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# Stretch-Induced Phosphorylation of ERK1/2 Depends on Differentiation Stage of Osteoblasts

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**Abstract** The goal of this study was to investigate the effect of mechanical loading on osteoblasts and extracellular signal-regulated kinase (ERK1/2) signaling in relation to osteoblast differentiation and mineralization. A human osteoblast cell line (SV-HFO) was triggered to differentiate to the advanced state of mineralization by addition of the osteogenic factors dexamethasone and β-glycerophosphate. Osteoblasts were subjected to cyclic, equibiaxial stretch for 5, 15, or 60 min at different stages of differentiation (days 7, 14, and 21). Baseline (static) phosphorylated ERK1/2 and total ERK1/2 levels gradually increased during osteoblast differentiation. Cyclic stretch induced a rapid increase in ERK1/2 phosphorylation with a maximum between 5 and 15 min. Prolonged stretching for 60 min resulted in a decrease of phosphorylated ERK1/2 towards baseline level, suggesting a desensitization mechanism. The effect of stretch on ERK1/2 phosphorylation was strongest at later stages of differentiation (days 14 and 21). At day 21, the increase of ERK1/2 phosphorylation in response to stretch was significantly lower in non-differentiating than in differentiating osteoblasts. This could not be explained by differences in cell density, but did correlate with the formation of extracellular matrix, collagen fibrils. Mineralization of the extracellular matrix did not lead to a further increase of ERK1/2 phosphorylation. In conclusion, the current study demonstrates that the extent of activation of the ERK1/2 pathway is dependent on the differentiation or functional stage of the osteoblast. The presence of an extracellular matrix, but not per se mineralization, seems to be the predominant determinant of osteoblastic response to strain. J. Cell. Biochem. 93: 542–551, 2004.


**Key words:** ERK1/2; stretch; mechanotransduction; human osteoblast; differentiation

Mechanical loading is essential for bone maintenance. In the normal adult skeleton, biomechanical signals are generated during dynamic loading and lead to an adaptation of bone structure and mass [Duncan and Turner, 1995]. This adaptation is mediated via the bone remodeling process, a dynamic equilibrium between bone formation by osteoblasts and bone resorption by osteoclasts. To initiate a cellular response, transduction of the mechanical stimulus into a biochemical signal (i.e., mechanotransduction) is required. Although the general concept of mechanotransduction is widely accepted, the exact mechanisms that enable bone cells to translate biomechanical signals into biochemical signals are far from being understood.

In search for the exact mechanism of mechanotransduction, an increasing number of studies focus on mechanical loading and its effect on intracellular biochemical processes. An important signaling candidate is the phosphorylation cascade involved in the activation of mitogen-activated protein kinases (MAPKs). The MAPK pathway plays a crucial role in cell proliferation and differentiation by transmitting extracellular signals from membrane to nucleus. Phosphorylation of extracellular signal-regulated kinase (ERK1/2, a MAPK subgroup) 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

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binding sites [Blenis, 1993], as well as the phosphorylation of Runx2/Cbfa1 [Xiao et al., 2002b], which are expressed at high levels in osteoblasts. Activation of ERK1/2, via phosporylation, 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 osteoblastic cells [Lai et al., 2001; Xiao et al., 2002a]. In vitro, bone cells can respond to stretch [Rawlinson et al., 1996; Kawata and Mikuni-Takagaki, 1998; Peverali et al., 2001; Rubin et al., 2002, 2003; Ziros et al., 2002], stretch and fluid flow using four point bending [Jones et al., 1991; Pitsillides et al., 1995; Owan et al., 1997; Stanford et al., 2000; Jessop et al., 2002], fluid flow alone [Jacobs et al., 1998; Ajubi et al., 1999; You et al., 2001; Jiang et al., 2002; Kapur et al., 2003], hydrostatic pressure [Klein-Nulend et al., 1995; Brighton et al., 1996], and gravity loading [Hatton et al., 2003]. Activation of the MAPK cascade and ERK phosphorylation has been shown in osteoblasts in the response to mechanical loading [Peverali et al., 2001; You et al., 2001; Jessop et al., 2002; Jiang et al., 2002; Rubin et al., 2002, 2003; Ziros et al., 2002; Hatton et al., 2003; Kapur et al., 2003]. However, there is a wide variety in cell types with different osteoblastic phenotypes being used ranging from MC3T3 cells, periodontal ligament cells, rat osteosarcoma cells, murine osteoprogenitor stromal cells, to human osteoblasts, and there is great diversity in biological responses. Differentiation stage of the osteoblasts has been shown to be an important determinant of osteoblast activity and responsivity [Chiba et al., 1993; Stein and Lian, 1993; Arts et al., 1997; Weyts et al., 2003]. Osteoblast differentiation could also prove to be an important factor in processing mechanical stimuli. As osteoblasts and osteocytes are biologically anchored to the organic bone matrix (via adhesion receptors), it is likely that these cells are more susceptible to mechanical forces when they become embedded in their own matrix. This is in line with the hypothesis that the terminally differentiated osteoblast, the osteocyte, is the important mechanosensory cell and is further supported by the observation that chicken osteoblast- and osteocyte-cultures differ in the levels of PGE2 secretion in response to fluid flow [Klein-Nulend et al., 1995]. However, in the latter study, osteocytes were seeded on plastic and were not embedded in a mineralized matrix. To our knowledge, detailed studies on mechanical sensitivity in a system where osteoblasts proceed through a differentiation process ending up embedded in a mineralized matrix are scarce. We have shown in a previous study that mechanical loading effects on apoptosis are dependent on osteoblast differentiation [Weyts et al., 2003].

The aim of the current study was to determine whether human osteoblasts respond to cyclic stretch with increased levels of phosphorylated ERK1/2, and to evaluate whether this response was dependent on differentiation stage and in particular related to the presence of a mineralized matrix. For this we used the human osteoblast cell line SV-HFO that shows ongoing matrix production, has highest alkaline phosphatase activity around day 14 (matrix maturation phase), and subsequently mineralizes the formed matrix up to at least day 21 (mineralization phase) of culture [Chiba et al., 1993; Iba et al., 1995; Arts et al., 1997; Janssen et al., 1999], and applied cyclic stretch at specific time points during differentiation.

## MATERIALS AND METHODS

### Cell Culture

At the start of experiments (day 0), approximately $2 \times 10^5$ SV40-immortalized human fetal osteoblast cells (SV-HFO) per well were seeded on flexible, collagen type I coated six-well plates (BioFlex, Flexercell, McKeesport, PA) to ensure proper adherence of the cells. Cells were cultured in αMEM medium without phenol red (Gibco, Paisly, UK), supplemented with 20 mM HEPES (Sigma Chemical Co., St. Louis, MI), 2% charcoal-treated fetal calf serum, 1.8 mM $CaCl_2$, 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco). To trigger differentiation of SV-HFO cells, culture medium was supplemented with osteogenic factors: 1 μM dexamethasone (Sigma) and 10 mM β-glycerophosphate (Sigma). For cell density experiments, also $1 \times 10^5$ SV-HFO cells per well were seeded (half cell density).

### Stretching

Stretching experiments were performed using a loading unit (Flexercell) inside a 37°C, 5% $CO_2$ incubator. In this setup, vacuum is applied to the area under six-well plates, causing the flexible silicon bottom to stretch over loading posts underneath. Vacuum was

generated through a Venturi valve using airflow regulated by a computer (using Labview software). The unit produces homogeneous biaxial stretch [Brown, 2000] and was adapted to produce physiological relevant strain levels with minimal vertical displacement of the silicon bottom to minimize build-up of fluid flow [Weyts et al., 2003]. Since we noticed an important effect of handling of the cells on the ERK1/2-P signal, each six-well plate was placed in the loading unit 90 min prior to mechanical loading (pre-incubation period). Wells were subjected to a vacuum at 650 mbar, 0.5 Hz, using Ø 32 mm diameter loading posts. This generated cyclic strains of 0.4% (4,000 μstrain) that was verified by a set of calibrating experiments [Weyts et al., 2003]. Cyclic strains were applied for 5, 15, or 60 min on day 7, 14, or 21 of culture. Static control groups were also placed in the loading unit for 90 min, but not stretched. Osteogenic factors that triggered osteoblast differentiation were either present or absent in the experiments performed.

Selected experiments were performed in the presence or absence of a selective mitogen-activated protein kinase kinase (MEK) inhibitor U0126 (Promega, Madison, WI). The inhibitor, or its vehicle DMSO, was added to the medium 90 min prior to stretching (at the start of the pre-incubation period).

Following stretching, cells were immediately placed on ice and washed once with ice-cold PBS. Cells were collected in appropriate lysis buffer for either western blotting or DNA and calcium assays.

### Western Blotting

Cells were lysed in buffer (200 μl/well) containing 25 mM HEPES, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM $Na_3VO_4$, 10 μg/ml leupeptin, and 2 mM β-glycerophosphate, and harvested with a cell scraper. After centrifugation at 11,000*g* for 15 min, supernatant was collected and stored at −80°C until further use. At a later stage, protein levels were determined using BCA protein assay kit (Pierce, Rockford, IL). Cell lysate (10 μg protein/lane) was separated by SDS–PAGE and transferred to Hybond+ nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ). After overnight blocking with TBS + 0.1% Tween 20 containing 5% bovine serum albumine, membranes were incubated with monoclonal anti-ERK1/2-P antibodies (1:2,000, Cell Signaling, Beverly, MA) for 3 h at room temperature and detected using horseradish peroxidase coupled secondary antibodies and the ECL detection system (Amersham Pharmacia Biotech). After exposure to Kodak HR film, immunoreactive bands were quantified using Quantity One (Bio-Rad) software. Finally, all blots were stripped and blocked again for incubation with monoclonal antibodies against ERK1/2 (1:2,000, Cell Signaling). ERK1/2-P levels were normalized for total ERK1/2 levels to correct for loading differences.

### DNA, Alkaline Phosphatase, and Calcium Content

SV-HFO cell lysates in 0.1% PBS-Triton X-100 were treated with heparin and RNase A (50 mg/ml in PBS) for 30 min at 37°C. DNA content was measured according to the ethidium bromide method by Karsten and Wollenberger [1977]. Alkaline phosphatase activity was determined by the colorimetric method of Lowry et al. [1954]. Calcium deposition into the matrix was determined after overnight extraction with HCL, using the Sigma calcium assay.

### Procollagen

Type I procollagen was measured in medium using the PICP RIA kit (Orion Diagnostica, Espoo, Finland), based on a competitive radioimmunoassay technique, and reflects the synthesis of type I collagen fibers.

### Statistics

Each condition was performed in triplicate ($n = 3$). Data are presented as means from at least three wells for each condition ± standard error of mean. Differences between groups were analyzed using the unpaired *T*-test and were considered significant when $P < 0.05$.

## RESULTS

### Cell Growth and Differentiation

Culturing SV-HFO cells on collagen I coated silicon bottom wells resulted in increased DNA and protein levels in time, both in the presence and absence of osteogenic factors. At all days, DNA levels in differentiating cell cultures were higher than in cell cultures without supplementation of osteogenic factors. Alkaline phosphatase activity in total cell lysates peaked at day

14 of culture. In HFO cells cultured without osteogenic factors, alkaline phosphatase activity remained below 1 nmol/min/μg DNA during culture. Mineralization was strongly induced after 21 days of culture only in the presence of osteogenic factors (Fig. 1).

### Differentiation Stage Affects Baseline Levels of Phosphorylated ERK1/2

In the static condition both ERK1/2-P and ERK1/2 levels increased, resulting in a constant ERK-P/ERK ratio (Fig. 2).

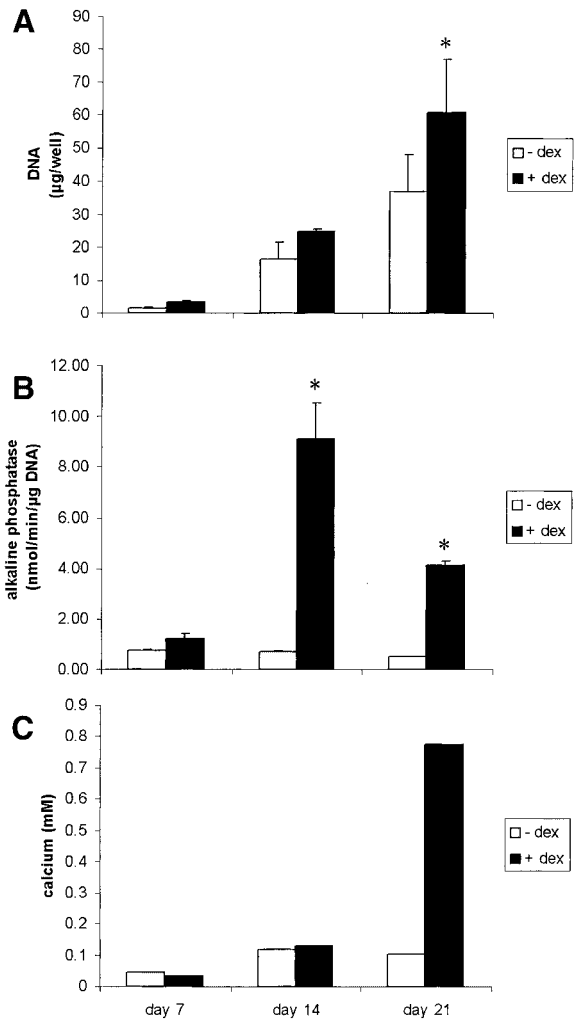


**Fig. 1.** Increasing DNA levels of SV-HFO cells during culture, either if cultured in the absence the osteogenic factors dexamethasone and β-glycerophosphate (−dex) or if cultured in the presence of osteogenic factors (+ dex). Significant differences due to osteogenic factors are marked by an asterisk. **A**: DNA levels, (**B**) alkaline phosphatase activity, (**C**) calcium accumulation.

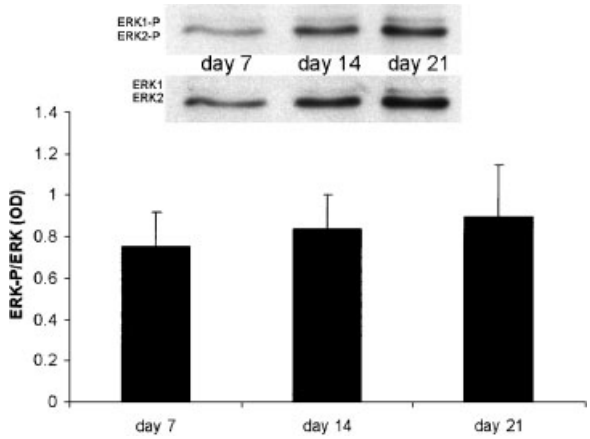


**Fig. 2.** Effect of osteoblast differentiation on basal phosphorylated and non-phosphorylated ERK1/2 levels. SV-HFO cells were cultured in the presence of osteogenic factors and collected for protein analysis at indicated days using Western blotting technique. Both phosphorylated ERK1/2 and total ERK1/2 levels increased, resulting in a constant ERK-P/ERK ratio.

### Stretch Temporarily Induces Differentiation Stage-Dependent ERK1/2 Phosphorylation

Biaxial stretch strains of 0.4% induced a rapid increase in ERK1/2 phosphorylation with a maximum between 5 and 15 min. Prolonged stretching up to 60 min, however, resulted in a decrease of phosphorylated ERK1/2 levels towards baseline (Fig. 3). Total ERK1/2 levels did not change upon stretching. Experiments at other days showed a similar temporospatial pattern (data not shown).

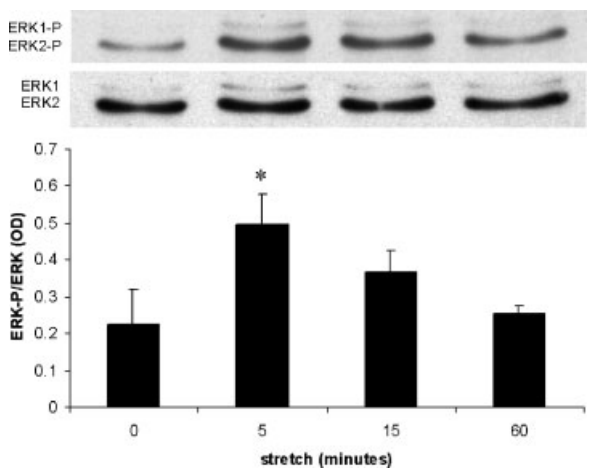


**Fig. 3.** Typical pattern ERK1/2 phosphorylation levels after cyclic stretch of 0.4%. Cells were cultured for 21 days in the presence of osteogenic factors and collected immediately after stretching for protein analysis. Experiments at other days showed a similar temporospatial pattern. ERK-P/ERK ratio after 5 min of stretch was significantly higher than baseline (0 min).

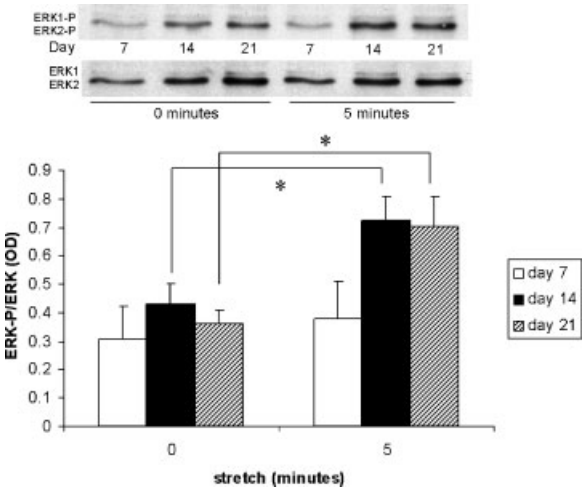


**Fig. 4.** Effect of glucocorticoid-induced osteoblast differentiation on stretch-induced ERK1/2 phosphorylation. Cells were cultured in the presence of dexamethone and β-glycerophosphate, and stretched for 5 min after 7, 14, or 21 days. Stretch-induced increase of ERK1/2 phosphorylation was significant at day 14 and 21.

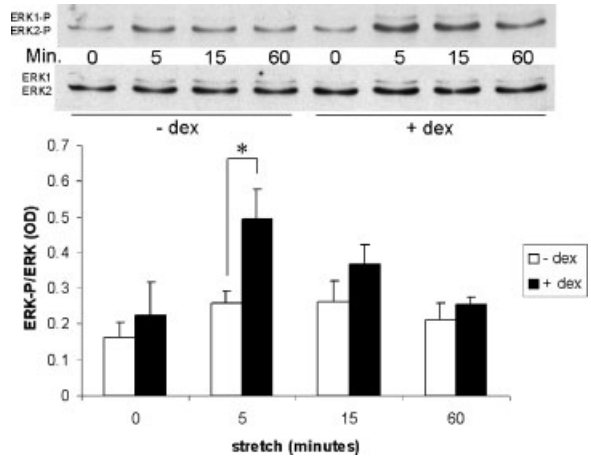


**Fig. 5.** Effect of glucocorticoid-induced osteoblast differentiation on stretch-induced ERK1/2 phosphorylation. Cells were cultured up to 21 days in the absence (−dex) or presence (+dex) of dexamethasone and β-glycerophosphate. Stretch-induced ERK1/2 phosphorylation levels were highest in differentiated osteoblasts, which reached significance after 5 min of stretch.

Next, we examined whether the magnitude of the ERK1/2 phosphorylation was dependent on osteoblast differentiation. Therefore, stretch was applied to SV-HFO cells for 5 min at day 7, 14, or 21 of culture. Static controls served as a reference. Figure 4 clearly shows that the effect of stretch on ERK1/2 phosphorylation was strongest at day 14 and 21 (Fig. 4).

Differentiation stage-dependent effects of strain were also assessed in a different manner. Cell cultures supplemented with or without osteogenic factors were cultured up to 21 days. When cultured in the presence of osteogenic factors, the differentiated osteoblasts showed a higher response of ERK1/2-phosphorylation following stretching than when cultured in the absence of osteogenic factors at the same time point (Fig. 5).

In presence of the specific MAPK pathway inhibitor U0126, the increased ERK1/2 phosphorylation after mechanical stimulation was effectively suppressed (Fig. 6).

### Role of Cell Density and Collagen Formation on Stretch-Induced ERK1/2 Phosphorylation

Total DNA content is different between cultures in osteogenic medium and non-osteogenic medium. To address whether cell densities could explain the observed differences in ERK1/2 phosphorylation after stretch, cultures were performed with various cell densities and osteogenic culture conditions. As shown in Figure 7A, cells initially seeded in normal density ($2 \times 10^5$ cells per well) and cultured without osteogenic factors resulted in higher density after 14 days as cells seeded in half density ($1 \times 10^5$ cells per well) and cultured in the presence of osteogenic factors. Basal ERK1/2 phosphorylation levels were not affected by these differences in cell density. However, most important, despite the lower cell density stretch-induced ERK1/2 phosphorylation was significantly higher in cells cultured in

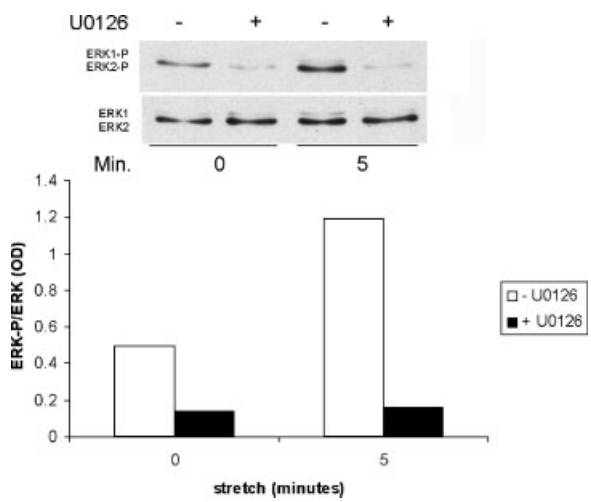


**Fig. 6.** The effect of the MAPK inhibitor U0126 on the basal and stretch-induced ERK phosphorylation. Cells were cultured for 21 days and then preincubated with U0126 for 90 min followed by 5 min of stretch.

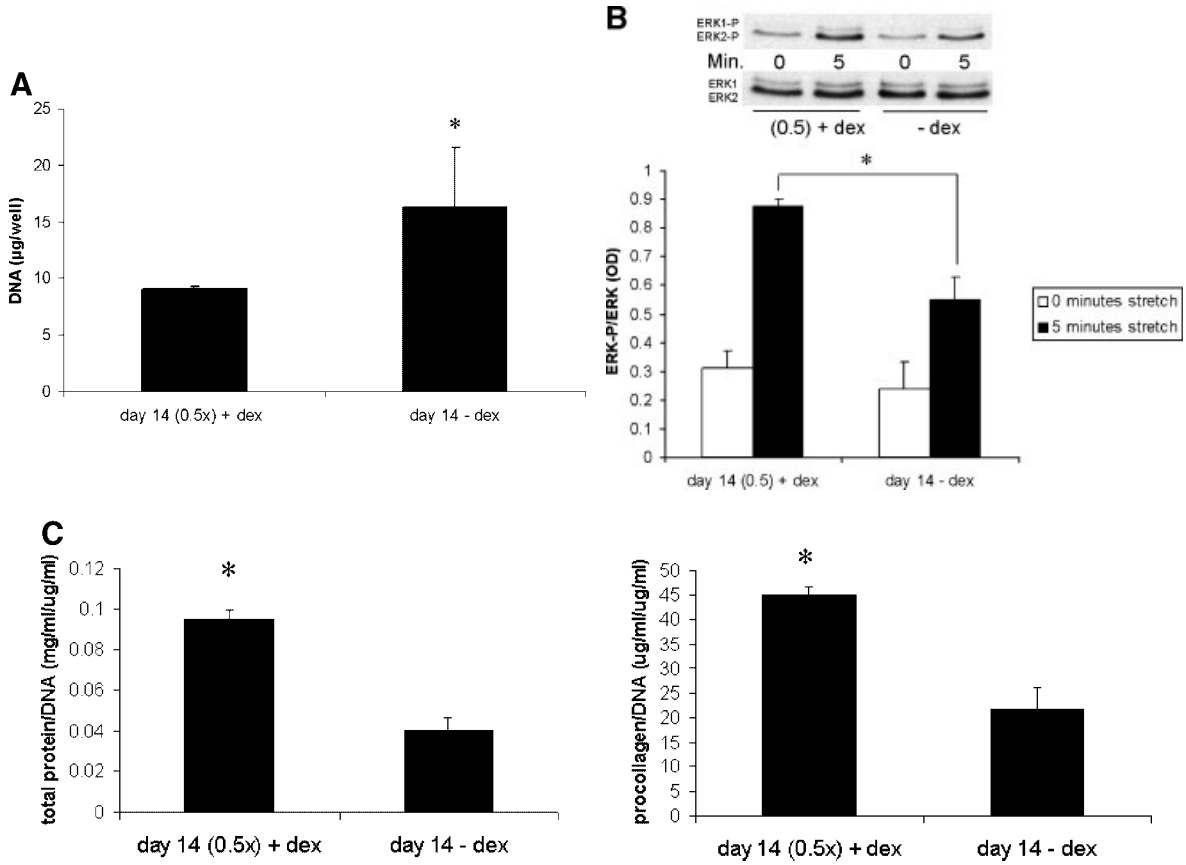


**Fig. 7.** Effect of cell density and glucocorticoid-induced osteoblast differentiation on stretch-induced ERK1/2 phosphorylation. **A**: DNA levels after 14 days of the group initially seeded in half density ($1 \times 10^5$ cells per well) while cultured in the presence of osteogenic factors (+dex) and the group seeded in normal density ($2 \times 10^5$ cells per well) while cultured without osteogenic factors (−dex). **B**: The ERK-P response after 5 min of stretch was significantly higher in the first, differentiated group. **C**: The amount of protein and procollagen per DNA at day 14 in the differentiated half-density seeded culture (+dex 0.5×) and the non-differentiated normal-density seeded culture (−dex). Protein (mostly procollagen) per DNA was highest in the differentiated culture supplemented with osteogenic factors (+dex).

osteogenic medium (Fig. 7B). Next, we investigated whether differences in extracellular matrix could explain the observed differences in ERK1/2 phosphorylation. Total protein as well as pro-collagen type I levels were similar between groups. After correction for DNA, however, Figure 7C shows that both total protein and pro-collagen levels were significantly lower when cultured in the absence of osteogenic medium. Intriguingly, a halving of collagen type I per DNA was paralleled by a similar reduction in ERK1/2 phosphorylation (compare Fig. 7B,C).

### Role of Mineralization of the Extracellular Matrix on Stretch-Induced ERK1/2 Phosphorylation

The level of phosphorylation of ERK1/2 after stretch increased during the differentiation process of the osteoblasts. To determine the role of mineralization of the extracellular matrix in this increase, cells were cultured in the presence of osteogenic factors. Phosphorylation levels of ERK1/2 after stretch were assessed at day 14 (differentiated osteoblasts surrounded by matrix, but no mineralization) and 21 (differentiated osteoblasts surrounded by mineralized matrix) (Fig. 8). Virtually no differences in ERK1/2-P levels could be observed between the two groups.

## DISCUSSION

This study demonstrates that ERK1/2 can be activated upon cyclic strain in human osteoblasts. This finding supports previous studies reporting similar results using mouse osteoblasts, rat osteosarcoma cells, human period-

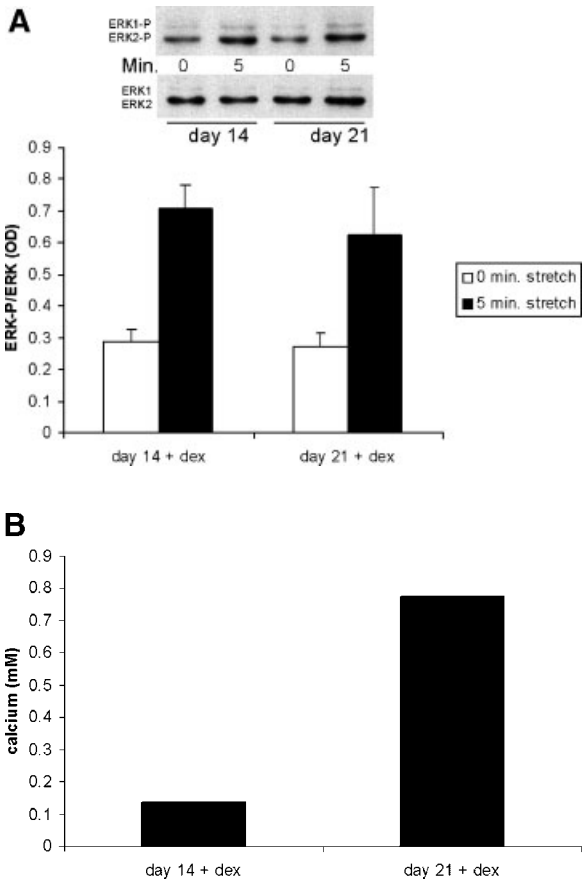


**Fig. 8.** Effect of mineralization of the extracellular matrix on stretch-induced ERK1/2 phosporylation. Cells were cultured in the presence of osteogenic factors. **A**: Phosphorylation levels of ERK1/2 after 5 min of stretch were assessed at day 14 (differentiated cells surrounded by matrix, but without mineralization) or 21 (differentiated cell surrounded by mineralized matrix). Both groups showed similar induction of ERK1/2-phosphorylation, although the amount of calcium was different (**B**).

ontal ligament cells, and murine stromal cells [Peverali et al., 2001; Jessop et al., 2002; Rubin et al., 2002, 2003; Ziros et al., 2002]. However, to our best knowledge this is the first study showing that differentiation of human osteoblasts affects their response to strain in vitro on a molecular level. The SV-HFO cell line was used as a well-defined model of osteoblastic differentiation. Cultured under the appropriate conditions these cells express many osteoblastic markers in a time-dependent fashion as has been described previously by us and others [Chiba et al., 1993; Iba et al., 1995; Arts et al., 1997; Janssen et al., 1999; Weyts et al., 2003]. We demonstrate a higher increase of phosphorylated ERK1/2 levels upon mechanical loading after 14 and 21 days of culture, when matrix has been formed and subsequently becomes mineralized. A specific MAPK pathway inhibitor was used to demonstrate that the increase is due to activation of this pathway.

Weyts et al. [2003] already showed the significance of the differentiation stage of osteoblasts for the translation of mechanical signals. A possible explanation is that interaction within the extracellular matrix is needed for osteoblasts to detect mechanical deformation. Furthermore, adhesion of bone cells to the extracellular matrix has been shown to be important for osteoblastic development and function [Lai et al., 2001]. As the osteoblasts are physiologically anchored to the organic bone matrix via adhesion receptors, it is likely that osteoblasts are more susceptible to mechanical forces when they become embedded in the matrix. We used two different approaches to demonstrate the importance of differentiation with regard to increased stretch-induced ERK1/2-P levels. First, we compared ERK1/2-P levels between different stages of the differentiation process in time. Higher ERK1/2-P levels in response to stretch were seen on day 14 and 21 as compared to day 7. In a second experiment, we compared the ERK1/2-P levels between the differentiated, mineralized cultures and the non-differentiated cultures at day 21. The phosphorylated ERK1/2 response was significantly higher in the differentiated cultures. Both experiments indicate a positive contribution of osteoblast differentiation to the ability of cells to activate the ERK pathway in response to stretch. We hypothesized a possible effect of higher cell density on the increased responsiveness of the osteoblasts caused by prolonged culturing. To exclude this possible effect, we compared ERK1/2-P levels in osteoblasts cultured in the absence of osteogenic factors with a normal cell density and osteoblasts cultured in the presence of osteogenic factors with half cell density. The difference in seeding density resulted in significant lower DNA levels in the last group at day 14. The ERK1/2-P response after strain was highest in this group, indicating a positive contribution of osteoblast differentiation, and not cell density, to its ability to activate the ERK1/2 pathway for processing mechanical signals. Intriguingly, when corrected for DNA, a halving of procollagen type I was paralleled by a similar reduction in ERK1/2 phosphorylation. This might indicate a direct relationship between the amount of collagen fibril formation

and the responsivity of osteoblasts to mechanical loading. A minimal amount of collagen per cell could be necessary to ensure proper cell–matrix interaction. This is in concordance with results of MacKenna et al. [1998] who found in rat cardiac fibroblasts that ERK2 was rapidly activated by stretch when the fibroblasts were allowed to synthesize their own matrices. They also demonstrated that integrins act as mechanotransducers, providing insight into potential mechanisms for in vivo responses to mechanical stimuli. Although we cannot exclude some other aspect of glucocorticoid-induced differentiation, the collagen formation showed a striking relation with the levels of ERK phosphorylation, that might indicate the importance of matrix synthesis.

When the extracellular matrix gets mineralized, the matrix will become more rigid and it is feasible that this will seclude the osteoblasts from physical deformation upon mechanical loading. This may result in a decrease of responsiveness of the cells. To verify this theory, the effect of mineralization of the matrix on responsivity of the osteoblasts was investigated, comparing two differentiating cell cultures. Differentiating osteoblast in a non-mineralized matrix at day 14 showed similar extent of ERK1/2 phosphorylation after stretch as differentiating osteoblasts in a mineralized matrix at day 21. This led to the conclusion that, although mineralization is part of the physiological pathway of osteoblast differentiation, it does not contribute towards increased responsiveness of the osteoblasts to stretch.

Hatton et al. [2003] found that phosphorylated ERK1/2 can translocate to the osteoblast nucleus upon mechanical loading, where it can affect gene expression through the activation of transcription factors of the AP-1 family [Hipskind and Bilbe, 1998]. Several genes like e.g. cbfa-1, c-fos, collagen I, osteopontin, osteonectin, and osteocalcin, that are pivotal for bone formation, reveal AP-1 promoter elements [Rodan, 1991; Tou et al., 2001] and may thus be regulated downstream of ERK1/2 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; You et al., 2001]. Recently, Rubin showed a mechanical regulation of RANKL via ERK1/2 in bone stromal cells, demonstrating the importance of the MAPkinase pathway in osteoblasts–osteoclast signaling [Rubin et al., 2002]. This suggests phosphorylation of ERK1/2 as a potential pathway regulating differential expression of these genes in response to mechanical stimulation.

We used 0.4% strain (4,000 μstrain) to stimulate the osteoblasts, which is considered to be towards the high-end level of physiological strain, but lower than in most other studies. The magnitude of strain to which bone-lining cells and osteocytes are subjected in vivo, however, remains controversial. In vivo peak strains on the surface of long bones, averaged over several millimeters by strain gauge measurement, range from 2,100 to 3,000 μstrain in most mammals [Rubin and Lanyon, 1985; Burr et al., 1996]. However, because bone is a composite material with many embedded cavities and a complex micro-architecture, strain magnitude within bone would not be expected to be the same as on the bone surface [Cowin and Sadegh, 1991].

In concordance with data from Rubin et al. [2002], this study also demonstrates that prolonged stretching for 60 min does not result in a further increase of ERK1/2-P levels, but rather a decrease towards baseline levels, suggesting desensitization of the cells to mechanical loading.

We cannot exclude that the current Flexercell setup generates a fluid flow that initiates the stimulus that leads to the activation of the MAPK pathway. However, it should be understood that flow and its subsequent shear forces on the cells also generate cell deformation. Thus, the mechanical loading devices that are currently in use always include both flow and strain at the same time and therefore are incapable to pinpoint fluid flow or deformation as the actual mechanotransduction signal. Furthermore, the ERK pathway is part of a very sensitive signaling cascade that responds to a wide variety of stimuli. We found that mounting the plate in the stretching device already induced significant increases in ERK-P levels. It is likely that this effect is the result of a mechanical stimulus (fluid flow or strain) caused during mounting. To abolish any effect of plate handling on ERK1/2 phosphorylation, we used a pre-incubation period of 90 min. This was based on the following observations. Pilot experiments showed a decrease in ERK1/2 phosphorylation towards baseline 60 min after cessation of stimulation, indicating that the effect of stimulation is lost within this period.

Cylcic stretching for 5 min after a rest period of 60 min induced again similar levels of ERK-P as the previous stretching episode, suggesting that the refractory period is also less than 60 min. Consequently, an incubation period of at least 60 min between loading of the six-well plates in the stretching device and initiation of stretching experiments is required to avoid possible activation of ERK by plate handling. In literature, this phenomenon is often neglected and can easily provide a bias in the experiments.

In conclusion, the current study demonstrates that physiological stretch levels (0.4%) can activate the MAPkinase signaling pathway in osteoblasts. The extent of activation of this signal pathway is dependent on the differentiation or functional stage of the osteoblast, and is highest when the extracellular matrix has been formed and the osteoblasts become entrapped in it. The presence of an extracellular matrix, but not per se mineralization, seems to be the predominant determinant of osteoblastic response to strain.

## ACKNOWLEDGMENTS

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