

Activation of extracellular signal-regulated kinase (ERK) and p38 kinase in shock wave-promoted bone formation of segmental defect in rats

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Abstract

Extracorporeal shock waves (ESW) have recently been used in bone repair. Extracellular signal-regulated kinase (ERK) and p38 kinase are found to act as important mediators for osteogenic factor and mechanical-stimulated proliferation and differentiation of bone-forming cells. A previous study reported that ESW promoted healing of segmental defects in rats by inducing bone morphogenetic proteins (Bone 32 (2003) 387–396) and stimulating osteogenic differentiation of mesenchymal stem cells. In this study, we found that ERK and p38 activation was involved in ESW-augmented bone regeneration of segmental defects. ESW treatment (0.16 mJ/mm², 1 Hz, 500 impulses) rapidly promoted [³H]-thymidine uptake in 1 day and progressively increased alkaline phosphatase activity, collagen I, II, and osteocalcin synthesis in callus organ culture within 14 days after treatment. Results of [³²P]-phosphotransferase activity assay showed that ERK and p38 in calluses were rapidly activated 1 day and 7 days after ESW treatment, respectively. Histological observation showed that segmental defects subjected to ESW treatment underwent typical bone formation (mesenchymal cell aggregation, hypertrophic cartilage, and endochondral/intramembrane ossification). Intensive bone formation coincided with evident expression of phosphorylated ERK and p38. Moreover, expression of phosphorylated ERK persisted in mesenchymal, chondral, and osteoblastic cells at newly developed bone and cartilage, and the expression of activated p38 was evident on chondral cells located at hypertrophic cartilage. Our findings suggest that mitogen-activated protein kinases (MAPK) regulate the stimulation of biophysical ESW, triggering mitogenic and osteogenic responses in the defects. ERK phosphorylation is active throughout the period of ESW-induced bone regeneration. p38 activation most likely plays an important role in signaling cartilage formation in callus.

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Introduction

Bone cells respond to mechanical stimulation via mechanoreceptor and convert biophysical stimulation into biochemical signals that alter gene expression and cellular adaptation [1]. Physical stimulation of fractures, which can elicit a proliferative adaptive modeling response for bone

repair, is a noninvasive strategy that includes mechanical distraction, ultrasound, and electromagnetic fields [2–4].

Mitogen-activated protein kinases (MAPK) have been reported to regulate cell growth, differentiation, and apoptosis [5]. The MAPK also act as important mediators of cell response to various extracellular stimuli [6–10]. Three subfamilies of MAPK, extracellular signal-regulated kinase (ERK), p38 kinase (p38) and c-Jun N-terminal kinase (JNK), have been identified in mammalian cells [11,12]. ERK has been found to be activated in the growth factor stimulation of osteoblast growth [13–16]. It has been suggested that this molecule also acts as a proliferative mediator for mechanical strain, fluid flow, and hypergravity

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augmentation of bone cell growth [17–19]. p38 phosphorylation has been reported to regulate chondrogenesis in mesenchyme and osteoblastic cell differentiation [20,21].

Extracorporeal shock waves (ESW), which release pulsed acoustic energy and pressure, have been used as a noninvasive biophysical strategy to accelerate healing of nonunion [22,23]. Animal studies have shown that segmental femoral defects subjected to ESW treatment undergo intense osteochondral cell growth, leading to the healing of these defects [24]. While ESW treatment has been found to have these benefits, we have not determined the molecular mechanism by which ESW promote osteogenic and chondrogenic cell proliferation in the defects. One of our recent studies has shown that ERK mediates ESW promotion of mesenchymal stem cell differentiation into osteogenic lineage [25]. The findings in that study suggest that physical ESW stimulation may provoke intracellular signaling transduction, which in turn activates mitogenic and osteogenic activities of bone cells, leading us to hypothesize that ESW treatment promotes bone regeneration by increasing MAPK activity in bone tissue.

The present study investigates the cell proliferation and bone matrix protein synthesis in the callus organ culture of segmental femoral defect after exposure to ESW treatment in an effort to clarify the spatial and temporal expression of activated ERK and p38 in the ESW promotion of bone regeneration.

Materials and methods

Segmental defect model

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. Three-month-old Sprague–Dawley rats (National Experimental Animals Production Center, Taipei, Taiwan) were caged in pairs and maintained on rodent chow and water ad libitum. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Nembutal® sodium, Abbott Laboratories, IL, USA). Segmental femoral defect in rats was created as previously described [26]. Briefly, after stripping off the periosteum, a four-hole AO/ASIF mini-plate was positioned on the anterolateral femoral shaft. Proximal and distal holes were drilled and tapped with 1.5-mm bicortical screws. A 6-mm long, full thickness disk of cortical and cancellous bone was resected from the middle of the diaphysis with a saline-cooled burr. The fixation plate was stabilized using a 0.45-mm Kirschner wire engaged with the trabecular bone in the proximal femoral diaphysis. The wound was closed in layers. Chloramphenicol (10 mg/kg, intraperitoneal injection) and analgesia (0.02 mg/kg buprenorphine, subcutaneous injection; Reckitt and Colman Pharmaceutical Inc., Richmond, VA, USA) were administered for 2

days. Animals were allowed unrestricted postoperative activity and weight bearing as tolerated. Wounds were topically treated with povidone iodine solution for 4 days and healed without infection. Animals ambulated freely within 5 days postoperatively. Body weights were measured weekly. All animals survived and gained weight weekly.

ESW treatment

Each rat with the segmental defect was re-anesthetized 8 weeks postoperatively and placed in a supine position with all four limbs stabilized in extension. To avoid interference by the anterolateral mini-plate, ESW were applied medially. A single ESW treatment (0.16 mJ/mm², energy flux density, 1 Hz, 500 impulses; Ossatron® HMT High Medical Technologies GmbH, Kreuzlingen, Switzerland) was applied to the fracture site for 10 min using a C-arm image intensifier and the treatment device's control guide [24]. The ultrasound transmission gel (Pharmaceutical Innovations Inc, New Jersey, USA) was used as contact medium between the ESW apparatus and skin.

Experimental design

One hundred and twenty-eight rats with segmental defects were randomly divided into ESW and control groups. Sixty-four rats were treated with ESW. Rats were killed using an overdose of intraperitoneal pentobarbital sodium 0 day ($n = 8$), 1 day ($n = 8$), 3 days ($n = 8$), 7 days ($n = 8$), 14 days ($n = 8$), 28 days ($n = 8$), 42 days ($n = 8$), 56 days ($n = 8$) after ESW treatment. The remaining 64 rats with a segmental defect received no ESW treatment and were used as controls. Eight rats were killed at the same times as those in the ESW group. Five femurs were dissected to harvest calluses, and three were to assess histologically. Calluses were harvested by dissection, cleaned of surrounding tissue and cut into two portions. One portion of the callus was used for organ culture, and one portion was snap-frozen in liquid nitrogen and stored at -70°C until protein extraction.

Cell proliferation in callus organ culture

A 1-mm slice of callus was harvested by transverse incision with two sharply parallel No. 20 scalpel blades, after careful exposure of the segmental defect site. Each callus specimen was separately cultured in 1% agarose-DMEM pre-layered medium and covered with culture medium containing DMEM, 10^{-8} M dexamethasone, 10^{-2} M β -glycerophosphate, 50 $\mu\text{g/ml}$ ascorbic acid, 1 μM proline, 1% antibiotics-antimycotics, and 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA) [27]. The specimens were cultured for 48 h in a humidified incubator with 5% CO_2 at 37°C . Cell prolifer-

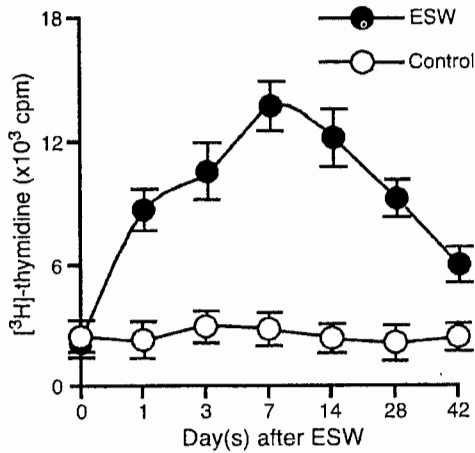


Fig. 1. ESW promoted cell proliferation of calluses. Segmental femoral defects were subjected to ESW treatment (0.16 mJ/mm² energy flux density, 1 Hz, 500 impulses). Calluses were harvested and cultured in organ culture medium. Cell proliferation of calluses was measured using [³H]-thymidine uptake. Results were presented with the mean values and standard errors calculated from experiments with five rats.

ation in the callus was determined by nuclear [³H]-thymidine uptake. Calluses were cultured for 24 h before 1 μCi [³H]-thymidine/well (Amersham-Life Science, Aylesbury, England) was added for a further 24-h of culturing. At the

end of culture period, calluses were washed twice with DMEM and processed to determine [³H]-thymidine uptake using a liquid scintillation analyzer (Tri-Crab 2100TR, Packard Inc, USA). Cultured medium was collected and stored at -70°C till ELISA determination. The [³H]-thymidine uptake of each callus was normalized with total protein of the callus slice.

Alkaline phosphatase activity

Aliquots of cultured supernatants were incubated with substrate buffer containing 50 mM glycine, 1 mM magnesium chloride, (pH 10.5) and 2.5 mM *p*-nitrophenyl phosphate (Sigma Inc. St. Louis, MO, USA). The reactions were incubated at 37°C for 30 min, and then stopped using 0.1 ml of 1 N sodium hydroxide. Results were read at OD_{405 nm} by a microplate reader. Alkaline phosphatase activity in each sample was normalized with protein concentration [28].

Collagen I, collagen II, and osteocalcin content measurement

Collagen I, collagen II, and osteocalcin levels in cultured supernatants released by callus organ cultures were determined using rat osteocalcin (Biomedical Technologies Inc.,

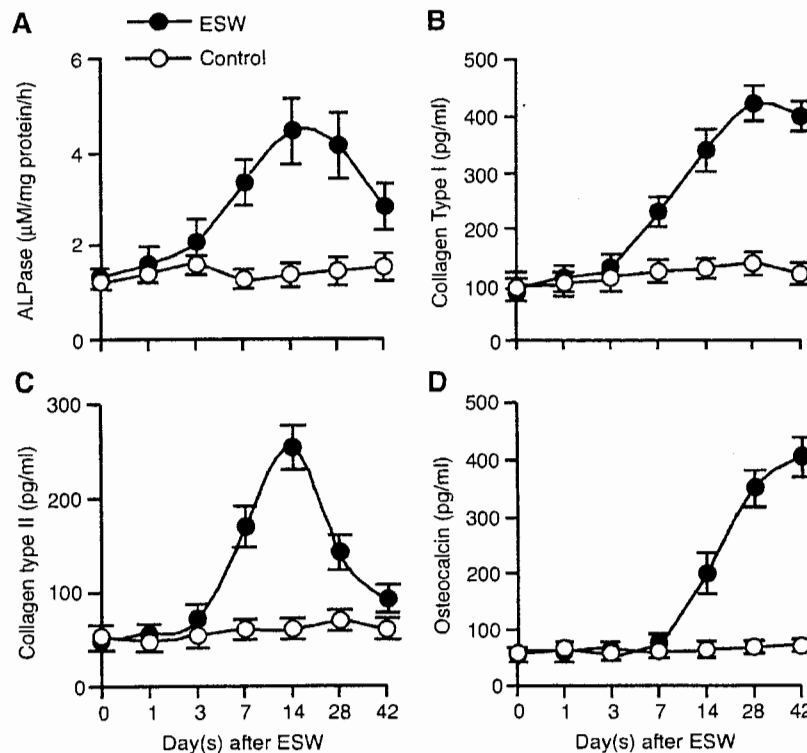


Fig. 2. Effect of ESW treatment on bone matrix protein production. ESW promoted (A) alkaline phosphatase activity, (B) collagen I, and (C) collagen II in 7 days. (D) ESW stimulated osteocalcin production in 14 days. Calluses were cultured in organ culture system. Cultured supernatants were subjected to ELISA assays. Results were presented with the mean values and standard errors calculated from experiments with five rats.

Stoughton, MA, USA), collagen I and collagen II ELISA kits (Chondrex Inc., Redmond, WA, USA) according to manufacturers' instructions, respectively. The collagen I, collagen II and osteocalcin levels were calculated using standard solutions prepared in parallel and normalized with total protein of callus, respectively.

Callus homogenate

Each callus was ground with a mortar and pestle in liquid nitrogen, lysed with ice-cold PBS containing 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 100 µg/ml PMSF, and 30 µg/ml aprotinin, and homogenized by ultrasonication [29]. Total protein concentration in each callus homogenate

was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratory, USA).

ERK and p38 activity

ERK and p38 phosphotransferase activities of calluses were measured using a MAP kinase assay kit (Transduction Biotechnology, NY, USA) according to manufacturer's instructions [6]. Callus homogenates were incubated with anti-ERK and anti-p38 antibodies (Upstate Biotechnology, NY, USA) for 1 h at 4°C. After incubation, the immune complexes were precipitated with protein A (Sigma Inc, St. Louis, MO, USA). The ERK- and p38-immunoprecipitates (10 µg) were subjected to incubation with MAP phosphor-

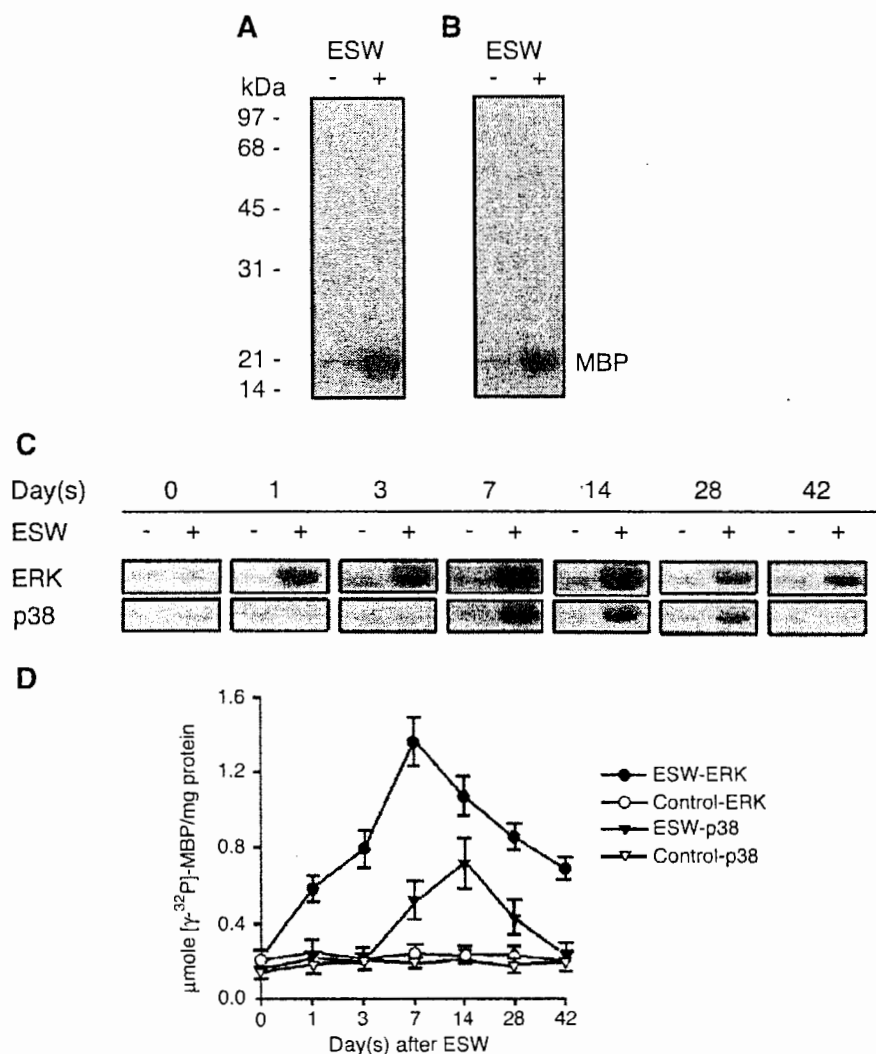


Fig. 3. Effect of ESW treatment on ERK and p38 phosphorylation in calluses. (A) ESW treatment activated (A) ERK and (B) p38 as implicated by increasing phosphorylated MBP band intensities. The 21-kDa bands corresponded to MBP. ERK and p38 immunoprecipitates were subjected to phosphotransferase activity assay using MBP as a substrate in the presence of [γ - 32 P]-ATP. (C) Kinetic autoradiographs of ERK and p38 phosphorylation in calluses with or without ESW treatment as demonstrated by phosphorylated MBP. ERK and p38 immunoprecipitates were subjected to phosphotransferase activity assay 0, 1, 3, 7, 14, 28 and 42 days after ESW treatment. (D) ESW treatment rapidly increased ERK activation in 1 day and progressive elevated p38 activation in 7 days. Results were presented with the mean values and standard errors calculated from experiments with five rats.

ylation buffer containing 20 mM ADB, 20 mM MOPS (pH 7.2), 25 mM β -glycerophosphate, 2 mg/ml myelin basic protein (MBP), 15 mM $MgCl_2$, 5 μ M protein kinase inhibitor, and 100 μ Ci [γ - ^{32}P]-ATP (Amersham Bioscience, Buckinghamshire, England) for 30 min at 32°C. Aliquots of reaction mixtures were spotted onto the P81 phosphocellulose paper and processed for liquid scintillation counting. The ERK and p38 activities were determined the phosphorylation of MBP in the presence of [γ - ^{32}P]-ATP and ERK and p38-immunoprecipitates and were expressed as μ mole [γ - ^{32}P]-MBP/mg protein/h. The reaction mixtures were

further added with Laemmli buffer and resolved by SDS-PAGE. The gels were dried and radioactive bands were visualized using Kodak Bio-Max films with a intensify screen at $-70^\circ C$ for 16 h.

Immunohistochemistry

Femurs were fixed in 4% PBS-buffered paraformaldehyde for 48 h and decalcified in PBS-buffered 10% EDTA. Decalcified tissues were embedded in paraffin. Bone specimens were longitudinally cut into 5- μ m sections and trans-

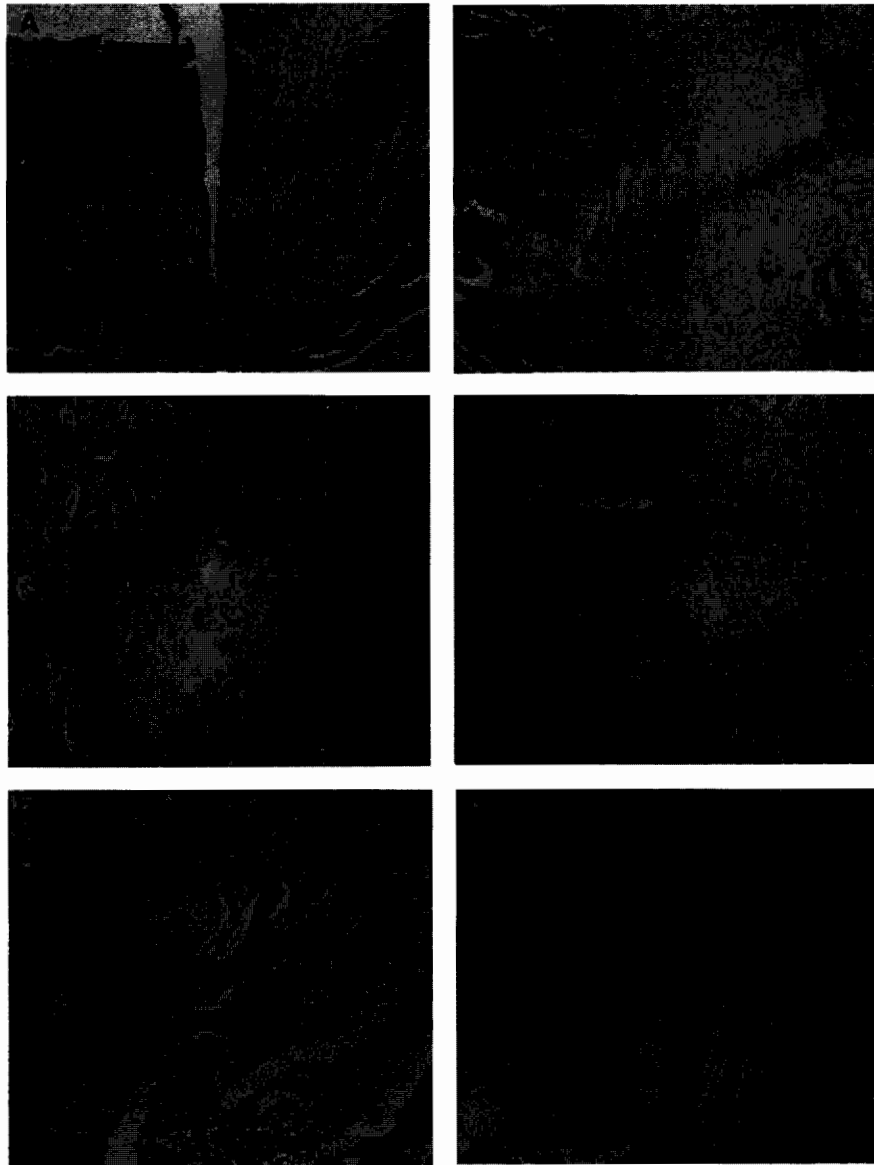


Fig. 4. Representative histological photographs of segment defect after exposure ESW treatment. (A) Fibrosis tissue in segmental defect underwent mesenchymal cell aggregation in 7 days. (B) Fibrous tissue filled the defect in control groups. (C) Twenty-eight days fate treatment, newly formed hypertrophic cartilage bridged the defects. (D) In control sections, the defects filled with fibrous tissue. (E) Fifty-six days following ESW, the defect was surrounded by hard callus. (F) The defects in control group were filled with fibrous tissue and skeletal muscle that had collapsed into the defects. Specimens were stained by conventional hematoxylin-eosin. Specimens were observed in magnification = 200 \times . Cb: cortical bone; Fs: fibrous tissue; Ms: muscle trissue; Cg: cartilage; Wb: woven bone; Bm: bone marrow.

ferred to poly-lysine-coated slides for conventional hematoxylin–eosin staining. Immunoreactivity in specimens was demonstrated using horseradish peroxidase (HRP)-3', 3'-diaminobenzidine (DAB) cell and tissue staining kit (R&D Systems, Inc. Minneapolis, MN, USA), in accordance with manufacturer's instructions. Monoclonal antibodies used for immunohistochemistry were anti-ERK, anti-p38, anti-phosphorylated ERK, anti-phosphorylated p38 (Santa Cruz Biotechnology Inc, CA, USA). Sections were further incubated with biotinylated anti-mouse IgG and then incubated with streptavidin conjugated to HRP. Immunoreactivity was determined by incubating the sections in a chromogen solution containing DAB and 0.1% hydrogen peroxide in

the dark, followed by counterstaining with hematoxylin. Dehydrate sections were mounted with mounting medium. Those without primary antibodies were enrolled as negative controls for the immunostaining.

Histomorphometry

Six regions within the segmental defects from three sections obtained from three rats were studied. For immunostaining quantification, sections were analyzed using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Gottingen, Germany). Areas (3 mm^2) containing positive immunostained cells were analyzed. Three random images of 0.75

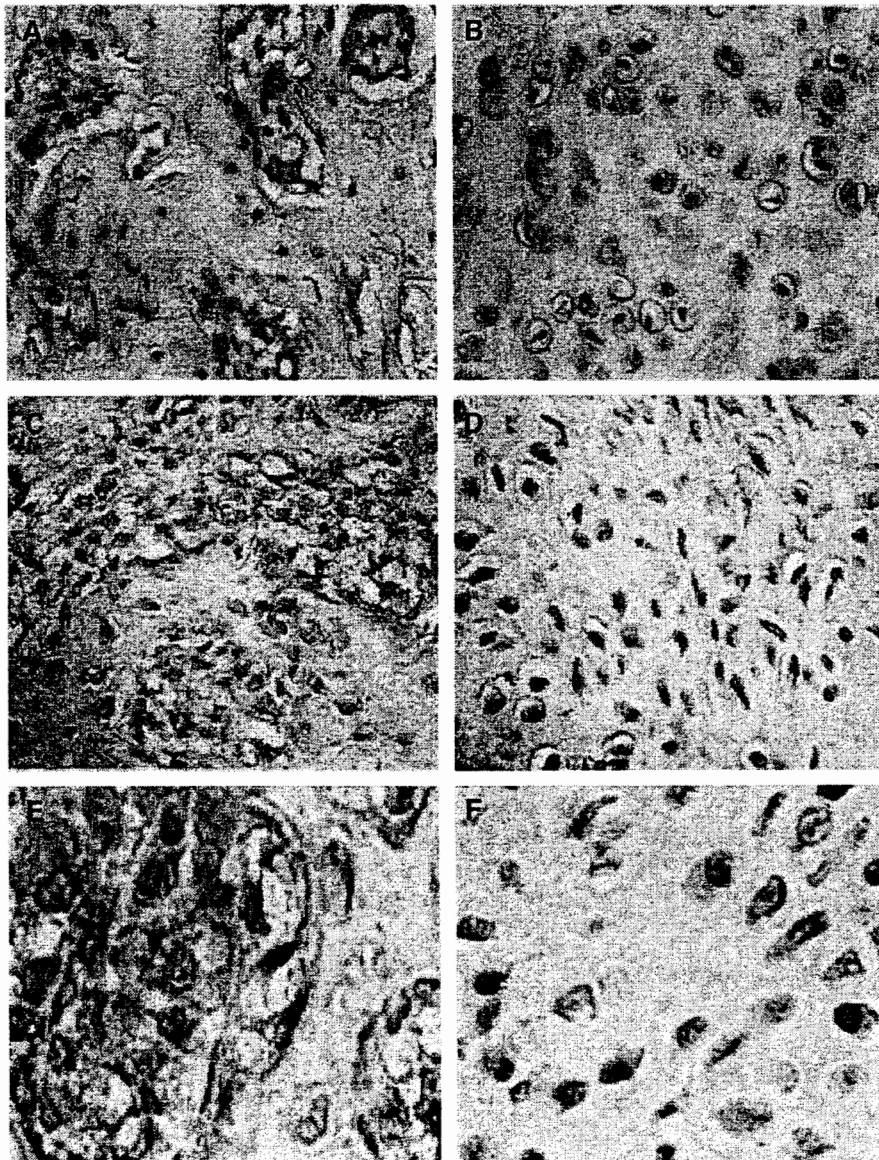


Fig. 5. Spatial expression of ERK and p38 in calluses with or without ESW treatment. Negative controls of (A) ERK and (B) p38 expression. No immunostaining in cells without primary antibodies. Cytoplasm in cells showed positive (C) ERK and (D) p38 expression. Nuclei in cells showed positive (E) phosphorylated ERK and (F) phosphorylated p38 expression. Specimens were observed in magnification = $400\times$ (A, B, C, and D) and $800\times$ (E and F).

mm² from each selected area (3 mm²) were then taken under 400× magnifications. All images of each specimen were captured using a Cool CCD camera (SNAP-Pro c.f. Digital kit; Media Cybernetics, Sliver Spring, MD, USA). Images were analyzed using Image-Pro® Plus image-analysis software (Media Cybernetics, Sliver Spring, MD, USA) as previously described [30]. The number of positive immunolabeled cells and total cells in each area were counted and percentages of positive-labeled cells were calculated. Fibroblasts, chondrocytes, and osteoblasts were identified morphologically. A pathologist, blinded to the treatment

regimen, performed measurements on all sections under 400× magnification.

Statistical analysis

Data were analyzed with a nonparametric analysis of variance followed by Student's *t* test to determine significance between treated and untreated groups. A *P* value of < 0.05 was considered statistically significant. Histologic data were analyzed using a general linear model followed by a Duncan's multiple range tests to determine the significance

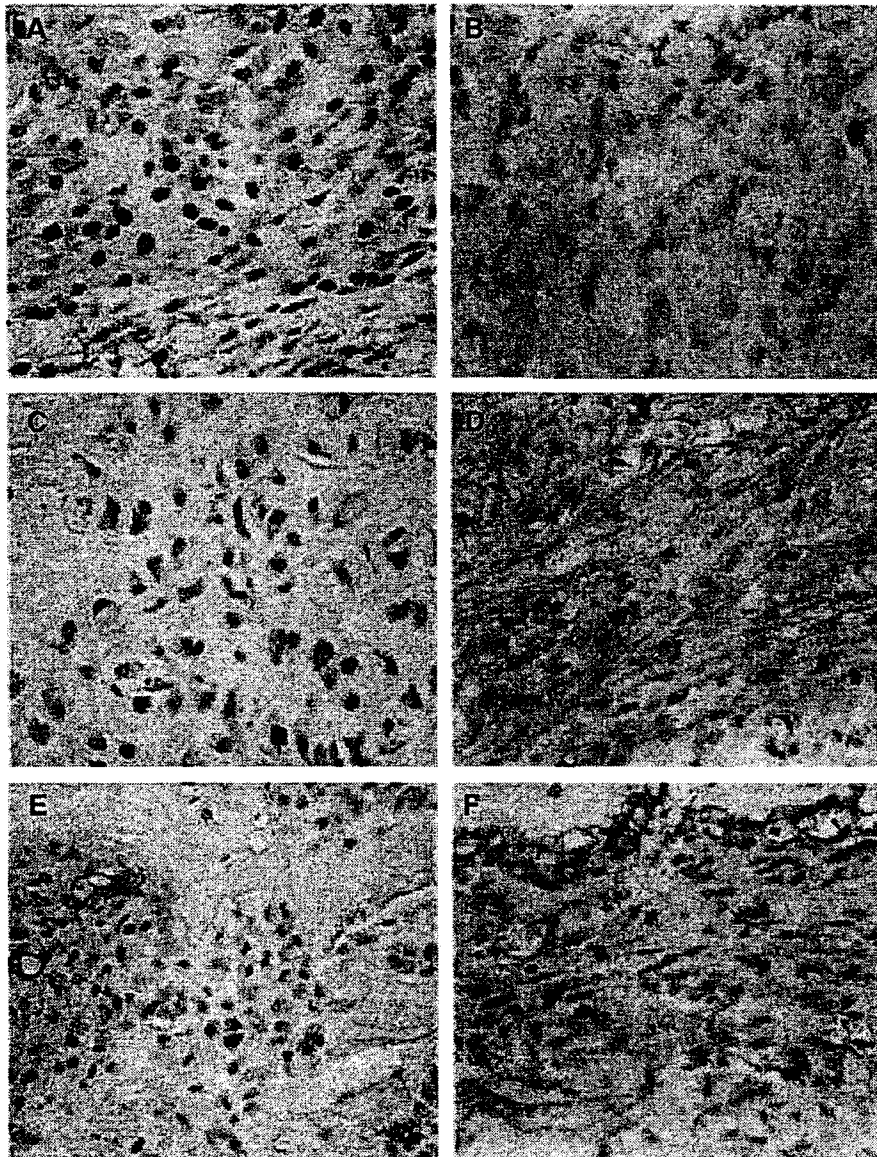


Fig. 6. Expression of cells with positive phosphorylated ERK. (A) Seven days after treatment, aggregated mesenchymal cells expressed intensive phosphorylated ERK expression. (B) In control groups, cells at the fibrous tissue showed limited positive phosphorylated ERK. (C) Within 14 and 56 days after ESW, chondral cells at hypertrophic cartilage, and (D) osteoblasts at ossified tissue displayed strong phosphorylated ERK expression. (E and F) In control sections, fibroblasts adjacent to fibrous tissue displayed very weak phosphorylated ERK expression at the same time. Specimens were observed in magnification = 400×.

between treatments. A *P* value of < 0.05 was considered statistically significant.

Results

Cell growth and bone matrix protein synthesis of callus organ cultures

We investigated whether ESW affected cell growth and the production of bone matrix protein in segmental defects.

Calluses with or without ESW treatment were harvested for organ culture assay. Our results showed that ESW significantly increased [³H]-thymidine uptake of callus organ cultures within 42 days after treatment, peaking at 7 days (Fig. 1). ESW treatment increased bone alkaline phosphatase activity (Fig. 2A), collagen I (Fig. 2B), and collagen II (Fig. 2C) production in 7 days. ESW stimulation subsequently increased osteocalcin synthesis in 14 days (Fig. 2C). No significant alteration of [³H]-thymidine uptake and bone matrix protein production was observed in control groups throughout the study period (Fig. 2).

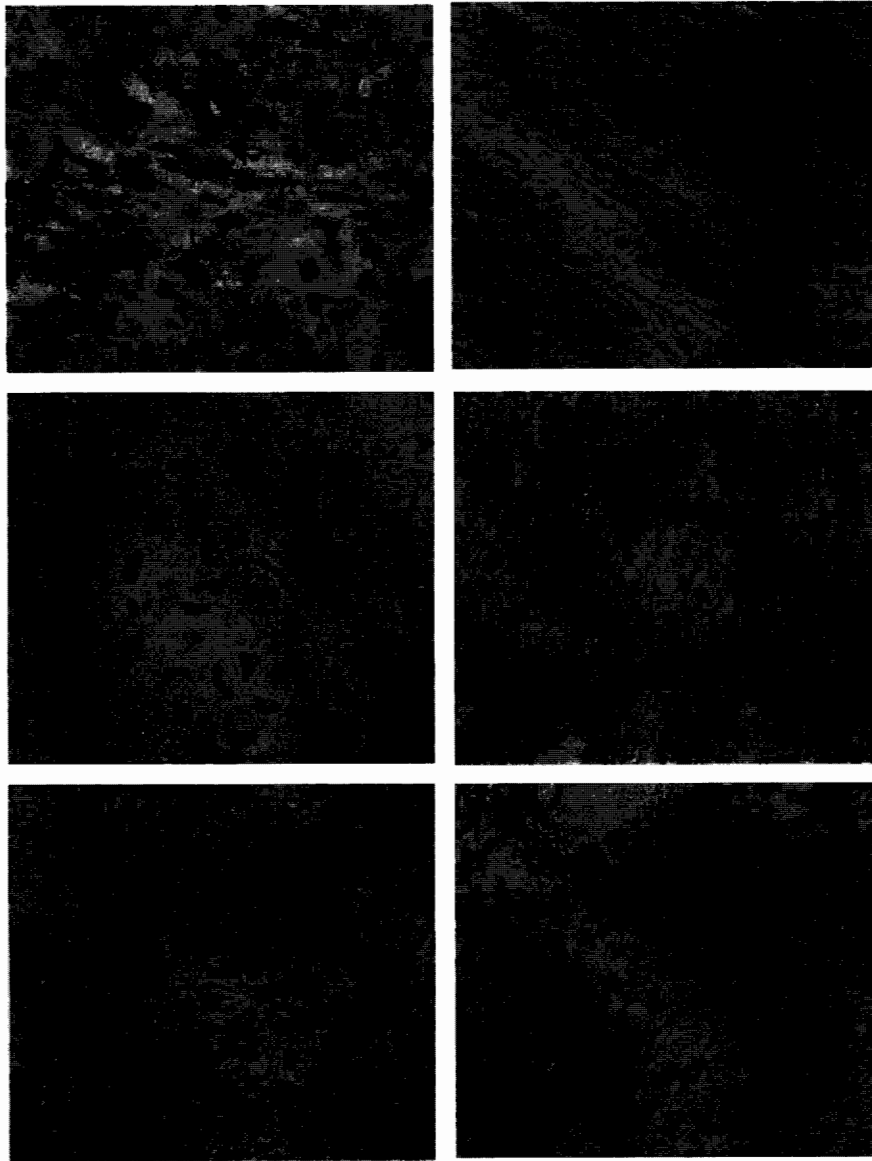


Fig. 7. Expression of cells with positive phosphorylated p38 expression. (A) Seven days after ESW, limited fibroblasts showed phosphorylated p38 expression. (B) In control groups, cells at defect site showed weak phosphorylated p38 expression. (C) Within 12 and 42 days following ESW, chondrocytes in the area of hypertrophic cartilage exhibited strong phosphorylated p38 expression. (D) In control sections, cells at the defect sites contained little phosphorylated p38 expression. (E) Fifty-six days after ESW, mature chondrocytes and osteoblasts showed weak phosphorylated p38 expression. (F) In control groups, cells at the osteotomy gap appeared slightly phosphorylated p38 expression. Specimens were observed in magnification = 400 \times .

ERK and p38 phosphorylation activity

We further investigated whether ERK and p38 were activated in bone tissue after physical treatment with ESW. Using MBP as a substrate, we subjected ERK and p38 immunoprecipitates, obtained from callus extracts, to phosphotransferase activity determination. Autoradiographs showed that ESW treatment significantly increased ERK (Fig. 3A) and p38 (Fig. 3B) phosphotransferase activities as demonstrated by increasing phosphorylated 21-kDa band intensities, which corresponded to MBP. Kinetic changes in ERK and p38 phosphotransferase activities as demonstrated by phosphorylated MBP are shown in Fig. 3C. ERK and p38 phosphorylation activities were also measured by scintillation counting. Within 1 day of ESW treatment, calluses rapidly underwent an increase in ERK activity, which continued for 42 days. The p38 phosphorylation subsequently increased in 7 days and then declined to baseline by day 42 (Fig. 3D).

Histology

Seven days after ESW treatment, segmental defects underwent intensive mesenchymal cell aggregation (Fig. 4A), while fibrous and fibrocartilaginous tissues filled the defect in the control group (Fig. 4B). Within 28 days after treatment, the defects were found to be bridged by newly formed cartilage and woven bone with intensive endochondral/intramembrane ossification (Fig. 4C). The defects in control group were filled with fibrous tissue (Fig. 4D). Fifty-six days following ESW treatment, the defect was surrounded by hard callus and bone remodeling was in process (Fig. 4E), while skeletal muscle had collapsed into the defects in the control group. Throughout the study period,

no callus formation was found around the defects in the control group (Fig. 4F).

Activated ERK and p38 expression

In the absence of the primary antibody, no immunostaining of ERK (Fig. 5A) and p38 (Fig. 5B) was visible. The cytoplasm of cells expressing positive ERK (Fig. 5C) and p38 (Fig. 5D) was visualized as brown. Fibroblastic cells, chondrocytes, and osteoblasts at segmental defect expressed ERK and p38. We also used anti-phosphorylated ERK and anti-phosphorylated p38 antibodies to identify the expression of activated ERK and p38. The nuclei of cells were positively stained for phosphorylated ERK (Fig. 5E) and phosphorylated p38 (Fig. 5F). Intensities of phosphorylated ERK and phosphorylated p38 expression varied with the different stages of bone regeneration and cell morphology. Seven days after ESW treatment, fibroblasts and aggregated mesenchymal cells at the osteotomy gap expressed intensive phosphorylated ERK (Fig. 6A), while cells at the fibrous tissue expressed limited positive phosphorylated ERK in the control sections (Fig. 6B). Within 14 and 56 days after ESW treatment, phosphorylated ERK was expressed in chondrocytes at hypertrophic cartilage (Fig. 6C) and osteoblasts adjacent to the newly formed woven bone (Fig. 6E). Fibroblasts adjacent to fibrous tissue in the control group displayed very weak phosphorylated ERK expression (Figs. 6D and 6F).

Seven days after ESW treatment, limited fibroblasts were found to express phosphorylated p38 (Fig. 7A), while cells at defect site in the control sections were found to only weakly express phosphorylated p38 (Fig. 7B). Within 14 and 42 days following ESW treatment, chondrocytes in the area of hypertrophic cartilage exhibited strong phosphory-

Table 1
Number of fibroblastic, chondral, and osteoblastic cells exhibiting positive phosphorylated ERK and phosphorylated p38 expression of segmental defects after receiving ESW treatment

Expression	Day(s)	Fibroblastic cells		Chondral cells		Osteoblastic cells	
		ESW	Control	ESW	Control	ESW	Control
Phosphorylated ERK	0	185 ± 23	162 ± 19	102 ± 19	114 ± 21	134 ± 20	121 ± 19
	1	562 ± 76	148 ± 21	112 ± 21	107 ± 18	115 ± 17	132 ± 24
	3	625 ± 68	163 ± 18	132 ± 24	122 ± 19	126 ± 24	116 ± 15
	7	731 ± 71	161 ± 22	368 ± 35	109 ± 21	296 ± 35	108 ± 21
	14	321 ± 39	152 ± 16	436 ± 46	126 ± 26	418 ± 46	136 ± 24
	28	289 ± 41	159 ± 23	321 ± 43	118 ± 19	485 ± 52	119 ± 21
	42	256 ± 34	157 ± 15	242 ± 39	125 ± 23	426 ± 34	108 ± 22
	56	236 ± 23	152 ± 24	152 ± 22	131 ± 25	314 ± 33	118 ± 19
Phosphorylated p38	0	78 ± 15	65 ± 16	114 ± 21	95 ± 18	63 ± 11	68 ± 14
	1	89 ± 21	76 ± 19	102 ± 19	92 ± 19	56 ± 21	61 ± 17
	3	113 ± 24	67 ± 17	127 ± 24	103 ± 23	71 ± 17	57 ± 21
	7	146 ± 27	72 ± 21	358 ± 37	107 ± 27	96 ± 23	72 ± 19
	14	121 ± 39	75 ± 19	486 ± 36	112 ± 25	113 ± 25	65 ± 20
	28	107 ± 41	66 ± 23	329 ± 34	106 ± 17	105 ± 22	69 ± 18
	42	116 ± 34	64 ± 18	207 ± 31	122 ± 24	98 ± 16	71 ± 20
	56	107 ± 23	78 ± 24	142 ± 24	115 ± 18	85 ± 23	73 ± 18

Data are mean ± standard error calculated from six segmental defect of areas from three sections of three rats after receiving ESW at 0.16 mJ/mm², 1 Hz, 500 impulses.

lated p38 expression (Fig. 7C), while cells at the defect sites in the controls exhibited little (Fig. 7D). Fifty-six days after ESW treatment, mature chondrocytes and osteoblasts weakly expressed phosphorylated p38 expression (Fig. 7E). In control groups, cells at the osteotomy gap appeared only slightly expressed phosphorylated p38 expression (Fig. 7F). Table 1 summarizes the number of fibroblasts, chondrocytes, and osteoblasts that during the process of ESW-promoted bone formation were found to have positive phosphorylated ERK and phosphorylated p38 expression. Results show that mesenchymal cells, chondrocytes cells, and osteoblastic cells displayed intensive phosphorylated ERK expression during bone repair (Table 1). Cells which displayed positive phosphorylated p38 expression were mainly chondral cells in the stage of hypertrophic chondrogenesis (Table 1).

Discussion

In this study, we demonstrate that segmental femoral defect subjected to ESW treatment promotes the formation of new bone. We found a trend toward increased bone protein biosynthetic activity in response to pulsed acoustic energy and pressure released by ESW. Increasing mitogenic and anabolic activities were associated with elevating ERK and p38 phosphorylation in calluses after exposure to ESW. While several studies have explored the involvement of ERK and p38 in bone cell growth and differentiation *in vitro*, few have been concerned with ERK and p38 activation in bone repair. This study provides the first evidence that ESW-promoted bone formation of segmental defect is linked to activation of ERK and p38. This study provides prominent molecular evidence that bone tissue converts physical ESW stimulation into mitogenic signaling for subsequent chondrogenesis and osteogenesis in bone repair. We propose that it is the increased mitogenic and osteochondrogenic responses of bone tissue that bring about the clinical success of ESW treatment in promoting healing of nonunion.

Segmental defects have been used as models of nonunion formation because they tend to form fibrous connective tissue during the healing process [31,32]. The 6-mm defect was of critical size since very little bone formed within the untreated defect, and no bone connected the ends of the untreated defects. This model has also widely been employed for elucidating the effects of tissue and genetic engineering strategies on bone repair, including mesenchymal stem cell implantation and BMP-2-producing bone-marrow cells [31–34]. Histological observation of current study revealed that the defects were filled with loose connective tissue. The defects clearly demonstrate that physical ESW stimulation can induce bone formation. An added advantage to this model is that the segmental defect provides an excellent model for assessing sequential stage of human fracture healing [32]. Our studies find the use of

segmental defect to an excellent model for clarifying the molecular and histological mechanism that biophysical strategies such as ESW treatment use to stimulate bone formation *in vivo*.

In this study, calluses respond to ESW treatment by raising [³H]-thymidine uptake and subsequently elevating bone matrix protein synthesis. These findings suggest that ESW stimulate intensive osteogenesis and chondrogenesis in calluses. Chondrogenesis, an essential component of endochondral fracture healing in long bone, is characterized by the development of cartilage and subsequent replacement by bone [35]. In this study, we found down-regulation of collagen II production to be followed by bone alkaline phosphatase of calluses. Our histological observation also shows that segmental defects after exposure to ESW, progressive hypertrophic cartilage coincided, with endochondral ossification. Our findings suggest that ESW treatment trigger mitogenic activities that remodel fibrosis tissue into new cartilage and bone and induces cascades of endochondral bone formation in segmental defects.

A rapid provocation of cell growth in bone tissue by physical ESW treatment raises the possibility that a certain mitogenic mechanism is responsible for this biological reaction. In this current study, increased ERK and p38 activity occurred with increasing [³H]-thymidine uptake of calluses after ESW treatment. MAPK are reported to be required for cell growth and involved in regulating shear/strain stress-, stretch-, and pressure overload-stimulated bone cell growth [36–39]. Our findings suggest that ERK and p38, at least in part, play important roles in communicating physical ESW stimulation into intracellular mitogenic responses. On the other hand, bone tissue is found to convert physical stimulation into biochemical signals via release of anabolic or cytokine molecules of bone tissue [40,41]. Our previous studies have shown that segmental defects respond to ESW treatment by elevating BMP, TGF- β 1, and IGF-1 levels [24,30]. The activation of ERK and p38 is involved in osteogenic factor-induced bone cell proliferation and differentiation [14,16,42,43]. In the present study, the persistent activation of ERK and the subsequent activation of p38 in calluses after a single ESW treatment suggest that osteogenic factors probably contribute to activating MAPK in ESW-stimulated bone regeneration.

A growing body of evidence has demonstrated that ERK and p38 are involved in regulating osteogenic differentiation of mesenchymal stem cells and alkaline phosphatase expression of osteoblast [21,37,41]. The p38 also mediates osteogenic factor- and parathyroid hormone-stimulated chondrogenesis of bone-derived cells [44,45]. Inhibition of ERK and p38 activity induces chondrocyte apoptosis and abrogates chondrogenesis of mesenchyme [1,46]. Few previous studies have focused on the involvement of MAPK in the ESW promotion of bone repair *in vivo*. Our current study provides the first immunohistochemical evidence that osteogenic and chondrogenic cells in the areas of aggregated mesenchymal, hypertrophic cartilage and ossified tissue

displayed intensive phosphorylated ERK expression. Chondrocytes at hypertrophic cartilage were found to clearly express phosphorylated p38 expression. We suggest that ERK and p38 are actively involved in fracture healing. Each has a distinct temporal expression pattern and potentially unique role in the healing of segmental defect.

Biophysical signals imparted by ESW to segmental defects have been shown to rapidly activate multiple intracellular and intercellular signaling molecules. Our study provides evidence that bone tissue responds to physical ESW treatment could raise ERK and p38 activities to regulate bone formation. Activation of these signals in turn leads to increasing cell proliferation and altering tissue structure and function. We cannot exclude the possibility of another subfamily of MAPK, the JNK, regulating bone regeneration of segmental defect. Nevertheless, it is presently unknown what molecule(s) is responsible for ESW activation of MAPK. We have found that ESW treatment induces membrane hyperpolarization and activated Ras kinase cascades of signal transduction for osteogenic differentiation of mesenchymal stem cells [47]. Our more recent study has demonstrated that membrane-bound pertussis toxin-sensitive proteins regulate ESW stimulation of bone formation [30]. Reactive radicals such as nitric oxide, calcium, and prostaglandin have, however, been reported to be critical mediators for mechanical stimulation of osteoblast and chondrocyte proliferation [18,19,37,39]. Perturbation of membrane potential and alternation of bioactive radicals influx may be involved in regulating MAPK activity during bone regeneration of segmental defect. Further studies are needed to explore what detailed upstream signaling pathways and what bioactive radicals are responsible for activation of ERK and p38 in ESW enhancement of fracture healing in vivo. In summary, our research findings provide evidence that ESW treatment promotes bone morphogenesis in segmental defects, with ERK and p38 playing important mitogenic and osteochondral roles in signaling physical ESW stimulation.

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