Review
Future Rheumatology

Role of osteocytes in the adaptation of bone to mechanical loading

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The mechanical performance of our skeleton is secured by the constant adaptation of bone to its mechanical loading environment. Mechanical adaptation of bone is brought about by the coordinated actions of osteoclasts and osteoblasts, which are orchestrated by the most mechano-sensitive cells in bone, the osteocytes. Loading on bone generates a flow of interstitial fluid through the lacuno-canalicular network in which the osteocytes are positioned. This flow is sensed by the osteocytes, which respond by the release of signaling factors such as nitric oxide, prostaglandins and Wnts, which alter the recruitment and activity of osteoblasts and osteoclasts, thereby affecting bone mass. Thus, any factor that alters the response of osteocytes to mechanical loading potentially affects bone mass. This paradigm could have implications for the field of rheumatology, where proinflammatory cytokines might affect bone mass by altering the response of osteocytes to mechanical loading.

The vertebrate skeleton performs a variety of functions, of which the relative importance will change depending on environmental circumstances. The functions of the skeleton include the provision of space for hematopoiesis, the protection of the soft organs and the regulation of calcium homeostasis. Lately, evidence is accumulating for an important role of the skeleton in phosphate homeostasis as well. The most important task of the skeleton, however, is to provide mechanical support, in order to withstand the force of gravity, and to support muscle forces allowing movement. This function is secured by the constant adaptation of bone to its mechanical loading environment. That bone adapts its mass and structure to mechanical loading has been extensively demonstrated [1–6]. Therefore, it is now common knowledge that increased mechanical loading (e.g., due to exercise) increases, and unloading (e.g., due to bedrest) decreases bone mass, mineral content and bone matrix protein production. This phenomenon is known as functional adaptation of bone, and it serves to obtain bones that combine a proper resistance against mechanical failure with a minimum use of material. More than 20 years ago, Frost postulated his mechanostat theory that predicts that there are threshold levels of mechanical strain, above which adaptive bone formation is activated, and lower levels, below which bone resorption is activated [7]. What is interesting about this mechanostat theory is that these threshold levels (or setpoints) can shift under the influence of agents such as hormones. Such agents would then affect bone mass in a way that resembles the effect of mechanical (un)loading [7]. According to the mechanostat theory, bone loss takes place owing to the activation of bone remodeling below a certain mechanical stimulation. Indeed, it is likely that the actual adaptation of bone to the ever changing mechanical demands takes place during the complicated process of bone remodeling [2,8]. However, bone remodeling takes place continuously, and depending on the balance between bone formation and resorpion within the newly formed osteons, bone mass can be lost or gained.

Bone remodeling

Often bone is thought of as a dead tissue, consisting solely of calcified matrix, but the opposite is true. Human bone contains over 15,000 osteocytes per mm^3, and mouse bone contains even more than 60,000 osteocytes per mm^3 of matrix [9,201]. Bone is not a static tissue either. As a matter of fact, old bone is constantly being removed by osteoclasts and replenished by osteoblasts in a coordinated fashion. This process, known as bone remodeling, presumably serves to prevent or remove microscopic damage in the matrix that occurs owing to continuous wear and tear. The constant renewal of bone also aids the adaptation of bone to its mechanical environment. It allows the osteoclasts to remove excess bone in places that are relatively unloaded, and the osteoblasts to add bone in places that are exposed to relatively high levels of loading.

Keywords
bone remodeling ■ fluid flow ■ mechanical adaptation ■ mechanosensing ■ mechanotransduction ■ osteocyte

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The microscopic damage that occurs owing to tissue fatigue is assumed to be the actual signal for activation of the process of bone remodeling [10–12]. Fatigue damage will likely promote osteocyte apoptosis, which attracts osteoclasts, thereby activating bone remodeling [11–13]. After activation, bone remodeling involves groups of osteoblasts and osteoclasts, which collaborate in a tightly coordinated fashion in so-called basic multicellular units (BMUs) [14]. Within these BMUs, the osteoclasts excavate a tunnel in the compact bone, or a trench along the surface of trabeculae. The osteoclasts are closely followed by osteoblasts that refill the tunnel [15]. The osteoblasts frequently bury one of themselves in the newly formed matrix, thereby forming the new osteocytes [16,17]. The entombed cells assume a stellate shape, with cell bodies positioned in lacunae in the matrix, from which slender cell processes radiate in all directions. The cell processes pass through the bone matrix via small canals, the canaliculi, in order to keep in contact with other osteocytes and the cells outside the bone matrix. The osteoblasts do not completely fill the tunnel that is excavated by the osteoclasts, but a space is left in the middle for blood vessels, providing the osteocytes with nutrients and oxygen [15]. This way, osteons are formed, containing osteocytes at a maximum of six cell layers deep, and a blood vessel in the middle (Figure 1).

Mechanical loading drives interstitial fluid flow

It is generally assumed that the coordinated cooperation of osteoclasts and osteoblasts during bone remodeling and mechanical adaptation is orchestrated by the osteocytes, which are able to respond to mechanical signals [18–20]. Yet, it has long been a matter of debate how mechanical loading of intact bone is transduced into a signal that is able to activate the osteocytes. The application of mechanical loads on bone during physical activity results in several potential cell stimuli. These include changes in hydrostatic pressure, direct cell strain, fluid flow and electric fields resulting from electrokinetic effects accompanying fluid flow [21]. In healthy, adequately adapted bone, strains as a result of physiological loads (e.g., resulting from normal locomotion) are quite small. Quantitative studies of the strain in bones of animals and humans found a maximal strain not higher than 0.2–0.3% [22,23]. It might be that these strains are high enough to to be sensed directly by osteocytes. Vatsa and colleagues hypothesized that if osteocytes can sense matrix strains directly, the cell shape, cytoskeletal alignment and distribution of adhesion sites in osteocytes *in situ* will bear alignment to the mechanical loading patterns [24,25]. Indeed, using confocal laser scanning microscopy, they found that the cell shape and distribution of actin

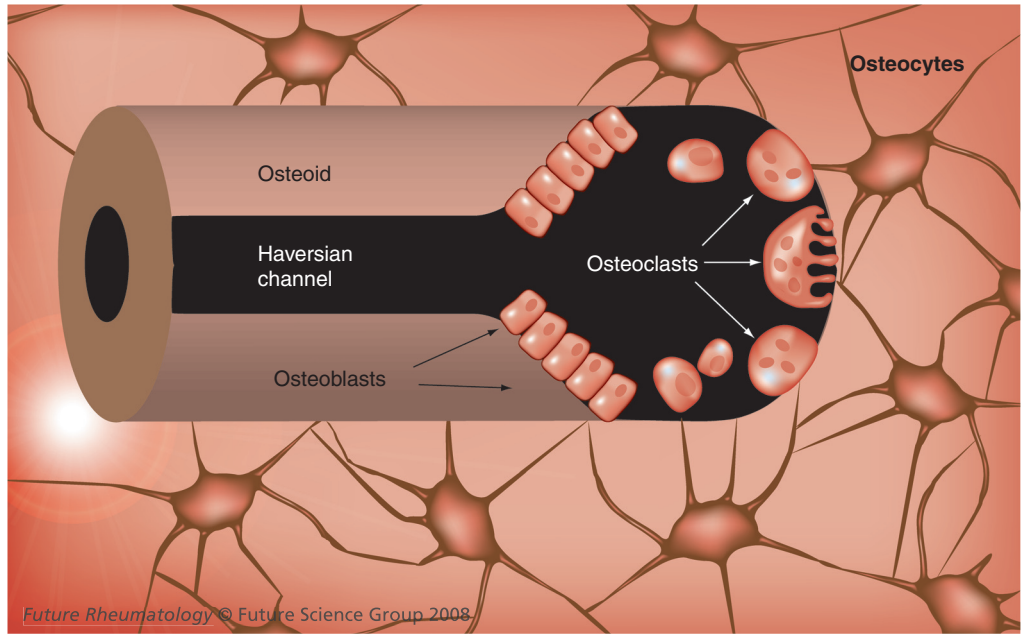

Figure 1. Osteonal tuneling. Fatigue damage promotes osteocyte apoptosis, which attracts osteoclasts, thereby activating bone remodeling. After activation, osteoclasts excavate a tunnel in compact bone and are closely followed by osteoblasts that refill the tunnel. The osteoblasts frequently bury one of themselves in the newly formed matrix, thereby forming the new osteocytes. The osteoblasts do not completely fill the tunnel that is excavated by the osteoclasts, but a space is left in the middle for blood vessels.

and paxillin in osteocytes of mouse tibiae and calvariae reflected the respective mechanical loading patterns in these bones [24,25]. This suggests that osteocytes might be able to directly sense matrix strains due to external loading of bone.

However, if we accept that bone organ strains are extremely small, then it is an attractive idea that bones possess an amplification system whereby small matrix strains are transduced into a larger signal that is easily detected by osteocytes. The canalicular flow hypothesis proposes such an amplification system. In this theory, the small matrix strains are sufficient to drive the thin layer of interstitial fluid surrounding the osteocytes to flow from regions under high pressure to regions under low pressure [18,19,26–31]. The flow of fluid gives rise to a mechanical signal that activates the osteocytes. The theoretical model, which makes the flow of extracellular tissue fluid through the lacuno-canalicular network as a result of bone tissue strains plausible, was described more than 30 years ago by Piekarski and Munro [32], and has meanwhile been shown to occur experimentally [30,33]. It is notheworthy that the kind of mechanical stimuli that would drive a fluid flow (i.e., high-amplitude, low-frequency strains) are extremely rare in the activities of daily life, whereas high-frequency (up to 40 Hz), low amplitude (<0.001%) stimuli are common [34]. This does not mean that interstitial fluid flow is not important for the osteogenic response of bone to mechanical loading. As a matter of fact, (static) loading regimes that do not elicit fluid flow through the lacuno-canalicular network do not seem to be osteogenic [35]. The most osteogenic are loading regimes that are dynamic, which elicit high strain rates and thereby presumably strong flow of interstitial fluid in bone [36]. As strain rate is determined by both the magnitude of the strain and the frequency of the stimulus, both magnitude and frequency of loading are important parameters for bone formation. Using strain sensors in hip implants of humans [37], it has been calculated that the frequency spectra of the forces on the hip shows a rich harmonic content ranging between 1 and 3 Hz for walking cycles and reaching 8–9 Hz for running cycles [38].

Fluid flow activates osteocytes

If osteocytes do not directly sense the loading-induced strain of the bone matrix, but rather respond to the strain-induced flow of interstitial fluid along the network of osteocytes, then what is the flow-derived mechanical stimulus that activates the cells? By comparing variations in fluid transport with variations in wall shear stress, it was shown that the stimulus that activates primary bone cells or osteoblasts *in vitro* is fluid shear stress rather than streaming potentials or chemotransport [39,40]. Whether these observations can be extrapolated towards osteocytes that are embedded in a 3D matrix is a matter of debate. In this light it is noteworthy to mention that results obtained with osteoblasts as a model for mechanosensitive bone cells subjected to oscillating fluid flow, suggested that depriving the cells of nutrients reduces the response to the mechanical stimulus. This indicates that chemotransport could also play a role in mechanotransduction [41]. In any case, it has been predicted that fluid induced shear stresses over the cell extensions of osteocytes *in vivo* will be in the order of magnitude of 8–30 dyn/cm^2 [29]. Although both the accuracy and the relevance of these calculations are currently under debate, it is noteworthy that bone cells *in vitro* seem to be highly sensitive to shear stress in the order of this magnitude.

Recently, insights into the flow-derived stimulus that activates the osteocyte *in vivo* have been evolving. While it is still generally assumed that the flow-derived stimulus is the wall shear stress over the cell membrane, the tensile forces on the cell due to the drag forces on the fibers that tether the osteocyte process to the wall of the canaliculus may be more important for transducing whole bone loads into a stimulus for osteocytes, since these tensile forces are many times greater than the fluid shear stresses [31,42]. Recently it was suggested that the osteocyte processes might be attached directly to the canalicular wall by β3 integrins at the apex of infrequent, previously unrecognized canalicular projections. A theoretical model was developed, which predicts that the tensile forces acting on these integrins are less than 15 pN, and thus provide stable attachment for the range of physiological loadings. The model also predicts that axial strains caused by the sliding of actin microfilaments are two orders of magnitude greater than whole-tissue strains. *In vitro* experiments indicated that membrane strains of this order are large enough to open stretch-activated cation channels [43]. Although it is yet to be determined what the exact mechanical stimulus is for osteocytes *in vivo*, there is a general consensus that this stimulus is likely derived from strain-derived flow of interstitial fluid.

Osteocytes transduce mechanical stimuli into chemical signals

After the fluid flow-derived mechanical stimulus has reached the osteocyte, it is translated into a

biochemical signal. Cellular features that likely play a role in the process of mechanotransduction are stretch-activated ion-channels, integrins, the cytoskeleton and possibly primary cilia.

As previously mentioned, the integrin–cytoskeleton complex may play a role as an intracellular signal transducer for stress signals. In addition to integrins, the nonintegrin adhesion receptor CD44 may attribute to the attachment of osteocytes to the surrounding matrix. CD44 is present in abundance on the osteocyte surface [44,45], and is also linked to the cytoskeleton. Several studies suggest that the attachment complex between intracellular actin cytoskeleton and extracellular matrix macromolecules, via integrins and CD44 receptors in the cell membrane, provides the site of mechanotransduction in osteoblasts as well as osteocytes [46–50].

Lately, evidence is accumulating underscoring the crucial role of the cytoskeleton for a multitude of cellular processes. The cytoskeleton, just like our bony skeleton, provides structure and support for the cell, is actively adapted and is highly responsive to external physical and chemical stimuli. The cytoskeleton is strongly involved in processes such as migration, differentiation, mechanosensing and even cell death, and largely determines the material properties of the cell (i.e., stiffness). Typical features of the osteocyte cytoskeleton are the prominent actin bundles in the osteocytic processes, together with the abundant presence of the actin-bundling protein fimbrin [51,52].

From the field of physics, it is known that the effect of stresses applied at different rates at an object are largely determined by the material properties of that object. For cells, the material properties are to a large extent determined by the cytoskeleton. For osteoblasts, it was shown that the production of signaling molecules correlated with the applied stress rate [38,53–55]. This suggests that the response of osteoblasts to loading is related to cytoskeletal properties. Although the cytoskeleton of osteoblasts and osteocytes show clear differences, for instance, in the expression of actin-binding proteins [51,56], it seems likely that the response of osteocytes to loading is also related to cytoskeletal properties. Indeed, it has been described that osteocytes under round-suspended morphology were an order-of-magnitude more elastic compared with flat-adherent cells, and required lower force stimulation in order to show an increase in nitric oxide (NO) production [57], indicating that cytoskeletal properties of the cell affect the ability for mechanosensing. This is confirmed by *in vitro* experiments demonstrating that cytoskeletal disruption reduces the response of osteocytes and osteoblasts to mechanical loading [48,58,59]. Since cytoskeletal properties of the cell affect the ability for mechanosensing, and since osteocytes are more responsive to mechanical stress than osteoblasts [26], this might be directly related to the different cytoskeletal properties of osteoblasts and osteocytes. The different cytoskeletal properties indeed seem to be reflected by the decrease in elastic modulus as bone cells mature from osteoblasts to preosteocytes and osteocytes [60]. On the other hand, a different study reported that flat adherent MLO-Y4 cells, primary chicken osteocytes, MC3T3-E1 osteoblasts, and primary chicken osteoblasts all showed a similar elastic modulus of less than 1 kPa [61]. This indicates that differences in mechanosensitivity between cells might not be directly related to the elasticity of the cell, but might be more related to other cell-specific properties, such as the presence of receptors or ion channels in the membrane.

Studies on the mechanical properties of osteocytes have been performed on flat, adherent cells. Under this condition, the properties of osteocytes might not reflect the true osteocyte properties when assuming their unique 3D morphology. Moreover, it is debatable whether mechanical properties of the cell body are of relevance, since mechanical loading *in vivo* might mostly affect the cell protrusions of the osteocytes, be it via drag forces on the cell membrane, hoop strain or direct activation of integrins. As such, the osteocyte processes can be considered parts of the cell that not only allow cell-to-cell communication and possibly matrix mineralization, but can also be considered the predominant cell organ that allows mechanosensing. The differentiation marker protein E11/gp38 is a protein expressed by osteocytes *in vivo* that appears to be responsible for the formation of dendritic processes [62]. This molecule might thus also be of utmost importance for the ability of osteocytes to sense mechanical loading. Culture experiments with isolated osteocytes have shown that although the cells lose their stellate shape in suspension, they re-express this morphology as soon as they settle on a support (**Figure 2**) [63]. Apparently, the typical stellate morphology is an intrinsic characteristic of terminal osteocyte differentiation.

In bone, gap junctions are present between the tips of the cell processes of connecting osteocytes [64], thereby forming a network of gap junction-coupled cells. Gap junctions are transmembrane channels connecting the cytoplasm of two adjacent cells and regulating the passage of molecules

less than 1 kDa [65,66]. Gap junction channels are formed by members of a family of proteins known as connexins. One of these members, connexion 43 (Cx43), appears to play an important role in bone cells, as Cx43-null mice have delayed ossification, craniofacial abnormalities and osteoblast dysfunction [67]. It has been proposed that gap junctions function through the propagation of intracellular signals contributing to mechanotransduction in bone, thereby regulating bone cell differentiation [68]. A dominant negative mutant of Cx43 diminishes fluid flow-induced release of PGE_2, but not Ca^{2+} responses [69]. Fluid flow-induced shear stress stimulates gap junction-mediated intercellular communication and increases Cx43 expression in MLO-Y4 cells [70], while oscillating fluid flow has been shown to upregulate gap junction communication by an ERK1/2 MAP kinase-dependent mechanism in MLO-Y4 osteocytes [71].

Besides gap juctions, osteocytes posess hemichannels, unapposed halves of gap junction channels, that localize at the cell surface [72]. Primary osteocytes and MLO-Y4 osteocyte-like cells [73] express very large amounts of Cx43 compared with other cell types such as osteoblasts, yet these cells are only in contact through the tips of their dendritic processes, raising the question regarding the function of Cx43 on the rest of the cell membrane. It has been shown that oscillating fluid flow activates hemichannels in MLO-Y4 osteocyte-like cells, but not in MC3T3-E1 osteoblast-like cells [74]. Hemichannels expressed in bone cells such as MLO-Y4 osteocytes appear to function as essential transducers of the anti-apoptotic effects of bisphosphonates [75], and serve as a portal for the exit of elevated intracellular PGE_2 in osteocytes induced by fluid flow shear stress [76].

Another cellular feature that might play a role in the process of mechanotransduction is the primary cilium. It has recently been shown that osteocytes express a single primary cilia [77], and that PKD1/PC1, a mechanosensory protein in the kidney that localizes to primary cilia, is known to play a role in normal bone structure. MC3T3-E1 osteoblasts and MLO-Y4 osteocytes possess primary cilia that project from the cell surface and deflect during fluid flow [78]. These primary cilia were required for osteogenic and bone resorptive responses to dynamic fluid flow, such as the expression of osteopontin, prostaglandins and RANKL. The translation of fluid flow into bone cellular responses appeared to be independent of calcium- and stretch-activated ion channels [78]. It should be noted, however, that the role of the osteocyte primary cilium for mechanosensing *in vivo* is still under debate, as the space between the osteocyte cell membrane and lacunar surface is several-fold smaller than the length of a cilium, making it unlikely that it can deflect.

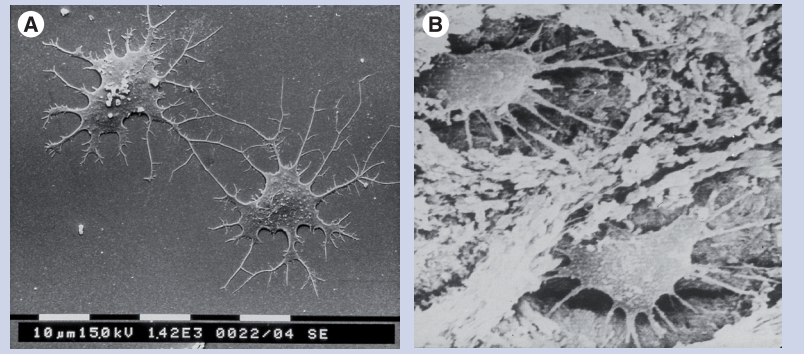

Figure 2. Osteocyte morphology. (A) Isolated osteocytes in culture maintain their typical *in vivo* phenotype, with cell fingers radiating in all directions. The typical stellate morphology might thus be an intrinsic characteristic of terminal osteocyte differentiation. **(B)** Osteocytes embedded in calcified bone. Note the many cell fingers, radiating from the osteocyte cell bodies that might have an important function in mechanosensing. Scanning electron microscopy pictures (original magnification: ×1000).

Osteocytes release signaling molecules in response to mechanical loading

So far we have described that bone remodeling serves to adapt bone mass and structure to the mechanical demands. We have emphasized that osteocytes play a pivotal role in this process, by sensing the mechanical stimulus and translating it into a chemical signal. We will now describe the nature of some of these chemical signals, and the effect of these chemical messenger molecules on the cells that actually change bone mass, in other words, the osteoclasts and osteoblasts.

An important early response to mechanical loading is the influx of calcium ions through mechanosensitive ion channels in the plasma membrane and the release of calcium from internal stores [31,49,79–82]. The resulting rise in intracellular calcium concentration is necessary for activation of calcium/calmodulin-dependent proteins, and activates many downstream signaling cascades, such as protein kinase C and phospholipase A_2. Calcium/calmodulin-dependent enzymes that are activated by flow-induced rises in intracellular calcium include endothelial NO synthase (NOS), the enzyme responsible for loading-induced NO production. The activation of phospholipase A_2 results in increased arachidonic acid release and PGE_2 production [49]. Calcium also affects prostaglandin release by

bone cells in an alternative manner, as it has been shown that fluid flow activates L-type voltage-sensitive Ca^{2+} channels to promote Ca^{2+} entry. This stimulates vesicular ATP release, which in turn mediates flow-induced prostaglandin release via activation of purinergic receptors [83].

Prostaglandins are abundantly produced by osteocytes, as well as by other cells of the osteoblastic lineage [84–87], and play a key role in the bone formation response to mechanical loading *in vivo* [88,89]. Several studies have shown that osteocytes rapidly increase their prostaglandin production in response to mechanical loading in the form of a fluid flow *in vitro* [46,89]. Cyclooxygenase (COX) is the key enzyme involved in the production of prostaglandins [89], and exists in a constitutive (COX-1) and an inducible form (COX-2). Fluid shear stress does not affect COX-1 mRNA expression in primary human bone cells [90], but mechanical loading induces a rapid rise in COX-2 mRNA in human bone cells and chicken osteocytes *in vitro*, as well as COX-2 protein expression in rat bone cells *in vivo* [90–92]. Importantly, inhibition of COX-2, but not COX-1, inhibits fluid flow induced prostaglandin production by primary bone cells *in vitro* [93]. In addition, COX-2 has been shown to mediate the anabolic response of bone tissue to mechanical loading [64], illustrating the importance of loading-induced prostaglandin production for the process of adaptive bone remodeling. Besides purigernic receptors, prostaglandins are released through hemichannels in response to a mechanical stimulus [76].

Besides prostaglandins, NO release has also been shown to be essential for load-induced bone formation *in vivo* [26,94,95]. Several studies have shown that NO production is rapidly increased in response to mechanical stress in bone cells, including isolated osteocytes [38,53,96–98]. NO production has been widely used as a marker for studying the mechanosensitivity of osteocytes [96]. The intracellular upregulation of NO after mechanical stimulation has been shown in single bone cells using DAR-4M AMchromophore [99,100]. These studies also demonstrated that a single osteocyte can disseminate a mechanical stimulus to its surrounding osteocytes via extracellular soluble signaling factors (NO), which reinforces the putative mechanosensory role of osteocytes [99]. The production of NO has been shown to be endothelial NOS dependent [101].

Another family of molecules that has very recently been identified as a mediator of the adaptive response of bone to mechanical loading is the Wnt family of proteins. The Wnt gene family represents a diverse group of secreted proteins that are potent modulators of cell behavior [102]. Wnt receptor complexes require the presence of a member of the Frizzled family of proteins [103], as well as the low-density lipoprotein receptor-related protein (LRP) 5 or 6 [104] for activation of canonical Wnt signaling. The simplified model for canonical Wnt signaling proposes that upon binding of the Wnts to both their receptors, β-catenin levels rise in the cytoplasm and form a complex with TCF/Lef1, which accumulates in the nucleus as a stable transcriptional regulator. Wnt signaling can also take place through kinases [105] and Wnts can activate GTPases, thereby modulating cytoskeletal organization [106]. In light of the role of the cytoskeleton in mechanosensing, it is noteworthy that Wnts may modulate cytoskeletal organization, and that β-catenin links cadherins to the actin cytoskeleton. Wnt signaling might be an important modulator of the process of mechano-regulated bone adaptation. This is illustrated by the finding that *in vivo* loading of mouse tibiae results in increased gene expression of Wnts and Wnt target genes including *Wnt10B*, *SFRP1* and connexion 43. In addition, loading of tibiae by means of 4-point bending leads to more bone formation in mice with a dominantly active LRP5 receptor (resulting in a continuous activation of the Wnt signaling cascade) than in wild-type mice [107]. Similarly, it was found that the anabolic response of bone to mechanical loading was enhanced in ulnae of mice lacking the Wnt inhibitor Frzb [108]. Strikingly, the bones of these mice seemed to be more sensitive to mechanical loading, responding to stimuli that were not sufficient to elicit a response in wild-type mice. These findings were also supported by *in vitro* studies showing that MC3T3-E1 osteoblasts increased Wnt gene expression after mechanical stimulation by substrate deformation [109], and that 1 h of a mechanical stimulus in the form of a pulsating fluid flow (PFF) (0.7 ± 0.3 Pa, 5 Hz) upregulated mRNA expression of β-catenin, APC and Wnt3a as well as the Wnt antagonist SFRP4 in MLO-Y4 osteocytes, at 0.5–3 h after cessation of the fluid flow stimulus [110], showing that osteocytes respond to mechanical loading with a modulation of expression of molecules involved in the wnt signaling cascade.

Sclerostin is a molecule that acts as a Wnt antagonist by binding the Wnt coreceptor LRP5 [111]. Sclerostin appears to be highly expressed in mature osteocytes compared with immature osteocytes [112]. Transgenic mice lacking sclerostin exhibit an increased bone mass, and the human condition of sclerostosis is due to

a premature termination of the *Sost* gene [113], suggesting that sclerostin inhibits bone mass accrual. It has been suggested that sclerostin protein may be transported through canaliculi to the bone surface, where it inhibits bone formation by osteoblasts. Interestingly, *Sost* transcripts and sclerostin protein levels were dramatically reduced in osteocytes after loading of mouse ulnae *in vivo*. The magnitude of the strain stimulus was associated with Sost staining intensity and number of sclerostin-positive osteocytes. Hindlimb unloading, on the other hand, yielded a significant increase in Sost expression in the mouse tibia [114]. Mechanical loading might therefore lead to an increase in Wnt signaling, thereby driving the mechanical adaptation of bone.

Interestingly, several molecules have been identified, of which the expression is modulated by mechanical loading and seems to be more or less specific for osteocytes. Matrix extracellular phosphoglycoprotein (MEPE), for instance, is highly expressed in osteocytes compared with osteoblasts. MEPE-null mice show increased bone formation and bone mass, as well as resistance to age-related trabecular bone loss [115], suggesting a role for MEPE in the regulation of bone homeostasis. It has been shown that MEPE expression is upregulated in a time-dependent fashion in alveolar osteocytes in response to mechanical loading applied by orthodontic tooth movement [116].

Dentin matrix protein 1 (DMP1) is another molecule that seems to be highly expressed in osteocytes compared with other cells types [117,118]. DMP1 expression increases two- to three-fold in osteocytes of the mouse ulna 24 h after a single 2.4 N load for 30 s at 2 Hz [119]. A potential role of DMP1 in osteocytes may be related to hydroxyapatite formation. DMP1 is specifically expressed along and in the canaliculi of osteocytes within the bone matrix [118]. The canaliculi and lacunae in bones of DMP1-null mice have a compromised structure, which can have implications for the amplification of load signals to the osteocytes [120].

Phex protein is found on the plasma membrane of osteoblasts and osteocytes [121]. The precise function of Phex is unclear, but it clearly plays a role in phosphate homeostasis and bone mineralization. Deletion of Phex, as well as deletion or mutation of the osteocyte marker DMP1, results in hypophosphatemic rickets [118]. Phex gene expression are increased in response to load [119], opening the possibility that not only bone mass, but also mineral metabolism is regulated by mechanical loading.

Osteocytes orchestrate osteoblast & osteoclast activity

Loading-induced production of signaling molecules by osteocytes, likely serves to modulate osteoblast and osteoclast recruitment and activity (Figure 3). It is known that prostaglandins and Wnts can stimulate osteoblast recruitment and activity [122–124] and that prostaglandins can stimulate osteoclast activity [86], while Wnts and NO inhibit osteoclast activity [125,126]. Vezeridis and colleagues demonstrated that PFF-treated osteocytes *in vitro* produce factors that inhibit osteoblast proliferation and stimulate alkaline phosphatase activity, suggesting a stimulation of osteoblastic activity [127]. Similar results were shown by Taylor and colleagues who found that osteocytes exposed to a continuous fluid flow rapidly stimulate alkaline phosphatase activity in osteoblasts [128]. They also showed that the ability to mediate osteoblastic alkaline phosphatase levels in response to the application of fluid shear is a phenomenon unique to osteocytes, and is not reproduced by other mesenchymal cell types [128]. Interestingly, the stimulation of osteoblast differentiation as described by Vezeridis and

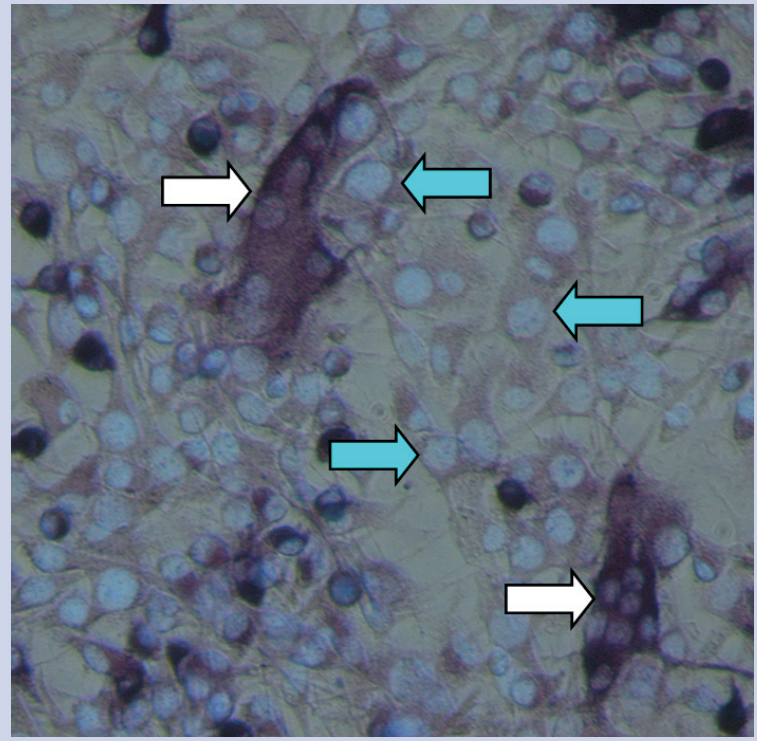

Figure 3. Osteocytes affect osteoclast development. Mouse bone marrow cells and MLO-Y4 osteocytes were co-cultured for 6 days. The number of multinucleated osteoclasts developing depends on the presence of live osteocytes.
Shaded arrows: osteocytes. White arrows: TRAP-positive multinucleated osteoclasts.

coworkers [127] seemed to be NO-dependent, and did not require direct cell–cell contact. By contrast, Taylor and colleagues described that osteocyte–osteoblast physical contact via gap junctions is a prerequisite for stimulation of osteoblast differentiation by mechanically stimulated osteocytes [128].

Regarding osteoclast activity, it has been reported that MLO-Y4 osteocytes produce M-CSF, RANKL and Opg, and are able to actually promote osteoclast formation and activity under static culture conditions. The promotion of osteoclast formation required cell–cell contact, possibly owing to requirement of cell-bound RANKL [129,130]. By contrast, both Tan and colleagues [131] and You and coworkers [130] have shown that osteocytes subjected to mechanical loading by PFF actually inhibit osteoclast formation and resorption via soluble factors. Using primary osteocytes, Tan and colleagues demonstrated that the release of these factors was at least partially dependent on activation of an NO pathway in osteocytes in response to fluid flow [131]. In addition, You and coworkers showed that mechanical loading also decreases the potential of the osteocyte to induce osteoclast formation by direct cell–cell contact [130].

Burger and colleagues have developed a theory that links osteocyte sensing of different loading-induced canalicular flow patterns around cutting cone and reversal zone during remodeling to the coordinated actions of osteoblasts and osteoclasts in a BMU [8,132]. Volumetric strain in the bone around a bone multicellular unit cutting cone has been related to canalicular fluid flow [133], and the predicted area of low canalicular flow around the tip of the cutting cone was proposed to induce local osteocyte apoptosis. Mechanical loading by fluid shear stress has been shown to promote osteocyte survival [134], while unloading is associated with osteocyte apoptosis [135]. Osteocyte apoptosis is a likely trigger for osteoclastic bone resorption, for instance via the release of alarmins such as HMGB1 [136], and very likely via apaoptotic bodies [137]. Osteocyte apoptosis at the tip of the cutting cone would thus attract osteoclasts, leading to further excavation of bone in the direction of loading. The model by Smit and colleagues further predicts that at the base of the cutting cone and further down the reversal zone, osteocytes receive enhanced fluid shear stress during loading [132]. This could prevent osteocyte apoptosis, but may also stimulate the

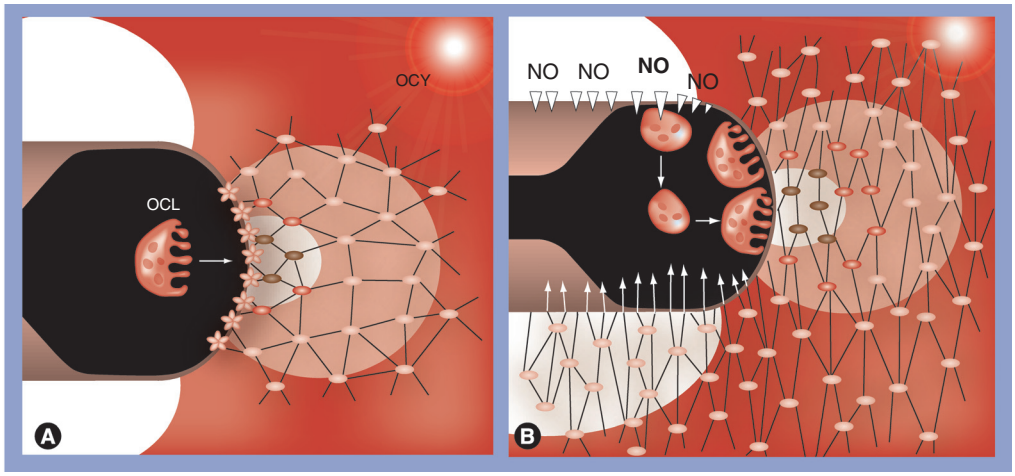

Figure 4. Basic multicellular unit-based bone remodeling orchestrated by mechanosensitive osteocytes. (A) Representation of a basic multicellular unit (BMU). Osteocyte apoptosis (indicated as dark lacunae) is caused by lack of fluid flow at the tip of the cutting cone, where the matrix strains are relatively low during normal loading. Osteoclasts are attracted towards apoptotic osteocytes, and as a result the osteoclastic attack follows the direction of loading. **(B)** Representation of postulated events in the cutting cone of a progressing BMU. Osteoclasts are attracted by apoptotic osteocytes in the cutting cone tip, but forced to withdraw again from the bone surface at the cutting cone base, as a result of high amounts of NO produced by well-stressed osteocytes. As NO production remains high further down the reversal zone, osteoclasts remain within the cutting cone and may even re-enter the resorption cycle, leading to a 'treadmill' of active and inactive osteoclasts that together dig the resorption tunnel or trench. Vertical arrows indicate direction and magnitude of canalicular fluid flow; vertical arrow heads indicate release of NO by well-stressed osteocytes. Light colours denote places in the matrix that experience low strains during loading in the normal direction, while white areas denote areas of high strain.
NO: Nitric Oxide; OCL: Osteoclast; OCY: Osteocyte network.
Modified from [132].

release of signaling molecules that promote the retraction and detachment of osteoclasts from the bone surface. These two mechanisms, attraction of osteoclasts to the cutting cone tip and induction of osteoclast detachment from the cutting cone base, together could explain the mechanically meaningful behavior of osteoclasts during remodeling (Figure 4). Recently, a finite element-based computer model was developed for bone adaptation to mechanical loading [138]. The model was based on the mechanosensory function of osteocytes, in other words, strain-induced osteocyte signals inhibit bone resorption and stimulate bone formation, in order to investigate how this paradigm would affect BMU-based bone remodeling. The simulations showed that a strain-induced osteocyte signal can direct resorbing osteoclasts in the dominant loading direction, while unloading leads to abnormal resorption directions, as was predicted by Burger and colleages [8]. Under normal loading conditions the osteocyte signal-induced indirect coupling of formation to resorption [138]. The computer model thus shows that BMU-based bone remodeling, driven by input from mechanosensitive osteocytes, could explain bone adaptation to both loading and unloading.

In summary, it is generally assumed that loading on bone generates a flow of interstitial fluid through the lacuno–canalicular network. This flow is sensed by the osteocytes, which respond by the release of signaling factors. These signaling factors, including NO, prostaglandins and Wnts, can alter the recruitment and activity of osteoblasts and osteoclasts, thereby affecting bone mass. Indeed, experiments employing targeted ablation of osteocytes in mouse bones revealed that viable osteocytes are required for transducing mechanical signals into a cellular response [139]. The osteocyte-less mice were resistant to unloading-induced bone loss, suggesting that viable osteocytes are necessary for activation of osteoclastic resorption in response to unloading. Strikingly, (re)loading-induced recovery of bone mass seemed to be unhampered, even when 80% of the osteocytes were dead [139]. If true, these remarkable results will have a great impact on current paradigms regarding loading-induced increase in bone mass.

If osteocytes indeed regulate bone mass by dictating the balance between bone formation and resorption in response to mechanical loading, any factor altering the response of osteocytes to mechanical loading potentially affects bone mass. Such an alteration of the mechanosensitivity would be analogous to a change in setpoint for activation of bone formation or resorption, as described in the mechanostat theory by Frost [7]. One agent that has been suggested to alter the sensitivity of osteocytes to mechanical loading is estrogen. Estrogens have a strong effect on bone mineral density in humans [140,141], which could be explained on the one hand by direct effects of estrogen on osteoblasts and osteoclasts [142,143], and on the other hand by an alteration of response of bone cells to mechanical loading [144]. Opposing this latter explanation are several *in vivo* studies that were unable to detect a synergistic effect of estrogen and mechanical loading on bone formation [140,141,145,146]. However, it is striking that the anabolic response of bones to mechanical loading *in vivo* requires ER-α [147]. Expression of ER-α is affected by estrogen, which would be in-line with the hypothesis that bone loss associated with estrogen deficiency is a consequence of reduction in ER-α expression, reducing the effectiveness of the anabolic response of bone cells to strain [148]. However, the response of primary human bone cells to a mechanical stimulus *in vitro* was similar in magnitude in the presence and absence of estrogen [149]. Thus, although there is convincing evidence available for the role of ER-α in mechanotransduction by osteocytes, it has yet to be proven that estrogen affects the mechanical setpoint of bone.

The role of osteocytes in inflammation-associated bone loss

During inflammatory diseases such as rheumatoid arthritis (RA), the balance between bone formation and resorption is often disturbed, resulting in localized bone loss around the affected joints as well as generalized osteoporosis [150–153]. This bone loss is multifactorial, and might be caused by the physiological adaptation of bone to reduced physical activity that is common in patients with RA, or the use of corticosteroids by the patients. In addition, it is likely that proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)1-β that play an important role in the etiology of RA also contribute to this bone loss [154].

If cytokines decrease the mechanosensitivity of osteocytes, as has been suggested for estrogen [7], they could thereby contribute to the generalized bone loss in patients with RA. Indeed we recently found that both TNF-α and IL-1β inhibit the upregulation of NO production after mechanical stimulation by PFF [155]. This inhibition was associated with a prevention of

fluid shear stress-induced $[Ca^{2+}]_i$ upregulation [155]. This provides a novel mechanism by which cytokines might interfere with bone remodeling during inflammatory diseases, via an effect on the mechanoresponse of osteocytes.

Conclusion

Significant progress has been made in the uncovering of the cellular and mechanical basis of the osteocyte's response to loading, and to our understanding of cellular mechanotransduction and bone remodeling. The 3D interconnected osteocyte network with its accompanying lacuno-canalicular porosity is the site of mechanosensing in bone tissue. Mechanotransduction then includes the translation by osteocytes of canalicular flow into cell signals that can alter the recruitment and activity of osteoclasts and osteoblasts. Osteoclastic bone resorption and osteoblastic bone formation could thus be related to different loading patterns within the bone, while the mechanosensitive osteocytes are of crucial importance for orchestrating the remodeling process.

Factors such as cytokines, which reduce the physiological response of osteocytes to mechanical loading, might lead to a disruption in the osteocyte-mediated balance between osteoblast and osteoclast activity thereby affecting bone mass. A reduction of cytokine levels might therefore be a priority in inflammatory diseases such as RA, not only to reduce the local deleterious effects of inflammation, but also to reduce the systemic effects on the skeleton.

Future perspective

The importance of mechanical stress for maintaining bone strength predicts that mechanical stimuli have great potential for prevention, and even treatment, of bone fractures. A logical therapeutic approach to prevent bone loss, for instance associated with osteoporosis or inflammatory diseases such as RA, would therefore be to enhance adaptive bone formation. To date, the signaling pathways activated by mechanical loading in osteocytes leading to changes in bone remodeling have been partially elucidated. One striking example is the Wnt signaling pathway, and more specifically, the inhibitor of this pathway, sclerostin. Sclerostin is a molecule that is strongly linked to bone mass disorders, and is currently under investigation for use in a clinical setting. Considering the role of the osteocytes as the professional mechanosensors of bone, and the importance of the cytoskeleton for the response of osteocytes to mechanical loading, much is to be expected from research focusing on the cytoskeletal components of the osteocyte. Moreover, future studies should be actively pursued to elucidate the molecular mechanism by which cytokines affect the osteocyte's mechanosensitivity, thereby affecting bone remodeling. However, one major limitation in current *in vitro* research on mechanosensing by osteocytes is the lack of multiple osteocyte cell models. Although many valid and valuable results have been obtained using models such as mechanically stimulation of cultured bone-like cells using a controlled fluid shear stress, we should keep in mind that bone cells in a 2D morphology might not always respond to this stimulus in an identical manner as cells in their unique 3D environment would. In addition, osteoblasts are often used as a surrogate for osteocytes, although there are obvious differences in, for instance, morphology. The difference between osteoblasts and osteocytes is clearly visible by looking at the unique morphological features of the osteocytes, in other words, the cell protrusions, which might be strongly related to the function of the osteocyte as mechanosensor. The many protrusions are probably of utmost importance for this mechanosensory function, although there is an ongoing discussion with regard to which part of the osteocyte, in other words, the cell body or cell fingers, are responsible for mechanosensing *in vivo*. A numerical study showed that application of fluid flow to cells on a 2D substrate results in maximum displacements at the apical surface of the cell body [156]. In contrast, *in vivo*, fluid shear stress will likely only occur over the cell processes, as the unique dimensions required for strain amplification are present in the unit made up by the cell process within its canaliculus, but not in the unit that is composed of an osteocyte cell body within its lacuna. This could be considered a major limitation of the methods currently used to study the response of bone cells to mechanical loading. Although theoretically promising, a much greater knowledge of the signal transduction pathways that govern the adaptive response of osteocytes in their natural 3D surroundings will be needed before the therapeutic option of mechanical stimuli is applicable in a clinical setting.

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Executive summary

Bone remodeling

- Bone adapts its mass and shape to mechanical loading.
- Mechanical adaptation of bone is facilitated by bone remodeling.

Mechanical loading drives interstitial fluid flow

- Bone loading causes matrix deformations that will result in fluid shifts within the bone matrix.
- Loading-derived fluid flow activates osteocytes.

Fluid flow activates osteocytes

- The mechanical stimulus that activates bone cells *in vitro* is fluid shear stress rather than streaming potentials or chemotransport.
- The mechanical stimulus for osteocytes *in vivo* is likely derived from strain-derived flow of interstitial fluid.

Osteocytes transduce mechanical stimuli into chemical signals

- Cytoskeletal properties of the osteocyte affect the ability for mechanosensing.
- The primary cilium might play a role in the process of mechanotransduction.

Osteocytes release signaling molecules in response to mechanical loading

- An early response of osteocytes to mechanical loading is a rise in $[Ca^{2+}]_i$.
- Upon mechanical stimulation, osteocytes release signaling factors such as nitric oxide, prostaglandins and Wnts, which can alter recruitment and activity of osteoblasts and osteoclasts.

Osteocytes orchestrate osteoblast & osteoclast activity

- Osteocyte sensing of different loading-induced canalicular flow patterns around cutting cone and reversal zone during remodeling explains the coordinated actions of osteoblasts and osteoclasts in a basic multicellular unit.
- Mechanical loading promotes osteocyte survival, while unloading is associated with osteocyte apoptosis, which triggers osteoclastic bone resorption.

The role of osteocytes in inflammation-associated bone loss

- Cytokines might interfere with bone remodeling during inflammatory diseases via an effect on the mechanoresponse of osteocytes.

Conclusion

- Mechanosensitive osteocytes are of crucial importance for orchestrating the remodeling process in bone.
- Significant progress has been made in the uncovering of the cellular and mechanical basis of the osteocyte's response to loading, and to our understanding of cellular mechanotransduction and bone remodeling.

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