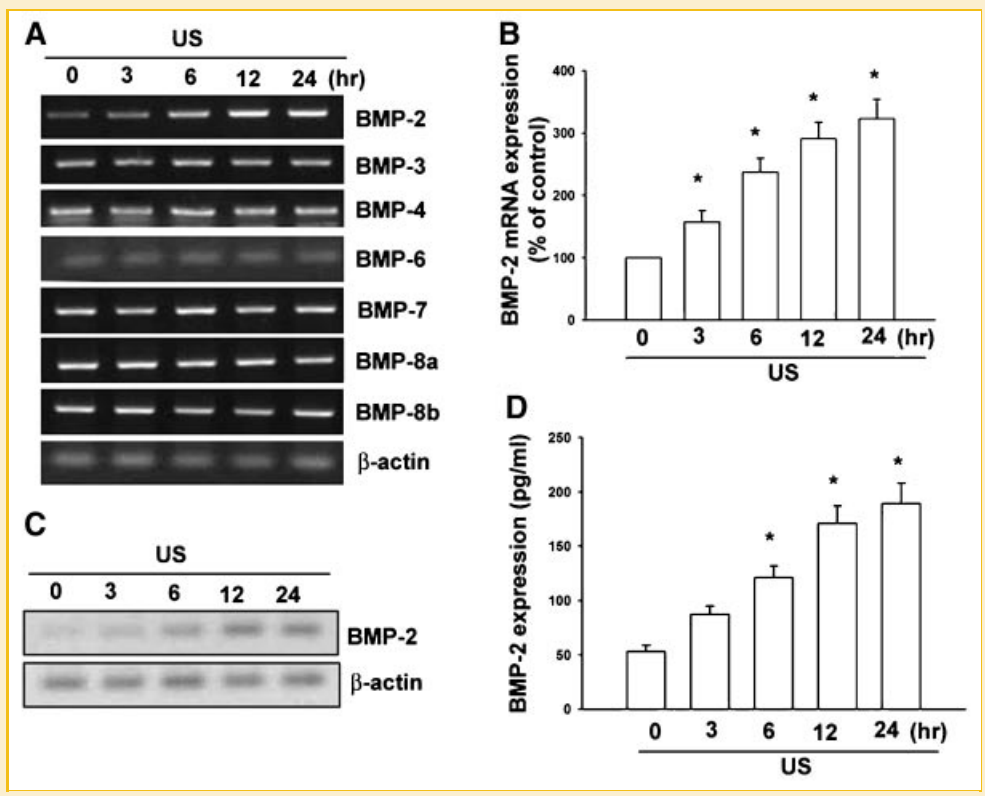

Fig. 1. US stimulation induces BMP-2 expression in osteoblasts. Osteoblasts were exposed to US for 20 min. mRNA levels of BMPs were analyzed by RT-PCR at various time intervals after US stimulation (A). Osteoblasts were exposed to US for 20 min. mRNA levels of BMP-2 were analyzed by qPCR at various time intervals after US stimulation (B). Osteoblasts were exposed to US for 20 min. Protein levels of BMP-2 were analyzed by Western blot (C) and ELISA (D) at various time intervals after US stimulation. Data are presented as mean ± SE. $^*P < 0.05$ as compared with control.

gel electrophoresis and visualized by UV.The primers: 5′-GGGTTGGAACTCCAGACTGT-3′ and 5′-GAAGAGTGAGTGGACCC-CAG-3′ were utilized to amplify across the BMP-2 promoter [Su et al., 2007].

TRANSFECTION AND REPORTER GENE ASSAY

Osteoblasts were co-transfected with 0.8 μg BMP-2 or AP-1-luciferase plasmid or 0.4 μg β-galactosidase expression vector. Cells were grown to 80% confluent in six-well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were then incubated with the indicated agents. After further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of

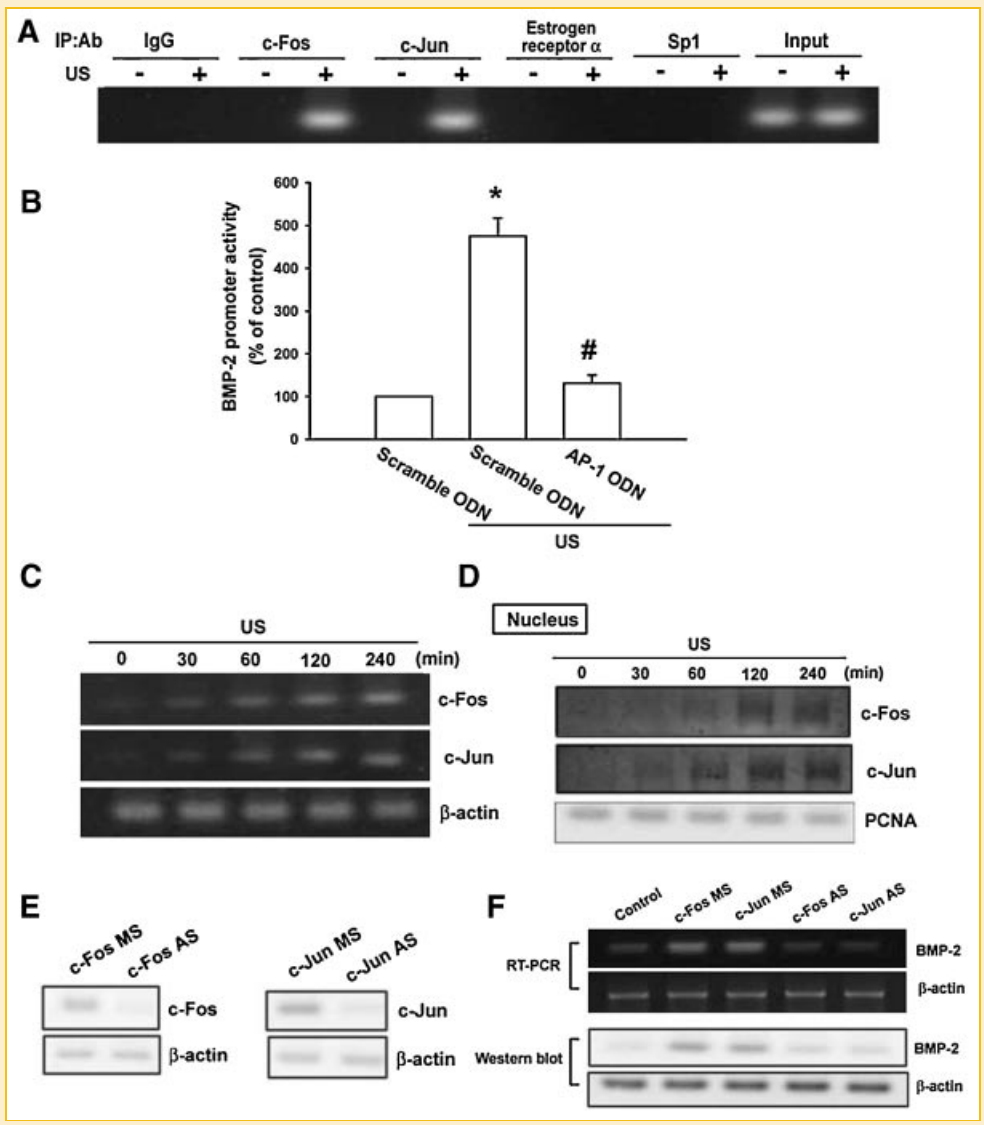

Fig. 2. AP-1 is involved in US-induced BMP-2 production. A: Osteoblasts were treated with US for the 20 min, and ChIP assay was then performed 120 min following US stimulation. Chromatin was immunoprecipitated with c-Fos, c-Jun, estrogen receptor α, SP1 antibodies or IgG control. One percent of the precipitated chromatin was assayed to verify equal loading (Input). B: Cells were cotransfected with BMP-2-Luc and the AP-1 or scramble ODN for 24 h then treated for 20 min with US. Luciferase activity was measured 24 h after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ compared with US-treated group. C: Osteoblasts were exposed to US for 20 min. mRNA levels of c-Fos and c-Jun were analyzed by RT-PCR at various time intervals after US stimulation. D: Osteoblasts were exposed to US for 20 min. The level of nuclear c-Fos and c-Jun was determined by immunoblotting with c-Fos and c-Jun-specific antibodies at various time intervals after US stimulation. E: Cells were transfected with c-Fos or c-Jun antisense-oligonucleotides (AS) or misense-oligonucleotides (MS) for 24 h, and the protein level of c-For or c-Jun was determined by using Western blot analysis. F: Cells were transfected with c-Fos or c-Jun (AS) and (MS) for 24 h followed by stimulation with US, and the mRNA and protein level of BMP-2 were determined by RT-PCR and Western blot analysis 24 h following US stimulation.

protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

STATISTICS

The values given are means ± SEM. The significance of difference between the experimental groups and controls was assessed by Student's *t* test. The difference is significant if the *P*-value is <0.05.

RESULTS

EFFECT OF US STIMULATION ON BMP-2 EXPRESSION IN OSTEOBLASTS

It has been reported that US enhanced the healing of bone fracture [Duarte, 1983; Wang et al., 1994]. BMPs play an important role on bone formation during facture healing [Szczesny, 2002]. We then investigated the effect of US stimulation on the BMP expression in osteoblasts. Osteoblasts were exposed to US for 20 min, and the cell lysates were collected at different time intervals. The results from RT-PCR analysis indicated that US time-dependently increased mRNA levels of BMP-2 but not other BMPs (Fig. 1A). The induction of BMP-2 expression in response to US in osteoblasts cells was also observed using quantitative real-time RT-PCR analysis (Fig. 1B). On the other hand, US stimulation also increased BMP-2 protein expression in a time-dependent manner as determined by Western blotting and BMP-2 ELISA assay, respectively (Fig. 1C,D).

INVOLVEMENT OF AP-1 ELEMENT IN US-INDUCED BMP-2 PRODUCTION

Several consensus sequences, including those for AP-1 element, estrogen receptor response element (ERE) and GC-rich Sp1 response element in the 5′ promoter region of the BMP-2 gene, have been identified as regulatory sequences [Helvering et al., 2000]. To ascertain whether US induces the association of the transcription factors with the BMP-2 promoter, a ChIP assay was employed. Using specific antibodies to various transcription factors, c-Fos and c-Jun, but not estrogen receptor α and SP-1, was found to associate with the BMP-2 promoter after US stimulation in osteoblasts (Fig. 2A). Therefore, the AP-1 element but not ERE or SP-1 plays critical function in US-induced BMP-2 production. We then examined the effect of US on the promoter activities using construct of BMP-2. As shown in Figure 2B, US stimulation increased the promoter activity of BMP-2 (p-BMP-2-Luc). Co-transfection with AP-1 ODN reduced US-induced BMP-2 promoter activity (Fig. 2B). These data are consistent with a significant role for the AP-1 element but not ERE or SP-1 element mediating the US-induced BMP-2 expression. We further examined the activation of AP-1 components c-Fos and c-Jun after US stimulation. Time-dependent increase in the c-Fos and c-Jun mRNA expression in osteoblasts after US stimulation (Fig. 2C). US-activated c-Fos and c-Jun were also evidenced by the accumulation of c-Fos and c-Jun in the nucleus (Fig. 2D). Transfection of cells with c-Fos or c-Jun AS-ODN specifically

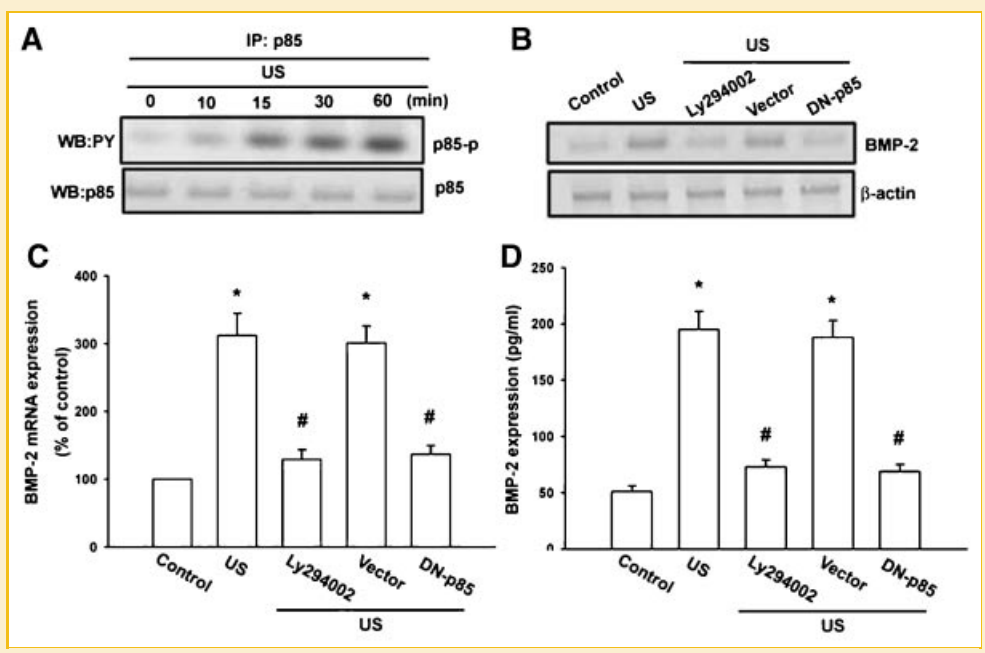

Fig. 3. Involvement of PI3K signaling pathway in response to US stimulation in osteoblast. A: Osteoblasts were stimulated with US for 20 min, and cell lysates were immunoprecipitated (IP) with an antibody specific for p85. Immunoprecipitated proteins were separated by SDS–PAGE and immunoblotted (WB) with anti-phosphotyrosine (PY) at various time intervals after US stimulation. B: Osteoblasts were pretreated with PI3K inhibitor Ly294002 (10 μM) for 30 min or transfected with p85 mutant for 24 h followed by stimulation with US for 20 min, and BMP-2 expression was determined by Western blot (B), qPCR (C) and ELISA (D) 24 h after US. Data are presented as mean ± SE. $^{*}P<0.05$ as compared with control. $^{\#}P<0.05$ compared with US-treated group.

blocked the c-Fos and c-Jun expression, respectively (Fig. 2E). US-induced mRNA and protein expression of BMP-2 were also inhibited by c-Fos and c-Jun AS-ODN but not by MS-ODN (Fig. 2F).

THE SIGNALING PATHWAY OF PI3K AND AKT ARE INVOLVED IN THE POTENTIATING ACTION OF US STIMULATION

It has been reported that PI3K and Akt are mediated the BMP-2 expression in osteoblasts [Ghosh-Choudhury et al., 2007]. We performed Western blot analysis to elucidate the signal transduction pathways involved in the US-induced up-regulation of BMP-2. Exposure of osteoblasts to US for 20 min led to a significant increase of phosphorylation of p85 subunit of PI3K, as assessed by the measurement of phosphotyrosine from immunoprecipitated lysates using p85 (Fig. 3A). To explore whether PI3K is involved in US-induced BMP-2 expression, PI3K inhibitors LY294002 were used. As shown in Figure 3B–D, pretreatment of osteoblasts with LY294002 or transfection with p85 mutant markedly attenuated the US-induced protein and mRNA expression of BMP-2. On the other hand, US stimulation also induced an increase in Akt phosphorylation in a time-dependent manner (Fig. 4A). US-induced BMP-2 expression was greatly reduced by treatment with Akt inhibitor (Fig. 4B–D). In addition, transfection of cells with Akt mutant also antagonized the US-induced BMP-2 expression (Fig. 4B–D). Pretreatment of cells with Ly294002 and Akt inhibitor markedly reduced the US-increased Akt phsophorylation (Fig. 4E). Taken together, these data suggest that the activation of the PI3K/Akt pathway is required for the US-induced increase of BMP-2 in osteoblasts.

US-INDUCED AP-1 ACTIVATION VIA PI3K, AKT, AND C-FOS/C-JUN PATHWAY

To further confirm the AP-1 element involved in the action of US-induced BMP-2 expression, transient transfection was performed using the AP-1 promoter-luciferase constructs. Exposure to US led to a 3.1-fold increase in AP-1 promoter activity in osteoblasts (Fig. 5A). The increase of AP-1 activity by US stimulation was antagonized by Ly294002 and Akt inhibitor (Fig. 5A). In cotransfection experiments, the increase of AP-1 promoter activity by US was inhibited by the dominant-negative mutant of p85 and Akt or c-Fos and c-Jun AS-ODN (Fig. 5B). Next we examined whether PI3K, Akt, c-Fos/c-Jun and AP-1 pathway is involved in

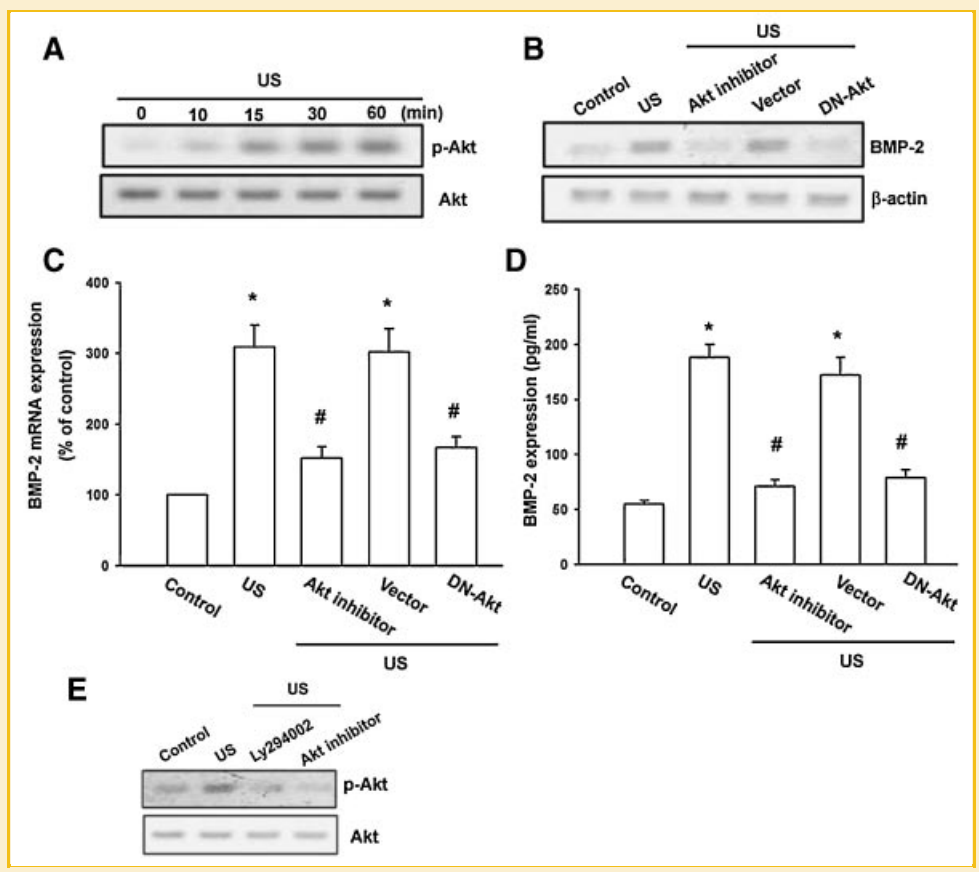

Fig. 4. Akt is involved in the potentiation of BMP-2 expression by US stimulation. A: Osteoblasts were stimulated with US for 20 min, and then Akt phosphorylation was determined at various time intervals after US stimulation. Osteoblasts were pretreated with Akt inhibitor (10 μM) for 30 min or transfected with Akt mutant for 24 h followed by stimulation with US for 20 min, and BMP-2 expression was determined by Western blot (B), qPCR (C) and ELISA (D) 24 h after US. (E) Osteoblasts were pretreated with Ly294002 (10 μM) or Akt inhibitor (10 μM) for 30 min followed by exposure to US for 20 min, and Akt phosphorylation was then determined 60 min after US stimulation. The protein level of Akt is shown for comparison. Data are presented as mean ± SE. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ compared with US-treated group.

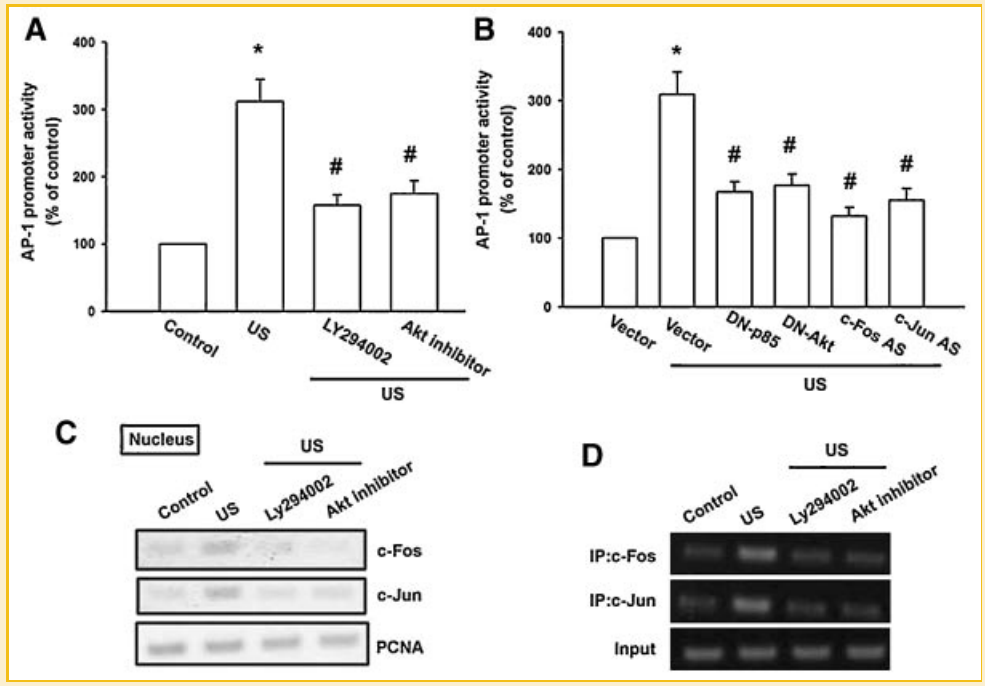

Fig. 5. PI3K and Akt pathway is mediated US-induced AP-1 activity. A: The AP-1 promoter activity was evaluated by transfection with the AP-1-Luc luciferase expression vector. Osteoblasts were pretreated with Ly294002 (10 μM) or Akt inhibitor (10 μM) for 30 min before stimulation with US. B: Cells were cotransfected with AP-1-Luc and the DN mutant of p85 and Akt or antisense-oligonucleotides (AS) of c-Fos and c-Jun then treated for 20 min with US. Luciferase activity was measured 24 h after US stimulation, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ compared with US-treated group. C: Osteoblasts were pretreated with Ly294002 (10 μM) or Akt inhibitor (10 μM) for 30 min followed by exposure to US for 20 min, and the accumulation of c-Fos and c-Jun in nucleus was then determined 240 min after US stimulation. D: Osteoblasts were pretreated with Ly294002 (10 μM) or Akt inhibitor (10 μM) for 30 min followed by exposure to US for 20 min, and ChIP assay was then determined 240 min after US stimulation.

US-induced BMP-2 production. The accumulation of c-Fos and c-Jun in the nucleus was antagonized by Ly294002 and Akt inhibitor (Fig. 5C). Furthermore, the binding of c-Fos and c-Jun to AP-1 element on BMP-2 promoter by US was attenuated by Ly294002 and Akt inhibitor (Fig. 5D). Taken together, these data suggest that the activation of the PI3K, Akt, c-Fos/c-Jun and AP-1 pathway is required for the US-induced increase of BMP-2 in osteoblasts.

DISCUSSION

Bone cells are equipped with mechanisms to sense diverse physical forces and transduce signals for adjustment of their microenvironment [Rubin et al., 1995]. The non-invasive nature of US provides many advantages in practical applications. Although US is clinically used as a treatment for fracture repair, the molecular mechanisms by which US alters cell function or protein synthesis are virtually unknown. In this study, we have discovered a new mechanism by which US can stimulate BMP-2 expression in osteoblasts via sequential activation of PI3K, Akt, c-Fos/c-Jun, and AP-1 pathway. These results represent the first detailed characterization of the US signaling pathway that stimulates BMP-2 expression via the activation of AP-1 element.

A variety of growth factors stimulate the expression of BMP-2 genes via signal transduction pathways that converge to activate AP-1 complex of transcription factors [Palcy et al., 2000; Ghosh-Choudhury et al., 2007]. PI3K and Akt pathways induce the expression of AP-1 transcription factors [Ghosh-Choudhury et al., 2007]. Phosphorylation of the p85 subunit is required for activation of the p110 catalytic subunit of PI3K. Pretreatment of osteoblast with PI3K inhibitor Ly294002 antagonized the increase of BMP-2 expression by US stimulation. This was further confirmed by the result that the dominant-negative mutant of p85 inhibited the enhancement of BMP-2 expression by US stimulation. On the other hand, Akt inhibitor and Akt mutant also reduced the US-increased BMP-2 expression. The cytoplasmic serine kinase Akt was found to be activated by US stimulation in osteoblastic cells. These effects were inhibited by Ly294002 and Akt inhibitor, indicating the involvement of PI3K-dependent Akt activation in US-mediated induction of BMP-2 expression.

The c-Fos and c-Jun genes are the best studied member of the cellular immediate-early genes whose transcription are activated rapidly and transiently within minutes of growth factor stimulation [Yang et al., 2004]. Since the c-Fos gene has been reported to be induced by a uniaxial and biaxial stretcher system [Kletsas et al., 2002; Yang et al., 2004]. In the present study, we also show that US stimulation increased c-Fos and c-Jun expression. Apparently the c-Fos and c-Jun induction become a common mechanoresponsive pattern despite the origin of the strain, be it static or dynamic, or generated by stretching [Kletsas et al., 2002; Ogata, 2003], vibration, or fluid flow. Such phenomenon may leave us to ponder whether a single application of strain above cellular activity is sufficient by

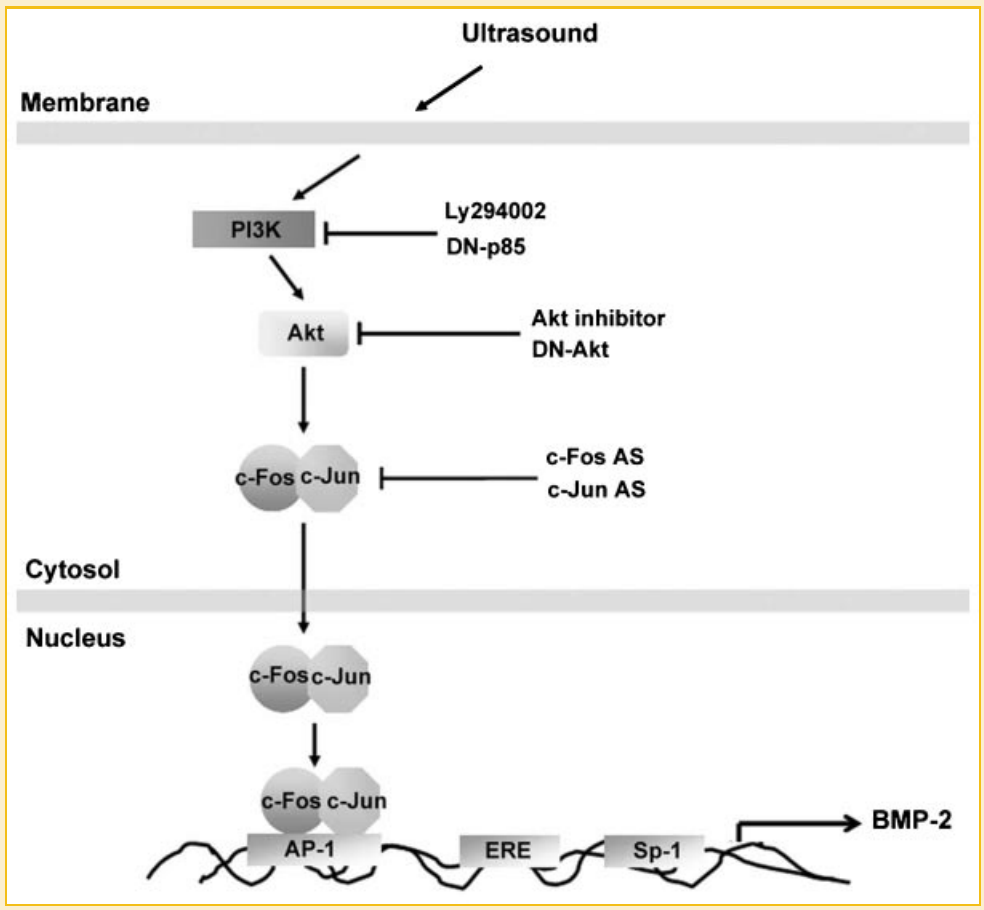

Fig. 6. Schematic diagram of the signaling pathways involved in US-induced BMP-2 expression in osteoblasts. Osteoblasts increases BMP-2 expression by activation of PI3K, Akt, which enhances binding of c-Fos and c-Jun to the AP-1 site, resulting in the transactivation of BMP-2 expression.

osteoblasts to initiate c-Fos and c-Jun related cellular responses. The c-Fos and c-Jun gene have been shown to be dependent either on a protein kinase C- or protein kinase A-mediated signaling pathway via gravitational loading at large or mild magnitude stress, respectively [Nose and Shibanuma, 1994; Fitzgerald et al., 2000]. Transfection of cells with c-Fos and c-Jun AS-ODN but not MS-ODN reduced the US-induced BMP-2 expression. US stimulation also increased the accumulation of c-Fos and c-Jun in nucleus. The binding of c-Fos and c-Jun to AP-1 element and the nuclear accumulation of c-Fos and c-Jun was attenuated by Ly294002 and Akt inhibitor, indicating that PI3K, Akt and c-Fos/c-Jun pathways are very important to mediate the action of US stimulation in osteoblasts.

During osteoblastic differentiation, BMP-2 mRNA is induced, and maintains the sustained phenotype of mature osteoblasts [Anderson et al., 2000]. Previous studies have indicated that the BMP-2 gene regulation during limb morphogenesis and osteoblast differentiation may involve multiple mechanisms and signaling pathways, such as ER, prostaglandin E_2, retinoic acid, Hoxa13, Gli2/3, interferon, and interleukins [Garrett et al., 2003; Zhao et al., 2006]. There are several binding sites for a number of transcription factors including ERE, SP-1 and AP-1 in the 5′ region of the BMP-2 gene [Zhao et al., 2006]. Our data on BMP-2 promoter analysis suggest that the enhancement of BMP-2 transcription induced by US is mediated by c-Fos/c-Jun but not ER and SP-1 through its binding site (AP-1) in the promoter. Using transient transfection with AP-1-luciferase as an indicator of AP-1 activity, we also found that US increased AP-1 activity in cultured osteoblasts. PI3K and Akt inhibitor also reduced the US-induced AP-1 promoter activity. Furthermore, PI3K and Akt mutant or c-Fos and c-Jun AS antagonized the US-mediated AP-1 promoter activity. These results indicate that US might act through the PI3K, Akt, c-Fos/c-Jun, and AP-1 pathway to induce BMP-2 expression in osteoblasts.

In conclusion, the signaling pathway involved in US-induced BMP-2 expression in osteoblasts has been explored. US increases BMP-2 expression by activation of PI3K and Akt, which enhances binding of c-Fos and c-Jun to the AP-1 site, resulting in the transactivation of BMP-2 production (Fig. 6).

ACKNOWLEDGMENTS

This work was supported by grants from the Department of Health, Executive Yuan, Taiwan (DOH97-PA-1001) and National Taiwan University Hospital, Yun-Lin Branch (NTUHYL97.S0012; NTUHYL97.S009 and NTUHYL 97.N004). We thank Dr. W.M. Fu for providing p85 and Akt mutant. We thank Dr. M. Zhao for providing BMP-2 luciferase plasmids. We also thank Smith & Nephew

Company (Brooks Road, Memphis, TN, USA) for providing six-well ultrasound devices.

REFERENCES

Anderson HC, Hodges PT, Aguilera XM, Missana L, Moylan PE. 2000. Bone morphogenetic protein (BMP) localization in developing human and rat growth plate, metaphysis, epiphysis, and articular cartilage. J Histochem Cytochem 48:1493–1502.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL. 1994. Transforming growth factor-beta gene family members and bone. Endocr Rev 15:27–39.

Chiu YC, Yang RS, Hsieh KH, Fong YC, Way TD, Lee TS, Wu HC, Fu WM, Tang CH. 2007. Stromal cell-derived factor-1 induces matrix metalloprotease-13 expression in human chondrocytes. Mol Pharmacol 72:695–703.

Cook SD, Ryaby JP, McCabe J, Frey JJ, Heckman JD, Kristiansen TK. 1997. Acceleration of tibia and distal radius fracture healing in patients who smoke. Clin Orthop Relat Res 337:198–207.

Duarte LR. 1983. The stimulation of bone growth by ultrasound. Arch Orthop Trauma Surg 101:153–159.

Fitzgerald J, Dietz TJ, Hughes-Fulford M. 2000. Prostaglandin E2-induced up-regulation of c-fos messenger ribonucleic acid is primarily mediated by 30,50-cyclic adenosine monophosphate in MC3T3-E1 osteoblasts. Endocrinology 141:291–298.

Garrett IR, Chen D, Gutierrez G, Zhao M, Escobedo A, Rossini G, Harris SE, Gallwitz W, Kim KB, Hu S, Crews CM, Mundy GR. 2003. Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. J Clin Invest 111:1771–1782.

Ghosh-Choudhury N, Mandal CC, Choudhury GG. 2007. Statin-induced Ras activation integrates the phosphatidylinositol 3-kinase signal to Akt and MAPK for bone morphogenetic protein-2 expression in osteoblast differentiation. J Biol Chem 282:4983–4993.

Heckman JD, Ryaby JP, McCabe J, Freym JJ, Kilcoyne RF. 1994. Acceleration of tibial fracture-healing by non-invasive, low-intensity pulsed ultrasound. J Bone Joint Surg 76:26–34.

Helvering LM, Sharp RL, Ou X, Geiser AG. 2000. Regulation of the promoters for the human bone morphogenetic protein 2 and 4 genes. Gene 256:123–138.

Kletsas D, Basdra EK, Papavassiliou AG. 2002. Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. J Cell Physiol 190:313–321.

Lian JB, Stein GS, Javed A. 2006. Networks and hubs for the transcriptional control of osteoblastogenesis. Rev Endocr Metab Disord 7:1–16.

Luu HH, Song WX, Luo X, Manning D, Luo J, Deng ZL, Sharff KA, Montag AG, Haydon RC, He TC. 2007. Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells. J Orthop Res 25:665–677.

Nose K, Shibanuma M. 1994. Induction of early response genes by hypergravity in cultured mouse osteoblastic cells (MC3T3-E1). Exp Cell Res 211:168–170.

Ogata T. 2003. Increase in epidermal growth factor receptor protein induced in osteoblastic cells after exposure to flow of culture media. Am J Physiol Cell Physiol 285:C425–C432.

Palcy S, Bolivar I, Goltzman D. 2000. Role of activator protein 1 transcriptional activity in the regulation of gene expression by transforming growth factor beta1 and bone morphogenetic protein 2 in ROS 17/2.8 osteoblast-like cells. J Bone Miner Res 15:2352–2361.

Reddi AH. 1994. Bone and cartilage differentiation. Curr Opin Genet Dev 4:737–744.

Reher P, Harris M, Whiteman M, Hai HK, Meghji S. 2002. Ultrasound stimulates nitric oxide and prostaglandin E2 production by human osteoblasts. Bone 31:236–241.

Rubin CT, Lanyon LE. 1985. Regulation of bone mass by mechanical strain magnitude. Calcif Tissue Int 37:411–417.

Rubin CT, Gross TS, McLeod KJ, Bain SD. 1995. Morphologic stages in lamellar bone formation stimulated by a potent mechanical stimulus. J Bone Miner Res 10:488–495.

Sakou T, Onishi T, Yamamoto T, Nagamine T, Sampath T, Ten Dijke P. 1999. Localization of Smads, the TGF-beta family intracellular signaling components during endochondral ossification. J Bone Miner Res 14:1145–1152.

Sapkota G, Alarcon C, Spagnoli FM, Brivanlou AH, Massagué J. 2007. BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25:441–454.

Su JL, Yang CY, Zhao CY, Kuo ML, Yen ML. 2007. Forkhead proteins are critical for bone morphogenetic protein-2 regulation and anti-tumor activity of resveratrol. J Biol Chem 282:19385–19398.

Szczesny G. 2002. Molecular aspects of bone healing and remodeling. Pol J Pathol 53:145–153.

Tang CH, Yang RS, Huang TH, Lu DY, Chuang WJ, Huang TF, Fu WM. 2006. Ultrasound stimulates cyclooxygenase-2 expression and increases bone formation through integrin, focal adhesion kinase, phosphatidylinositol 3-kinase, and Akt pathway in osteoblasts. Mol Pharmacol 69:2047–2057.

Tang CH, Hsu TL, Lin WW, Lai MZ, Yang RS, Hsieh SL, Fu WM. 2007a. Attenuation of bone mass and increase of osteoclast formation in decoy receptor 3 transgenic mice. J Biol Chem 282:2346–2354.

Tang CH, Lu DY, Tan TW, Fu WM, Yang RS. 2007b. Ultrasound induces hypoxia-inducible factor-1 activation and inducible nitric-oxide synthase expression through the integrin/integrin-linked kinase/Akt/mammalian target of rapamycin pathway in osteoblasts. J Biol Chem 282:25406–25415.

Turner CH, Forwood MR, Otter MW. 1994. Mechanotransduction in bone: Do bone cells act as sensors of fluid flow? FASEB J 8:875–878.

Urist MR. 1965. Bone: Formation by autoinduction. Science 150:893–899.

van der Horst G, van Bezooijen RL, Deckers MM, Hoogendam J, Visser A, Lowik CW, Karperien M. 2002. Differentiation of murine preosteoblastic KS483 cells depends on autocrine bone morphogenetic protein signaling during all phases of osteoblast formation. Bone 31:661–669.

Wang SJ, Lewallen DG, Bolander ME, Chao EY, Ilstrup DM, Greenleaf JF. 1994. Low intensity ultrasound treatment increases strength in a rat femoral fracture model. J Orthop Res 12:40–47.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. 1998. Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.

Yang CM, Chien CS, Yao CC, Hsiao LD, Huang YC, Wu CB. 2004. Mechanical strain induces collagenase-3 (MMP-13) expression in MC3T3-E1 osteoblastic cells. J Biol Chem 279:22158–22165.

Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D. 2002. Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. J Cell Biol 157:1049–1060.

Zhao M, Qiao M, Harris SE, Chen D, Oyajobi BQ, Mundy GR. 2006. The zinc finger transcription factor Gli2 mediates bone morphogenetic protein 2 expression in osteoblasts in response to hedgehog signaling. Mol Cell Biol 26:6197–6208.