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MECHANICAL LOADING AND HOW IT AFFECTS BONE CELLS: THE ROLE OF THE OSTEOCYTE CYTOSKELETON IN MAINTAINING OUR SKELETON

J. Klein-Nulend[1,*], R.G. Bacabac[2] and A.D. Bakker[1]

[1] Department of Oral Cell Biology, ACTA-University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Amsterdam, The Netherlands
[2] Department of Physics, Medical Biophysics Group, University of San Carlos, Cebu City, Philippines

Abstract

Lack of physical activity causes bone loss and fractures not only in elderly people, but also in bedridden patients or otherwise inactive youth. This is fast becoming one of the most serious healthcare problems in the world. Osteocytes, cells buried within our bones, stimulate bone formation in the presence of mechanical stimuli, as well as bone degradation in the absence of such stimuli. As yet, we do not fully comprehend how osteocytes sense mechanical stimuli, and only know a fraction of the whole range of molecules that osteocytes subsequently produce to regulate bone formation and degradation in response to mechanical stimuli. This dramatically hampers the design of bone loss prevention strategies. In this review we will focus on the first step in the cascade of events leading to adaptation of bone mass to mechanical loading, i.e., on how osteocytes are able to perceive mechanical stimuli placed on whole bones. We will place particular emphasis on the role of the osteocyte cytoskeleton in mechanosensing. Given the crucial importance of osteocytes in maintaining a proper resistance against bone fracture, greater knowledge of the molecular mechanisms that govern the adaptive response of osteocytes to mechanical stimuli may lead to the development of new strategies towards fracture prevention and enhanced bone healing.

Keywords: Mechanical loading; mechanotransduction; fluid shear stress; osteocyte; bone cell; cytoskeleton; inflammatory cytokines; cell mechanics; cell shape; cell stiffness.

*Address for correspondence:
J. Klein-Nulend
Department of Oral Cell Biology
Research Institute MOVE,
ACTA-VU University Amsterdam, Gustav Mahlerlaan 3004,
1081 LA Amsterdam, The Netherlands
Telephone Number: +31 205980881
FAX Number +31 205980333
E-mail j.kleinnulend@acta.nl

Bones adapt to mechanical loading

Throughout life bone is constantly remodelled by the coordinated action of bone-resorbing osteoclasts and bone-forming osteoblasts in basic multicellular units. This continuous remodelling likely serves to prevent and remove fatigue-related micro-damage and allows adaptation of the bone mass and structure. The balance between the amount of bone resorption and bone formation determines whether the process of bone remodelling leads to a net gain or loss of bone mass. The number and activity of osteoclasts and osteoblasts are determined by a multitude of factors, such as hormones and cytokines, as well as by locally produced signalling molecules under the influence of mechanical stimuli (Vezerides *et al.,* 2006; You *et al.,* 2008; Onal *et al.*, 2012). The osteocyte is a source of soluble factors not only to target cells on the bone surface but also to target distant organs, such as muscle, kidney, and other tissues (Bonewald, 2011).

During physical activity, mechanical forces are exerted on the bones through ground reaction forces and by the contractile activity of muscles (Lanyon *et al.*, 1975; Usui *et al.*, 2003). These physical forces result in a maintenance or gain of bone mass, but also drive adaptation of bone structure. The adaptation of trabecular bone architecture according to the demands of mechanical usage is evident in the vertebrae, where the trabeculae are predominantly oriented in the longitudinal direction, providing the best possible resistance to compression fracture of the vertebrae with a minimal use of material. A classic example of the stimulating effect of mechanical stimuli on bone mass is provided by the bones in the forearm of tennis players. The ulna and radius in the arm that holds the racket are exposed to high impact forces, leading to tiny deformations in the stiff bone matrix and an increase in bone mass of 5 to 10 % compared to the ulna in the contra-lateral arm (Ducher *et al.*, 2004). The deformations that occur in bones as a result of physical forces are expressed as strain, where 1,000 microstrain equals a 0.01 % change in length of the bone compared to its original length. Vigorous exercise induces bone strains up to 1,000 microstrain in humans (Lanyon *et al.*, 1975). By comparison, controlled bouts of whole bone loading resulting in 1,000 to 3,000 microstrain are anabolic in experimental animal models of bone-loading, demonstrating the potential for appropriate physical exercise routines as a means to enhance bone mass (Rubin and Lanyon, 1987; Turner *et al.*, 1994; Reijnders *et al.*, 2007).

In contrast with the increase in bone mass with vigorous physical exercise, we lose bone mass in the absence of mechanical stimuli. Bone mass is rapidly lost under unloading conditions, e.g., during bed rest, hind-limb unloading in mice, or local denervation of muscles (Globus *et al.*, 1986; Vandamme *et al.*, 2012). Interestingly, strains resulting from habitual activity suffice to prevent bone loss compared to complete unloading, even though these habitual strains hardly ever exceed 400 microstrain. Thus, either mechanical loads that lead to tiny strains within the bone matrix are somehow sensed by the cells within bone, which then act to preserve bone mass, or sporadic mechanical stimuli that exceed 2,000 microstrain still occur often enough to prevent bone loss. We have shown that cultured bone cells release nitric oxide (NO) in response to mechanical stimulation in the form of a fluid shear stress, and that the amount of NO released linearly correlates to the *rate* of the applied fluid shear stress (Bacabac *et al.*, 2004). Since the rate at which a mechanical stimulus is applied is the product of the magnitude (amplitude) and the frequency of the stimulus, this supports the notion that low-magnitude, high-frequency mechanical stimuli are as potent in evoking a response in bone cells as high-amplitude, low-frequency stimuli (Ozcivici *et al.*, 2010). In other words, very small mechanical stimuli may elicit a cellular response only if applied fast enough. The rate dependent-response to stress provides a possible explanation why adaptive bone formation *in vivo* may occur despite the sporadic occurrence of high-amplitude strains in daily life. The question *how* daily mechanical loads preserve bone mass is clinically highly relevant, as with our ageing population more and more people suffer from fragile bones.

Osteocytes sense mechanical stimuli and direct mechanical adaptation of bone

The cells likely responsible for sensing the physical stimuli derived from mechanical forces exerted on bones are the osteocytes, which comprise over 90 % of the bone cells. Osteocytes are stellate cells that are embedded within the calcified bone matrix. They form a large number of cell-cell contacts through their long slender cell processes, forming a syncytium capable of rapid transduction of signals (Fig. 1). Osteocytes are highly mechanosensitive, likely more so than periosteal fibroblasts or osteoblasts, and alter the production of a multitude of signalling molecules when triggered by a mechanical stimulus. Mechanically activated osteocytes produce signalling molecules like bone morphogenetic proteins (BMPs), Wnts, prostaglandin E_2 (PGE_2), and NO, which can modulate the recruitment, differentiation, and activity of osteoblasts and osteoclasts (Robling *et al.*, 2006; Tan *et al.*, 2007; You *et al.*, 2008; Santos *et al.*, 2009). Thus, osteocytes are theoretically capable of orchestrating bone adaptation in response to mechanical stimuli. That osteocytes are indeed essential mediators of osteoclastic bone resorption was confirmed in an elegant experiment by Tatsumi *et al.* (2007). They showed that the loss of bone mass following hind limb unloading of mice was prevented when ~80 % of the osteocytes were ablated. Osteocytes thus seem to stimulate osteoclast activity in the absence of daily mechanical loads, a capability that has been confirmed in *in vitro* studies (You *et al.*, 2008; Kulkarni *et al.*, 2010). Indeed it has been shown recently by two independent groups that RANKL production by osteocytes determines bone mass in adult mice, demonstrating the importance of osteocytes in the regulation of bone mass (Nakashima *et al.*, 2011; Xiong *et al.*, 2011). Interestingly, the same study demonstrating the requirement of osteocytes for mediating unloading-induced bone loss also showed that the anabolic response of bone to (re)loading does not require the presence of living osteocytes (Tatsumi *et al.*, 2007). However, this does not eliminate the role of osteocytes in mediating the anabolic response of bone to loading under normal conditions.

Fluid flow, strain, and hydrostatic pressure as stimuli for osteocytes

If osteocytes are the professional mechanosensing cells of bone, then how do these cells sense whole bone loads? One popular theory entails that matrix strains surrounding the osteocyte cell processes drive a thin layer of extracellular fluid surrounding the osteocyte cell processes to flow across a pressure gradient. This flow of fluid "amplifies" local strains, and is thereby the mechanical signal that is ultimately sensed by the osteocytes. There is ample experimental evidence to support the idea that deformations of the bone matrix drive an interstitial fluid flow. Knothe-Tate *et al.* have shown experimentally a flow of extra-cellular fluid around the osteocytes as a result of bone tissue strains, by loading of sheep tibiae and following the distribution of tracers through the lacuno-canalicular network (Knothe-Tate *et al*., 1998; Knothe-Tate *et al.,* 2000). More recently, Price *et al.* (2011) used fluorescence recovery after photobleaching for imaging fluid displacement synchronised with mechanical loading, to show that the mechanical loading of mouse tibia enhanced fluid transport through the lacuno-canalicular system, demonstrating the correlation of canalicular fluid flow with mechanical load. In addition, several investigators reported that it is not the amount of strain applied to a whole bone that influences bone formation, but the rate at which the strain is applied (Price *et al.*, 2011). Dynamic bone loading, which enhances fluid flow, has also been demonstrated to induce an osteogenic response (Lanyon and Rubin, 1984; Luo *et al.*, 1995; Mosley and Lanyon, 1998). Static loading on the other hand has little effect on lacuno-canalicular fluid flow, and has only a minor effect on bone formation (Lanyon and Rubin, 1984). It has been extensively demonstrated that osteocytes *in vitro* are sensitive to a flow of fluid when seeded as a monolayer on flat, 2-dimensional (2D) substrates (Klein-Nulend *et al.*, 1995a; Klein-Nulend *et al.*, 1995b; Ajubi *et al.*, 1996; Bakker *et al.*, 2001; Bacabac *et al.*, 2004; Bakker *et al.*, 2009; Litzenberger *et al.*, 2010; Juffer *et al.*, 2012; Kulkarni *et al*., 2012). One could argue that interstitial fluid is driven to flow within the canaliculi over only osteocyte processes *in vivo* while a laminar fluid flow over cells seeded on a flat substrate will deform the cell body as well as the cell

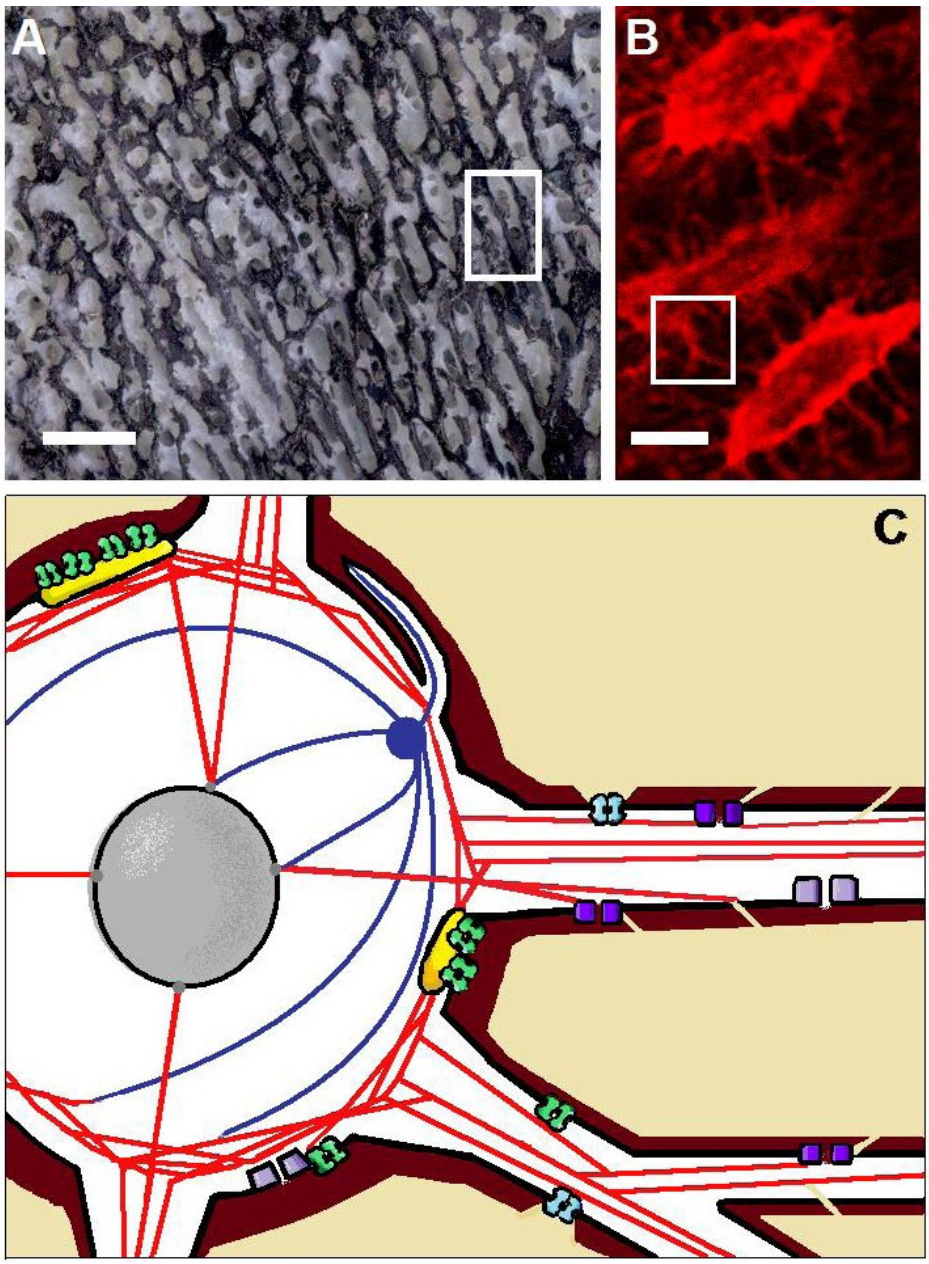

Fig. 1. In osteocytes, all is connected. (**A**) Macroscopic photograph of a transversely sectioned human femoral head, dipped in black ink to highlight the orientation of the bone trabeculae. Scale bar indicates 5 mm. (**B**) High magnification osteocytes embedded within their matrix. The osteocytes with their many cell processes are visible (red). Scale bar indicates 5 μm. (**C**) Schematic drawing of part of an osteocyte cell body with processes (white) residing within the calcified matrix (ECM; beige). The osteocyte cell membrane is surrounded by interstitial fluid and proteoglycans (ECM; brown). Microtubules (blue lines) originate from the centrosome and form a scaffold along which numerous molecules shuttle. Microtubules also form the core of the primary cilium. Osteocyte cell processes contain mostly actin (red lines), cross-linked by fimbrin (not shown) at places where processes bifurcate. The processes contain αvβ3 integrins (blue), possibly located on top of collagen "hillocks", and protein tethers. These tethers may deform as a result of interstitial fluid flow and subsequently "tug" at the actin cytoskeleton, or pull open stretch-activated ion channels (purple), allowing calcium and other ions to enter the cell and activate a multitude of chemical signalling cascades. The fluid flow may also tug directly at integrins, such as α5β1, present along the cell processes and body and associated with hemi-channels. Activated hemi-channels release signalling molecules such as ATP, triggering a signalling cascade. The mechanical signal may be transduced from sites where integrins are triggered, *via* the actin cytoskeleton, to distant sites, including the nucleus, which is connected to actin *via* LINC complexes. Forces applied to actin fibres can likely also open stretch-activated ion channels. Integrins are often clustered together at focal adhesions (yellow), which in osteocytes are predominantly located at places where the cell processes intersect with the cell body. Focal adhesions contain tyrosine kinases, such as focal adhesion kinase, which make focal adhesions a prime location for transducing mechanical signals into a chemical response. Considering that the cytoskeleton interconnects virtually every part of the mechanosensing machinery, one can easily imagine that changes in cytoskeletal properties affect mechanosensing.

processes, thereby eliciting a response that otherwise would not be provoked (McGarry *et al.*, 2005a; Fritton and Weinbaum, 2009). However, it has been shown recently using a Stokesian fluid stimulus probe that electrical signalling is provoked at much lower forces when applied to a cell process of osteocytes compared to the cell body, thus suggesting that *in vitro* fluid flow experiments provide a valid confirmation of the sensitivity of osteocytes to fluid flow (Wu *et al.*, 2011).

In addition to responding to strain-driven fluid flow, it is also possible that osteocytes respond to matrix strains directly. MC3T3-E1 osteoblasts increase their production of signalling molecules in response to substrate deformations as low as 3,400 microstrain (Robinson *et al.*, 2006), and osteocytes are more mechanosensitive than osteoblasts (Klein-Nulend *et al.*, 1995a). The relative flat and spread shape of isolated bone cells in 2D culture may greatly hamper their sensitivity to a mechanical stimulus (Bacabac *et al.*, 2008), and strains that are not able to elicit a response in bone cells adhered to a flat and stiff surface may be perfectly able to elicit a response in cells in their natural 3-dimensional (3D) conformation. Thus, direct sensation of bone strains may already suffice to activate mechanosensitive bone cells *in vivo*. It has also been proposed that matrix strains can be locally amplified up to 3-fold by the inhomogeneities in the matrix that are formed by the osteocyte lacunae (Bonivtch *et al.*, 2007). Matrix strains around the osteocyte cell bodies may thus exceed whole bone strains, especially at points where cell processes intersect with the cell body, and could be sufficient to directly activate the osteocytes (Bonivtch *et al.*, 2007). In this regard the observation by Vatsa *et al*. (2008a), that molecules involved in cellular mechanotransduction such as F-actin and paxillin are concentrated at these intersections, is highly interesting (Fig. 1).

Bones can be considered as a material containing large (vasculature) and small (canaliculi) interconnected fluid-filled pores. The permeability of the lacuno-canalicular porosity is several orders of magnitude lower than that of the vascular porosity (Gardinier *et al.*, 2010). Since the lacuno-canalicular porosity has a low permeability, rapidly placed load on bone causes strains that first pressurise the interstitial fluid around the osteocytes, and then drive fluid flow causing dissipation of the build-up of hydraulic pressure (Wang *et al.*, 1999). The magnitude of pressure experienced by osteocytes *in vivo* may reach up to 5 MPa according to recent calculations (Gardinier *et al.*, 2010). Cyclic hydraulic pressures of 68 kPa can modulate signalling molecule production in cells of the mouse MLO-Y4 osteocyte cell line, and a pressure of 13 kPa was sufficient to stimulate prostaglandin production by primary osteocytes isolated from chicken calvariae (Klein-Nulend *et al.*, 1995a; Liu *et al.*, 2010). This suggests that, besides substrate strain and fluid shear stress, the loading-induced hydraulic pressure could potentially serve as a mechanical stimulus for osteocytes. Whatever the mechanical load-derived stimulus is that activates the osteocytes, the question remains which osteocyte feature enables the perception of the physical stimulus and subsequent transduction into a chemical signal. This question is not necessarily restricted to osteocytes, as eukaryotic cells in general are sensitive to mechanical stress.

Cellular features enabling mechanotransduction in osteocytes

A multitude of sensory elements exist that allow cells to detect mechanical stimuli. Mechanosensing is enabled by force-induced conformational changes in cellular structures, such as stretch-activated ion channels, integrin complexes, and cell-cell adhesions. The conformational changes enable the influx and efflux of ions or the activation of signalling cascades, resulting in altered cell shape and altered activity and production of proteins (Hoffman *et al.*, 2011). The cytoskeleton, which can be considered a composite gel-like material (of actin, microtubules, intermediate filaments and their cross-linkers) is the scaffold determining cellular shape and stiffness (Sugawara *et al.*, 2008). Molecules like integrins anchor to the extracellular matrix (ECM) and mechanically link the exterior of the cell to the cytoskeleton, forming trans-membrane complex structures. These complexes, often clustered in so-called focal adhesions, are therefore prime suspects as mechanotransducers.

With respect to osteocyte mechanosensing, the focal adhesion kinase inhibitor-14 has been shown to abolish fluid flow-induced stabilisation of β-catenin and consequent activation of the Wnt/β-catenin pathway in osteocytes, suggesting that focal adhesions and integrins play an important role in osteocyte mechanosensing (Santos *et al.*, 2010). Indeed, β1-integrins on osteocytes *in vivo* have been shown to mediate specific aspects of mechanotransduction, confirming the importance of integrins for mediating mechanical stimuli in osteocytes (Litzenberger *et al.*, 2010). Interfering with β1-integrin signalling *in vitro* reduced the upregulation of cyclooxygenase-2 normally observed after mechanical stimulation of osteocytes, but did not affect mechanically induced intracellular calcium mobilisation (Litzenberger *et al.*, 2010). In addition, α5β1 integrins interact directly with connexin 43 (Cx43), and this interaction is required for mechanical stimulation-induced opening of Cx43-containing hemi-channels (Batra *et al.*, 2012). Cx43-containing hemi-channels are readily expressed on osteocytes and affect the response of osteocytes to mechanical loading *in vivo* (Zhang *et al.*, 2011). Direct mechanical perturbation of α5β1 integrins leads to the opening of the Cx43-containing hemi-channels (Batra *et al.*, 2012). Integrin attachments thus likely serve as the mechanotransducing units that potentiate the opening of hemichannels (Burra *et al.*, 2010). Other integrins that may mediate osteocyte mechanotransduction are αvβ3 integrins. Although αvβ3 integrins are not essential for the attachment of osteocytes to ECM *in vitro* (Aarden *et al.*, 1996), they may play a role in osteocyte physiology, since it has been shown recently that αvβ3 integrins mediate signalling *via* DMP-1, a molecule that is almost exclusively produced by osteocytes (Wu *et al.*, 2011). Interestingly, *in vivo* αvβ3 integrins are present along the

osteocyte processes which are thought to be the main sites of mechanotransduction in osteocytes (McNamara *et al.*, 2009). The glycocalyx of the osteocyte dendritic process is required for the formation of strong integrin attachments (Burra *et al.*, 2010; Burra *et al.*, 2011), demonstrating that the role of integrins should be considered in the context of the complex structures in which they reside before we can fully comprehend how mechanical stimuli are transduced to osteocytes. It should also be noted that as a consequence of osteocytes expressing their own specific set of integrins and the selectivity of integrins for substrates, the nature of the ECM likely affects the mechanoresponse of osteocytes.

Although stretch-activated ion channels have long been suspected to play a role in osteocyte mechanotransduction, it is currently unclear which molecules act as stretch-activated ion channels in osteocytes. The osteocyte response to mechanical loading can clearly be inhibited by gadolinium chloride, which is a non-specific blocker of TRP channels (Ajubi *et al.*, 1999; Bakker *et al.*, 2009). However, which member of the extensive family of TRP channels is responsible for transducing mechanical signals in the osteocyte has not been elucidated. It is unlikely that TRPV6 is a candidate as it is only present at low levels in murine osteoblasts and osteocytes and plays a minor functional role in calcium uptake by osteoblasts (Little *et al.*, 2011).

To understand how the cellular features mentioned above act as a mechanotransduction complex, one needs to regard the cellular context of these features. It would be difficult, if not impossible, for forces acting on a cell to induce conformational changes in cellular molecules that are freely floating in a semi-liquid cell membrane. However, structures that are anchored to neighbouring cells, the ECM or the glycocalyx, as well as to the cytoskeleton, form a direct mechanical link between the extracellular environment and the intracellular compartment (Fig. 1). Such structures are in an excellent position to sense mechanical forces. Integrins are coupled to the cytoskeleton *via* molecules such as vinculin, talin, and α-actinin. Though non-trivial, one may imagine three non-linear springs in a series representing the mechanical link between the ECM, the transmembrane proteins (including the focal adhesions), and the cytoskeleton (Fig. 2), to conceptualise the transfer of forces between these protein structure clusters (Fig. 3). Note, however, that each protein network is expected to become stiffer in response to applied forces (Storm *et al.*, 2005), which could support an amplified force transfer. Hence, the importance of anchoring mechanotransduction complexes that connect the ECM to the cytoskeleton predicts that the osteocyte cytoskeleton plays a key role in osteocyte mechanotransduction.

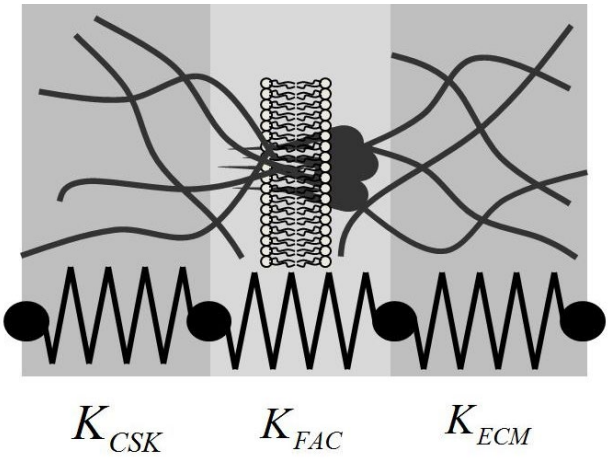

Fig. 2. Spring-series conceptualisation of mechanical linkage between the cytoskeleton (CSK), focal adhesion complexes (FAC), and the extracellular matrix (ECM), with respective non-linear spring constants, K_{CSK}, K_{FAC}, K_{ECM}.

Structure of the osteocyte cytoskeleton

The cytoskeleton is a scaffold made out of protein components that provide mechanical structure to cells. The viscoelastic properties of the cytoskeletal structure provide cells with resistance to shear or compression, enable cell migration, enable transport of intracellular molecules, determine the mechanical properties of the cells, and allow for mechanosensing. Eukaryotic cells contain three main kinds of cytoskeletal structures, each built up out of their own proteins: actin, intermediate filaments, and microtubules. The distribution of each of these structures seems to change when osteoblasts differentiate into osteocytes. In parallel, the stiffness of osteoblasts decreases during their differentiation towards osteocytes (Sugawara *et al.*, 2008). Microtubules are limited in distribution to the proximal region of osteocyte processes but extend the entire length of cell processes of MC3T3-E1 osteoblasts grown in 3D in collagen gels (Murshid *et al.*, 2007). Microtubules are essential for the integrity and formation of osteoblast cell processes grown in 3D, but processes of primary osteocytes in 3D are dependent on actin (Murshid *et al.*, 2007). Actin filaments are also crucial for maintaining the shape of primary chicken osteocytes when grown on flat substrates (Tanaka-Kamioka *et al.*, 1998). Not surprisingly, osteocytes also contain a set of actin-bundling proteins distinctive from that in osteoblasts. Fimbrin and α-actinin are predominantly present in the processes of osteocytes, with especially strong signals of fimbrin at the sites of bifurcation of the processes (Kamioka *et al.*, 2004). Compared to osteoblasts, osteocytes also contain high amounts of villin, which is present within the osteocyte cytoplasm but not within the processes. Osteoblasts immunostained with anti-spectrin show punctate signals on their cytoplasmic membranes, whereas spectrin is co-localised with actin from the distal portion of the cytoplasmic processes to the cell centre (Kamioka *et al.*, 2004). The typical morphology of the osteocyte, determined by its cytoskeleton, was originally thought to be imposed on differentiating osteoblasts during their incorporation in the bone matrix. Osteocytes have to remain in contact with other cells and ultimately with the bone surface to ensure access to oxygen and nutrients. Culture

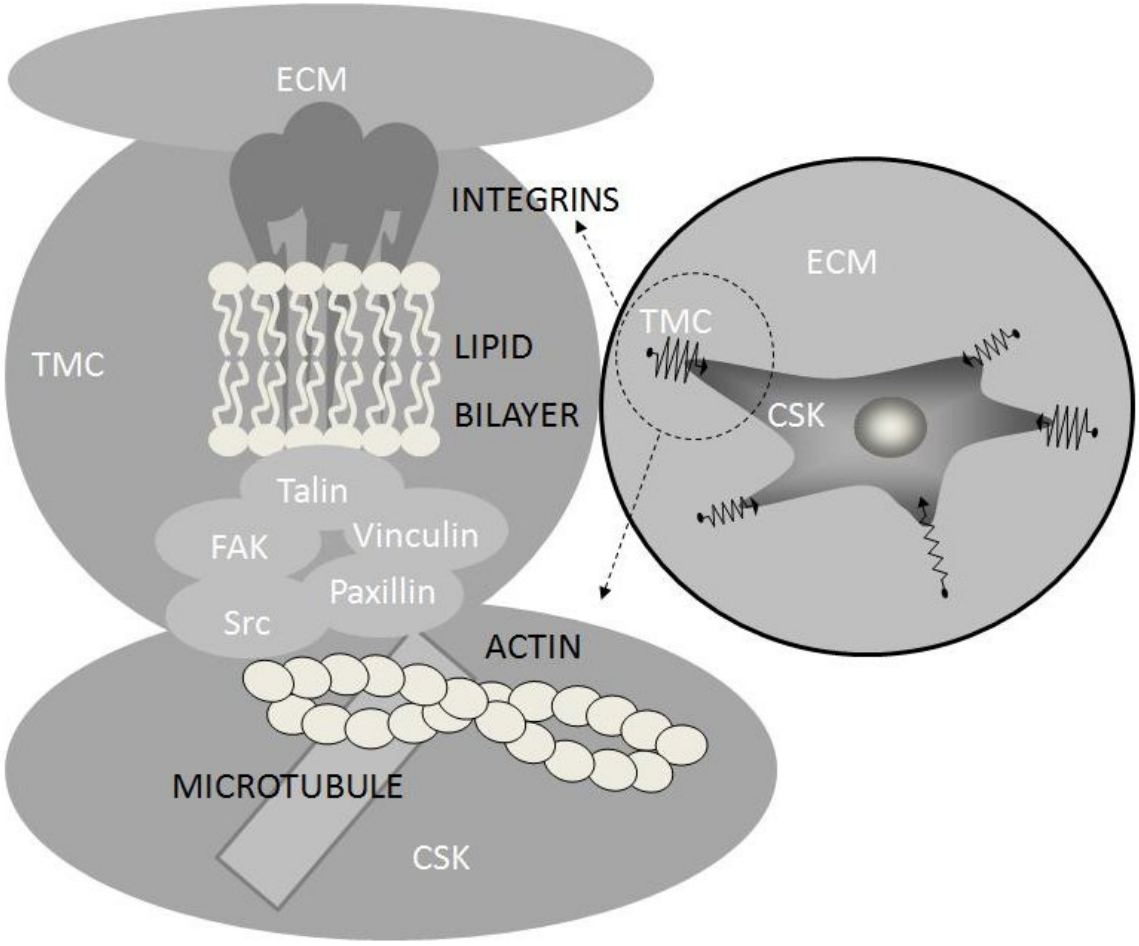

Fig. 3. Protein network clusters across the extracellular matrix, trans-membrane proteins, and the cytoskeleton regions of a spread cell. ECM, extracellular matrix; TMC, transmembrane complex; FAK, focal adhesion kinase.

experiments with isolated chicken osteocytes have shown, however, that although the cells lose their stellate shape in suspension, they re-express this morphology as soon as they settle on a support (Van der Plas and Nijweide, 1992). Apparently, the typical stellate morphology and the need to establish a cellular network are intrinsic characteristics of terminal osteocyte differentiation.

Although the stellate shape of osteocytes seems innate, by no means does this shape represent a fixed or static feature. Dallas and co-workers (Veno *et al.*, 2006) have shown cell body movement and the extension and retraction of cell processes over time using dynamic imaging of living osteocytes within their lacunae. Calvarial explants from transgenic mice with green fluorescent protein expression targeted to osteocytes revealed that the osteocyte is highly dynamic. Therefore the osteocyte processes, rather than being permanent connections between osteocytes as well as between osteocytes and bone surface cells, may have the capacity to connect, disconnect, and re-connect (Bonewald, 2006; Zang *et al.*, 2006). This phenomenon implies a complex role for intercellular connection in bone, and may indicate an adaptive information transfer facility through the ostecytic syncytium beyond mere force transfer mechanisms. Whereas signalling molecule information is diffusion-limited, intercellular communication provides a more efficient means for information transfer.

Since the osteocyte cell processes are of predominant importance for sensing mechanical stimuli, and the osteocyte processes apparently contain mainly actin, the actin cytoskeleton rather than the microtubule cytoskeleton could be important for osteocyte mechanosensing. However, disruption of both the actin cytoskeleton with cytochalasin B and disruption of the microtubule cytoskeleton with colchicine inhibits mechanical stimulation-mediated release of PGE_2 in the mouse osteocyte cell line MLO-Y4 (McGarry *et al.*, 2005b). Besides the cell processes, osteocytes may possess a single primary cilium that contributes to mechanosensing (Malone *et al.*, 2007). The primary cilium is a solitary organelle that emanates from the cell surface of most mammalian cell types, and is a key coordinator of signalling pathways during development and in tissue homeostasis (Berbari *et al.*, 2009). Primary cilia consist of an axoneme of nine doublet microtubules that extends from a basal body. This could explain why disruption of actin as well as of microtubules may upset the osteocyte response to mechanical stimulation. In the mouse osteoblast cell line MC3T3-E1, which has been shown to depend on microtubules rather than actin for their cytoskeletal integrity, inhibition of actin polymerisation did not inhibit intracellular calcium mobilisation or PGE_2 release in response to a mechanical stimulus (McGarry *et al.*, 2005b; Malone *et al.*, 2007). In contrast, in calvarial

osteoblasts derived from chicken, disruption of the actin cytoskeleton did strongly inhibit mechanical loading-stimulated PGE_2 release (Ajubi *et al.*, 1996).

Cytoskeletal mechanics and stress-response signatures

Fluid flow over dendrites in the lacuno-canalicular porosity has been suggested to induce strains in the actin filament bundles of the cytoskeleton that are more than an order of magnitude larger than tissue level strains (You *et al.*, 2001). Using ultrastructural data for the cell process cytoskeleton and the tethering elements that attach the process to the canalicular wall, a 3D model was created for the osteocyte process. Using this model the deformed shape of the tethering elements and the hoop strain on the central actin bundle as a result of loading-induced fluid flow was predicted. It was found that tissue-level strains of >1,000 microstrain at 1 Hz result in a hoop strain of >0.5 % (You *et al.*, 2001). The tethering elements of the osteocyte process can thus act as a strain-amplifier. Tethering filaments appear to be absent in the pericellular space surrounding the cell body, likely due to the wide pericellular space (~1 μm) between the cell membrane and the wall of the lacuna, in contrast to the pericellular space surrounding the cell process (~80 nm) (You *et al.*, 2004). Potentially CD44 serves as the tethering molecule since it is expressed in osteocyte cell processes and has an attachment site for hyaluronan (Noonan *et al.*, 1996). Alternatively, a protein tether involved in transduction of mechanical stimuli has recently been identified in cutaneous mechanoreceptors. This molecule is a protein filament with a length of ~100 nm (Hu *et al.*, 2010). It is possible that the osteocyte tether is similar to this protein tether.

More recently, a new theoretical model has been developed that predicts that integrin-based attachment complexes along osteocyte cell processes would dramatically and focally amplify small tissue-level strains (Wang *et al.*, 2007). Using rapid fixation techniques it was observed that osteocyte cell processes seem attached directly at canalicular projections emanating from the canalicular wall, *via* αvβ3 integrins (McNamara *et al.*, 2009). The theoretical model predicts that the tensile forces acting on these integrins are <15 pN and that axial strains caused by the sliding of actin microfilaments about the fixed integrin attachments are an order of magnitude larger than the radial strains in the earlier proposed strain-amplification theory (Wang *et al.*, 2007). *In vitro* experiments indicated that a Newtonian flow exerting a force of 1 to 2 pN on osteocyte cell processes is able to activate cell signalling to osteocytes, especially when applied around focal contacts (Wu *et al.*, 2011).

To simulate exercise loading on bone tissue, we stimulated bone cells with noisy fluid shear stress and demonstrated that noise enhances the molecular response (Bacabac *et al.*, 2009). An optimum noise intensity stimulated a maximum NO release, which diminished upon the addition of higher intensities (Bacabac *et al.*, 2009). A noise-amplified response implies a threshold-activated mechanism. Thus, despite the meagre strains experienced at normal daily activities, bouts of noisy strains (during strenuous activities as in sports) might provide enough stimuli for enhanced bone cell activity. We also showed in another study that bone cell responses to vibration stress demonstrated a NO release which positively correlated with the cube of the vibration frequency (Bacabac *et al.*, 2006). The response to vibration stress was fundamentally different from the response to fluid shear stress, which evoked a rate-dependent response (i.e., linear to the frequency) (Bacabac *et al.*, 2004). Considering that the cell nucleus is in bulk denser than the cytoplasm containing the cytoskeleton, the cube of the vibration stress stimulation is theoretically linear to the nuclear displacement by momentum transfer, suggesting that the molecular responses observed were linear to nuclear displacement (Bacabac *et al.*, 2006). In other words, the nucleus seemed to vibrate within the cytoplasm. As indicated earlier the extracellular matrix, transmembrane adhesion molecules, and the cytoskeleton can be considered a continuum. This continuum is mechanically coupled to the nucleus *via* the LINC (linker of nucleoskeleton and cytoskeleton) complex, and forces exerted on integrins by shear stress could be indirectly transferred all the way to the nucleus (Lombardi and Lammerding, 2011; Kardas *et al.*, 2012). Taken together, vibration directly stimulates the cell nucleus, which is probably not the case for fluid shear stress loading. The nucleus thereby contributes to how signalling molecule dosage is controlled by the type of stress sensed, whereas the cytoskeletal structure mediates force transfer (Bacabac *et al.*, 2006).

Several groups suggest that the deformation of the nucleus can be mediated *via* shear stresses on the cytoskeleton, which in turn might influence gene regulation (Davies *et al.*, 1995; Ingber, 1997; Janmey, 1997). Recently, it has been shown in endothelial cells that nesprin-1, included in the complex linking of the cytoskeleton to the nucleus, is crucial in cytoskeleton-mediated nuclear deformation (Anno *et al.*, 2012). It is therefore implied that for similar shear deformations in osteocytes, nuclear deformation could directly transfer mechanical load to the nucleus by shear stresses on the cytoskeleton, which may re-direct gene expression.

The previous paragraph nicely illustrates that the osteocyte is not just an on-off system, either activated by a mechanical stimulus or not, but rather that it is able to discern different stimuli applied at different frequencies (Li *et al.*, 2012a). The highly dynamic nature of the attachment sites and cytoskeleton, which are continuously undergoing turnover, likely explains how mechanical stimuli of varying magnitude and frequency regulate distinct signalling pathways, as elegantly described in a recent review by Hoffman *et al.* (2011).

Studies on microtubule self-organisation *in vitro* under microgravity (simulated by clinostats or by space flight), indicate that polymer network structures are fundamentally different compared to microtubules subjected to gravitation (Papaseit *et al.*, 2000). Although conditions within living cells are totally different, such studies could at least provide generic structural tendencies in the presence or absence of gravitation for a network of biopolymers. To characterise networks of biopolymers, the thermal fluctuation of probe

particles can be monitored using optical tweezers with sub-nm resolution using backfocal plane interferometry techniques (Gittes and Schmidt, 1998; Addas *et al.*, 2004). The mechanical properties of the network can be described by the probe fluctuations when quantified using the power-law of the spectral signature. Considering that the dynamic signature of the mechanical properties of semi-flexible polymer networks is essentially thermal at high frequencies (i.e., the power law of G above 1 Hz), we expect a non-negligible change in the dynamics only at the low frequency end where the non-thermal (or out-of-equilibrium) dynamics reflect structure and reaction rates of the underlying molecular processes. A change in the power-law itself (especially at the low frequency end) would indicate a more fundamental rheological change in cellular mechanics. Thus, an experimental approach correlating changes in the effective stiffness of the cells to that of the ECM under different gravitational states is a promising procedure towards understanding how the ECM and the cytoskeleton mechanically relate to each other. If gravitation, or its loss, provides enough stimulus (purely mechanical or biophysical), a biologically meaningful change in the slope of correlation between the stiffness of the cell and that of the ECM should be measurable. In turn, this implies that mechanosensing is either impaired or enhanced depending on the specific cell function.

Recently, we probed the mechano-activity and mechano-sensitivity of various bone cell types and fibroblasts, using a two-particle *in vitro* assay for measuring the viscoelasticity of cells, with two-particle micro-rheology (Bacabac *et al.*, 2008). Mechano-activity was characterised by the induction of force traction on attachment sites by cells, and mechano-sensitivity is the ability of cells to sense minute forces. We found that osteocytic cell types induce a relatively higher traction force on attached particles than osteoblastic cells. Fibroblastic CCL-224 cells are even more mechano-active compared to MLO-Y4 cells. The force fluctuations on the attached probes reflect intracellular movement, which might include actin (and microtubule) polymerisation, as well as motor and crosslinker dynamics. Since cell migration involves these dynamic processes, the lower magnitude of force fluctuation might reflect a lower capacity of osteocytes for motility compared to fibroblasts (Bacabac *et al.*, 2008).

In a two-particle *in vitro* assay, MLO-Y4 cells release NO simultaneous with increasing force application (Bacabac *et al.*, 2008). Furthermore, a typical behaviour observed in response to increasing force application was the predictable occurrence of force traction by cells on the attached beads, which was simultaneous with the specific morphological adaptation from a spherical to a polar shape defining ends at the attachment points. It would seem quite clear that force traction, morphology change, and possibly the release of signalling molecules are quantitatively coupled in response to micro-environmental stress conditions. Osteocytes under round-suspended morphology required lower force stimulation in order to show an NO response, even though they were an order of magnitude more elastic compared to flat-adherent cells (Bacabac *et al.*, 2008). Apparently, elastic osteocytes seem to require less mechanical force in order to respond than stiffer cells. On the other hand, 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 (Bacabac *et al.*, 2008), even though osteocytes are known to be more responsive to mechanical stress than osteoblasts (Klein-Nulend *et al.*, 1995a). This indicates that differences in mechano-sensitivity among cells are partly a property of elasticity, which reflects the underlying cytoskeletal structure that supports cell geometry. However, the observation that minute forces (~5 pN) are enough to stimulate soft round cells in contrast with stiff flat ones that require higher forces, suggests that molecular transducers (e.g. ion channels in the membrane) play a role where efficient force transfer across the cytoskeleton is lost (Bacabac *et al.*, 2008). Thus, mechanosensing is not solely a property of how cells globally deform under stress but is a complex property that may incorporate the contribution of suspected molecular sensors. The integrity of the cytoskeleton is therefore a prerequisite to mechanosensing mechanisms where intracellular force transfer is required.

Simultaneous with the increased NO release in response to mechanical stimulation, MLO-Y4 osteocytes show increased force traction on the attached beads (Bacabac *et al.*, 2008). The osteocytes generate a force up to 30 pN, which is within the order of magnitude capacity of activating integrins. Whether there is a causal link between loading-induced NO production by the cells and force generation is still under investigation. Attachment to opposing colloidal particles, trapped optically, simulates polarised attachment by cells spreading within a modelled ECM (e.g. fibrin or collagen gels), where the trap stiffness corresponds to ECM stiffness (Mizuno *et al.*, 2009). The observation that force fluctuation increased at higher trap stiffness (while cell stiffness remain constant), suggests a mechanism where the cell adapts itself to the stiffness of the surroundings. This adaptation is a direct way by which an osteocyte could "feel" an applied mechanical load. "Feeling" and adapting to the mechanical properties is by no means a unique property of osteocytes (An *et al.*, 2009). ECM mechanical properties strongly affect the behaviour of cells, which will remodel their internal arrangement and adapt cellular traction forces to match their own mechanical properties to those of the environment. Cells on stiff ECM will pull harder than cells on soft ECM, thereby increasing the tension on force-bearing elements such as F-actin (Trepat *et al.*, 2007). Interestingly, those force-bearing elements are the same as the elements involved in mechanosensing. Hence, ECM properties and the sensitivity of osteocytes to mechanical stimuli are coupled. Physical properties of the ECM are determined by its composition, the organisation of its components and their crosslinking. Cells produce ECM and crosslinkers, and by applying traction forces determine the architecture of ECM molecules and their degradation. Thus, interactions between the cell and the ECM are reciprocal.

Taken together, evidence has been provided demonstrating that the cytoskeleton affects the osteocyte response to stress, implying that the cytoskeleton is directly

involved in cellular mechanotransduction. Thus, any factor that significantly alters the osteocyte cytoskeleton in principle changes the response of osteocytes to mechanical stimuli, and could thereby affect bone mass.

Molecular signalling and the cytoskeleton in health and disease

It has been hypothesised often that the osteocytes in the skull have a different sensitivity to mechanical stimulation from the osteocytes in long bones, based on their difference in mechanical environment. Different mechanosensitivity of skull and long bone osteocytes could not be confirmed *in vitro* (Soejima *et al.*, 2001), where osteocytes were cultured on flat and stiff substrates and unlikely to replicate their site-specific cytoskeletal arrangement and thus mechanosensitivity (Vatsa *et al.*, 2008b). In addition, osteocytes were not grown on their native ECM before being mechanically stimulated. The ECM of skull bones differs significantly from that of long bones and, as already mentioned above, changes in ECM drive cytoskeletal changes and thereby possibly tune osteocyte mechanosensing. Osteoporosis is a bone disease leading to an increased risk of fracture in long bones and vertebrae, but not in the skull bones. Osteoporosis has so far not been connected to a difference in ECM composition or cytoskeletal structure of osteocytes, although it is a long standing hypothesis that changes in osteocyte mechanosensitivity contribute to the imbalance between bone mass and required strength (Bakker *et al.*, 2006).

The osteocyte cytoskeleton might be altered during osteoporosis, since enhanced circulating levels of cytokines are present in postmenopausal osteoporosis, and cytokines can modulate the cytoskeleton in several cell types. Cytokines are also highly expressed during inflammatory diseases such as Crohn's disease and rheumatoid arthritis, and are associated with a loss of bone mass. The cytokines tumour necrosis factor (TNF)α and interleukin (IL)-1β inhibit the increase in NO production and intracellular calcium that is normally observed in cultured osteocytes after application of a mechanical stimulus in the form of a fluid flow (Bakker *et al.*, 2009). TNFα and IL-1β strongly reduce F-actin content, which results in a reduction of osteocyte stiffness as indicated by the elastic moduli determined by twisting magnetic beads attached to the cell, providing a possible mechanism through which inflammation contributes to loss of bone mass (Bakker *et al.*, 2009).

Several bone mass disorders have been linked to mutations in a Wnt receptor and in a Wnt antagonist that is specifically expressed by osteocytes (Little *et al.*, 2002; Van Bezooijen *et al.*, 2007). Hence, molecules involved in Wnt signalling are of interest to the field of osteocyte biology. Wnts are a family of secreted glycoproteins with members that activate various intracellular pathways after binding to frizzled receptors or to a complex comprised of frizzled and LDL receptor-related proteins 5/6 (LRP5/6). The best studied Wnt pathway is the Wnt/β-catenin pathway. In the absence of Wnt ligands, β-catenin is recruited into a destruction complex and targeted for degradation. A protein named Dishevelled (DVL) is activated upon binding of Wnts to its receptors. Activated DVL recruits a destruction complex containing adenomatous polyposis coli (APC) to the plasma membrane, which prevents degradation of β-catenin. Because of the incapacitation of the destruction complex after binding of Wnts to the receptors, newly formed β-catenin accumulates in the cytoplasm and subsequently translocates to the nucleus, where it activates target gene transcription (Lai *et al.*, 2009; Li *et al.*, 2012b). Interestingly, DVL not only plays a central role in coordinating Wnt signalling, it also plays a role in various physiological and morphogenetic processes involving cytoskeletal interactions. DVL co-localises with microtubules and stabilises them (Krylova *et al.*, 2000). APC also interacts with proteins linked to the cytoskeleton, and several observations have highlighted the potential importance of the interaction between APC and microtubules (Matsumoto *et al.*, 2010). β-catenin has been suggested to alter the sensitivity of bone cells to mechanical loading (Robinson *et al.*, 2006). When MC3T3-E1 osteoblasts are treated with molecules that stabilise β-catenin and then subjected to mechanical loading, a synergistic up-regulation of Wnt gene expression is observed (Robinson *et al.*, 2006). This suggests that activation of the Wnt signalling cascade enhances the sensitivity of bone cells to mechanical loading. β-catenin is not only an important component of the Wnt signalling pathway, it also links cadherins (cell-cell adhesion molecules) to the actin cytoskeleton. A model has been proposed in which β-catenin is launched from its sites of cell-cell attachment towards target genes in the nucleus *via* cytoskeletal components upon mechanical stimulation (Bidwell and Pavalko, 2010). Although this model is not applicable to osteocytes with regard to β-catenin since osteocytes do not express cadherins, it would still be applicable to sites of focal adhesions (Bidwell and Pavalko, 2010).

Wnt signalling not only stabilises β-catenin, it also activates several members of the Rho family of GTPases to affect cellular function (Matsumoto *et al.*, 2010). Rho GTPases and their regulators regulate cytoskeletal remodelling. In light of the role of the cytoskeleton in mechanosensing, it is noteworthy that Wnts may modulate cytoskeletal organisation. Wnt signalling might indeed 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 Cx 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 signalling cascade) than in wild type mice (Johnson *et al.*, 2004). Similarly, the anabolic response of bone to mechanical loading is enhanced in ulnae of mice lacking the Wnt inhibitor Sfrp3 (Lories *et al.*, 2007). 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 (Lories *et al.*, 2007).

Mechanical loading leads to an increase in Wnt protein production by osteocytes, which activates the canonical Wnt signalling pathway in a paracrine fashion (Santos *et al.*, 2009; Tu *et al.*, 2012). Mechanical loading might thus lead to Wnt production by osteocytes, thereby tuning their own sensitivity to mechanical loading in a feedback loop.

The mechanosensitivity of osteocytes has been suggested to diminish with aging, although reports about a diminished anabolic response of bone with age are conflicting (Kohrt, 2001; Srinivassan *et al.*, 2003). There is evidence that aging is associated with changes in the cytoskeleton, which lead to apoptosis and changes in autophagy in chondrocytes, and aging induces changes in the actin and microtubules of primary human osteoblasts (Ankersen *et al.*, 1994). Unfortunately, nothing is known yet about age-related changes in the osteocyte cytoskeleton.

Aging and osteoporosis may not be considered special conditions of illness for bone tissue, considering that no organism is exempt. However, the lack of mechanical load (as in microgravity or bed rest as forms of disuse) may stimulate similar signalling, which in turn provides a special condition towards loss of bone mass and compromised bone structure. Similarly, apoptosis may contribute towards local disuse in terms of the recruitment of osteoclasts towards the vicinity of apoptotic osteocytes by responding to apoptosis-induced signalling (Kogianni *et al.*, 2008). Such a specialised condition could be short-lived as micro-cracks, a pre-condition for osteoclastic resorption, and is also a possible trigger for the onset of bone growth suggested by cellular distribution in the cutting cone (Burger and Klein-Nulend, 1999; Burger *et al.*, 2003).

Conclusion

The osteocyte cytoskeleton, which ultimately determines cell shape, is a key factor in determining how osteocytes feel stresses, whether by a local or bulk deformation, or *via* a transfer of forces inside of the cell. As osteocytes orchestrate bone remodelling, any factor affecting the osteocyte cytoskeleton and thereby the osteocyte response to mechanical stimuli, potentially affects bone mass. Accumulated depth in the study of molecular mechanisms involved in bone cell mechanosensing, while slowly completing the "big picture", draws a complex diagram where the role of mechanics is not independent of molecular signalling. Here, the need for computation becomes relevant in linking clusters of possible pathways that promise a practical grasp of correlations between biological function, signalling, and stress sensing. Computation is however a very important tool that will always require experimental verification, that is, predictions ensuing from theory must provide measurable parameters. Given the crucial importance of osteocytes for maintaining a proper resistance against bone fracture, it seems obvious that a much greater knowledge of the molecular mechanisms that govern the adaptive response of osteocytes is needed.

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Editor's Note: All questions/comments by the reviewers were answered by text changes, hence there is no "**Discussion with Reviewers**" section.