
Effect of Mechanical Loading on Cells: An Update

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Introduction

It is well known that aerobic weight-bearing exercise significantly improves bone strength, increases bone mineral density, and reduces fracture risk. How biomechanical forces on the skeleton are translated into increased bone strength is less well understood, but recent studies have given insight into many aspects of the processes involved.

The osteoblasts, osteoclasts, and osteocytes that regulate the acquisition and loss of bone mineral density are each responsive to biomechanical forces on the skeleton. During skeletal growth and development, these bone cells respond to changes in biomechanical forces on the bone by contributing to modeling, in which the architecture and microarchitecture of the bone is reworked in response to alterations in biomechanical forces across the skeleton. Throughout life, osteoblasts, osteoclasts, and osteocytes respond to biomechanical forces by the process of remodeling, in which old bone with microdamage is resorbed, and newly mineralized bone is formed to fill in microstructural defects that might lead to fracture. Modeling and remodeling transform the skeleton into the structure necessary to maintain maximum bone strength for the loads it is called upon to carry.

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Skeletal Anatomical Sites and Mechanical Loads

Mechanical loads applied simultaneously to the ends of long bones lead to increased forces on various sites within the bone as the length of the bone is compressed, and the bone bends to some degree in order to compensate for the increased stress. Compression and bending of the bone creates shear stress transmitted through Haversian canals and the pericellular matrix around osteocytes and osteocytic processes within the canalicular network, as fluid is displaced due to the applied load [1]. Models of this type of skeletal loading predict oscillatory fluid flow (OFF) patterns within the canalicular network, leading to shear stresses on osteocytes of 0.8 to 3.0 Pascal (Pa). High-frequency (>30 cycles per second), low-magnitude (<1 MPa) loads appear to be sufficient to generate osteocytic responses. Within the intramedullary bone marrow cavity, hydrostatic pressure compresses, and fluid flow exerts shear stress, on bone marrow-derived osteoprogenitor cells. Within blood vessel walls, mechanical strain is exerted on mesenchymal tissue-derived osteoprogenitor cells. The effect of mechanical loads on the skeleton, which increases compressive or bending forces, is to transiently deform bones, unless the forces applied exceed the ability of the bone to withstand the force, in which case fracture may occur.

The skeleton experiences relatively few high-strain (2000–3000 microstrain), low-frequency (1–3 cycles per second) events during the course of daily activity, but is exposed to nearly constant low-strain (<5 microstrain), high-frequency (10–50 cycles per second) events. These frequent low-strain, high-frequency events occur with muscle contractions necessary to maintain erect posture. As the skeleton ages and sarcopenia develops, the frequency of low-strain, high-frequency events decreases with time, possibly contributing to the bone loss that accompanies age-related deterioration of muscle function.

Sensors, Signaling Pathways, and Responses

The cells within the bone mediate bone tissue remodeling. These cells include osteocytes embedded within the bone that function as mechanosensors, osteoblasts that synthesize and secrete new bone matrix, and osteoclasts that resorb old bone affected by microdamage and their progenitors [2]. In order to fully understand the control of bone mechanobiological responses, improved understanding of the cellular and molecular bases of bone functional adaptation to stress is required.

Intracellular Signaling Pathways

Mechanical forces detected by bone cells are ultimately translated into biochemical signals that lead to downstream signaling pathway events such as phosphorylation, transcription factor translocation, or alterations in gene expression. Distal responses that occur in signaling pathways are associated with mechanotransducers that include protein kinase cascades, nuclear translocation of regulatory proteins, G-protein-regulated messengers, and second messenger systems such as intracellular Ca^{2+} and

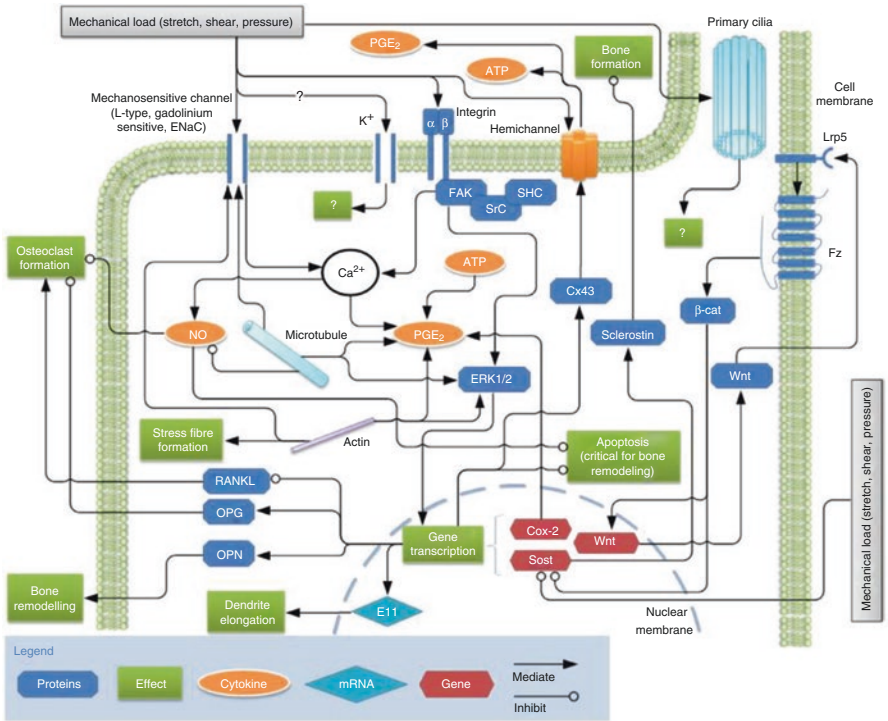


Fig. 2.1 Schematic of sensors, signaling pathways, and responses involved in osteocyte mechanobiology. Much of the current state of knowledge regarding osteocyte mechanobiology is represented in the schematic, although details are lacking in many cases. The detailed signaling mechanisms involved in osteoprogenitor mechanobiology are poorly defined. While osteoprogenitor cells appear to use many of the same sensors (e.g., integrins, ion channels, gap junctions, and primary cilia) and pathways (e.g., ERK1/2 and other MAPKs, Ca^{2+} , and Wnt) as osteocytes, their responses to distinct mechanical stimuli often differ and are not as well defined. Used with permission from Chen J-H, Liu C, You L, Simmons CA. Boning up on Wolff's Law: mechanical regulation of the cells that make and maintain bone. *J Biomech.* 2010;43:108–18

cAMP (Fig. 2.1). While mechanical signals are capable of activating nearly all types of signal transduction pathways within osteoblasts, osteocytes, and osteoclasts in the bone, several of the better understood pathways will be described here to give a sense of the multiple avenues by which mechanical signals regulate adaptive responses.

Protein Kinase Activation

Mechanical forces are particularly effective at activating mitogen-activated protein kinase (MAPK) cascades in nearly all types of bone cells evaluated. MAPKs are serine/threonine protein kinases necessary for bone cell differentiation, proliferation, and survival. In endothelial cells within bone, mechanical factors activate not only extracellular signal-related kinases 1 and 2 (ERK1/2) but also p38, beta-cell myeloid kinase 1 (BMK-1), and c-Jun terminal kinases (JNK). Mechanical activation of ERK1/2 has been shown to be critical for certain strain responses in bone stromal and osteoblastic cells [3]. ERK1/2 causes downregulation of receptor

activator of nuclear factor kappa-B ligand (RANKL) and upregulation of endothelial nitric oxide synthase (eNOS) protein after bone cells are exposed to strain. Activation of these pathways leads to decreased bone resorption and increased bone formation.

Activation of ERK1/2 is also necessary for transforming growth factor- β (TGF β)-induced osteogenic differentiation of mesenchymal stem cells (MSCs) into preosteoblasts and osteoblasts. ERK1/2 activation in bone cells also occurs during mechanical stimulation of voltage-sensitive calcium channels (VSCCs). Activation of ERK1/2 in osteoblastic cells by fluid shear requires Ca^{2+} influx through VSCCs and is ATP dependent. Osteocyte-like MLO-Y4 cells require association of the $\alpha_2\delta_1$ auxiliary subunit with VSCCs in order for mechanical signals to activate ERK1/2.

Protein kinase B (Akt) is a serine/threonine kinase involved in a broad range of cellular functions that is activated by a variety of growth factors, cytokines, and mechanical signals [4]. Mechanical signals that activate Akt lead to increased β -catenin activity, which causes inhibition of MSC adipogenesis, thereby causing differentiation of bone marrow MSCs toward the osteoblast lineage. Exposure to mechanical strain leading to Akt activation results in increased focal adhesion assembly in bone cells. Skeletal loading resulting in increased bone strength is influenced by refractory periods that allow regeneration of enzymes, recycling of molecules to the cell surface, and the numbers and arrangement of cytoskeletal platforms initiating the signaling cascade.

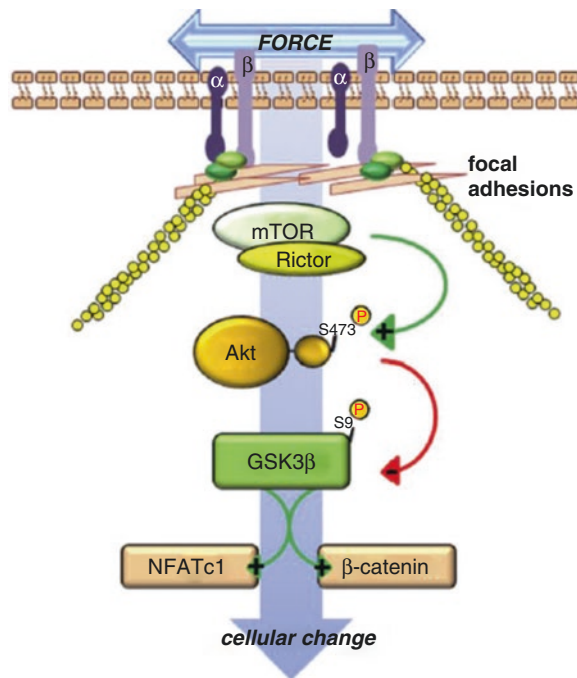
Focal adhesion kinase (FAK) is a non-receptor cytoplasmic protein tyrosine kinase (PTK) that is found in higher concentrations near focal adhesions between cells. This kinase plays an important role in signaling events involving growth factors, extracellular matrix (ECM) molecules, and stress signals [5]. FAK is found in proximity to signaling proteins including sarcoma gene (Src) family PTKs, phosphatidylinositol-3-kinases (PI3K), and paxillin. Interactions with these associated signaling proteins enable FAK to form a functional network of integrin-stimulated signaling pathways that result in activation of downstream targets, including MAPK pathways. Activation of FAK leads to autophosphorylation of FAK tyrosine 397, which leads to interactions with Src-family proteins and other molecules containing Src homology 2 (SH2) domains. Phosphorylation of FAK leads to MAPK activation by interacting with chicken sarcoma gene (c-Src), growth factor receptor-bound protein 2 (Grb2), and rat sarcoma gene (Ras), explaining why most biophysical stimuli in cell culture cause activation of MAPK pathways. Oscillatory fluid flow (OFF) has been demonstrated to cause sustained association of Src and FAK with $\alpha_v\beta_3$ integrin. Stimulation of FAK caused by this integrin association upregulates PI3K activity and modulates downstream ERK and Akt/mammalian target of rapamycin (mTOR)/p70S6K pathways, leading to increased osteoblast proliferation. FAK has also been shown to contribute to OFF-induced stimulation of osteopontin (OPN) and cyclooxygenase-2 (COX-2) expression in osteoblasts. This effect is critical for fluid shear stress-mediated increased expression of osteocalcin (OCN), core-binding factor subunit $\alpha 1$ (Runx2), and Osterix (Osx), demonstrating that FAK is important for osteoblast differentiation and bone formation.

β -Catenin

β -Catenin is critical for bone formation by osteoblasts, but plays other roles in bone biology. A mutation in the Wingless (Wnt) co-receptor lipid-related protein receptor-5/6 (LRP5/6) that causes constitutive activation of Wnt stimulates bone formation leading to a high bone mass phenotype known as sclerosteosis, and a dominant negative mutation of the same LRP5/6 receptor results in a low bone mass phenotype known as osteoporosis-pseudoglioma syndrome. Multiple studies have demonstrated the role of β -catenin in osteoblasts and osteocytes. β -Catenin regulates both bone formation and resorption [6, 7]. Skeletal loading has been shown to regulate levels of β -catenin in bone cells in animals and humans.

While the LRP5/6 receptor may function as a mechanoreceptor in osteoblasts, mechanical strain has been shown to control MSC differentiation via non-LRP5/6 regulation of β -catenin. Two percent mechanical strain at 10 cycles/min and continuous fluid flow at 8 dyn/cm² and OFF at 10 dyn/cm² have each been shown to stimulate β -catenin activity in osteoblasts in spite of blocked LRP5/6 receptors. Low-intensity vibration at <10 μ ε and 90 cycles per second stimulates β -catenin and suppresses fat cell differentiation, indicating that both high- and low-magnitude mechanical stimuli alter MSC fate through β -catenin. Focal adhesion-based connections with substrate mediate glycogen synthase kinase-3 β (GSK3 β) inhibition after loading of MSCs and osteoblasts, resulting in increased β -catenin due to reduced proteosomal degradation. Proximal signaling results in mechanical activation of mTORC2, leading to phosphorylation of Akt serine 473, leading to Akt-dependent inhibition of GSK3 β (Fig. 2.2). If

Fig. 2.2 Force-induced activation of β -catenin. Application of mechanical force to cells induces focal adhesion-dependent activation of mTORC2. mTORC2 then activates Akt, which inactivates GSK3 β via phosphorylation. Inhibition of GSK3 β leads to multiple downstream events, including preservation and nuclear translocation of β -catenin, as well as prolonging the nuclear residence of NFATc1. Used with permission from Thompson WR, Rubin CT, Rubin J. Mechanical regulation of signaling pathways in bone. *Gene*. 2012;503:179–93



mTORC2 functions as a mechanical target, there may be interactions between the cytoskeleton and metabolic responses to exercise since mTORC2 responds to insulin signaling.

GTPases and G-Protein-Coupled Receptors

Guanosine triphosphatases (GTPases) are a large family of enzymes that bind to and hydrolyze GTP. These enzymes function as switches regulating a wide variety of physiological processes. Mechanical stimuli activate heterotrimeric GTPases via G-protein-coupled receptors, resulting in increased intracellular calcium ($[Ca^{2+}]_i$), cyclic AMP (cAMP), and cyclic GMP (cGMP) (Fig. 2.3). Nitric oxide (NO) is produced by protein kinase G (PKG) after mechanical shear stress in osteoblasts [8]. PKGII was shown to be necessary for Src upregulation by phosphorylating Src homology 2 domain-containing tyrosine phosphatase-1 (SHP-1). Subsequent fluid shear stress led to recruitment of PKGII, Src, SHP-1, and SHP-2 to a β 3-integrin-containing mechanosome.

Estrogen Receptors

Estrogen deficiency after menopause is a major cause of osteoporosis, and estrogen receptors (ERs) play a key role in postmenopausal bone loss. ER α mediates bone formation in response to load-bearing in vivo and regulates how osteoblasts and osteocytes respond to mechanical stimulation [9]. ER also modulates mechanically

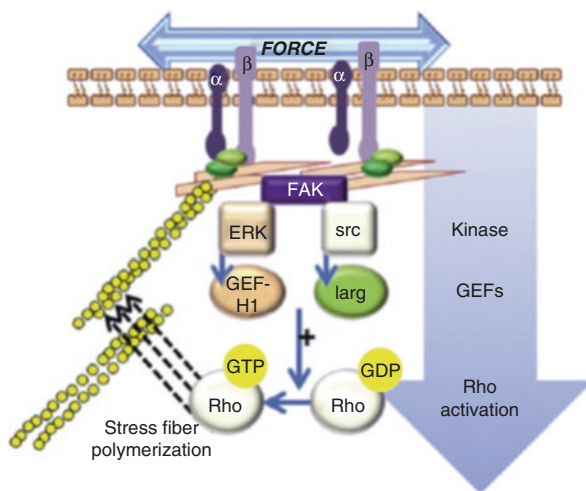


Fig. 2.3 Mechanical activation of Rho via focal adhesions. Forces transmitted through focal adhesions are known to activate signaling cascades, possibly through force-induced conformational change. Involved signaling pathways include focal adhesion kinase (FAK), ERK1/2, and Src. ERK and Src can initiate Rho signaling via activation of GEFs (here shown as GEF-H1 and Larg). GDP-bound RhoA is critical for assembly of new focal adhesions and stress fiber polymerization. Used with permission from Thompson WR, Rubin CT, Rubin J. Mechanical regulation of signaling pathways in bone. *Gene*. 2012;503:179–93

activated signaling pathways. ER α knockout (KO) mice were shown to have reduced response to tibial loading, with severely reduced and delayed transcriptional response and a broad range of effects compared to wild-type littermates. Three hours following a brief loading regimen, wild-type mouse tibiae showed altered transcription of 642 genes, while only 26 genes were modified in ER α KO tibiae. For example, sclerostin gene (*SOST*) expression was significantly reduced after loading in wild-type mice, but unchanged in loaded tibiae of ER α KO mice.

Bone cells respond to mechanical stimuli via ER α through both genomic and nongenomic actions. ER α nongenomic actions depend on direct interaction with insulin-like growth factor-1 receptor (IGF-1R), leading to sensitization of IGF-1R and upregulation of early strain-regulated genes including COX-2. β 1-Integrin expression, which is important for load-induced bone formation, is also upregulated by ER α . These interactions help explain why estrogen may help upregulate osteogenic target genes in response to mechanical stimuli.

Calcium Signaling

A rapid increase in $[\text{Ca}^{2+}]_i$ is the earliest detectable response in mechanically activated bone cells [10]. Calcium channels in the plasma membrane and intracellular organelles help regulate $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ concentrations are tightly regulated to maintain a very low level of free $[\text{Ca}^{2+}]_i$, making $[\text{Ca}^{2+}]_i$ an excellent second messenger system. $[\text{Ca}^{2+}]_i$ serves as an initial signal in bone cell proliferation, mitosis, differentiation, and motility. $[\text{Ca}^{2+}]_i$ mobilization is initiated by cell membrane strain, pressure, fluid flow, and osmotic swelling. The frequency of $[\text{Ca}^{2+}]_i$ spiking is more important in bone cell response to loading than the magnitude of Ca^{2+} spikes, and a rest period between loading enhances Ca^{2+} response in osteoblastic cells.

Changes in $[\text{Ca}^{2+}]_i$ are linked to several mechanically regulated signaling cascades, including inositol-3-phosphate (IP3), adenosine triphosphate (ATP), and NO. $[\text{Ca}^{2+}]_i$ mobilization stimulates downstream signaling through protein kinase A (PKA), MAPK, and c-Fos. Prostaglandin E₂ (PGE₂) release plays an important role in mechanical bone formation, but is activated by a Ca^{2+} -independent mechanism. Other studies show that PGE₂ release is dependent on Ca^{2+} entry through the L-type VSCC, followed by release of ATP.

Influence of Mechanical Loading on Mesenchymal Stem Cell Differentiation

Mechanical strain applied to mesenchymal stem cells causes these cells to differentiate toward osteoblasts and suppresses differentiation toward adipocytes [11]. Bone formation occurs during embryonic growth and development and during postnatal bone modeling, remodeling, and repair. In order for osteoblasts to continue to fulfill their role, the skeleton or other tissues must contain a sufficiently large reservoir of osteoprogenitor cells to generate new osteoblasts from birth until death. Osteoprogenitor cells maintain their ability to differentiate from a less differentiated precursor state, or to transdifferentiate from other differentiated cell types, into

osteoblast-like cells that produce a variety of proteins characterizing osteoblasts, such as Runx2, alkaline phosphatase (ALP), ON, and OCN, and synthesize bone matrix. Because osteoprogenitor cells have not yet been identified to express specific protein markers, they are difficult to identify, and the precise anatomical location of their niche remains unknown. Osteoprogenitor subpopulations have been identified in bone marrow, but also in cardiovascular tissues, including the aortic heart valve, vascular smooth muscle, and capillary beds. Mechanical signals are also thought to regulate the function and differentiation of osteoprogenitor cells in tissues other than the bone.

Substrate Deformation Effects on Osteoprogenitor Cells

Mechanical strain induces differentiation of osteogenic precursors and suppresses differentiation of osteoprogenitor cells into adipocytes. After being stretched for over 24 h, mRNA transcripts for ALP, Runx2, BMP2, BMP4, and collagen type I are significantly increased in osteogenic precursors. After cyclic stretching for 1–2 weeks, osteogenic precursor OCN and Runx2 protein levels increase, and mineralization of deposited matrix occurs. The extent of the osteogenic response depends on the size of the strain detected and mediated through FosB activation [12]. How osteoprogenitor cells respond to mechanical strain depends on their stage of differentiation. Mechanical strain at less differentiated stages leads to increased Runx2 and type I collagen mRNA expression, decreased proliferation of osteoprogenitors, and increased apoptosis, compared to exposure at more differentiated stages.

Stretching the extracellular matrix applies force to cell focal adhesions to the matrix, which results in increased FAK activity, activating downstream MAPK signaling pathways. Mechanical strain induces rapid phosphorylation of MAPK. Although mechanical strain increases MAPK activity, and inhibition of MAPK in cell culture decreases mineralization, inhibition of these pathways has little to no effect on strain-induced osteogenic differentiation in osteoprogenitor cells found in blood vessels. These findings suggest that mechanical strain also activates other signaling pathways to stimulate osteogenic differentiation.

Certain calcium channels may be activated by mechanical strain. Blockage of these calcium channels pharmacologically, or culture of osteoprogenitor cells in calcium-free media, leads to decreased strain-induced ALP upregulation. The Wnt signaling pathway may be stimulated mechanically to promote bone formation. Mechanical strain upregulates Wnt 10B and frizzled-2 receptor mRNA, which leads to increased phosphorylated β -catenin nuclear translocation in osteoprogenitor cells.

Fluid Flow Effects on Osteoprogenitor Cells

Fluid flow in the intramedullary space and Haversian canals occurs in response to mechanical strain, suggesting that fluid flow stimulates osteoprogenitor cells in these compartments also. Osteoprogenitor cells appear to be more responsive to shear

stress than matrix deformation when these are both applied at the physiological ranges seen in the bone.

Although most studies have reported that fluid flow promotes osteogenic differentiation, some studies have not. In parallel plate flow chamber studies, flow-induced shear stress stimulated mRNA expression of some preosteoblast markers, but had no effect or inhibited other preosteoblast markers such as ALP, Runx2, or osteocalcin. These findings were attributed to differences in cell substrate surface chemistry and different flow patterns employed. ECM proteins and substrate materials are known to influence osteogenic differentiation, but studies have not thoroughly explored the effect of matrix proteins in combination with mechanical strain. One study demonstrated that the calcium phosphate coating on glass slides was associated with less flow-stimulated OPN and bone sialoprotein mRNA synthesis, suggesting that matrix properties affect osteoprogenitor cell mechanosensation. Osteopontin mRNA expression and ERK1/2 phosphorylation are variable, depending on whether the flow pattern is continuous, intermittent, or oscillatory and the magnitude of shear stress and flow frequency. Variable osteoprogenitor response to flow pattern may also explain some of the discrepancies seen between different studies.

The effect of flow on osteoprogenitor cells has also been evaluated in three-dimensional cell culture systems. In this case, cells are seeded into hydrogels or scaffolds and then cultured during spinning or flow perfusion. Similar to what is found in 2D systems, fluid flow stimulates preosteoblast differentiation in 3D systems. Fluid-induced preosteoblast differentiation in 3D systems also depends on substrate material. Even though 3D systems better reflect the *in vivo* cellular environment, the effects of shear stress and mass transport are difficult to separate. In addition, variability in pore size and interconnectivity of the scaffolds make it difficult to quantitate shear stress experienced by the cells.

Primary cilia, gap junctions, and the cytoskeleton are important in osteoprogenitor cell mechanosensing of fluid flow [13]. Primary cilia are nonmotile and project from the cell membrane into the extracellular space and move with the flow. Mechanosensing through primary cilia in osteoblast precursors is independent of calcium flux and stretch-activated channels and required for flow-induced stimulation of osteoblast differentiation, as pharmacological removal of primary cilia prevents flow-induced stimulation of OPN mRNA and PGE₂ secretion. Pharmacological inhibition of gap junctions in osteoprogenitor cells caused similar results.

Actin stress fiber formation in osteoprogenitor cells was stimulated by steady fluid flow (SFF), whereas OFF did not. Responsiveness of osteoprogenitor cells to fluid flow may depend in part on cytoskeletal tension. Osteoprogenitor cells that are more spread out on substrate surfaces are less responsive to mechanical strain than cells that are less spread out.

Fluid flow releases intracellular stored calcium and increases intracellular calcium concentration in osteoprogenitor cells, independent of activation of mechanosensitive calcium channels. This is in contrast to mature osteoblasts, in which fluid flow recruits mechanosensitive calcium channels. Fluid flow also activates ERK1/2 and p38 MAPK to stimulate osteogenesis, as inhibition of either of these kinases

reduces flow-induced osteogenesis. Depletion of intracellular calcium stores pharmacologically results in decreased osteogenic response, but reduction in intracellular calcium does not affect ERK1/2 activity. These findings collectively indicate that fluid flow stimulates ERK1/2 independently of calcium signaling, but that p38 MAPK is a potential target of downstream flow-induced calcium signaling. Additionally, actin cytoskeleton disruption did not reduce intracellular calcium release in osteoprogenitor cells subjected to OFF. Instead, these cells showed increased calcium responses and increased PGE₂ release compared to untreated cells. These results demonstrate that PGE₂ release and mobilization of intracellular calcium does not depend on an intact actin cytoskeleton. One possible explanation for these results is that cytoskeleton compromise may allow greater cell deformation, which indirectly contributes to increased intracellular calcium and PGE₂ release.

Hydrostatic Pressure and Compression Effects on Osteoprogenitor Cells

Osteoprogenitor cells are able to sense and respond to mechanical stress due to hydrostatic pressure or compressive loading, likely in part through membrane integrins [14]. Mechanical stress induces ALP activity and mRNA expression of Runx2, BMP2, Osx, collagen I, OCN, osteonectin, and OPN in osteoprogenitor cells. After mechanical loading for 1–2 weeks, mineralization and OCN and OPN protein expression are significantly increased compared to nonloaded cells. Osteogenic response depends on mechanical stress in a biphasic manner, suggesting that there is an optimal range of stress for induction of osteogenic differentiation. The optimal range of stress has not yet been fully characterized, as reported optimal stresses vary from 49 Pa to 11.8 kPa. Osteogenic response to hydrostatic pressure also depends on the extent of differentiation of osteoprogenitor cells, with less differentiated cells being more sensitive to hydrostatic pressure.

ERK1/2 and p38 MAPK appear to play a positive but nonsignificant role in mechanotransduction of hydrostatic pressure. Mechanical stress stimulates phosphorylation of ERK1/2 and p38 MAPK, but pharmacological inhibition of these kinases partially inhibits mechanical stress-induced osteogenic differentiation, suggesting that other signaling pathways are involved. Mechanical loading causes activation of the Wnt signaling pathway, with Wnt10B and Wnt4 mRNA expression stimulated. Stimulation of Wnt10B mRNA partially depends on ERK1/2 phosphorylation, but Wnt4 mRNA expression does not.

Substrate Stiffness Effects on Osteoprogenitor Cells

Osteoprogenitor cells sense stiffness of the ECM and change the way they respond to various stimuli, including how rapidly they proliferate [1], differentiate, and undergo apoptosis. The mechanism affecting this variable responsiveness to ECM stiffness is as yet unknown, but cellular response to matrix stiffness depends on

osteoprogenitor cell differentiation. More differentiated osteoprogenitor cells appear to be more responsive to ECM stiffness. Some studies have shown that stiffer ECM promotes greater osteogenic differentiation and mineralization. One study showed that osteoprogenitor cells have a biphasic dependence on matrix stiffness, in which physiologically relevant stiffness in the range of 25–40 kPa was optimal. Another study demonstrated more significant osteogenic differentiation in this same stiffness range, compared to stiffer matrices. Differences between studies are likely due to differences in ECM surface chemistry and osteoprogenitor differentiation. Differences in differentiation potential between cell systems might also result in the biphasic response observed in primary osteoprogenitor cells, which often differentiate to myofibroblasts on stiffer ECM.

Inhibition of non-muscle myosin II decreases osteogenic differentiation on substrate of variable stiffness. MAPK activation downstream of the Ras homolog gene family member A (RhoA)-Rho-associated, coiled-coil-containing protein kinase (ROCK) signaling pathway also affects how matrix stiffness influences osteogenic differentiation. Runx2 mRNA expression by MC3T3-E1 preosteoblasts increases on stiffer substrates and correlates with increased ERK1/2 activity. Inhibition of RhoA, ROCK, and MAPK decreases Runx2 activity and inhibits osteogenesis, with resultant decreased OCN, bone sialoprotein, gene expression, ALP activity, and mineralization.

Influence of Use and Disuse on Bone Turnover

Mechanical loading significantly influences bone remodeling. Disuse or lack of loading causes an acceleration of bone turnover, with bone resorption exceeding bone formation, and subsequent rapid bone loss. This type of bone loss is observed in astronauts who spend extended periods of time in low-gravity environments or patients confined to bed rest after stroke or head injury. Bone overuse causes damage to the tissue, which in turn stimulates bone remodeling to repair microfractures. One of the important roles of bone turnover is to continuously replace and repair damaged bone tissue. Osteoclasts preferentially target regions of microdamage and remove damaged bone tissue and replaced it with new bone tissue. If damage accumulates faster than the tissue can be repaired, larger microfractures may develop and propagate to form a stress fracture.

The increased number of bone remodeling sites with skeletal disuse or overuse is preceded by programmed cell death in osteocytes [15]. The factors that initiate osteocyte apoptosis are not well understood, but these may include damage to osteocytes via microfractures in the bone matrix or lack of fluid flow during disuse. The effects of loading on bone remodeling are described by a U-shaped curve. Remodeling increases with disuse due to insufficient loading. Overuse results from overloading leading to damage. Similar to the optimal range of strain on bone cells described above, there is an optimal range of skeletal loading within which bone remodeling is reduced to a nadir. Periosteal new bone formation typically increases with increased skeletal loading.

Lack of physical use during growth limits the development of characteristic cross-sectional shape of long bones. An immobilized tibia develops a fairly round cross-sectional area, rather than the typical triangular shape. Disuse or low stress on the bone reduces bone formation on periosteal surfaces and increases bone resorption on endocortical and trabecular surfaces. These effects are associated with rapid bone loss. The effects of disuse on bone surfaces, differential response to disuse in growing and mature skeletons, and regional differences in bone loss with disuse have been evaluated in many studies. Studies in dogs have shown that casted forelimbs undergo profound bone loss resulting from disuse. In growing dogs, disuse is most apparent at the periosteal surfaces of long bones, with decreased normal appositional bone formation, resulting in smaller cross-sectional bone area and reduced second moment of inertia. In older dogs, disuse results in bone loss due to accelerated bone resorption, with loss occurring mainly on the endosteal surface of long bones. Reduction of loading of a bone in an older dog leads to a rapid expansion of the marrow cavity and a substantial worsening of cortical porosity. In both young and older dogs, bone loss is greatest in the most distal bones of the casted forelimb, indicating that skeletal sites closest to the ground lose the most bone with disuse. The osteogenic effects of mechanical loading are greatest in regions closest to the ground, likely due to direct loading of the bones. Greater pressure within the medullary cavity in the distal bones due to gravity enhances appositional bone growth. Distal regions of weight-bearing limbs have the largest anabolic response to exercise and the most severe catabolic response to disuse. These regions are the greatest distance below the heart and therefore subject to higher interstitial fluid pressure.

Human volunteers at long-term bed rest gain bone mass in their cranial bones [16]. This is due to change in the relative effect of gravity on the body when a person lies supine. Bed rest causes body fluids to shift so that dependent regions like the feet that are typically under high fluid pressure are subject to lower pressure. Sites typically under lower fluid pressure, like the head, adapt to higher pressure, which may increase bone formation or decrease bone resorption in the cranial bones. Bone loss is not equal throughout the skeleton at bed rest. Bone loss is increased in the lower extremities, particularly the calcaneus, where fluid pressure decreases substantially with bed rest.

Exercise generally stimulates an increase in bone density. Bone density measurements in professional athletes compared with non-athletes show increased bone density at the hip, or throwing arm, or kicking leg, but decreased bone density in the upper skull. Therefore, bone density in the skeleton is preserved because changes in bone density at weight-bearing sites caused by disuse or exercise are partially offset by changes in bone density at skeletal sites that do not bear weight.

Microdamage, Osteocyte Apoptosis, and Bone Resorption

Cortical and trabecular bone damage in the form of microfractures occurs throughout the skeleton each day, with the number of microfractures depending on the magnitude of the forces to which the skeleton is exposed. Immunostained iliac crest

bone biopsies demonstrate microfractures that develop due to fatigue damage to the bone [2]. Microfractures may accumulate such that the bone weakens sufficiently for a fracture may occur. Microfractures typically occur along the lamellar surfaces of trabecular bone and cortical bone, and some cross osteonal units in the Haversian structure of the bone. How far microfractures propagate depends on the force applied and the mechanical properties of the affected bone.

Microfractures are believed to serve as a stimulus for osteoclast-mediated bone resorption, likely preceded by osteocyte apoptosis [17]. Osteocytes adjacent to microfractures are typically apoptotic, presumably because the adjacent microfracture disrupted the canalicular network affecting osteocyte nutrient supply. Microdamage generally correlates with strain within the bone, and bone resorption correlates with microdamage removal and repair. The osteocyte network is therefore critical for physiologic response to bone microdamage. Removal of apoptotic osteocytes during the bone resorption process, and synthesis of new bone in the area of previous microdamage, leads to the generation of new osteocytes.

Mechanotransduction in Bone

The human skeleton is both strong and tough enough to prevent fractures, but light enough to maintain mobility and functionality. Osteocytes embedded within mineralized bone sense mechanical loads applied to the skeleton. Mechanical loads are transformed into biological signals which result in bone resorption or bone formation at appropriate sites within the skeleton. Osteocytes stimulated by mechanical loads release signals coordinating the response of osteoclasts and osteoblasts, leading to adaptation of the skeleton to biomechanical forces. Wilhelm Roux first proposed that functional adaptation of the skeleton is the end result of self-organized bone cellular processes in 1881.

Osteocytes produce OCN, osteonectin, OP, and sclerostin, but have reduced expression of AP. Following mechanosensation and conversion of mechanical signals into biochemical signals, osteocytes coordinate the formation and activity of osteoblasts and osteoclasts. The intercellular communication required for this coordination involves signals including NO, PGs, BMPs, Wnts, and others.

Application of mechanical loads to the skeleton leads initially to intracellular movement of extracellular calcium through ion channels in the plasma membrane, with subsequent release of calcium from intracellular stores. The increase in intracellular calcium activates downstream signaling cascades involving phospholipase C and phospholipase A2 and is necessary for activation of calcium-calmodulin-dependent proteins such as constitutive forms of NOS. Activation of phospholipase A2 results in arachidonic acid production and PGE₂ release. Gene transcription of c-Fos, MEPE, and IGF-1 is also modified by mechanical loading.

NO is produced by NOS, molecular oxygen, NADPH, and other cofactors during conversion of L-arginine to L-citrulline. Mechanical stimulation causes increased production of NO. NO modulates the activity of osteoblasts and osteoclasts, and inhibition of NO production by osteoblasts inhibits new bone formation. Activity of

eNOS is not necessary for mechanical stimulation-induced production of NO. It is not yet clear which NOS is responsible for producing NO in mechanically stimulated osteocytes.

PGs are widely expressed in osteocytes and cells of the osteoblastic lineage and play a significant role in mechanical stimulation of new bone formation. Mechanical loading causes a rapid increase in PG production by osteocytes. Osteocyte COX produces PGs and is found in a constitutive form (COX-1) and inducible form (COX-2). Fluid shear stress on primary human bone cells does not stimulate COX-1 mRNA, but stimulates a rapid rise in COX-2 mRNA in human bone cells *in vitro*. Inhibition of COX-2, but not COX-1, inhibits fluid shear stress-induced PG production by primary bone cells *in vitro*. COX-2 has been shown to mediate the anabolic response of bone tissue to mechanical loading. PGs are released through hemichannels and purinergic receptors in response to mechanical stimuli [18].

Wnt signaling is an important modulator of mechanical stimuli-regulated bone adaptation. Wnt signaling is mediated by β -catenin pathways, kinases, or GTPases that modulate cytoskeletal organization. Activation of β -catenin signaling in response to fluid shear stress is mediated by PGE_2 in osteocytes [19]. Wnts modulate cytoskeletal organization, and β -catenin links cadherins to the actin cytoskeleton. MC3T3-E1 osteoblasts increase Wnt gene expression after mechanical stimulation by substrate deformation. Pulsating fluid flow upregulates mRNA expression of β -catenin, APC, and Wnt3a, as well as the Wnt antagonist soluble frizzled-related protein 4 (SFRP4) in MLO-Y4 osteocytes. LRP5/6, a co-receptor for Wnt signaling, functions locally in osteocytes.

Sclerostin is highly expressed in mature osteocytes and at lower levels in immature osteocytes. Sclerostin is transported to the bone surface via the canalicular network in the bone, where it inhibits new bone formation by osteoblasts. Loading of mouse ulnae *in vivo* results in reduced expression of sclerostin mRNA and protein by osteocytes. The magnitude of the strain stimulus is associated with sclerostin staining intensity and the number of sclerostin-positive osteocytes. Hind limb unloading resulted in a significant increase in sclerostin expression in mouse tibiae.

Mechanoreceptors

A variety of candidate mechanoreceptors have been identified that mediate the effects of mechanical strain on bone cells. Mechanosensors include any molecule, protein complex, or biological structure that can sense alterations in force on a cell. Mechanoreceptors must be able to directly contact the extracellular space or be able to detect changes in pressure or fluid shear on the cell membrane.

Proposed mechanoreceptors for bone cells include ion channels and connexins, integrins, the cytoskeleton, focal adhesions, lipid rafts, cadherins, ephrins, and primary cilia (Fig. 2.4). Ion channels in osteoblasts are stimulated by stretch or strain of the cell membrane or by parathyroid hormone. At least three classes of ion channels sensitive to mechanical signals have been identified, including gadolinium-sensitive stretch-activated cation channels, transient receptor potential channels,

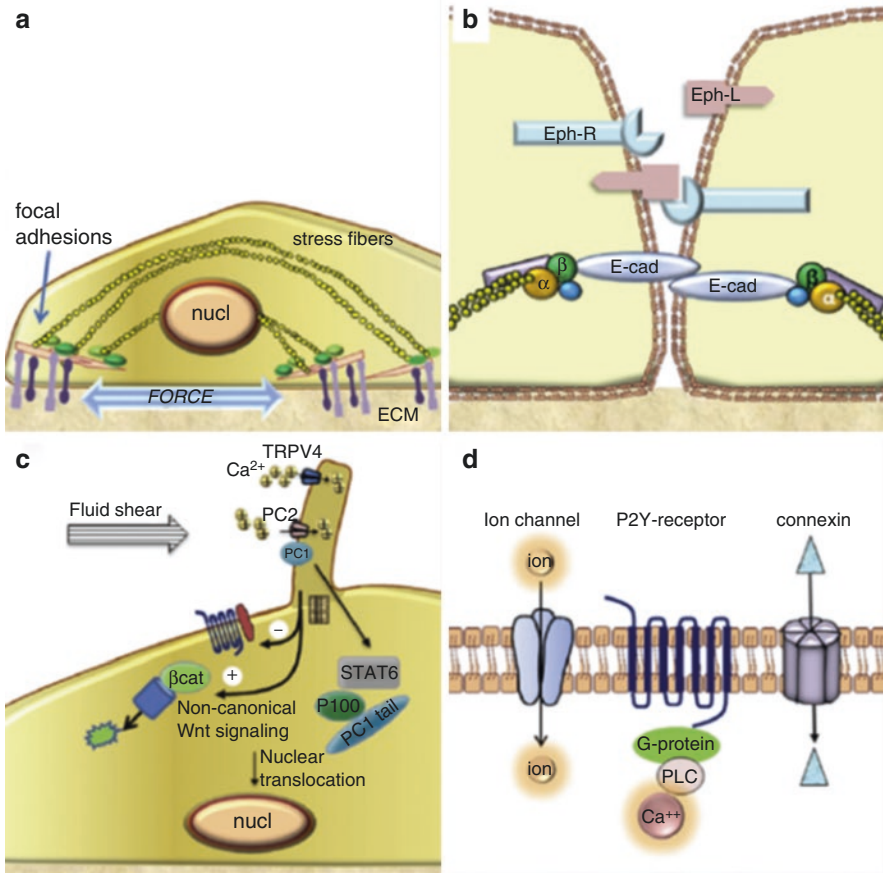


Fig. 2.4 Candidate mechanotransducer systems. (a) Cell cytoskeleton senses loading at the membrane through integrins that transmit force through focal adhesions and F-actin stress fibers. (b) Cadherins, which connect to the cytoskeleton, are examples of outside-in signaling modifiers. Ephrins exemplify an intercellular signaling system regulated by movement of components within the plasma membrane. (c) Primary cilia may sense flow, pressure, and strain, activating ion flux through PC1 and TRPV4, which can activate Stat signals. Cilia also modulate Wnt signaling via noncanonical antagonism that leads to β -catenin degradation. (d) Membrane-spanning proteins such as ion channels, purinergic receptors, and connexins can be regulated through shear and strain. Used with permission from Thompson WR, Rubin CT, Rubin J. Mechanical regulation of signaling pathways in bone. *Gene*. 2012;503:179–93

and multimeric L-type and T-type voltage-sensitive calcium channels in osteoblasts and osteocytes, respectively.

Membrane perturbation or shear across the cell membrane, as well as transient changes in pressure, is transmitted to the cell cytoskeleton and finally to cell adhesion proteins that attach the cell to the bone matrix. Both membrane-spanning integrins that connect the cell to the extracellular matrix, and a large number of adhesion-associated linker proteins, are potential molecular mechanotransducers.

Lipid rafts on the cell membrane are composed of various proteins that compartmentalize signals within the membrane containing several phases of liquid-ordered and liquid-disordered lipid, which may sense mechanical signals. Shear stress may cause signaling molecules in the cell membrane to translocate to caveolar lipid rafts. Degradation of caveolae internally leads to termination of both proximal and downstream signals, including those in the MAPK pathway.

Cadherins are integral membrane glycoproteins that have a large extracellular domain, single transmembrane domain, and short intracellular domain. The intracellular domain links the cadherin to the cytoskeleton by associating with multiprotein complexes that include vinculin and α - and β -catenin. Fluid shear stress reduces that amount of β -catenin bound to N-cadherin, thereby increasing the cytoplasmic availability of β -catenin. Cadherins may therefore serve to release β -catenin for nuclear translocation after mechanical stimuli are received [20].

Ephrin ligands and their cognate ephrin receptors are regulated by the cytoskeleton. Limitation of ephrin clustering within the cell membrane may modulate cellular responses to mechanical stimuli.

Osteoblasts and osteocytes possess nonmotile, microtubule-based cilia derived from centrioles. Fluid flow-induced PGE₂ signaling in osteoblasts and osteocytes, independent of intracellular Ca²⁺ changes, is dependent on cilia. Primary cilia regulate Wnt signaling by causing β -catenin degradation by noncanonical pathways, such that the loss of ciliary function leads to increased canonical Wnt signaling.

Conclusion

Mechanical loading of bone cells prevents bone loss and reduces fractures. Mechanical loads applied to the bone increase fluid flow and hydraulic pressure on bone marrow-derived osteoprogenitor cells and mechanical strain on blood vessels leading to effects on mesenchymal tissue-derived osteoprogenitor cells within the intramedullary canal and bone marrow space and shear forces within Haversian canals and the canalicular network, leading to effects on osteocytes. Mechanical loads involving stretch, shear force, or pressure stimulate bone cell mechanosensitive L-type, gadolinium-sensitive, ENaC channels, integrins, connexin-43 hemi-channels, primary cilia, and possibly potassium channels. Intracellular calcium, Wnt/ β -catenin, sclerostin, Src, and ERK signaling pathways appear to mediate cellular responses to mechanical loading. Mechanical loading diverts mesenchymal stem cell differentiation from adipocytes to osteoblasts, leading to increased bone formation and reduced marrow fat. Bone use increases bone strength by stimulating bone formation, whereas disuse reduces bone strength by increasing bone turnover leading to bone resorption. Bone microdamage results in adjacent osteocyte injury and apoptosis, leading to bone resorption. Mechanotransduction in the bone by multiple mechanisms, and multiple potential mechanosensors, provides the skeleton with the flexibility and adaptability it requires to sustain loads of different weight and to avoid fracture.

Summary

Osteoporosis is a systemic skeletal disorder characterized by compromised bone strength predisposing to increased risk of fracture. Bone strength reflects integration of bone density and bone quality, and bone quality reflects architecture, turnover, damage accumulation (microfractures), and mineralization. In the USA, 44 million persons are estimated to have low bone mass, based on the 2000 census and projections from the National Health and Nutrition Examination Survey (NHANES) data, with 55% of the US population over age 50 affected. Of this number, 33.6 million are estimated to have osteopenia and ten million, osteoporosis.

The bone cells that regulate gain and loss of bone density in the skeleton are responsive to biomechanical forces. During growth and development, bone cells respond to changes in biomechanical forces by modeling the shape and architecture of the bone in response to alterations in biomechanical forces on the skeleton. During aging and senescence, these same bone cells respond to biomechanical forces by the process of remodeling, in which old bone is resorbed and new bone is laid down. The effect of both processes is reworking of the skeleton to gain or maintain maximum bone strength to support the loads carried by the skeleton.

The focus of this chapter will be the mechanisms by which mechanical loading affects bone cells. The chapter will briefly review mechanical loads borne by different skeletal anatomical sites and summarize current knowledge regarding bone cell sensors, signaling pathways, and responses to mechanical loading. The influence of mechanical loading on mesenchymal stem cell differentiation will be discussed, as will the influence of use and disuse on bone turnover. The influence of microdamage on osteocyte apoptosis and bone resorption will be summarized, and current knowledge regarding mechanotransduction in bone explored in some detail. Finally, what is known regarding potential mechanoreceptors will be briefly summarized.

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