Journal of Cellular Biochemistry 84:497–508 (2002)

Predominant Integrin Ligands Expressed by Osteoblasts Show Preferential Regulation in Response to Both Cell Adhesion and Mechanical Perturbation

R.S. Carvalho,[1,2] A. Bumann,[3] J.L. Schaffer,[4] and L.C. Gerstenfeld[1]*

[1]Orthopaedic Research Laboratory, Department of Orthopaedic Surgery, Boston University Medical Center, 715 Albany Street, R-205, Boston, Massachusetts 02118-2526
[2]Department of Orthodontics, School of Dental Medicine, Boston University, 100 East Newton St., Boston, Massachusetts 02118
[3]Department of Experimental Dentistry, Free University, D-14197, Berlin, Germany
[4]Department of Orthopaedic Surgery, The Cleveland Clinic, 9500 Euclid Avenue, Desk A-41, Cleveland, Ohio 44195

Abstract Previous studies have demonstrated that both mechanical perturbation and cell adhesion induced the expression of osteopontin (*opn)* by osteoblasts (Carvalho et al. [1998] J. Cell. Biochem. 70:376–390). The present study examined if these same stimuli on osteoblasts would induce the expression of other integrin binding proteins, specifically fibronectin (*fn*) and bone sialoprotein (*bsp*). All three genes showed three- to four-fold maximal induction in response to both cell adhesion and a single 2-h period of an applied spatially uniform, dynamic biaxial strain of 1.3% at 0.25 Hz. Each gene, however, responded with a different time course of induction to mechanical strain, with *bsp*, *fn*, and *opn* showing their maximal response at 1, 3, and 9 h, respectively, after the perturbation period. In contrast, peak induction to cell adhesion was observed at 24 h for *bsp* and *opn*, while *fn* levels peaked at 8 h. Interestingly, while both *opn* and *fn* mRNA expression returned to base line after cell adhesion, *bsp* mRNA levels remained elevated. Examination of collagen type I and osteocalcin mRNAs showed unaltered levels of expression in response to either type of perturbation. A common feature of the signal transduction pathways, which mediate the gene expression in response to both cell adhesion and mechanical perturbation, was the activation of specific tyrosine kinases based on the ablation of the induction of these genes by the tyrosine kinase inhibitor genistein. While cycloheximide blocked the induction of all three mRNAs in response cell adhesion, it failed to block the induction of any of these genes in response to mechanical perturbation. Such results suggest that the induction of these genes after mechanical perturbation was mediated by an immediate response to signal transduction, while cell adhesion mediated effects secondary to signal transduction. Depolymerization of microfilaments with cytochalasin D had no effect on the overall expression of any of these genes in response to cell adhesion and only blocked the induction of *opn* expression in response to mechanical perturbation. These results suggest that cytoskeletal integrity is only selectively important in the signal transduction of certain types of stimuli and for the regulation of certain genes. In summary, both mechanical perturbation and cell adhesion stimulated the expression of integrin binding proteins. Furthermore, while there are common features in the signal transduction processes that mediate the induction of these genes in response to both stimuli, specific genes are separately regulated by precise mechanisms that are unique to both forms of stimuli. J. Cell. Biochem. 84: 497–508, 2002.

Key words: integrin; osteopontin; osteoblasts; mechanical stimulation

The mediation of cellular responses to mechanical stimuli depends in part on the recognition and interaction of selected cell-surface receptors with the extracellular matrix (ECM). Integrins are one class of cell surface receptors that mediate cell adhesion to the ECM. The integrins are a family of heterodimeric membrane receptors composed of multiple α and β isotypes. Variations in the extracellular

Grant sponsor: The Department of Defense, Bone Health and Military Readiness Program; Grant number: DAMD17-98-1-8510.

*Correspondence to: L.C. Gerstenfeld, Ph.D., Department of Orthopaedic Surgery, Orthopaedic Research Laboratory, Boston University Medical Center, 715 Albany Street, R-205, Boston, MA 02118. E-mail: lgersten@bu.edu

Received 21 March 2001; Accepted 4 September 2001

DOI 10.1002/jcb.10031

domains of the individual isotypes of integrin receptors impart their specificity for unique ECM proteins [Hynes, 1992; Miyauchi et al., 1995]. The interaction of many integrin isotypes with their specific ligands is mediated by their recognition of the amino acid sequence, arginine–glycine–aspartic acid–serine (RGDS) [Hynes, 1992]. It has been demonstrated that the binding of specific adhesion proteins to their integrin receptors generates a cascade of intracellular signals that are responsible for the regulation of a wide variety of cellular responses [Damsky and Werb, 1992; Juliano and Haskill, 1993]. Such interactions facilitate the appropriate functioning of essential cell functions such as cell adhesion, cell migration, and survival of many cell types. These many functions are in turn mediated through integrin receptor activation of specific signal transduction mechanisms or by their mediation of structural alterations in the cytoskeletal architecture of cells [Juliano and Haskill, 1993].

In bone tissue, osteoblasts express high levels of several different RGD-containing proteins, the most predominant being osteopontin (*opn*), bone sialoprotein (*bsp*), and fibronectin (*fn*) [Puleo and Bizios, 1992; Gotoh et al., 1995; Winnard et al., 1995]. Osteopontin has been shown to interact with both osteoblasts and osteoclasts [Oldberg et al., 1988; Gotoh et al., 1990; Ross et al., 1993], and it is thought to play a role in mediating osteoclast resorption of bone tissue [Reinholt et al., 1990; Denhart and Guo, 1993]. The expression of *opn* is seen concurrently with alkaline phosphatase, and it has been identified as an early marker of osteoblast differentiation [Gerstenfeld et al., 1990]. Bone sialoprotein is another specific integrin ligand that is expressed by osteoblasts. It has a very restricted expression to only cells within the skeletal lineage and is seen predominantly in areas of mineralized growth cartilage and osteoid [Chen et al., 1994; Yang and Gerstenfeld, 1996, 1997]. Bone sialoprotein has been shown to initiate calcification through its binding properties to collagen, calcium, and hydroxyapatite [Hunter and Goldberg, 1994]. Unlike the former two proteins, fibronectin is expressed ubiquitously in most connective tissues. However, this protein appears to play an important role in the mechanisms of cell attachment, spreading, and migration during early osteoblast differentiation [Curtis, 1987; Winnard et al., 1995]. In addition to the RGDS peptide, fibronectin contains a synergistic adhesion site to the RGD sequence. It also contains two adhesion sites in the type III connecting segment of the whole molecule, the CS1 portion and the arginine–glutamic acid–aspartic acid–valine (REDV) sequence within the CS5 portion of fibronectin [Puleo and Bizios, 1992]. Some studies suggest that fibronectin also acts as an activator of cell adhesion rather than as a direct adhesion molecule [Curtis et al., 1992]. Thus fibronectin may mediate multiple interactions and responses by cells. Currently, there is a large body of data to suggest that the RGD-containing proteins *opn*, *bsp*, and *fn* play essential roles in the cellular differentiation and migration of osteoblasts during skeletal growth and/or in the initiation of spatial deposition of mineral in the ECM [Curtis et al., 1992; Hunter and Goldberg, 1993; Gerstenfeld et al., 1995; Schaffer et al., 1996].

Previous studies have shown that both the mechanical environment of osteoblasts and cell adhesion induce *opn* gene expression [Toma et al., 1997; Carvalho et al., 1998]. These studies suggest that the interactions of cells with the ECM are integral components to the mediation of these stimuli. However, cellular perturbation through receptors may occur both through the occupancy of the receptor as well as the deformation through the engagement of the receptors with matrix attachment [Miyauchi et al., 1995]. Furthermore, it has been shown that precise reverse phosphorylation of specific proteins appears to regulate various intracellular pathways exclusively upon cellular attachment [Guan et al., 1991]. These results raise the possibility that signal transduction may be dependent on the matrix composition and that the cellular matrix components themselves may function as autocrine factors that regulate their own expression [Gerstenfeld, 1999]. Mechanical perturbation through receptor deformation and stimuli through receptor-ligation then may share overlapping molecular elements that mediate intracellular signal transduction and lead to common genomic responses. Such modulations may also involve cytoskeletal integrity and its relation with the integrin receptors. It has been hypothesized that cellular shape changes determine signal transduction pathways through the direct deformation of cellular membranes and reorientation of the microfilament network, thereby affecting integrin behavior [Ingber, 1991].

In previous studies from our laboratory, we have shown that the continuous application of a dynamic, spatially uniform biaxial mechanical perturbation to osteoblasts leads to cytoskeletal rearrangement as well as changes in the ECM composition cellular response [Meazzini et al., 1998]. Other studies have shown that mechanical strain in cells of osteoblastic lineage only showed increased DNA synthesis when the cells were attached to specific ligands such as fibronectin [Wilson et al., 1995]. Thus, it seems that mechanical perturbation leads to both physical alteration of ECM proteins and alterations in cellular architecture, which suggests that cells reach a homesostatic balance that is regulated through a complex set of receptor-mediated interactions between the cells and the ECM proteins. In this study we examined if genes such as *opn*, *bsp*, and *fn*, which specifically interact with integrins, would be commonly induced following either mechanical perturbation and/or cell-ligand binding. It was further examined if there are common signal transduction pathways by which the expression of these proteins were modified following cellular attachment or mechanical perturbation, and if these pathways are unique or have overlapping mechanisms.

MATERIALS AND METHODS

Materials

All tissue culture supplies, cytochalasin D, colchicine, and cycloheximide were from Sigma Chemical Company (St. Louis, MO). H89 genistein was from LC Laboratories (Woburn, MA). Nylon membranes for Northern blots were from Biotrans, ICN Corp. (Aurora, OH).

Cell Culture

Seventeen-day embryonic chicken calvaria osteoblasts were isolated and grown in culture as previously described [Gerstenfeld et al., 1988]. These cells were plated at a density of 2×10^6 cells in 100-mm tissue culture dishes either left uncoated or coated with purified fibronectin (1 mg/ml) as previously described [Schaffer et al., 1994]. Cultures were grown for 2 weeks until they reached confluence in minimum essential media supplemented with 10% fetal bovine serum (FBS). The medium was changed to BGJ_b supplemented with 10% FBS with the addition of 10 mM β-glycerophosphate and 12.5 μg/ml ascorbic acid. All analyses were performed on at least three separate preparations of cells, and all data are presented as a percent increase in expression over that of the controls which were determined from parallel cultures grown under identical conditions. All error bars represent the standard deviation (SD) of the determinations from separate experiments and the number of replicates that were used for each measurement is denoted in each figure.

Mechanical Perturbation/Attachment Assays

The mechanical stretch apparatus used for these experiments was as previously described [Schaffer et al., 1994]. The design of the device imposes a verified temporal and spatial displacement profile to an optically transparent elastomeric membrane in which the strain magnitude was experimentally demonstrated to be homogeneous and isotropic (i.e., radial strain = circumferential strain = constant over the culture surface) [Schaffer et al., 1994]. A polyurethane membrane (a generous gift from Dow Chemical Corporation, Midland, MI) was used in the culture dishes allowing for a constant 1.3% uniform biaxial strain at 0.25 Hz to be applied for a single 2 h period. For each experiment, non-stimulated controls were performed on identical culture surfaces at the same time and from the same preparation of cells grown at identical conditions as the mechanically stimulated cultures. In all experiments for mechanical stretch (perturbation), determinations were carried out 6 h after the end of the 2 h period of active cellular perturbation. For the attachment/integrin ligation assays, the cells were allowed to attach to fibronectin coated (1 mg/ml) dishes at the same concentration as those of mechanical stimulated cultures for 24 h. Fibronectin served as the basic ligand and uncoated plastic plates as controls.

Signal Transduction Studies

Signal transduction pathways that mediate the cell responses of the mechanical perturbation and/or attachment/ligation were investigated by the use of specific chemical inhibitors. The final concentration for each of these compounds was: 50 mM cycloheximide, 20 mg/ml genistein, 1 μM H89 (Sigma), 50 μM cytochalasin D (Sigma), and 1 μM colchicine (Sigma). Cycloheximide and genistein were incubated for 30 min, while cytochalasin D was incubated for

1 h and colchicine for 6 h. Controls were separately determined for each compound in cultures treated identically with the various compounds but in which the cells were either not mechanically stimulated or were attached to uncoated dishes.

Isolation and Analysis of RNA

Total RNA was isolated using tri-Reagent™ (Molecular Center, Cincinati, OH) according to the manufacturer's instructions. RNA was resolved on 1% agarose gels containing 2.2 M formaldehyde [Toma et al., 1997] and 5 mg of total RNA was loaded per gel per lane. Chicken cDNAs used for these studies were pro α1[I] collagen [Lehrach et al., 1979], osteocalcin [Neugebauer et al., 1995], *opn* [Moore et al., 1991], and *bsp* [Yang et al., 1995]. Northern blots with ^{32}P cDNA-labeled probes were carried out at 65°C in 2.5 × SSC, 50 mM Na-phosphate buffer, 100 μg/ml single stranded salmon sperm DNA, and for 18–24 h in a rotating hybridization oven (Robins Scientific, Sunnyvale, CA). Autoradiograms were quantified using an LKB Ultra II scanning densitometer (LKB, Broma, Sweden), and values were normalized against 18S ribosomal RNA obtained by hybridization of each blot to a conserved nucleotide sequence probe of 18S ribosomal subunit (Ambion Corp., Austin, TX). All analyses were performed at least three times, and all data are presented as a percentage in expression over that of the control, which were determined from parallel cultures. All data were evaluated as a mean ± 2 standard deviations with a minimum of three experiments from different populations of primary cells, and appropriate statistical analysis were performed.

RESULTS

Osteoblast-Adhesion and Mechanical Perturbation Increase Levels of RGD-Containing Proteins

Initial studies were carried out to assess the expression of mRNA levels for *opn*, *bsp*, and *fn* osteoblasts following either cell adhesion or mechanical perturbation. Fibronectin was used as the adhesion substrate in these experiments. The temporal profiles of *opn* expression were shown to peak at 24 h. This induction was three- to four-fold above that of control samples. At time periods beyond 24 h, there was a sharp reduction in *opn* expression, which returned to its baseline levels of expression (Fig. 1). Bone sialoprotein expression followed a similar profile to that seen for *opn* also peaking at 24 h (three-fold). However, there was no marked decrease in the expression of *bsp*, which remained elevated (two-fold) at 48 h and even 72 h (Fig. 2). In contrast, the expression of *fn* mRNA started at significantly higher levels when compared to the other two mRNAs (Fig. 3). Expression of *fn* peaked at 8 h from the onset of the perturbation (two-fold) and showed a sharp reduction to its baseline levels soon thereafter.

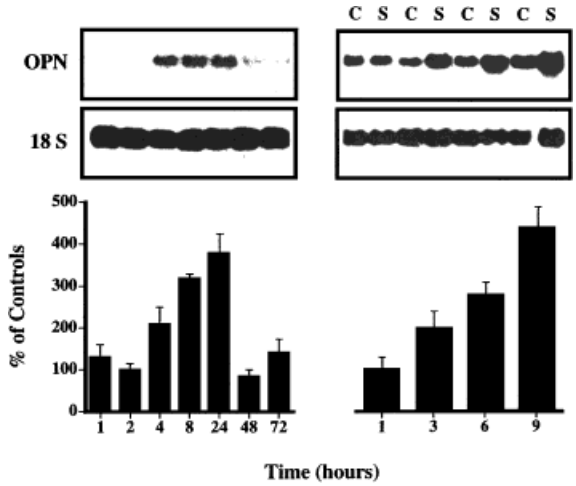

Fig. 1. Effect of cell adhesion and mechanical perturbation on the temporal expression of osteopontin (*opn*) mRNA expression by osteoblasts. Northern blot analysis of *opn* mRNA expression following cell adhesion of osteoblasts to fibronectin is seen on the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48, and 72 h after an initial 24-h period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6, and 9 h post-mechanical perturbation. Controls = C and strained samples = S. All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the SD determined from at least three experiments.

The expression of these mRNAs was then examined after the application of mechanical perturbation. As expected, mechanical perturbation of osteoblasts increased *opn* expression by two to threefold, peaking at 9 h post-stretch (Fig. 1). This clearly contrasted with adhesion, which showed a peak induction in *opn* expression at 24 h. In the case of *fn*, mechanical perturbation also showed an increase in expression peaking at 3 and 6 h from the onset of the perturbation (Fig. 3). It is interesting to note, however, that mechanical perturbation was

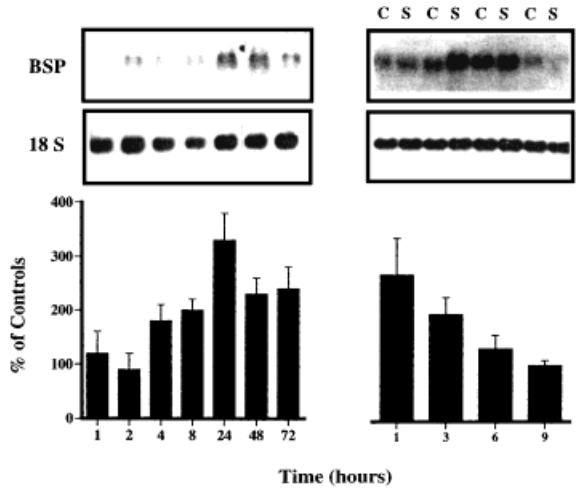

Fig. 2. Effect of cell adhesion and mechanical perturbation on the temporal expression of *bsp* mRNA expression by osteoblasts. Northern blot analysis of bone sialoprotein mRNA expression following cell adhesion of osteoblasts to fibronectin is seen on the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48, and 72 h after an initial 24-h period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6, and 9 h post-mechanical perturbation. Controls = C and strained samples = S. All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the SD determined from at least three experiments.

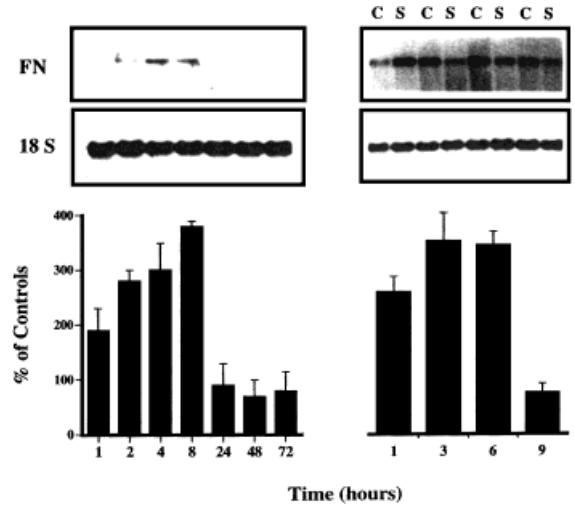

Fig. 3. Effect of cell adhesion and mechanical perturbation on the temporal expression of *fn* mRNA expression by osteoblasts. Northern blot analysis of fibronectin mRNA expression following cell adhesion of osteoblasts to fibronectin is seen in the left and that of induction in response to mechanical perturbation is seen on the right. The expression of the 18S rRNA is seen in the lower panel of each figure. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 2, 4, 8, 24, 48, and 72 h after an initial 24-h period of attachment relative to the control samples at identical times. The graphic representation of the temporal expression for the mRNA levels is shown for 1, 3, 6, and 9 h post-mechanical perturbation. Controls = C and strained samples = S. All data are presented as percent induction of expression of the mRNAs relative to their control samples. Error bars are the SD determined from at least three experiments.

inhibitory for *bsp* expression. In Figure 2, *bsp* levels started at twofold of control samples at 1 h post-stretch and quickly decreased to baseline levels at 9 h post-stretch.

These results would suggest that integrin binding-ECM molecules are selectively responsive to perturbation via either mechanical perturbation or cell adhesion-mediated signal transduction. Two other prevalent ECM genes, collagen type I and osteocalcin, were then examined as a comparison to these RGD containing integrin ligands. Both collagen type I (*col1*) and osteocalcin (*oc*) mRNAs were examined after mechanical perturbation. Interestingly, neither of these genes showed alterations in their expression when the cells were subjected to mechanical perturbation (Fig. 4).

Different Matrix Proteins Require Different Signal Transduction Pathways Following Cell Adhesion

In order to further understand if common signal transduction processes mediated the

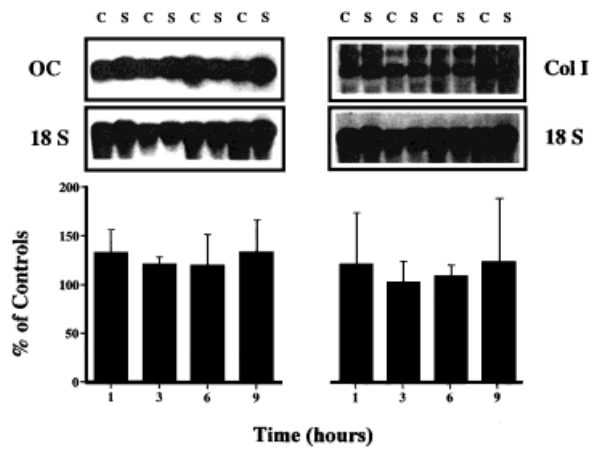

Fig. 4. Effect of mechanical perturbation on the temporal expression of *col1* and *oc* mRNA expression by osteoblasts. Northern blot analysis of osteocalcin and collagen type I mRNA expression following mechanical perturbation of osteoblasts are denoted in the figure. The graphic representation of the temporal expression for these mRNAs is shown by the times of 1, 3, 6, and 9 h post-perturbation. All the panels show the percent induction of expression of the steady mRNA levels relative to their control samples. Controls = C and strained samples = S. Error bars are the SD determined from at least three experiments.

induction of the three RGD integrin ligand genes, pharmacological inhibitors of specific signal transduction pathways were used. From the slow increase in *opn* levels following cell adhesion, it appears that this protein is involved in a secondary, down-stream event to other genomic changes. Indeed, the compound cycloheximide, a known inhibitor of de novo protein synthesis, blocked the induction of *opn* mRNA expression following adhesion to fibronectin (Fig. 5). Cycloheximide also inhibited the expression levels of *fn* and *bsp* genes (Figs. 6 and 7), showing that these genes were also dependent on new protein synthesis following cellular adhesion. Even though the maximum levels of *fn* occurred at 9 h post cellular adhesion, this finding was consistent with the relative long period for maximum induction of *opn* and *bsp* (Figs. 1–3).

Previous observations had shown that changes in *opn* mRNA expression in response to mechanical perturbation were dependent on the integrity of the microfilament structure of the cell [Toma et al., 1997]. This finding is consistent to the results seen in this study (Fig. 5). The role of the cytoskeleton in the signal transduction pathways for each of these mRNAs was examined in these studies. Incubation of the osteoblast cultures with the microtubule depolymerizing agent colchicine did not affect the expression of any of the mRNAs (Figs. 5–7). However, cultures treated with cytochalsin-D, a microfilament disruption agent, inhibited the levels of *opn* mRNA below those of control levels (Fig. 5), following mechanical perturbation. This change was not seen for either *bsp* or *fn* mRNAs (Figs. 6 and 7).

Finally, specific inhibitors for second messenger systems were used. The use of genistein, a potent inhibitor of tyrosine kinase phosphorylation, was shown to significantly inhibit the expression of *opn*, *bsp*, and *fn* mRNAs. As shown in Figure 5, this finding has also been observed previously for *opn* expression in mechanically stimulated cells [Toma et al., 1997; Carvalho et al., 1998]. Genistein treatment in cells subjected to mechanical perturbation also inhibited the levels of *fn* and *bsp* mRNAs (Figs. 6 and 7). The pharmacological inhibitor of PKA-like kinases, H-89, also caused an

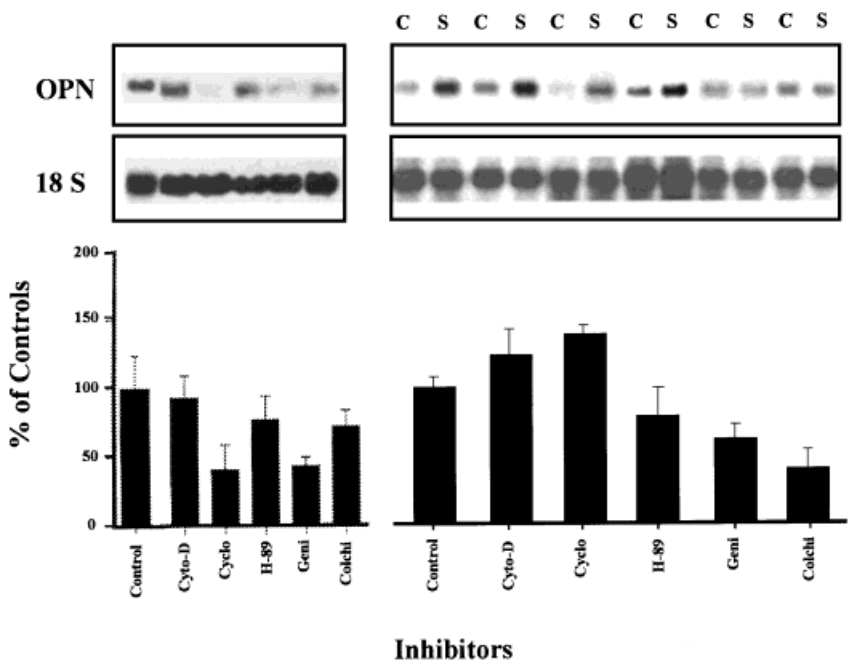

Fig. 5. Effect of pharmacological inhibitors on the mRNA expression of *opn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *opn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89), and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the SD of three experiments.

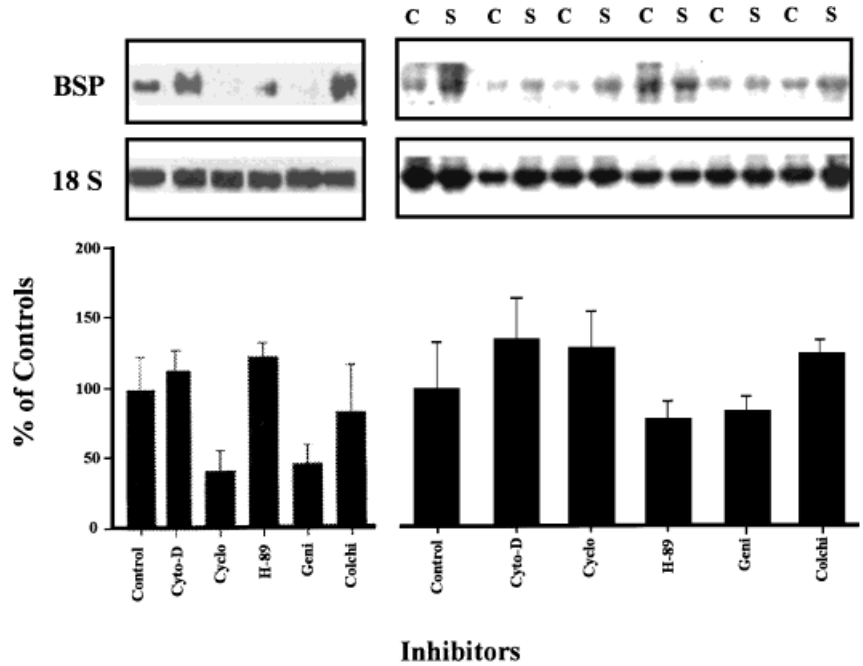

Fig. 6. Effect of pharmacological inhibitors on the mRNA expression of *bsp* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *bsp* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89), and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18 S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the SD of three experiments.

inhibition of *fn* levels, but it did not effect the expression of *opn* or *bsp* following cell adhesion. However, when H-89 was given to cells that had undergone mechanical perturbation, the expression of both *bsp* and *opn* mRNAs were inhibited, but not that of *fn* mRNA (Figs. 5–7). These findings suggest that the perturbation in expression of the RGD-containing extracellular proteins, which are studied here in response to cell adhesion, is distinct from that of mechanical perturbation, yet both responses appear to be uniquely dependent on the activation of specific subsets of kinases.

DISCUSSION

In previous studies, we have shown that both mechanical perturbation and cell adhesion led to the induction of increased *opn* expression. While both types of stimuli were dependent on integrin receptors, each of them was mediated by a specific set of intracellular signals that were distinct for each type of perturbation [Carvalho et al., 1998]. The present study provides further evidence that both cellular adhesion and mechanical perturbation lead to the selective induction of multiple integrin binding proteins within osteoblasts. Furthermore, while these data suggest that there may be some common mechanisms of signal transduction that stimulate the increased expression of these genes, each of the genes was uniquely and separately regulated by both stimuli (Table I).

In the bone extracellular matrix, RGD-containing glycoproteins including *opn*, *bsp*, and *fn* are presumed to interact with cell adhesion receptors (integrins) on the surface of the bone cells [Grzesik and Robey, 1994]. Studies suggest that that these extracellular components directly affect gene expression [Pienta et al., 1991], which takes place following mechanical perturbation [Resnick et al., 1993; Toma et al., 1997; Carvalho et al., 1998]. When considering adhesion separately from mechanical perturbation, one needs to take into account the effects of the former over the latter, as it may be speculated then that adhesion or integrin-ligation acts as a "primer" prior to any response due to the mechanical perturbation. In particular, it is interesting to note that the effects of the dynamic,

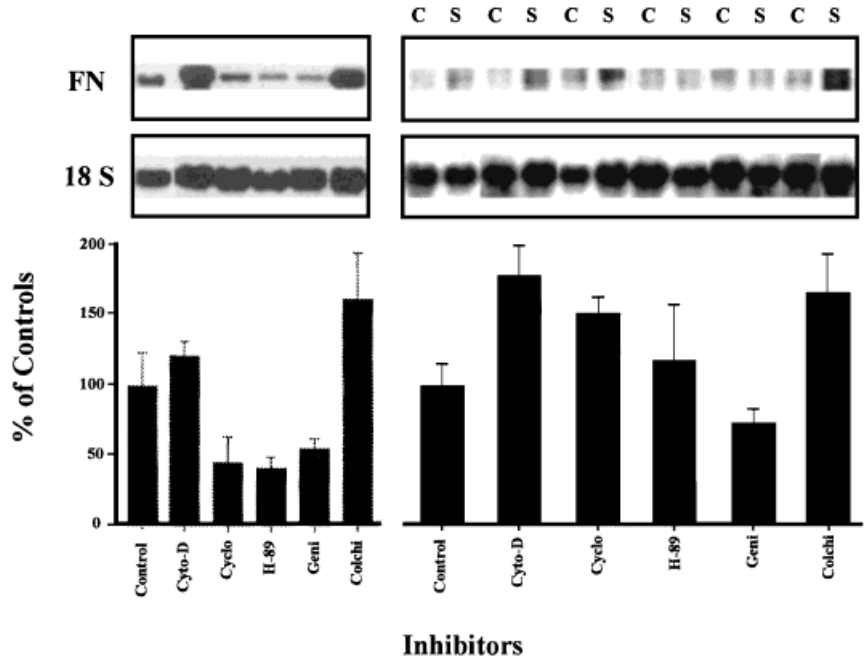

Fig. 7. Effect of pharmacological inhibitors on the mRNA expression of *fn* by osteoblasts in response to cell adhesion and mechanical perturbation. Effects of various second messenger inhibitors and cytoskeletal disrupters on the expression of *fn* mRNA in response to cell adhesion and mechanical perturbation were examined. Cells were treated with the microfilament depolymerizing agent cytochalasin-D (Cyto-D), the microtubule disrupting agent colchicine (Colchi), the protein synthesis inhibitor cycloheximide (Cyclo), the PKA inhibitor H-89 (H-89), and the tyrosine kinase inhibitor genistein (Geni). Autoradiographs for the Northern blot analysis of the steady state levels of each mRNA and of the 18S RNA are presented separately. Graphic analysis shows the percent induction or inhibition of the various mRNAs compared to that of controls. Error bars are the SD of three experiments.

spatially uniform mechanical perturbation on cells that are in the process of adhesion enhances the formation of new receptor-ligand bonds. In studies by Kuo et al. [1977], mechanical perturbation was shown to alter the kinetic regulation of cell adhesion, thus interfering with binding rate. However, once the cells are adherent, specific RGD tri-peptides inhibited both adhesion and mechanical perturbation responses [Carvalho et al., 1998]. Wilson et al. [1995] also presented evidence that RGD peptides, fibronectin, and certain integrin antibodies disrupted integrin-ligand interaction, which in turn ablated strain induced mechanotransduction responses, without disrupting adhesion of the same cells.

It has been proposed that receptors such as integrins can behave as a homeostatic system for modulating the ECM structure and organization in response to the structural needs of the

TABLE I. Comparison of Signal Transduction Pathways for the mRNA Expression of Osteopontin (*opn*), Bone Sialoprotein (*bsp*), and Fibronectin (*fn*) Between Cell Adhesion and Mechanical Stretching

	Genes					
	opn		*bsp*		*fn*	
Perturbation	Adhesion	Stretch	Adhesion	Stretch	Adhesion	Stretch
Time after peak expression (h)	24	9	24	1	8	3
De novo protein synthesis	Yes	No	Yes	No	Yes	No
Tyrosine kinase-mediated	Yes	Yes	Yes	Yes	Yes	Yes
PKA-mediated	No	Yes	No	Yes	Yes	No
Requires microfilaments	No	Yes	No	No	No	No
Requires microtubules	Yes	No	No	No	No	No

cell [Werb et al., 1989; Gerstenfeld, 1999]. In this context, it is interesting to note that the mechanical forces that are applied to tissues should be structurally deformed at their anchorage points to the extracellular matrix. These sites should be the focal points at which mechanical strains are specifically transmitted to the cells. The strains to which a cell is subjected, therefore, will be effected by both the compositional properties of the matrix and the receptors on the surfaces of the cells, which can interact with these ECM proteins. The importance of extracellular interactions for the process of mechanosensation has been shown by Du et al. [1996]. They demonstrated in their experiments that isolated genes of the touch receptor neurons in *C. elegans* encoded for extracellular proteins. These authors [Du et al., 1996] further hypothesized that the ECM mediates the anchoring properties of specialized cells, enabling their mechanosensory response.

Concerning the intercellular components of mechanosignal transduction, both Davies and Tripathi [1993] and Ingber [1997] have suggested that the transduction of mechanical stimuli in anchorage-dependent cells is due to a combination of signal transduction processes via the cytoskeleton through integrin receptors that interact with the cytoskeleton, as well as through biochemical signals. In order for mechanosignal transduction to occur through the cytoskeleton, it is necessary that cooperative interactions occur between the three component parts of cytoskeleton: the micofilaments, intermediate filaments, and microtubles. Studies of osteoblasts have also shown that the cytoskeleton changes both its structural architecture and its composition in response to mechanical perturbation [Meazzini et al., 1998]. However, signal transduction of different types of signals may not depend on the integrity of all of these structural elements. In this study, this is shown by following the selective inhibition of microtubules and microfilaments. The results presented in Figure 5 and Table I demonstrate that *opn*−cell adhesion dependent mRNA expression was inhibited following colchicine treatment, yet adhesion was not affected after the addition of cytochalasin-D. The inverse effect was seen when the cells were mechanically stimulated [Toma et al., 1997]. In contrast, alterations in cytoskeletal architecture via pharmacological manipulation did not change the expression of *bsp* or *fn* mRNAs in response to either mechanical perturbation or cell adhesion. These latter results suggest the involvement of microfilaments in the selective regulation of some genes but not others. Indeed, recent studies have shown that the induction of COX 2 enzyme expression within osteoblasts in response to fluid flow mediated shear stress was also regulated via alteration in the cells cytoskeletal architecture [Pavalko et al., 1998]. Such results suggest that there may be specific subsets of genes that are commonly regulated and are dependent on the cytoarchitecture of the cell, yet others may not be dependant on the cytoarchitecture, even though they are regulated by cell adhesion or mechanical perturbation.

This differentiation between mechanical perturbation and adhesion appears to follow unique mechanisms. Since each ECM ligand interacts with different integrins, it may be speculated that selective interactions or mediation of the various signals occur through specific integrin receptors. For instance, while $\alpha v\beta 3$ deficient cell populations were not capable of migrating in response to *opn*, these same cells did migrate significantly in response to fibronectin and vitronectin [Liaw et al., 1995]. Furthermore, specific ligand interactions may mediate a variety of intracellular signals ranging from ion flux to selective G-protein kinase and/or phosphatase activation. The transduction of the mechanosignal at the cellular membrane leads to a cascade of downstream signaling events, many of which are mediated by tyrosine kinases, which in turn phosphorylate other kinases [Berk et al., 1995]. Kinases that have been associated with mechanotransduction include mitogen-activated protein kinase (MAPK). MAPK activation has been shown to follow mechanical perturbation in cardiac cells [Yamazaki et al., 1993] and fluid flow in endothelial cells [Tseng and Berk, 1993]. It has been suggested that such responses are part of a multiplicity of pathways and might be grouped functionally into those that are either calcium-dependent or -independent [Berk et al., 1995; Ishida et al., 1997]. The presence of calcium is important for the activation of a putative shear stress receptor (membrane level), which regulates a pertussis toxin-sensitive G protein-coupled $K+$ channel (SSR) [Ohno et al., 1993] and the enzyme phospholipase C [Nollert et al., 1990]. The levels of PIP2, in turn will be regulated by rho, a small GTP-binding protein [Chong et al., 1994]. The calcium-independent

pathway involves the activation of MAPK [Berk et al., 1995]; however, other calcium-independent tyrosine kinases such as src and FAK may also be involved in the shear stress transduction.

A common feature in the signal transduction processes that regulate the expression of all the integrin binding genes in response to either adhesion or mechanical perturbation was the inhibition of the induction of their expression by genistein. This suggests that a tyrosine kinase(s) is involved in the signal transduction, which stimulates the expression of all of these proteins. In contrast, PKA inhibition through H-89 treatment demonstrated a selective effect for *opn* and *bsp* in response to mechanical perturbation but not with cell adhesion (Figs. 5 and 6). As for *fn*, H-89 inhibited the effects of adhesion but not those of mechanical perturbation (Table I; Fig. 7). In the experiments reported herein, there were no changes in the non-RGD containing proteins collagen type I and osteocalcin (Fig. 4). Integrin receptors have been described as potential mediators of mechanical perturbation [Ingber et al., 1994; Ishida et al., 1997]. Activation of integrins has been shown to induce the tyrosine phosphorylation of FAK at focal adhesion complexes [Schaller et al., 1994]. In addition, other proteins within these focal adhesion contacts, such as paxillin and src, will also be phosphorylated when exposed to flow [Girard and Nehem, 1993; Bull et al., 1994]. Our laboratory has shown that FAK phosphorylation was regulated by mechanical perturbation [Toma et al., 1997], also suggesting that the disruption of microtubules does not affect the expression of any gene studied following mechanical perturbation. This is an interesting finding as it relates to MAPK, as this kinase has been shown as the earliest signal activated by flow at physiological stress [Tseng and Berk, 1993]. MAPK is also known as a microtubule-associated kinase [Sabe et al., 1994], suggesting a role in the cytoskeleton. However, as disruption of microtubules did not affect gene expression following perturbation, one may only speculate on the role of MAPK as a mechanical perturbation-dependent kinase. On the other hand, we have observed that adhesion alone in the presence of the microtubule-disrupting drug colchicine, blocked the induction of *opn* expression in particular. Thus, it is conceivable that MAPK plays a role in this mechanism, since this kinase has been shown to be activated by cell binding to fibronectin [Morino et al., 1995]. It has been suggested that activation of integrins is associated with the same signal events that occur when cells are exposed to flow [Vuori and Ruoslahti, 1993; Schwartz and Deninghoff, 1994; Berk et al., 1995]. However, further study is needed, as the complexity of such a response can not be understood if the responses of mechanical perturbation and adhesion are not taken into account individually.

Integrin-ligation is thought to stimulate the same signal events as mechanical perturbation [Berk et al., 1995]. Indeed, we have shown here that this is the case, even though the mechanisms that mediate both responses are uniquely different. If integrins are the mediators for mechanotransduction in both forms of activation, then there maybe several different integrin receptors acting in concert with other sensors that are specific to the mechanical activation. The dependency of mechanical perturbation effects on RGD-containing proteins in this study and the lack of response in either collagen type I or osteocalcin further demonstrate an active role of integrins in adhesion and perturbation. It is clear that integrins and focal contacts play important roles in mechanotransduction. It remains to be determined, however, how the mechanisms of adhesion cross talk with those of mechanical perturbation and which kinases and second signals are common in regulating downstream events prior to any activation in gene expression.

ACKNOWLEDGMENTS

The authors acknowledge the expert technical assistance of Irina Simkina in preparation and maintenance of the osteoblast cell cultures. We thank Becton Dickinson Labware and Dow Chemical Company for their generous support of this project. R.S.C. acknowledges the support of the American Association of Orthodontists Foundation.

REFERENCES

Berk BC, Corson MA, Peterson TE, Tseng H. 1995. Protein kinases as mediators of fluid shear stress stimulated signal transduction in endothelial cells: a hypothesis for calcium-dependent and calcium-independent events activated by flow. J Biomech 28:1439–1450.

Bull HA, Brickell PM, Dowd PM. 1994. Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. FEBS Let 351:41–44.

Carvalho RS, Schaffer JL, Gerstenfeld LC. 1998. Osteoblast induction of osteopontin expression in response to cell attachment or mechanical perturbation shows common mediation through integrin receptors. J Cell Biochem 70:376–390.

Chen J, McKee MD, Nanci A, Sodek J. 1994. Bone sialoprotein mRNA expression and ultrastructural localization in fetal procine calvarial bone: comparisons with osteopontin. Histochem J 26:67–78.

Chong LD, Traynor-Kaplan A, Bokoch GM, Schwartz MA. 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell 79:507–513.

Curtis ASG. 1987. Cell activation and adhesion. J Cell Sci 87:609–611.

Curtis ASG, McGrath M, Gasmi L. 1992. Localised application of an activating signal to a cell: experimental use of fibronectin bound to beads and the implications for mechanisms of adhesion. J Cell Sci 101:427–436.

Damsky CH, Werb Z. 1992. Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. Curr Opin Cell Biol 4:772–781.

Davies PF, Tripathi SC. 1993. Mechanical stress mechanisms and the cell: an endothelial paradigm. Circ Res 72:239–245.

Denhart DT, Guo X. 1993. Osteopontin: a protein with diverse functions. FASEB J 17:1476–1481.

Du H, Gu G, William CM, Chalfie M. 1996. Extracellular proteins needed for *C. elegans* mechanosensation. Neuron 16:183–194.

Gerstenfeld LC. 1999. Osteopontin in skeletal tissue homeostasis: an emerging picture of autocrine/paracrine functions of the extracellular matrix. J Bone Min Res 14:850–855.

Gerstenfeld LC, Chipman S, Kelly C, Lee DD, Landis WJ. 1988. Collagen expression, ultrastructural assembly, and mineralization in cultures of chicken embryo osteoblasts. J Cell Biol 106:979–989.

Gerstenfeld LC, Gotoh Y, Mckee MD, Nanci A, Landis WJ, Glimcher MJ. 1990. Expression and ultrastructural localization of the major phosphoprotein synthesized by chicken osteoblasts during in vitro mineralization. Anat Rec 228:93–103.

Gerstenfeld LC, Uporova T, Ashkar S, Salih E, Glimcher MJ. 1995. Regulation of avian osteopontin pre- and post-translational expression in skeletal tissues. Annals NY Acad Sci 270:67–82.

Girard PR, Nehem RM. 1993. Endothelial cell signalling and cytoskeletal changes in response to shear stress. Front Med Biol Eng 5:121–125.

Gotoh Y, Gerstenfeld LC, Glimcher MJ. 1990. Identification and characterization of the major chicken bone phosphoprotein. Analysis of its synthesis by cultured embryonic chick osteoblasts. Eur J Biochem 187:49–58.

Gotoh Y, Salih E, Glimcher MJ, Gerstenfeld LC. 1995. Characterization of the major non-collagenous proteins of chicken bone: identification of a novel 60 kDa non-collagenous phosphoprotein. Biochem Biophys Res Commun 208:863–870.

Grzesik W, Robey PG. 1994. Bone matrix RGD glycoproteins: immunolocalization and interaction with primary osteoblastic bone cells in vitro. J Bone Min Res 9:487–496.

Guan J-L, Trevithick JE, Hynes RO. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. Cell Regul 2:951–964.

Hunter GK, Goldberg HA. 1993. Nucleation of hydroxyapatite by bone sialoprotein. Proc Natl Acad Sci USA 90:8562–8565.

Hunter GK, Goldberg HA. 1994. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J 302:175–179.

Hynes RO. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11–25.

Ingber DE. 1991. Integrins as mechanochemical transducers. Curr Opin Cell Biol 3:841–848.

Ingber DE. 1997. Tensegrity: the architectural basis of cellular mechanotransduction. Ann Rev Physiol 59:575–599.

Ingber DE, Dike L, Hansen L, Karp S, Liley H. 1994. Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulated cell growth, migration, and tissue pattern during morphogenesis. Int Rev Cytol 150:173–224.

Ishida T, Takahashi M, Corson MA, Berk BC. 1997. Fluid shear stress-mediated signal transduction: how do endothelial cells transduce mechanical force into a biological response? Ann NY Acad Sci USA 811:12–23.

Juliano RL, Haskill S. 1993. Signal transduction from the extracellular matrix. J Cell Biol 120:577–585.

Kuo SC, Hammer DA, Lauffenburger DA. 1977. Simulation of detachment of specifically bound particles from surfaces by shear flow. Biophys J 73(1):517–531.

Lehrach H, Frischauf AM, Hanahan D, Wozney J, Fuller F, Boedtker H. 1979. Construction and characterization of pro alpha 1 collagen complementary deoxyribonucleic acid clones. Biophys J 73(1):517–531.

Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM. 1995. The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. J Clin Invest 95:713–724.

Meazzini MC, Schaffer JL, Toma CD, Gray ML, Gerstenfeld LC. 1998. Osteoblast cytoskeletal modulation in response to mechanical strain in vitro. J Orthop Res 16:170–180.

Miyauchi AJ, Alvarez JI, Greenfield EM, Teti A, Grano M, Colucci S, Zambonin-Zallone A, Ross FP, Teitelbaum SM, Cheresh D, Hruska KA. 1995. Recognition of osteopontin and related peptides by an avb3 integrin stimulates immediate cell signals in osteoclasts. J Biol Chem 266: 20369–20374.

Moore MA, Gotoh Y, Rafidi K, Gerstenfeld LC. 1991. Characterization of a cDNA for chicken osteopontin: expression during bone development, osteoblast differentiation, and tissue distribution. Biochemistry 30: 2501–2508.

Morino N, Mimura T, Hamasaki K, Tobe K, Ueki K, Kikuchi K, Takehara K, Kadowaki T, Yazaki Y, Nojima Y. 1995. Matrix/integrin interaction activates the mitogen activated protein kinase pp44*erk-1* and p42*erk-2*. J Biol Chem 270:269–273.

Neugebauer BM, Moore MA, Broess M, Gerstenfeld LC, Hauschka PV. 1995. Characterization of structural sequences in the chicken osteocalcin gene: expression of osteocalcin by maturing osteoblasts and by hypertrophic chondrocytes in vitro. J Bone Miner Res 10:157–163.

Nollert MU, Eskin SG, McIntire LV. 1990. Shear stress increases inositol trisphosphate levels in human endothelial cells. Biochem Biophys Res Commun 170: 281–287.

Ohno M, Cooke JP, Gibbons GH. 1993. Shear stress induced TCF-beta-1 gene transcription via a flow activated potassium channel. Circulation 88:103i.

Oldberg A, Franzen A, Heinegard D, Pierschbacher M, Ruoslahti E. 1988. Identification of a one sialoprotein receptor in osteosarcoma cells. J Biol Chem 263:19433–19436.

Pavalko FM, Chen NX, Turner CH, Burr DB, Atkinson S, Hsieh YF, Qiu J, Duncan RL. 1998. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. Am J Physiol 275: C1591–C1601.

Pienta KJ, Murphy BC, getzenmber RH, Coffey DS. 1991. The effect of extracellular matrix interaction on morphologic transformation in vitro. Biochem Biophys Res Comm 179:333–339.

Puleo DA, Bizios R. 1992. Mechanisms of fibronectin-mediated attachment of osteoblasts to substrates in vitro. Bone Min 18:215–226.

Reinholt FP, Hultenby K, Oldberg A, Heinegard D. 1990. Osteopontin: possible anchor of osteoblasts to bone. Proc Natl Acad Sci USA 87:4473–4475.

Resnick NT, Collins W, Atkinson DT, Bonthron CF, Dewey CF, Gimbrone MA. 1993. Platelet-derived growth factor B chain promoter contains a cis-acting fluid shear stress-responsive element. Proc Natl Acad Sci USA 90:4591–4595.

Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. 1993. Interaction between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem 5:9901–9907.

Sabe H, Hata A, Okada M, Nakagawa H, Hanafusa H. 1994. Analysis of the binding of the src homology 2 domain of Csk to tyrosine-phosphorylated protein in the suppression and mitotic activation of c-Src. Proc Natl Acad Sci USA 91:3984–3988.

Schaffer JL, Rizen M, L'ltalien GJ, Megerman J, Gerstenfeld LC, Gray ML. 1994. A Device for the application of a dynamic biaxially uniform and isotropic strain to a flexible cell culture membrane. J Orthop Res 12:709–719.

Schaffer JL, Toma CD, Meazzini MC, Gray ML, Gerstenfeld LC. 1996. Mechanical perturbation of osteopontin gene expression and its relationship to microfilament structure. In: Davidovitch Z, Norton L, editors. The biological mechanisms of tooth movement and cranial facial adaptation, Boston, MA: Harvard society for the advancement of orthodontics. p 113–121.

Schaller MD, Hildebrand JD, Dhannon JD, Fox JW, Vines RR, Parsons JT. 1994. Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH 2-dependent binding of pp60src. Mol Cell Biol 14:1680–1688.

Schwartz MA, Deninghoff K. 1994. αv Integrins mediate the rise in intracellular calcium in endothelial cells on fibronectin even though they may play a minor role in adhesion. J Biol Chem 269(11):133–137.

Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC. 1997. Mechano-induction of osteopontin expression in osteoblasts: dependency of signal transduction on microfilanment integrity. J Bone Min Res 12:1626–1636.

Tseng H, Berk BC. 1993. Fluid shear stress stimulates mitogen-activated protein kinase in bovine aortic endothelial cells. Circulation 88:1–184.

Vuori K, Ruoslahti E. 1993. Activation of protein kinase C precedes alpha 5 beta 1 integrin-mediated cell spreading to fibronectin. J Biol Chem 268:459–462.

Werb Z, Tremble PM, Behrendtsen O, Crowley E, Damsky CH. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J Cell Biol 109:877–889.

Wilson E, Sudhir K, Ives H. 1995. Mechanical strain of rat vascular smooth muscle cells is sensed by specific extracellular matrix/integrins interactions. J Clin Invest 96:2364–2372.

Winnard RG, Gerstenfeld LC, Toma C, Franceschi RT. 1995. Fibronectin gene expression synthesis and accumulation during in vitro differentiation of chicken osteoblasts. J Bone Min Res 12:1969–1977.

Yamazaki TK, Tobe E, Maemura K, Kaida T, Komuro I, Tamemoto H, Kadowaki T, Nagai R, Yazaki Y. 1993. Mechanical loading activates mitogen-activated protein kinase and S-6 peptide kinase in cultured rat cariac myocites. J Biol Chem 268:12069–12076.

Yang R, Gerstenfeld LC. 1996. Signal transduction pathways mediating parathyroid hormone perturbation of bone sialoprotein gene expression in osteoblasts. J Biol Chem 271:29839–29846.

Yang R, Gerstenfeld LC. 1997. Structural analysis and characterization of tissue and hormonal responsive expression of the avian bone sialoprotein (*BSP*) gene. J Cellular Biochem 64:77–93.

Yang R, Gotoh Y, Moore MA, Rafidi K, Gerstenfeld LC. 1995. Characterization of an avian bone sialoprotein (*bsp*) cDNA. Comparisons to mammalian *BSP* and identification of conserved structural domains. J Bone Miner Res 10:632–640.