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Mechanotransduction in Human Bone: In Vitro Cellular Physiology That Underpins Bone Changes with Exercise

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Abstract

Bone has a remarkable ability to adjust its mass and architecture in response to a wide range of loads, from low-level gravitational forces to high-level impacts. A variety of types and magnitudes of mechanical stimuli have been shown to influence human bone cell metabolism in vitro, including fluid shear, tensile and compressive strain, altered gravity, and vibration. Therefore, the current systematic review aims to synthesize *in vitro* data regarding the cellular mechanisms underlying human bone cells' response to mechanical loading. Current data demonstrates commonalities in response to different types of mechanical stimuli on the one hand, along with differential activation of intracellular signaling on the other. A major unanswered question is, how do bone cells sense and distinguish between different types of load? The studies included in the present review suggest that the type and magnitude of loading may be discriminated by overlapping mechanosensory mechanisms including (1) ion channels, (2) integrins, (3) G-proteins, and (4) the cytoskeleton. The downstream signaling pathways identified to date appear to overlap with known growth factor and hormone signals, providing a mechanism of interaction between systemic influences and local mechanical environment. The implications for exercise prescription in the context of osteoporosis are discussed.

Keywords

bone; cytokines; growth factors; mechanical loading; osteoblasts; osteoporosis; paracrine signaling; stress/strain

Introduction

The adaptability of human bone to different load levels is both a blessing and a curse. Following appropriate exercise, an improvement in bone density can be tracked *in vivo*, as can the decline following its cessation (1). The downside of this economical approach to bone is that prolonged unloading -- during times of illness, immobilization or exposure to microgravity -- leads to an over exuberant resorption of bone and a high risk of fracture (2).

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Conflict of Interest

All authors have no conflict of interest.

Early theories to explain the dynamic relationship between the structure of bone (form) and its mechanical environment (function) invoked notions of a piezoelectric effect within the mineralized matrix under loading (discussed in(3)). More current research supports a 4-stage cell-mediated theory of mechanotransduction: (i) **Mechanocoupling** describes the conversion of physiologic loads applied to tissues into a local mechanical signals experienced by bone cells; (ii) in **Biochemical Coupling**, cells sense that load using mechano-responsive structures and transform it into a biochemical response; (iii) **Signal Transmission** represents the resultant downstream signaling within and between cells; finally, (iv) the **Effector Response** of osteoblasts and osteoclasts describes the cellular outcomes that lead to build-up, remodeling or resorption of bone matrix.

The mechanostat theory developed by Harold Frost hypothesized that “bone’s biologic machinery would make healthy postnatal human load-bearing bones and their trabeculae strong enough to keep typical peak voluntary mechanical loads from breaking them suddenly or in fatigue” (4). According to this hypothesis, bone mass is adjusted when the typical loads (resulting in particular tissue strain values) diverge from a physiologic “set-point” (approximately 1000 – 1,500 microstrain or 1%–1.5%) much like a thermostat. The theory predicts that the balance of bone formation in healthy adults is influenced by systemic factors (cytokines and growth factors, metabolites, endocrine and neuroendocrine signals) but is mainly determined by the mechanical load history (4). Although powerful, an inherent limitation of the theory is that the actual cellular mechanisms of load sensing and its systemic integration by networks of interacting cell types are not detailed.

With the recent explosion of knowledge about cell signaling in the fields of cancer and immunological research, many signaling pathways have been examined in the context of mechanotransduction experiments. Experiments using *in vitro* mechanical stimulation of widely differing parameters have generated some controversy with respect to the types and magnitudes of load to which different bone cell types actually respond. Indeed, mounting data from various connective tissue cell types indicates that *in vitro* and *in vivo* responses may be divergent with respect to the influence of loading on matrix remodeling (see discussion in (5)). An additional variable is that certain cell lines and species differ from primary human osteoblasts in key elements of the biochemical coupling and signal transduction machinery (6–8). Therefore, the purpose of this comprehensive review is to synthesize, for the first time, the *in vitro* data regarding mechanotransduction in human bone cells.

Methods

Medline was searched (1966 to June 2006) using the following systematic search terms: [Mechanotransduction, mechanoreceptors, mechanical stress] combined with [bone, osteocyte, osteoblast, osteocyte]. The abstracts of studies resulting from this search were examined according to the following criteria.

Inclusion criteria

1. Involved the direct application or manipulation of mechanical stimulus to human bone cells.
2. Primarily addressed cell signaling pathways activated by mechanical loading

Exclusion criteria

1. Related to particular tissues or materials relevant to specialized disciplines (e.g. periodontal ligament, experimental biomaterials)

2. Review article
3. Animal studies

The following information was extracted from each study: cell type, experimental loading procedure, loading parameters including intensity, frequency and duration, and objective outcomes. This information was tabulated according to the type of loading protocol used. Studies which used more than one type of loading protocol were included in each appropriate table. The results of studies were elaborated and summarized in the results section according to the category of the main outcomes: 1. cell proliferation, differentiation and death; 2. altered extracellular matrix; 3. load-sensing and cell signaling.

Results

Seventy-two studies were identified that aimed to determine the function and involvement of mechanotransduction pathways and responses in human bone cells *in vitro* and that fit the criteria for review. All of the studies were conducted with cultured human osteoblasts or osteoblast-like cells or cell lines, including primary cells (derived from bone explants), bone-marrow-derived osteoblasts, or immortalized cell lines (MG-63, TE-85, G292, SaOS-2, and HOBIT). Only one study involved the application of load to other human bone cell types (osteoblasts and osteoclasts)(9).

The human bone cells in the reviewed studies varied by age, gender, presence of pathology, menopausal status, biopsy location and depth, differentiation status, and length of time in culture (including both primary and immortalized cells). Despite this variation the studies were essentially consistent in demonstrating that mechanical strain, vibration or fluid flow can induce an adaptive response in osteoblasts represented by proliferation, and secretion and mineralization of the extracellular matrix (detailed under Effector Response, below).

Mechanocoupling

The studies applied mechanical stimulation directly to bone cells using a wide variety of models which are grouped into 6 categories according to experimental protocol (Tables 1, 2, 3, 4, 5, and 6). The types of stimuli included fluid flow, substrate strain, membrane deformation or integrin stimulation, vibration, altered gravity, and compressive loading (increased hydrostatic pressure). In the studies reviewed, each of these different types of mechanical stimuli was able to induce osteogenic activity through similar pathways (see Biochemical Coupling, below), reinforcing the notion of a common system which can respond to a broad range of mechanical stimuli (Figure 1).

In contrast to the conclusions of a recent review (10), there was ample evidence that osteoblasts respond to levels of fluid flow and substrate strain which are within the range of what may be considered physiologic (4). Addressing the question of which type of these two types of loading is most osteogenic, You et al. found that both cyclic strain and fluid flow could induce calcium transients in osteoblasts, but fluid flow was 5 times more likely to induce calcium transients than a substrate strain at presumed physiologic levels. Fluid flow also had a greater effect on osteopontin expression than substrate strains (11).

Biochemical coupling

How are physical loads integrated into cellular responses as diverse as the decision to modulate the constitution and architecture of extracellular matrix, to differentiate, proliferate or undergo apoptosis? The present systematic review found that in osteoblasts, signaling is transduced into a biochemical response by at least 4 independent and interacting mechanisms including integrins, G-proteins, the cytoskeleton and ion channels. These initial

triggers activate or modulate enzymes that influence nuclear transcription, which in turn promote a variety of responses.

Ion channels—The earliest data on biomechanical coupling in human bone cells came from membrane stretch studies by Davidson et al. demonstrating the existence of several classes of ion channels in MG63 osteoblast-like cells (12, 13). The principal stretch-activated channel was a potassium channel whose probability of being open was proportional to the degree of membrane stretch applied. Whether this channel was activated during physiologic types of loading has never been reported; the membrane stretches that were applied were likely to have been supraphysiologic, and may be more relevant to studies of osmoregulation rather than physiologic loading.

Subsequent data on load-sensing mechanisms came from experiments conducted by McDonald and coworkers (14) using the U2 OS osteoblast cell line. In flow-stimulated cells, a rapid increase in intracellular calcium levels, $[Ca^{2+}]_i$, resulted from the influx of extracellular calcium. This was followed by a mobilization of intracellular calcium, which was independent of potassium currents. There was a measurable delay in the elevation in $[Ca^{2+}]_i$ which, additionally, was abolished by a G*i*-/G*o* protein inhibitor (PTX). Therefore, McDonald et al proposed a multi-step model where mechanical stimulation, perhaps transmitted by an integrin to an associated G protein, led to G protein-mediated opening of a calcium channel. The resulting calcium influx activated phospholipase (PLC) to generate inositol triphosphate (IP_3) which diffused through the cell and created the observed wave of calcium release from internal stores.

A limitation of the McDonald model is that the experiments were conducted with U2 0S cells which lack voltage-gated calcium channels. More recent experiments with adult primary osteoblasts demonstrated that the increase in intracellular calcium could be partially blocked by inhibiting voltage-gated calcium channels (15). Thus, the initial influx of extracellular calcium could result from the opening of both G-protein-activated and voltage-gated calcium channels. A potential role of both calcium channel types in osteoblasts was underscored by studies in which load-induced responses were negated when either the L-type (voltage activated) channels or the “stretch-activated” (i.e. G-protein mediated (14)) channels were blocked. A dependence on both calcium channel types was demonstrated for load-induced TGF-β up-regulation (16). The down-regulation of HB-GAM in fetal osteoblast precursors (a critical event in osteoblast differentiation) was also dependent on both types of calcium channel (17).

Despite the importance of different classes of calcium channels in osteoblast mechanosensation, their structure and mechanism of action have not been determined in human osteoblasts. An intriguing study recently demonstrated that a calcium-binding protein (Annexin V, which interacts with phospholipid bilayers to form Ca2+ selective channels) plays a substantial role in flow-induced calcium responses and downstream transcriptional activation (18), and further studies in human bone cells may shed light on this question.

G proteins—From other cell types, it is known that the ability of G*i* proteins to modulate calcium channels depends on intact linkages between integrin-β1 and the cytoskeleton, with which G*i* proteins co-localize (19). However, the precise manner in which G proteins are activated at the integrin-cytoskeleton complex and how they would modulate ion channels remain unknown. G proteins are required in some instances of integrin-mediated, calcium- and cytoskeleton-independent signaling, thus they may play a variety of possible roles (19) that have not yet been fully described in human bone cells. Despite the lack of mechanistic understanding, a vital role of G proteins in proliferation and extracellular matrix production was shown in the present review. *In vitro* ultrasound-treated fetal pre-osteoblasts displayed a

rapid activation of membrane-bound Gαi-1/3 protein (20). In this study, a G*i* protein inhibitor (PTX) blocked ERK1/2 activation (a key requirement of survival, proliferation and extracellular matrix production in osteoblasts). However, in another study, PTX had no effect on ERK1/2-dependent, fluid-flow induced proliferation (21). The conflicting results on the role of PTX-dependent ERK1/2 activation and subsequent proliferation may speak to the variety of calcium-entry mechanisms (G-protein dependent- and independent) reported above, as well as the large number of G proteins which may be activated in different scenarios.

Integrins—Integrins are transmembrane receptors consisting of α and β subunits which bind to, and are activated by, specific elements of the extracellular matrix (i.e. osteopontin, collagen, fibronectin). Binding and activation are both required to effectively transduce mechanical forces in the matrix into a signaling response in the cell (19). Primary osteoblasts express α2, α3, α4, α5, αv, α6, β1, β3 and β5 (22, 23).

In human osteoblasts, integrin signaling was studied in response to fluid shear and substrate deformation. Fluid flow led to α5β3 clustering (24) and an increased association between α5β3 integrin and Shc (an adaptor protein known to play a role in the activation of Ras, upstream of ERK1/2). This suggests that integrins may be directly responsible for some fluid-flow induced osteoblast effector responses (detailed below).

Several studies showed calcium to be a player in integrin-mediated mechanical forces. Hughes et al (25) showed that a rise in $[Ca^{2+}]_i$ was induced when integrins were clustered on beads and mechanically stimulated, more so than clustering alone. Pommerenke et al. compared different duty cycles of integrin stimulation, and found that 1Hz cyclic stress was more effective at causing an increase in $[Ca^{2+}]_i$ than continuous or low frequency (0.1Hz) stimulation. Interestingly, osteoblasts and bone marrow stromal cells displayed different kinetics of $[Ca^{2+}]_i$ following integrin mechanical stimulation. The bone marrow cells demonstrated a single peak, whereas osteoblasts showed single and oscillating transients, suggesting important differences in calcium-dependent mechanotransduction according to differentiation status.

In terms of events downstream of integrins, load-enhanced binding of an early response transcription factor (c-fos) was abolished by anti-β1 integrins, as well as by inhibitors of calcium channels and a calcium-dependent kinase (PKC)(26). Similar attempts to modulate or block integrin signaling using various substrates and antibodies did not affect ERK-1/2 activation, suggesting that integrin-independent mechanosensory paths may be responsible for ERK-1/2 activation. Salter et al. used an extensive panel of antibodies and peptides to block various integrins and integrin subunits and showed that a variety of α and β integrins are involved in load-induced changes in membrane potential, but that different duty cycles of stimulation act through different integrin subunits (27).

Cytoskeleton—In addition to their signaling role in mechanotransduction, integrins also play a mechanical function by transmitting forces to the cytoskeleton, whose independent role as a mechanosensor has been shown (28). Pommerenke et al. found that cyclic stress delivered via integrins led to an increase in the levels of cytoskeletal-bound phopshotyrosines, and a transolocation of FAK from the cytosol to the cytoskeleton (29, 30). Intact microtubules were shown to be a requirement for load-induced differentiation (17) and proliferation (31). A precise analysis of load-bearing and –sensing by distinct cytoskeletal elements awaits further study: the careful use of cytoskeletal inhibitors may generate useful data, as in animal cells (32).

Signal transmission

Intracellular signaling—Although the precise interactions of calcium channels, G proteins, integrins and the cytoskeleton have yet to be established, downstream osteogenic signaling was shown to converge on the activation of several key intracellular enzymes.

A key load-sensing event in many cells is the generation of NO. From animal experiments, NO is essential in the maintenance of bone mass (33). In primary osteoblasts, Klein-Nulend et al. found that fluid flow stimulated an early burst of NO release (peaking within 5 min), as well as an increase in eNOS mRNA. NO production was also examined in flow-stimulated or cyclically stretched primary osteblasts at a longer time point (24 hrs) (34). Both types of loading stimulated NO by equivalent amounts. NO could potentially stimulate proliferation and extracellular matrix production through the Ras-Raf-MEK-ERK cascade by binding to a regulatory site on Ras, but this has not yet been shown in human bone cells (35–37).

The downstream effects of NO were examined in a key study by Kapur and coworkers, in which primary osteoblasts were subjected to constant fluid flow (21). An NO inhibitor prevented ERK-1/2 activation at 30′ as well as the increase in proliferation and ALP activity at 24 hr (see Effector Response, below), suggesting that the Raf-Mek-ERK-1/2 cascade and its downstream osteogenic effects are primarily activated by an NO-dependent pathway. Kapur et al. found that blocking COX-1 and -2 with indomethacin abolished the load-induced proliferation and ALP activity while leaving ERK-1/2 levels unaffected. This implies that COX-1/2 induction, downstream of NO and ERK-1/2, may be a required chain in the sequence of events leading to flow-induced bone formation.

Intercellular signaling – autocrine and paracrine mechanisms—A crucial role was demonstrated for direct intercellular communication mediated by gap-junctions, as well as indirect communication via diffusible messengers.

Examination of changes in the concentration of free intracellular calcium demonstrated that gap junctions and diffusible messengers may cooperate to transmit mechanical signals. For example, blocking the ATP (P2Y) receptors in HOBIT osteoblast-like cells reduced the radius of the cell-cell calcium wave in response to membrane deformation. However, a smaller calcium wave persisted despite the reduction in response to ATP receptor blockade. This ATP-independent calcium wave could be abolished by a gap-junction inhibitor (38).

Further experiments in response to fluid flow (induced by displacing cell culture medium with a pipette) found a 4–5 fold increase in ATP release into the medium (39, 40). When the ATP-rich medium from stimulated cells was added to resting cultures, calcium transients were again observed. In other cell types, ATP release has been proposed to involve either G-protein- and integrin-dependent vesicle trafficking (41) or membrane-bound ATP-synthases (42), but the mechanisms in human bone cells have not yet been examined.

The question of cell-cell communication also gave rise to an interesting study of how osteoblasts and osteoclasts may signal to one another. Calcium waves originating in mechanically stimulated osteoblasts could be passed directly to adjacent osteoclasts, and vice verse (9). This represents the first direct evidence of load-induced intercellular communication between two different human bone cell effector cell types. Unlike osteoblasts, osteoclast calcium waves were not dependent on gap junctions but only on the purinergic (P2X7) receptors.

The majority of studies which examined PGE_2 release in response to loading demonstrated that this is a prominent response to loading in primary and transformed osteoblasts. Downstream of PGE_2, Sakai et al. found TGF-β mRNA and protein were both elevated

following fluid shear stress (16). However, in addition to this osteogenic signaling, IL-11 was released by flow-stimulated osteoblasts in sufficient quantities to differentiate osteoclasts from precursors (16). This study suggests that the crucial cooperation between these two effector cell types in bone remodeling may be mediated by load-induced paracrine effects.

Regarding the different mechanosensory roles of various bone cell populations, a question which was unaddressed by any of the studies in the present systematic review is how human osteocytes signal to osteoblasts and osteoclasts. Given that osteocytes may be the most important mechanosensory cell in bone, clearly a method for culturing human osteocytes would allow potentially relevant mechanisms to be more closely examined.

Effector response

Mechanical stimuli enhance synthesis and mineralization of the extracellular matrix—A large number of studies examined the effect of cyclic strain on extracellular matrix synthesis. Bone consists largely of a mineralized extracellular matrix whose mass and architecture result from a balance between production and resorption. The major components of the organic matrix (Type I collagen, osteopontin, and osteocalcin) and its associated minerals (hydroxyapatite) were examined in response to loading, as was the expression and activity of ALP, an enzyme whose activity corresponds closely to the onset of mineralization (43). In general, these studies demonstrated that several key stages of extracellular matrix assembly including transcription, secretion, and mineralization, could be stimulated by physiologic levels of strain (see Tables). The activity and mRNA levels of ALP were enhanced, as was matrix calcification by 16 days of intermittent cyclic strains (44). The enhancement of ALP could be stimulated by as small a stimulus as 15 minutes of 0.06% strains per day (45). Collagen I, osteocalcin, and osteopontin secretion or mRNA levels were also generally higher in strained as opposed to unstrained cultures (see Tables for citations).

The effect of fluid flow on the matrix was examined in five studies. Kapur and coworkers (21) showed that 30 minutes of constant fluid flow at 20 dynes/cm^2 resulted in approximately a 30% increase in ALP activity. This ability of fluid flow to enhance ALP activity was later confirmed and shown to be dependent on both PGE_2 and TGF-β(46). In a third study, You et al. reported a 100% increase in osteopontin mRNA by fetal osteoblasts three days after 1 hour of pulsatile fluid flow (2 N/m^2, 1 Hz). This effect was subsequently confirmed in bone marrow-derived stromal cells (47). A fifth report showed a 50% decrease in collagen I protein levels 24 hours following 1 hr of cyclic fluid flow (5Hz, 0.6 Pa)(34). Taken together, the studies suggest a stimulatory effect on bone matrix production and mineralization in response to fluid flow. However, the effect of fluid flow on collagen I – an essential element of the bony matrix – requires further study.

The influence of intermittent compressive loading on osteoblasts was examined in a single study. Messenger RNA levels of MMP-1 and -3 were enhanced by mechanical stimulation, whereas collagen I and osteocalcin were decreased. This suggests that compressive loading may negatively modulate important aspects of bone matrix remodeling (5). The coupling mechanisms have apparently not yet been examined for this type of loading stimulus.

Vibration of osteoblasts through acoustic or mechanical energy resulted in a similar variety of responses as cyclic strains, but the variation could apparently be attributed to the stimulation characteristics. A dose response was found in the effect of gross vibration on ALP activity in osteoblast cultures; lower frequencies (20 Hz) depressed ALP activity whereas higher frequencies (30 – 60 Hz) progressively increased ALP activity. Similarly, ALP and osteopontin mRNA levels were increased by 3.0 MHz only at higher intensities

(0.39 W/cm^2 and higher)(48). Although the optimal combination of frequency and intensity remain to be determined, clearly the magnitude of extracellular matrix stimulation in response to gross or ultrasound vibration can reach levels similar to those induced by fluid flow or substrate strain.

Finally, the effect of actual and simulated microgravity were investigated in human osteoblast-like cells, and demonstrated significant decreases in ALP activity and mRNA expression, as well as osteocalcin and collagen expression (49, 50). These studies demonstrated that gravitational stimulation of mechanotransduction pathways is a normal requirement for high levels of extracellular matrix production by osteoblasts.

Mechanical stimuli cause osteoblast proliferation—Proliferation of osteoblasts is a key event in bone formation (51) and remodeling (52). There was ample evidence that primary osteoblasts were induced to proliferate both by relatively short periods of fluid flow, or by cyclic substrate strains at physiologic levels.

Ten of the 25 studies using substrate deformation examined proliferation. Of these, 8 reported an increase in proliferation in response to strains ranging from 0.06% to 2.5%. One study (53) reported a dose-response to strain magnitude, with a progressive decline in proliferation in response to strains higher than 1%. Cheng et al. reported a peak proliferative response at strains of 0.3% (6). Of the two negative studies, one used experimental orthopaedic substrates (54). The other used a higher duration of loading (2.78 hrs vs 15 to 60 min, i.e.> 10,000 cycles) (55). The latter study implies that excessive cycle number may have an inhibitory rather than a stimulatory effect on osteoblast proliferation.

Three studies examined the effect of constant fluid flow on proliferation. All three studies reported a significant (50–100%) increase in proliferation or DNA content of primary osteoblasts in response to 30 min or 1 hour of constant fluid flow (21) (56, 57). Studies examining pulsatile fluid flow found no influence on proliferation in cells from post-menopausal women (58, 59). However, two other studies, one using bone marrow derived stromal cells and one using primary osteoblasts, did show an increase in proliferation (46, 47). This suggests that some aspect of the post-menopausal osteoblast phenotype may negatively influence their ability to respond to some types of mechanical stimulation.

Sensory (20 – 60 Hz) and ultrasound levels of vibration were also reported to significantly affect osteoblast proliferation. Standford et al. (55) reported no change in proliferation in response to 20 Hz vibration, however the results were based on cells from a single biopsy. In contrast, Rosenborg et al. (31, 60) showed that 20 Hz stimulated proliferation by approximately 50%, whereas 30–60 Hz caused either no change or a slight decrease in DNA synthesis. Doan et al. reported a similar magnitude of proliferation (50%) in response to several types and intensities of ultrasound-induced vibration (61). Thus, the rather limited evidence suggests that osteoblasts can respond favorably or negatively to a wide range of vibration frequencies.

Physiologic loading enhances survival signals—Physiologic loading was shown to induce survival signals in human osteoblasts. Fluid shear increased the expression of a pro-survival protein Bcl-2, whereas an important pro-death protein (Bax) remained unchanged. Ogata and coworkers showed that fluid flow can enhance the levels of a cell surface growth factor receptor (EGF-R) which could also potentially impact cell survival (62). Other potentially survival-enhancing effects observed (although not consistently) included clustering and upregulation of β1-integrins (63), release of autocrine / paracrine survival factors like IGF-I (64) or IGF-II (6) and activation of the estrogen receptor (6). In no case, however, were the effects of loading on cell survival assessed directly. Therefore, the impact

of these events on the susceptibility to cell death requires further study. This may be particularly relevant given that excessive osteocyte apoptosis is a prominent feature of osteoporosis (65), and apoptosis can be prevented by fluid shear *in vitro* in some animal models (66).

Inappropriate load levels impair survival signals—The issue of mechanical factors leading to cell death vs survival or proliferation received surprisingly little attention given the association of osteocyte apoptosis with osteoporosis. Lacouture and coworkers (67) observed significant levels of cell detachment and rupture of cell adhesions in response to high levels of strain (10% – 20%) on cells plated on a variety of physiologic substrates. The formation of integrin-containing focal adhesions was necessary for optimal strain resistance. It is known that detachment of adherent cell types can lead to rounding and apoptosis (a phenomenon known as “anoikis” (68)), but there is no evidence yet that this phenomenon occurs *in vivo* in response to high loads in bone.

Lack of load was also shown to influence osteoblast survival pathways. Gravitational unloading was shown to reduce the DNA binding of a survival-promoting transcription factor (NF-κB) in response to cytokine stimulation (TNF-α) (69). The effect on viability was not directly assessed, but the results suggest that gravitational unloading may sensitize cells to undergo apoptosis as shown in other cell types (70). Finally, the Bcl/Bax ratio was decreased in unloaded as compared to loaded osteoblasts suggesting that modulation of the mitochondrial pathway may be decisive in preventing cell death from lack of loading (69).

Osteoblast differentiation is a determinant of mechanical loading—The process of differentiation could be triggered or enhanced by mechanical loading. Levels of the osteoblast differentiation marker Cbfa-1 were increased in fetal osteoblasts and bone marrow-derived stromal cells in response to cyclic strain or ultrasound (20, 71). Oscillatory fluid flow also resulted in stromal cell proliferation and increased osteopontin and osteocalcin expression (47), which are key osteoblast products. Similarly, physiological cyclic strain reduced HB-GAM expression (typical of undifferentiated pre-osteoblasts) in primary bone cells. The load-induced reduction of HB-GAM in cultured osteoblasts is consistent with the hypothesis that cultured osteoblasts tend to dedifferentiate when expanded in cell culture (7) but the mature phenotype can be restored by mechanical loading (17).

In fetal pre-osteoblastic cells, cyclic strains caused apoptosis of undifferentiated cells, whereas the same level of strain caused proliferation when applied during differentiation (72). Although limited, such studies begin to provide a picture in which the fate of bone cells is determined by an integration of past and present mechanical and biochemical/ hormonal signals, with the state of differentiation of the cell emerging as a key factor determining the load response. This was highlighted by studies in a human osteoblast cell line (SV-HFO), which revealed that the effect of stretch on ERK1/2 phosphorylation was strongest at later stages of differentiation (73).

Henriksen and co-workers shed some light on possible mechanisms that may underlie the different effector responses observed with osteoblasts at early or later stages of differentiation (74). They observed that human bone-marrow-derived osteoblasts which are at an earlier stage of differentiation rely predominantly on purinergic ATP receptors for the propagation of intracellular calcium waves to their neighbors, whereas in more fully differentiated osteoblasts, direct transmission via gap junctions was the main mechanism (74).

Implications and future directions

In vitro studies have begun to pinpoint molecular mechanisms by which the mechanostat may operate, and have focused in particular on mechanisms that contribute to the osteogenic effect of mechanical loading.

The majority of studies were conducted on osteoblasts. A dearth of studies in osteocytes (believed to be the primary load sensor) represents a major gap in current understanding of mechanotransduction in human bone. Signaling between fluid-flow stimulated mouse osteocytes and human osteoblasts has recently been reported (57, 75). Fluid-flow-stimulated osteocytes signaled via gap junctions to osteoblasts, which increased their ALP activity in an ERK-1/2 dependent manner (57) Evidence was also reported for ATP-dependent communication of load-induced calcium waves between human osteoblasts and osteoclasts (9) as well as osteoblast-induced differentiation of osteoclasts (76). These studies prove in principle that load can induce a co-coordinated response among the various cell types of bone, but many questions remain, particularly with regard to the specific signals leading to bone breakdown or build-up in human bone.

Most interestingly, the results from several studies support a hypothesis that pathology may result from a fault in the mechanostat mechanism. Osteoblasts from post-menopausal women did not demonstrate the same proliferative effector response to fluid flow as did non-menopausal cells (see Table 1). Further, osteoblasts derived from patients with osteoporosis were shown to be unresponsive in terms of two key signal transmission events; the release of PGE_2 (59) and downstream induction of TGF-β secretion (77). A possible explanation is that the upstream process of biochemical coupling may be dysfunctional. One specific possibility is the estrogen receptor (ER), whose expression is diminished in osteoblasts during the post-menopausal period (78). Cheng et al. showed that load-induced signal transmission was dependent on the activation of the ER, but not on the release of estrogen (6). An intriguing hypothesis is that internal activation of the ER receptor by load-induced ERK-1/2 activity is a requirement for osteogenic cell signaling. In animal studies, ERK-1/2 has been shown to phosphorylate the ER at a regulatory site (ser 122) in response to cyclic strain (79).

This type of “cross-talk” between mechanical and growth factor signals might also underlie the enhanced bone growth seen in children, whose higher systemic levels of IGF-I could potentiate the ERK-1/2 path and thereby amplify the osteogenic effect of loading.

The intra- and inter-cellular pathways used in the biochemical coupling and signal transmission phases are not dedicated to load alone, but instead use much of the same molecular machinery as growth factors, hormones and integrins. McDonald showed that calcium channel opening could be potentiated when cAMP levels were experimentally elevated, which points to a possible role of cAMP-inducing agents like PTH -- which is itself increased in response to exercise (14, 78). From animal studies, PTH is also known to influence Cx43 expression and cell-membrane localization (80, 81), which could enhance cell-cell coupling and thereby sensitize bone cells to loading. These findings support an integrated model (82, 83) with load and systemic influences as “vectors” integrated by the cell at the biochemical couple and signal transmission phases.

That mechanical stimulation activates many of the same pathways as growth factors underscores the enormous therapeutic potential for therapeutic exercise to maintain or restore normal cellularity and matrix of bone, and points to the importance of further study into how mechanical and biological signals are integrated into the “yin and yang” of bone balance.

An unanswered question which arises from the current review—and one with immediate implications for exercise prescription—is, why is excessive cycle number associated with a diminishing rather than increasing response to cyclic strain? At this point, the down-regulation of mechanically induced signals remains essentially unexamined in human bone cells. Cytoskeletal stiffening or upregulation of cell-cell and cell-matrix adhesions could lead to acute load-dampening responses at the level of the mechanical couple (63, 84, 85). Alternately, the biomechanical couple may be responsible. A normal part of any activation event is that a down-regulation of the initial signal occurs in order to return the signal to normal. For example, a recent study demonstrated a loss of ERK-1/2 phosphorylation with prolonged cyclic strain of human osteoblasts, implicating phosphatases in the down-regulation of mechanical signaling (73). Under conditions of excessive or prolonged mechanically induced signals, the net effect may be an overabundance of negative feedback signals.

Finally, it will be critical to determine whether there is a good correlation between the *in vitro* findings in osteoblasts and those derived from the more complex *in vivo* environment where cell types, matrix, physiology and mechanical loading intersect (for in depth discussion of the divergence between *in vitro / in vivo* findings, see(86)). It should also be kept in mind that excessive loading, or prolonged unloading, can have detrimental effects not just on bone, but on closely related connective tissues including cartilage, ligament, tendon, meniscus and joint capsule. Bone is unique in having dedicated anabolic and catabolic cell types, whereas the other connective tissues tend to use a single cell type to balance both sides of tissue homeostasis. Thus, whether other connective tissue have a similar mechanostat function is not a given, and much insight will be gained from a comparative study of load-sensing in a variety of human cell types.

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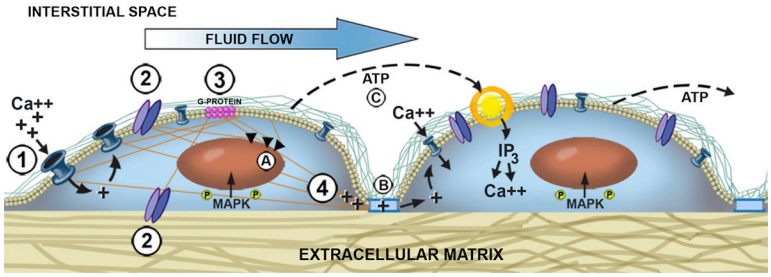

Figure 1.

A variety of independent but interacting mechanosensors have been identified in osteoblasts. (1) Stretch activated Ca^{++} channels open, activating intracellular enzymes (e.g. PLC, PKC) and causing membrane depolarization with subsequent voltage-gated channel opening and further Ca^{++} entry. (2) Integrins are activated by deformation of their extracellular binding partners (e.g. collagen, osteopontin) by fluid shear or substrate strain. (3) G-proteins in the lipid bilayer are activated. (4) The cytoskeleton is deformed, providing enhanced docking and activation sites for kinases. These are the four “primary” mechanosensors that are believed to directly sense mechanical perturbations. From here, mechanically-sensed signals are transmitted by intracellular enzyme activity to the nucleus (A). Signalling is propagated to neighbouring cells via (B) gap junctions (resulting in influx of extracellular Ca^{++}) or (C) ATP (resulting in mobilization of intracellular Ca^{++}) or other diffusible messengers (cytokines, NO). *MAPK = mitogen activated protein kinases (e.g. ERK-1/2). IP3 = inositol triphosphate.*

Table 1

Fluid Flow

Reference	Cell Type	Loading Regime	Major Outcomes
(18)	Osteoblast-like cells (MG-63)	Type: Fluid flow Intensity: 20 dynes/cm^2 Frequency: 1 Hz Duration: 1 hr	↑ $[Ca^{2+}]_i$ and ↑ c-fos expression (Annexin-V-dependent)
(47)	Bone marrow derived stromal cells	Type: Fluid flow Intensity: 10 dynes/cm^2 Frequency: 1 Hz Duration: 2 hr	↑ $[Ca^{2+}]$ ↑ proliferation, ↑ osteocalcin and osteopontin mRNA
(66)	Primary osteoblasts	Type: Fluid flow Intensity: 0.6 Pa Frequency: 5 Hz Duration: 1 hr	↑ Bcl-2 mRNA ↑ Bcl-2 / Bax ratio
(57)	Primary osteoblasts	Type: Fluid flow Intensity: 4.4 dynes/cm^2 Frequency: Continuous Duration: 1 hr	↑ proliferation
(46)	Primary osteoblasts	Type: Fluid flow Intensity: 26.6 μPa Frequency: 1hr on, 5 hr off Duration: 10 – 48 hrs	↑ proliferation ↑ PGE_2 and TGF-β ↑ ALP activity (TGF-β and PGE_2 dependent) ↑ fibronectin (FN) and FN-receptor expression
(87)	Primary osteoblasts	Type: Fluid flow Intensity: 0.6 Pa Frequency: 5 Hz Duration: 1 hr	↑ PGE_2 secretion, abolished by a COX-2 inhibitor ↑ COX2 mRNA
(56)	Osteoblast-like cells (MG-63)	Type: Fluid flow Intensity: 1–30 dynes/cm^2 Frequency: Continous Duration: 1 hr	↓ ALP and osteocalcin, ↑ PGE_2 ↑ proliferation, ↓ TGF-β Results varied by surface roughness
(58)	Primary post-menopausal female osteoblasts	Type: Fluid flow Intensity: 0.7 Pa, 12.2 Pa/s Frequency: 5 Hz Duration: 1 h	No proliferation ↑ PGE_2 and PGI_2 secretion ↑ COX-2 mRNA
(59)	Primary post-menopausal female osteoblasts	Type: Fluid flow Intensity: 0.6 Pa, 8.4 Pa/s Frequency: 5 Hz Duration: 1 h +/− estrogen	No proliferation ↑ PGE_2 and PGI_2 secretion Estrogen enhanced PGE_2 secretion in loaded but not unloaded cells
(21)	Primary osteoblasts	Type: Fluid flow Intensity: 20 dynes/cm^2 Frequency: Continuous Duration: 30 min	↑ DNA synthesis and ↑ ALP activity, both fully dependent on MEK, NO and COX-1/2 NO inhibition blocked ERK1/2 activation at 30′
(88)	Primary osteoblasts	Type: Fluid flow Intensity: 0.7 Pa, 12.2 Pa/s Frequency: 1 Hz Duration: 1 hr	↑ NO release peaked at 5 minutes ↑ eNOS mRNA post-incubation
(14)	Osteoblast-like cells (U2/OS)	Type: Medium displacement with pipetter Intensity: NA Frequency: NA Duration: NA	Deformation results in G-protein mediated influx of extracellular calcium, followed by IP_3 / PLC dependent intracellular calcium wave
(34)	Primary osteoblasts	Type: Fluid flow Intensity: 0.6 Pa Frequency: 5 Hz Duration: 1 hr	↑ NO and PGE_2 ↓ collagen I

Reference	Cell Type	Loading Regime	Major Outcomes
(62)	Osteoblast-like cells (SV-HFO)	Type: Fluid flow Intensity: 3 cm amplitude rotation of culture plates Frequency: 1 Hz Duration: 0.1 to 10 min	Fluid flow caused ↑ in EGF-R protein level
(40)	Osteoblast-like cells (HOBIT, MG-63 & SaOS-2)	Type: Medium displacement with pipetter Intensity: NA Frequency: NA Duration: NA	↑ ATP release (4 fold) ↑ $[Ca^{2+}]_i$ and ↑ Egr-1 expression (ATP-dependent)
(39)	Osteoblast-like cells (HOBIT)	Type: Medium displacement with pipetter Intensity: NA Frequency: NA Duration: NA	↑ ATP release (4–5 fold)
(16)	Osteoblast-like cells (SaOS-2)	Type: Fluid flow Intensity: 1.7 to 2.0 Pa Frequency: Continuous Duration: 1 – 24 hrs	↑ TGF-β1 mRNA and protein via a stretch- and voltage-activated Ca^{2+} channels No change in IL-6, PDGF-A, IGF-I or IGF-II mRNA levels
(76)	Osteoblast-like cells (SaOS-2)	Type: Fluid flow Intensity: 1.7 to 2.0 Pa Frequency: Continuous Duration: 1 – 24 hrs	↑ PGE_2 production ↑ IL-11 mRNA and protein via a PGE_2-dependent pathway; independent of stretch- or voltage-gated Ca^{2+} channels. ↑ differentiation of osteoclasts via IL-11 in response to fluid flow
(89)	Pimary osteoblasts (normal & osteoporotic)	Type: Fluid flow Intensity: 0.7 Pa Frequency: 5 Hz Duration: 1 hr	↑ NO ↓ TGF-β ↑ COX-2 expression ↑ PGE_2; osteoporotic cells were un-responsive in terms of sustained PGE_2 production
(72)	Osteoblast-like cells (MG-63)	Type: Fluid flow Intensity: 1.2 Pa Frequency: Continuous Duration: 15 min – 16 hrs	ERK phosphorylation was rapid (by 15 min), sustained (at least 16 hrs) and reduced by a pTyr inhibitor ↑ clustering of α5β3 integrins ↑ SHC-α5β3 integrin association Blocking with anti-α5β3 integrin, anti-β1 or RGD peptides did not block pERK
(11)	Fetal osteoblast-like cells	Type: Fluid flow Intensity: 2 N/m^2 Frequency: 1 Hz Duration: Up to 3 minutes	↑ $[Ca^{2+}]_I$ ↑ OPN production

"↑" or "↓" denote a change relative to mechanically unstimulated controls

Table 2

Cyclic Substrate Deformation

(44)	Bone marrow stromal cells	Type: Four point bending Intensity: 0.0044 – 0.2151 % Frequency: 250 cycles / 24 hours Duration: 16 days	↑ ALP mRNA and activity ↑ osteopontin and osteocalcin mRNA ↑ matrix calcification
(90)	Osteoblast-like cells (MG-63)	Type: Equiaxial Intensity: 0.5% Frequency: 0.05 Hz, 10 min on, 10 min off Duration: 1, 2, 4 hrs	↑ osteopontin secretion
(63)	Osteoblast-like cells (TE-85)	Type: Equiaxial Intensity: - Frequency: 0.05 Hz Duration: 15 min – 28 days	↑ proliferation ↑ integrin β1 clustering and expression
(6)	TE-85 & Primary osteoblasts	Type: Four point bending Intensity: 0 – 0.4%, Frequency: 1 Hz Duration: 10 min	↑ DNA synthesis ↑ IGF-II release (but not IGF-I) (peaked at 0.3%); blocked by anti-IGF-II, IGF-R or ER antibodies
(64)	Osteoblast-like cells (SaOS-2)	Type: Equiaxial Intensity: - Frequency: 0.05 Hz Duration: 8–24 hours	↑ mRNA for IGF-I, TGF-β, bFGF, and IL-6.
(85)	Osteoblast-like cells (MG-63)	Type: Biaxial Intensity: 0.06 % Frequency: 0.25 Hz Duration: 15 min, 3×/day × 5 days	↓ Collagen I pro-peptide ↑ fibronectin ↑ N-cadherin No change in osteocalcin or β1 integrin expression
(45)	Primary osteoblasts	Type: Biaxial Intensity: 0.06 % Frequency: 0.25 Hz Duration: 15 min, 3×/day × 5 days	↑ proliferation ↑ ALP activity
(91)	Primary osteoblasts	Type: Uniaxial Intensity: 0.1–0.4 %; 1.6%/s Frequency: 0.5 Hz Duration: 16.6 min	↑ DNA synthesis ↑ PGE_2 secretion
(92)	Osteoblast-like cells (OHS-4)	Type: Equiaxial Intensity: 0–12% Frequency: 0.05 Hz Duration: 1–4 days	↑ Type I collagen mRNA ↑ osteopontin and osteocalcin
(71)	Bone marrow stromal cells	Type: Uniaxial Intensity: 2% or 8% Frequency: 1 Hz Duration: (3 × 2 hours) × 3 days	↑ ALP (8% > 2%) ↑ osteocalcin (8% > 2%) ↑ collagen I and III and Cbfa1 mRNA, enhanced by dexamethasone
(93)	Primary osteoblasts	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 30 min	↑ proliferation ↑ collagen I production ↓ ALP activity and osteocalcin release
(94)	Primary osteoblasts	Type: Four point bending Intensity: 0.1% Frequency: 0.1 – 30 Hz Duration: 4–3,600 cycles	↑ proliferation with all cycle regimes (except for 30Hz × 5min) with a window of optimum cycle number (between 3,000 and 1,800)
(67)	Osteoblast-like cell line	Type: Biaxial Intensity: 10% or 20% Frequency: 0.25 Hz Duration: 6–24 h	Prolonged cycles caused progressive rupture of cell adhesions ↑ stress fibres and nuclear / cytoplasmic ratio with strain reorganization of β_1 integrins into adhesion sites ↑ osteopontin mRNA with strain
(17)	Primary osteoblasts & SaOS-2	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 30 min	↓ pleiotrophin (HB-GAM) mRNA which was inhibited by microtubule disruption (nocodazole) and by inhibition of voltage-gated calcium channels (nifedipine)

			↑ c-fos mRNA
(34)	Primary osteoblasts	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 1 hr	↑ NO (but not PGE_2) and ↑ collagen I with cyclic strain
(53)	Primary osteoblasts	Type: Biaxial Intensity: 1% to 8.8% Frequency: 1 Hz Duration: 15 min	1% strain gave greatest ↑ in proliferation; 8.8% strain caused decrease in proliferation No ↑ in ALP activity
(77)	Primary osteoblasts (normal & osteoporotic)	Type: Biaxial Intensity: 1% Frequency: 1 Hz Duration: 30 min /day × 3 days	↑ proliferation and TGF-β secretion in normal but not osteoporotic bone cells No ↑ in ALP activity
(95)	Primary osteoblasts	Type: Biaxial Intensity: 4 – 12 % Frequency: 1 Hz Duration: 24 hrs	↑ alignment of cells to minimize axial strain levels
(26)	Primary osteoblasts & MG-63	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 30 min	↑ c-fos expression which was dependent on integrin β1 signaling, stretch-activated Ca^{2+} channels and PKC but not on PGE2. Not blocked by cytochalasin D
(27)	Primary osteoblasts	Type: Equiaxial Intensity: 0.1% Frequency: 0.10 – 0.33 Hz Duration: 20 minutes	β1 and α5 integrins are involved in the transduction of strain at 0.33 Hz, which resulted in a hyperpolarization response via Ca^{2+} activated K+ channels. 0.10 Hz caused a depolarization response mediated by a Na+ channel and αv, β1 and αvβ5 (osteopontin, vitronectin, fibronectin) integrins.
(96)	Primary osteoblasts	Type: Equiaxial Intensity: 0.1% Frequency: 0.10 – 0.33 Hz Duration: 20 minutes	Hyperpolarization in response to 0.33 Hz, (but not 0.10 Hz) resulted from an IL-1β autocrine loop
(55)	Primary osteoblasts	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 2.78 hrs / day × 3 days	No change in proliferation or protein expression (ALP, collagen I, bone sialoprotein) in response to strain
(24)	Fetal osteoblast-like cells (HFO)	Type: Biaxial Intensity: 0.4% – 2.5% Frequency: 0.5 Hz Duration: 72 hrs	↑ proliferation and ↑ apoptosis at all strain levels. Results varied by differentiation status (see text)
(97)	Bone marrow stromal cells	Type: Biaxial Intensity: 0–12% Frequency: 0.05 Hz Duration: 48 hrs	↑ hydroxyapatite formation via strain-induced surface plaques of αvβ3 integrins, osteopontin, tissue transglutaminase and FAK
(54)	Primary osteoblasts	Type: Four point bending Intensity: 0.1% Frequency: 1 Hz Duration: 1 hr	↑ collagen I and ALP production ↓ DNA synthesis
(11)	Fetal osteoblast-like cells	Type: Uniaxial Intensity: 0.1 – 10% Frequency: 1 Hz Duration: 30s	↑ $[Ca^{2+}]_i$ in response to stretch no change in OPN production
(73)	Osteoblast-like cell line (SV-HFO)	Type: Equiaxial Intensity:0.4% Frequency:0.5 Hz × 5–60 min Duration: 1, 2 or 3 weeks	↑ ERK 1/2 phosphorylation, higher in more differentiated cells

Table 3

Vibration

Reference	Cell Type	Loading Regime	Outcomes
(20)	Fetal pre-osteoblastic cells	Type: Ultrasound Intensity: 30 mW/cm^2 Frequency: 1.5 MHz (pulsed) Duration: 20 min	↑ Cbfa mRNA ↑ Osteocalcin mRNA ↑ ERK activation ↑ Gαi-1 levels All were PTX sensitive; Cbfa and Osteocalcin increases were blocked by ERK inhibition
(61)	Primary osteoblasts	Type: Traditional or long-wave ultrasound Intensity: 0.1 – 1.0 W/cm^2 or 5–50 mW/cm^2 Frequency: 1 MHz (pulsed) or 45 kHz (continuous) Duration: 5 min	↑ DNA synthesis ↑ Collagen synthesis ↑IL-1 production ↑ IL-8, bFGF and VEGF Results varied by type and intensity
(48)	Osteoblast-like cells (MG-63)	Type: Ultrasound Intensity: 0.12, 0.39, or 1.42 W/cm^2 Frequency: 3 MHz Duration: 10 min	↑ ALP and OPN mRNA at 390 and 1420 mW/cm^2 ↓ OPN mRNA at 120 mW/cm$_2$
(98)	Primary osteoblasts	Type: Traditional or long-wave ultrasound Intensity: 0.1 – 1.0 W/cm^2 or 5–50 mW/cm^2 Frequency: 1 MHz (pulsed) or 45 kHz (continuous) Duration: 5 min	↑ NO Results varied by type and intensity
(60)	Primary osteoblasts	Type: Mechanical vibration Intensity: 25 +/− 5 μm amplitude Frequency: 20 – 60 Hz Duration: 2 min /day × 4 days	Low frequencies ↑ proliferation but ↓ ALP activity High frequencies ↓ proliferation and ↑ ALP activity.
(31)	Primary osteoblasts	Type: Mechanical vibration Intensity: 10 μm amplitude Frequency: 20 Hz Duration: 2 min /day × 4 days	↑ DNA content (36–51%) in response to vibration, which was dependent on intact microtubules
(55)	Primary osteoblasts	Type: Mechanical vibration Intensity: 0.1% Frequency: 20 Hz Duration: 8.3 min/ day × 3 days	No change in proliferation or expression of ALP, collagen I, bone sialoprotein

Table 4

Membrane deformation

Reference	Cell Type	Loading Regime	Outcomes
(12),(13)	Osteoblast-like cells (G292)	Type: Membrane stretch (micropipette) Intensity: pressure ↑ in 0.5 cmHg increments to rupture Frequency: 2–3s per increment Duration: 0.75 – 5 min	Osteoblasts express a variety of mechanosensitive ion channels.
(25)	Primary osteoblasts & bone marrow stromal cells	Tyoe: Magnetic beads bound to integrins Intensity: 10–30 pN Frequency: Continuous Duration: 5–15 min	↑ calcium transients in both cell types ↑ calcium oscillations in osteoblasts
(8, 99)	Bone marrow-derived osteoblasts	Type: Deformation with micropipette Intensity: NA Duration: Instantaneous	Two mechanisms of intercellular Ca^{2+} waves, fast (15 μm/s; ATP dependent) and slow (2–4 μm/s; gap-junction dependent) (see text)
(9)	Bone marrow-derived osteoblasts (and osteoclasts)	Type: Deformation with micropipette Intensity: NA Duration: Instantaneous	Osteoclasts and osteoblasts both experience intercellular Ca^{2+} wave The wave can initiate in one cell type and pass to the other Osteoclast intercellular communication does not require gap junctions
(29)	Osteoblast-like cells (U2/OS)	Type: Cyclic or continuous horizontal stress via integrin- or transferrin-coated microbeads Intensity: up to 2.2×10^{-11} N Frequency: 0.5 Hz Duration: 10 min	↑ $[Ca^{2+}]i$ in all stimulated cells, integrin > transferrin
(30)	Primary osteoblasts & U2/OS	Type: Cyclic or continuous horizontal stress via integrin-coated microbeads Intensity: from 2.5×10^{-11} N to 2×10^{-10} N Frequency: 1 Hz or 0.1 Hz Duration: 30 min	↑ $[Ca^{2+}]i$ 1 Hz cyclic stress showed stronger and faster increases in $[Ca^{2+}]i$ than 0.1 Hz or continous ↑ cytoskeletal pTyr Relocalization of FAK from cytosol to cytoskeleton. Activation of FAK and ERK1/2
(38)	HOBIT	Type: Deformation with micropipette Intensity: NA Duration: Instantaneous	Intercellular spread of Ca^{2+} waves are dependent both on P2Y receptors and gap junctions.
(100)	Primary osteoblasts	Type: Cyclic or continuous horizontal stress via integrin-coated microbeads Intensity: up to 2.2×10^{-10} N Frequency: 0.5 Hz Duration: 10 min	↑ accumulation of vinculin and talin at sites of mechanical stress
(101)	Osteoblast-like cells (U2/OS)	Type: Cyclic or continuous horizontal stress via microbeads coated with antibodies vs integrins (β1 or α2) or CD71 Intensity: 10 dyne / cm^2 per bead Frequency: 1 Hz Duration: 30 min	Cyclic strain through β1 and α2 integrins caused ↑ in total tyrosine phosphorylation Cyclic strain through integrin β1 caused greater ↑ in cytoskeletal pTyr than continuous strain or integrin clustering. Cyclic strain and clustering through integrin α2 induced equivalent amounts of cytosketletal pTyr. ERK1/2 were tyrosine-phosphorylated in response to cyclic stress
(102)	Primary osteoblasts	Type: Deformation with micropipette Intensity: NA Frequency: NA Duration: Instantaneous	↑ $[Ca^{2+}]_i$ which was enhanced by pre-treatment with a gap-junction enhancer (ZP123)
(15)	Primary osteoblasts	Type: Optical tweezers Intensity: 7 pN Frequency: NA Duration: Instantaneous	↑ $[Ca^{2+}]_i$ within 1 s Partially inhibited by blocking voltage gated Ca^{2+} channels.

Table 5

Gravitational / centrifugal force

Reference	Cell Type	Loading Regime	Outcomes
(49)	MG-63	Type: Spaceflight Duration: 9 days	↓ mRNA levels of collagen I, ALP, osteocalcinin
(103)	Primary osteoblasts	Type: Centrifugal force Intensity: 13g Duration: up to 24 h	↑ ERK 1/2 phosphorylation ↑ collagen I protein and mRNA which were inhibited by a MEK inhibitor (PD980589)
(69)	Osteoblast-like cells (TE-85)	Type: Simulated microgravity Duration: 72 hrs	↓ DNA binding and transactivation of NF-κB in response to TNF-α ↓ IL-8 secretion in response to TNF-α
(50)	Osteoblast-like cells (HuO9)	Type: Simulated microgravity Duration: 1 – 7 days	↓ ALP activity and osteocalcin production

Table 6

Compression

Reference	Cell Type	Loading Regime	Outcomes
(5)	Osteoblast-like cells (MG-63)	Type: Intermittent cyclic hydrostatic pressure Intensity: 0–0.8 MPa Frequency: 1Hz, 1 min on, 14 min off Duration: 4 – 12 hrs	↑ MMP-1 and MMP-3 mRNA ↓ collagen I and osteocalcin mRNA no change in c-fos mRNA