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ERK Activation and αvβ3 Integrin Signaling Through Shc Recruitment in Response to Mechanical Stimulation in Human Osteoblasts

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Abstract Osteoblast growth and differentiation are critical processes for bone development and maintenance, and are regulated by both humoral and mechanical factors. Humoral (hormonal) factors can affect gene transcription via MAPkinases, e.g., extracellular signal-regulated kinase (ERK). We studied whether the ERK pathway is also involved in processing mechanical inputs in human bone cells. Exposing MG63 cells to physiologically relevant levels of fluid flow resulted in ERK phosphorylation. Genistein blocked this response, indicating that it is dependent on tyrosine phosphorylation. Furthermore, αvβ3 integrins were activated in response to fluid flow, as shown by recruitment of adaptor molecule Shc and clustering of αvβ3 in focal adhesion-like structures. Antibodies blocking formation of β1 or β3 integrin-matrix interactions or RGD peptides could not inhibit fluid flow-induced ERK phosphorylation, suggesting that formation of new integrin-matrix interactions is not essential for this response and that other upstream mechanosensors regulate induction of ERK phosphorylation in response to fluid flow in human bone cells. J. Cell. Biochem. 87: 85–92, 2002.

Key words: MAPkinase; fluid flow; mechanotransduction; human osteoblast; integrin; intracellular signaling

Proliferation and differentiation of osteoblasts is regulated by orchestration of hormonal and mechanical inputs [Stein and Lian, 1993; Matsuda et al., 1998]. Mechanical control of osteoblast proliferation and differentiation enables bone to adapt its architecture to applied mechanical forces, through a process called modeling. Unraveling the mechanisms by which cells perceive mechanical information and process it into biochemical signals through the actions of a potential membrane-coupled mechanosensor links researchers from different fields with a common interest in mechanosensitive cells, e.g., cells in bone, cartilage, endothelium, or muscle.

There is increasing evidence that chemical and mechanical stimuli share intracellular signaling pathways. The MAPkinase pathway plays a crucial role in cell proliferation and differentiation by transmitting signals from the cell membrane to the nucleus. Phosphorylated extracellular signal-regulated kinase (ERK, one of the three identified MAPkinases) regulates the expression and phosphorylation of transcription factors from the fos and jun families that control downstream transcription of genes with promoters containing AP-1 elements [Blenis, 1993]. Activation of ERK has been shown to regulate differentiation of mesenchymal stem cells towards the osteogenic lineage [Jaiswal et al., 2000] and to be essential for growth and differentiation of human osteoblasts [Lai et al., 2001]. Furthermore, ERK is activated in bone cells in response to a wide range of stimuli including estrogen, phorbol esters, and

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the growth factors PDGF, EGF, and bFGF (reviewed in [Hipskind and Bilbe, 1998]). In addition to activation by these 'classical' MAP-kinase regulators, ERK phosphorylation has recently been implicated in the response of mouse osteoblasts and human periodontal ligament cells to mechanical stimuli [You et al., 2001; Ziros et al., 2002]. Also in another cell system, the endothelial cell, MAPkinases are activated by both humoral stimuli (e.g., vascular endothelium growth factor (VEGF) [Stein and Lian, 1993]) and by mechanical stimuli including adhesion, shear induced by fluid flow, and stretch [Tseng et al., 1995; Chien et al., 1998].

Although intracellular pathways involved in processing of mechanical stimuli by osteoblasts are beginning to be clarified, molecules responsible for the initial detection of mechanical forces and triggering of the ensuing intracellular signaling events remain elusive. Integrins, which are membrane spanning receptors involved in cellular adhesion to the extracellular matrix, are intriguing candidates for this mechanosensing function. They are ideally located to perceive mechanical stimuli as they connect the extracellular matrix to the intracellular cytoskeleton and have been shown to act as mechanosensors in endothelial cells [Shyy and Chien, 1997]. Furthermore, integrin-mediated signaling can result in activation of the MAPkinase pathway through recruitment of a signaling complex of proteins to the cytoplasmic tail of integrin molecules [Schlaepfer and Hunter, 1998]. The adaptor protein Shc [Pelicci et al., 1992] is a pivotal factor in this complex and is both necessary and sufficient for the activation of ERK in response to integrin ligation in A-431 carcinoma cells [Wary et al., 1996]. Whether Shc is involved in integrin-mediated signaling in osteoblasts is not known.

In rat calvarial osteoblasts, integrin-mediated signaling regulates AP-1 transcription factors through a protein kinase-sensitive pathway [Cowles et al., 2000], which is indicative of a role for integrin-matrix interactions in regulating ERK phosphorylation. Osteoblastic cells express several integrin subunits, including high levels of β1- and β3-containing dimers [Saito et al., 1994; Anselme, 2000]. Classical bone ligands for integrins are matrix proteins, such as collagen I, fibronectin, and osteopontin [Anselme, 2000] most of which contain the integrin-recognition sequence RGD (Arg-Gly-Asp). Involvement of integrins in bone cell mechanotransduction has been suggested by studies using RGD peptides or specific integrin antibodies to block downstream responses to mechanical stimulation in vitro [Salter et al., 1997; Peake et al., 2000; Wozniak et al., 2000]. To our knowledge, no data are available on whether human bone cells require integrin signaling to connect a mechanical stimulus to MAPkinase activation.

In this study, we characterized MAPkinase activation in human bone cells in reponse to a physiologically relevant mechanical stimulus. Cells were exposed to fluid flow and both levels and dynamics of ERK phosphorylation in response to this stimulus determined. Fluid flow is proposed as a physiologically relevant mechanical stimulus in bone, since the interstitial fluid flows through the lacuno-canalicular network upon in vivo bone loading, resulting in estimated shear on the bone cells ranging between 0.8 and 3 Pa [Burger and Klein-Nulend, 1999; You et al., 2000]. Subsequently, the hypothesis that integrin-matrix interactions are involved in the response to fluid flow was tested by monitoring the association of Shc to αvβ3 integrin as a marker for integrin activation and a potential mechanism resulting in ERK phosphorylation. Finally, by blocking specific matrix-integrin interactions, the requirement of these interactions for the ERK phosphorylation response to fluid flow was tested.

MATERIALS AND METHODS

Cell Culture and Pre-Treatment

MG63 cells [Billiau et al., 1977] were cultured to confluence on non-coated (unless indicated differently) glass slides in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1 mM of both penicillin and streptomycin. Before fluid flow treatment, cells were starved in medium containing 0.5% FCS for 16 h. To block specific integrin-matrix interactions, cells were pretreated with blocking agents for up to 2 h before the application of fluid flow. Anti-human β1 or anti-human αvβ3 (MAB2253 or MAB1976, Chemicon, Temecula, CA) was used at 10 μg/ml. RGD peptides (GRGDSP, 100 μg/ml, Gibco Invitrogen, Carlsbad, CA) were used to block all RGD-integrin interactions. These protocols have been shown to block fluid flow-induced ERK phosphorylation in endothelial cells [Li et al., 1997].

Fluid Flow

Cells were subjected to physiological levels of fluid flow at 1.2 Pa, using laminar fluid flow in a set up a previously described by Frangos et al. for times indicated. Briefly, a rectangular flow chamber (0.025 cm high, 2.5 cm wide, and 5.0 cm long), with an inlet and outlet for exposing the cells to fluid flow-induced shear stress, was created by sandwiching a silicon gasket between a glass slide that contained the cells and an acrylic plate. Steady, laminar flow across the chamber was generated by the difference in height between a high and low medium reservoir. The system was kept at 37°C and equilibrated with 95% humidified air plus 5% CO_2. Static controls were kept in the same incubator, but not loaded in the flow chamber, and therefore, not exposed to fluid flow or hydrostatic pressure.

Immunostaining

Confluent cell cultures were starved in 0.5% FCS overnight and exposed to 15 min of fluid flow. The cells were washed with cold PBS twice and fixed in 4% paraformaldehyde for 10 min. Incubation in 0.5% Triton X-100 for 10 min to permeabilise the cells was followed by blocking nonspecific binding sites in 1% BSA for 30 min. The cells were then incubated with 10 μg/ml anti-human αvβ3 monoclonal antibodies (MAB1976, Chemicon Temecula, CA) for 2 h and the staining was visualized by using a FITC conjugated goat-anti-mouse IgG (Molecular Probes, Eugene, OR) and a fluorescence microscope.

Immunoprecipitation

For immunoprecipitation, the cells were lysed in a 'low salt' lysis buffer: 25 mM HEPES, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.125 M NaCl, 5mM EDTA, 50 mM NaF containing 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 2 mM BGP. Lysates were centrifuged and supernatant containing 75 μg total protein was immunoprecipitated overnight at 4°C using anti-human αvβ3 monoclonal antibodies (MAB1976, Chemicon Temecula, CA) coupled to protein-A sepharose beads (Amersham, Uppsala, Sweden). Coupling was performed by mixing wet beads (100 μl of a 30% slurry in PBS) for 1 h with 30 μg antibody, followed by two washes in 0.2 M sodium borate, pH 9.0 and incubation in 20 mM dimethylpimelimidate in 0.2 M sodium borate for 30 min. The coupling reaction was stopped by washing with 0.2 M ethanolamine prior to incubation in 0.2 M ethanolamine for 2 h. Antibody coupled beads were washed and resuspended in the lysis buffer and 10% of this reactant was used for each immunoprecipitation. Immunoprecipitated complexes were washed and used for immunoblotting.

Immunoblotting

Immunoprecipitated complexes (for Shc association experiments) or 20 μg total protein containing total cell lysate supernatants (for ERK phosphorylation experiments) were transferred to nitrocellulose following SDS–PAGE on 10% gels. The membranes were blocked in a wash buffer (TBS + 0.1% Tween 20) containing 5% skim milk powder for 2 h. The primary antibodies used were polyclonal anti-ERK1/2 (1:2,000 Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-ERK-P (1:2000 New England Biolabs, Berverly, MA), or polyclonal anti-Shc (1:1,000, Transduction Laboratories, Lexington, KY). The ERK-P antibody recognizes predominantly ERK2-P (42 kDa). At high protein concentration or longer exposure time ERK1-P (44 kDa) can also be detected on blots. Membranes were incubated with diluted antibodies in the wash buffer containing 1% BSA and incubated for 2 h. The bound primary antibodies were detected using appropriate secondary antibodies coupled to horseraddish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL detection system (Amersham, Uppsala, Sweden).

Data Analysis

Quantification of bands on blots are presented as means from at least three blots for each condition ± standard error. Differences between treatments were analyzed using the Students' *t*-test and were considered significant, when $P < 0.05$.

RESULTS

Fluid Flow Induces ERK Phophorylation

Laminar fluid flow resulted in an induction of ERK phosphorylation in MG63 cells (Fig. 1). The ERK-P level increased to approximately four times relative to static controls after 15 min of fluid flow, reaching a maximum of five times control values at 30 min, declined slightly at 60 min, and was still significantly higher than

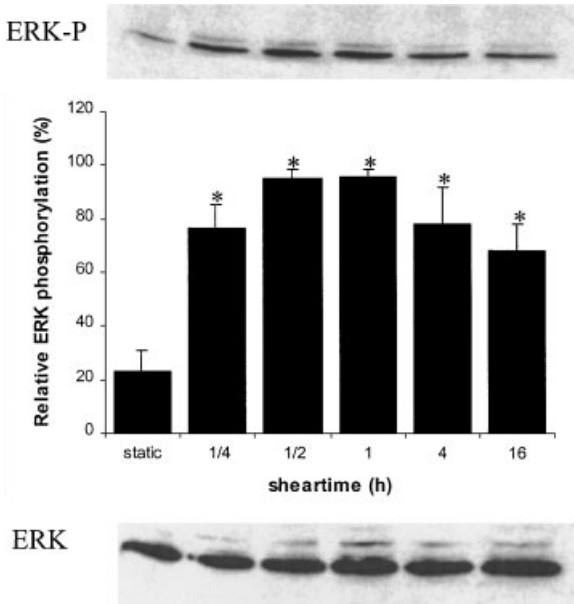

Fig. 1. ERK phosphorylation in MG63 cells in response to fluid flow. Cells were treated for the times indicated. Bars represent the mean of three experiments ± SE. Asterisks (*) indicates a significant ($P < 0.05$) difference from static controls.

static controls after 16 h of continuous fluid flow. The total level of ERK (phosphorylated and non-phosphorylated) was not changed by fluid flow (Fig. 1). Pre-treatment of MG63 cells with 100 μM genistein for 2 h markedly inhibited the ERK-P response to fluid flow (Fig. 2), indicating the involvement of tyrosine kinase.

Fluid Flow Induces Integrin αvβ3 Activation

The results of two types of experiments show that αvβ3 integrins are activated by fluid flow in MG63 cells. First, following the application of 15 min of fluid flow to starved MG63 cells, integrin αvβ3 was detected in focal adhesion-like clusters (Fig. 3A), whereas this did not occur in static controls (Fig. 3B). Second, there was a time-dependent increase in the association of the adaptor protein Shc with αvβ3 integrin (Fig. 4), indicating a fluid flow-induced integrin activation. Association was maximal after 30 min

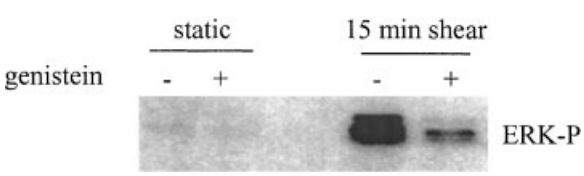

Fig. 2. Effect of pre-treatment with genistein on the ERK phosphorylation response of MG63 cells to fluid flow (15 min). The fluid flow-induced response was markedly attenuated after a 2 h pre-treatment with 100 μM genistein (+).

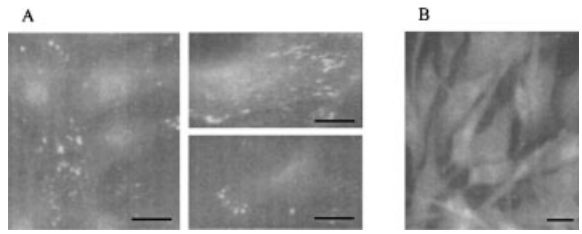

Fig. 3. Localisation of αvβ3 integrin in MG63 cells cultured overnight in medium containing 0.5% FCS and then exposed to fluid flow for 15 min in the same medium (**A**), or kept as static controls (**B**). Bars are approximately 20 μm.

of fluid flow and returned to basal levels in 4 h. The low molecular weight Shc isoform was found to be the most abundant in MG63 cells. There was no evidence for isotype-specific regulation by fluid fluid flow.

Formation of New RGD-Integrin Interactions Is Not Essential for ERK-P Response to Fluid Flow

Although integrins were shown to be activated upon fluid flow, pretreatment of MG63 cells with antibodies blocking formation of new β1 or β3 integrin-matrix interactions, or RGD peptides, which block formation of a broad range of integrin-matrix interactions, did not

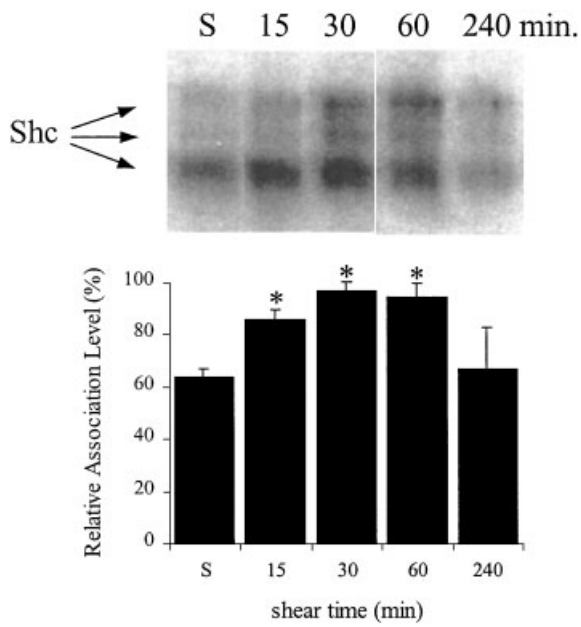

Fig. 4. Association of αvβ3 integrin with Shc in MG63 cells in response to fluid flow. Cells were treated for the times indicated and lysates were immuneprecipitated with αvβ3 antibodies followed by immunoblotting with Shc antibodies. Bars represent the mean of three experiments ± SE. Asterisks (*) indicates a significant ($P < 0.05$) difference from static controls.

inhibit the ERK-P response to fluid flow (Fig. 5). The adhesion of MG63 to glass slides coated with collagen I, which specifically favors β1-matrix interactions, in a medium containing 0.5% FCS for 1 h did not change the level of fluid flow-induced ERK phosphorylation when compared to cells adhered to uncoated glass slides (not shown). Even in this condition that favors β1-matrix interactions, anti-β1 antibodies or RGD peptides did not inhibit the fluid flow-induced ERK-P response (Fig. 6).

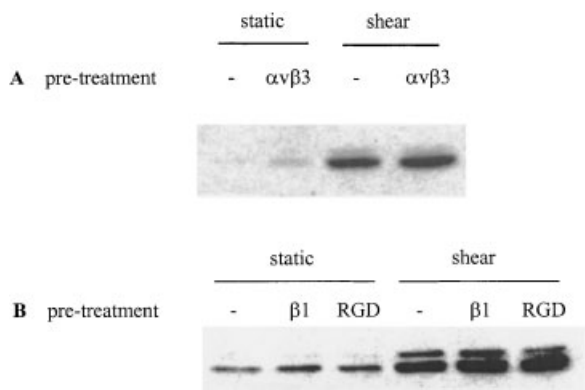

Fig. 5. Lack of effect of blocking formation of new integrin-matrix interactions on the fluid flow-induced ERK phosphorylation response of MG63 cells. The cells were subjected to a 2 h pre-treatment with αvβ3 (**A**) or β1 (**B**) blocking antibodies (10 μg/ml), or RGD peptides (B, 100 μg/ml) prior to the application of fluid flow for 15 min. The higher Mw band appearing in panel B is phosphorylated ERK-1 (p44) that is weakly recognized by the antibody and only visible in panel B due to longer exposure of the film, when compared to the film shown in panel A.

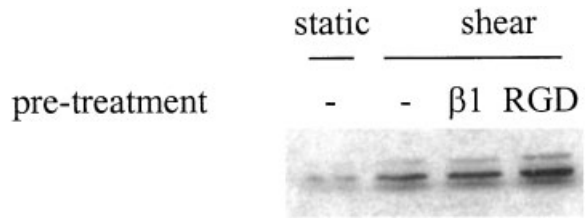

Fig. 6. ERK phosphorylation response of MG63 cells cultured on collagen I coated (10 μg/cm^2) glass slides to 15 min fluid flow following a 2 h pre-treatment with antibodies (10 μg/ml) that block formation of new β1 integrin-matrix interactions or RGD peptides (100 μg/ml), blocking new formation a wide range of integrin-matrix interactions.

DISCUSSION

The rapid increase in ERK phosphorylation in MG63 cells in response to laminar fluid flow shows that extracellular mechanical signals can be processed by human bone cells to trigger the same intracellular signaling pathway as extracellular chemical signals (hormones, growth factors). Phosphorylation of ERK in response to fluid flow has also been detected in the mouse MC3T3-E1 cell line, although the response was more transient [Ogata, 2000; You et al., 2001] and in human periodontal ligament cells in response to static stretch [Ziros et al., 2002]. Phosphorylated ERK can translocate to the osteoblast nucleus, where it affects gene expression through the activation of transcription factors of the AP-1 family [Hipskind and Bilbe, 1998]. Several genes that are pivotal in bone formation carry AP-1 elements in their promoters, including cbfa-1, c-fos, collagen type I, osteopontin, osteonectin, and osteocalcin [Rodan and Noda, 1991; Tou et al., 2001] and can thus be regulated downstream of ERK activation. In addition, all of these genes have been reported to be differentially regulated by mechanical stimulation [Raab-Cullen et al., 1994; Peake et al., 2000; Wang et al., 2001; You et al., 2001]. Our data suggest phosphorylation of ERK as a potential pathway regulating differential expression of these genes in response to mechanical stimulation.

The mechanism by which ERK phosphorylation is induced in response to mechanical stimuli in bone cells is not known. We assessed the potential role for integrin-matrix interactions in the bone cell response to mechanical stimulation for several reasons: (1) integrins are ideally located to perceive mechanical stimuli, (2) integrin-mediated signaling can result in activation of the MAPkinase pathway [Schlaepfer and Hunter, 1998] and regulates AP-1 transcription factors through a protein kinase-sensitive pathway in osteoblasts [Cowles et al., 2000], and (3) integrins have been shown to act as mechanosensors in endothelial cells [Shyy and Chien, 1997]. We observed clustering of αvβ3 integrin in response to fluid flow, representing activation and formation of new matrix-integrin interactions. The correlation between fluid flow-induced ERK phosphorylation and activation (clustering) of αvβ3 integrin is indicative for a possible functional coupling of the two responses.

Furthermore, ERK phosphorylation in response to fluid flow was also found to correlate with integrin signaling, as the adaptor protein Shc was recruited to αvβ3 integrin. Shc contains a Src homology domain-2 (SH-2) domain through which it can associate with receptor tyrosine

kinases (RTKs) following tyrosine-phosphorylation, resulting in down-stream activation of Ras and subsequent ERK phosphorylation [Rozakis-Adcock et al., 1992]. Shc is also implicated in integrin-mediated signaling as it was shown to be both necessary and sufficient for the activation of ERK in response to integrin ligation in A-431 carinoma cells [Wary et al., 1996]. In endothelial cells, dynamic matrix-integrin associations are required for Shc recruitment, implying that formation of new integrin-matrix interactions (and thus ligand binding) is essential for integrin mediated mechanotransduction in these cells [Jalali et al., 2001]. The high level of αvβ3 integrin-Shc association in static MG63 cells compared to the human endothelial cells in the Jalali study may indicate a basal level of signaling in the osteosarcoma cells. Fluid flow-induced clustering of αvβ3 integrin and recruitment of Shc by αvβ3 integrin in human bone cells represents new formation of integrin-matrix interactions and initiation of downstream signaling in response to the mechanical stimulus. This together with a fluid flow-induced increase in Shc phosphorylation in mouse osteoblastic cells [Ogata, 2000] suggests that Shc is involved in integrin-mediated signaling and mechanosensing in bone cells.

Although αvβ3 integrin clustering and signaling in MG63 cells upon fluid fluid flow correlate with ERK phosphorylation, suggesting a role for this integrin in processing the mechanical signal, pre-treatment of cells with antibodies blocking the formation of new matrix-αvβ3 integrin interactions did not block fluid flow-induced ERK phosphorylation. Furthermore, antibodies blocking formation of β1-matrix interactions, or RGD peptides blocking formation of RGD-dependent integrin-matrix interactions, had no effect on ERK phosphorylation in response to fluid flow. The capacity of these antibodies and RGD peptides to block ERK phosphorylation in response to shear using identical pre-treatment protocols has been shown in endothelial cells [Li et al., 1997]. Our observations are corroborated by the observation that RGD peptides could not block the increased transcription of c-fos in response to a mechanical stimulus (4-point bending) in the same cell line [Peake et al., 2000], given that ERK phosphorylation and c-fos activation in osteoblasts are strongly linked [Evans et al., 1996; Hipskind and Bilbe, 1998]. Obviously, these observations show that although mechanical stimulation activates integrins (as evidenced by clustering and Shc recruitment), formation of matrix interactions with these integrins is not essential for the ERK-phosphorylation response to fluid flow in MG63 cells. Therefore, integrin-matrix interactions tested in this study are not the essential mechanosensors responsible for coupling the mechanical fluid flow stimulus to the activation of the ERK pathway.

The observation that fluid flow-induced ERK phosphorylation does not require formation of new integrin-matrix interactions, does not rule out involvement of integrin-matrix interactions in mechanosensing in bone cells in general. MG63 cells may not use a single (integrin) regulator of ERK phosphorylation, thus rendering each individual integrin non-essential. However, non-integrin membrane associated potential mechanosensors may be involved as well. In this light, the class of receptor tyrosine kinases (RTKs) are of interest, since next to the well-known RTK-mediated activation of the ERK pathway upon ligand binding [Howe et al., 2002], RTKs have also been shown to act as mechanosensors in endothelial cells by recruiting Shc to their cytoplasmatic tails in response to fluid flow in a ligand-independent way [Chen et al., 1999]. Furthermore, other potential mechanisms of inititation of intracellular signaling in response to mechanical stimulation which are studied in different types of mechanosensitive cells include G-protein coupled receptors [Jo et al., 1997], the cytoskeleton [Pavalko et al., 1998], and the lipid bilayer [Butler et al., 2001]. In conclusion, it is evident from our experiments that mechanical stimuli regulate ERK signaling in human bone cells, which can provide a cellular mechanism through which bone adapts to mechanical loading via effects on bone cell proliferation and differentiation. Identification of the responsible upstream mechanosensor(s), however, requires additional studies.

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