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The role of osteocytes in bone mechanotransduction

A. Santos · A. D. Bakker · J. Klein-Nulend

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Introduction

Bones are subjected to a variety of mechanical loads during daily activities. In the nineteenth century, Julius Wolff proposed that bones adapt their mass and 3D structure to the loading conditions in order to optimize their load-bearing capacity, and that this process is driven by mechanical stress [1]. For the past centuries, an increasing number of theoretical and experimental results reveal that osteocytes are the pivotal cells orchestrating this bio-mechanical regulation of bone mass and structure, which is accomplished by the process of bone remodeling [2–5]

Osteocytes are terminally differentiated cells of the osteogenic lineage that are derived from mesenchymal precursor cells. A number of molecules have been identified as important markers of osteocytes, such as matrix extracellular phosphoglycoprotein [6] sclerostin [7], dentin matrix protein-1 [8], and plex protein [8]. The osteocytes are the most abundant cells in adult bone and are constantly spaced throughout the mineralized matrix. Mature osteocytes have a characteristic dendritic cell shape, with processes radiating from the cell body through the canaliculi in different directions. These processes form an intercellular network through gap and adherent junctions with surrounding osteocytes, the cells lining the bone surface and bone marrow. Through this unique 3D network, osteocytes are

anatomically placed in a prime position not only to sense deformations driven by stresses placed upon bone, but also to respond with passage of signals to the neighboring cells [9].

For more than a decade now, it is known that the osteocytes are very sensitive to stress applied to intact bone tissue [10–16]. Computer simulation models have shown that mechanosensors lying at the surface of bone, as osteoblasts and bone lining cells do, would be less sensitive to changes in the loading pattern than the osteocytes, lying within the calcified matrix [3]. Interestingly, targeted ablation of osteocytes in mice disturbs the adaptation of bone to mechanical loading [16].

Osteocytes as key players in the process of bone mechanotransduction

It is currently believed that when bones are loaded, the resulting deformation will drive the thin layer of interstitial fluid surrounding the network of osteocytes to flow from regions under high pressure to regions under low pressure [17, 18]. This flow of fluid is sensed by the osteocytes which in turn produce signaling molecules that can regulate bone resorption through the osteoclasts, and bone formation through the osteoblasts, leading to adequate bone remodeling [17, 18]. This concept is known as the fluid flow hypothesis. Evidence has been increasing for the flow of canalicular interstitial fluid as the likely factor that informs the osteocytes about the level of bone loading [2, 5, 17, 18]. Nevertheless, Vatsa and colleagues [19, 20] proposed that if osteocytes could sense matrix strains directly, the cell shape, cytoskeletal alignment and distribution of adhesion sites in osteocytes *in situ* would bear alignment to the mechanical loading patterns. Indeed, it was shown that the

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cell shape and distribution of actin and paxillin staining in osteocytes of mouse tibiae and calvariae were orientated accordingly to the respective mechanical loading patterns applied in these bones, suggesting that osteocytes might be able to directly sense matrix strains in bone [19, 20]. In accordance with these results, Wang and colleagues [21] developed a theoretical model that predicts that integrin-based attachment complexes along the osteocyte cell processes would amplify small tissue level strains. It was shown that osteocyte cell processes are directly attached to canalicular projections in the canalicular wall via $\alpha v \beta 3$ integrins [21]. The theoretical model predicts that the tensile forces acting on these integrins are <15 pN. Axial strains caused by actin microfilaments on fixed integrin attachments are an order of magnitude larger than the radial strains in the previously proposed strain amplification theory [21]. In vitro experiments indicated that membrane strains of this order are large enough to open stretch activated cation channels [21], thus theories regarding shear stress within lacunae and osteocyte signaling need further investigation.

Osteocyte structures involved in mechanosensing: cell processes, cell body, and cilia

Up to now it has not been determined which of the osteocyte cell parts are most important for the function of the osteocyte as mechanosensor. It has been suggested that fluid flow over dendritic processes in the lacunar–canalicular porosity can induce strains in the actin filament bundles of the cytoskeleton that are more than an order of magnitude larger than tissue level strains [22]. Vatsa and colleagues [23] developed a method which enabled the quantification of mechanically induced intracellular nitric oxide (NO) production of the cell body and the cell process in single MLO-Y4 osteocytes using DAR-4M AM chromophore [23]. NO released by nitric oxide synthase (NOS) is a known early mediator of the response of osteocytes to mechanical loading and it mediates the induction of bone formation by mechanical loading in vivo [24, 25]. In single osteocytes, mechanical stimulation of both cell body and cell process resulted in up-regulation of intracellular NO production [23]. These results indicate that both cell body and cell process might play a role in mechanosensing and mechanotransduction in bone [23]. In addition, it was shown that a mechanically stimulated single osteocyte can communicate the information of the local mechanical stimulus to the other cells in the vicinity independent of intercellular connections, suggesting that this communication occurs via extracellular soluble factors [9]. Furthermore, an alternative mechanosensing structure has been proposed, i.e., osteocytes project a single cilia from their

cell surface [26]. This structure can translate fluid flow stimuli into a cellular response, indicating that primary cilia might act as a mechanosensitive structure within the osteocyte [27].

The role of the cytoskeleton in mechanosensing

Lately, evidence is emerging highlighting the crucial role of the cytoskeleton as a structure that is highly responsive to external physical and chemical stimuli. The cytoskeleton is involved in processes such as mechanosensing and largely determines the material properties of the cell (i.e., stiffness). It is known that the effect of stresses applied at different rates at an object is largely determined by the material properties of that object. Low magnitude ($<10 \mu\epsilon$) and high frequency (10–100 Hz) loading can stimulate bone growth and inhibit disuse osteoporosis, while high loading rates have been shown to increase bone mass and strength after jumping exercises in middle-age osteopenic ovariectomized rats [28]. For bone cells, Bacabac and colleagues [29–31] have shown that the production of signaling molecules in response to an in vitro fluid shear stress (at 5 and 9 Hz) and vibration stress (5–100 Hz) correlated with the applied stress rate [29–31]. The faster the stress was applied, the stronger the observed response of the cells [32], suggesting that the bone cellular response to loading and mechanical properties of the cell are related, which implies that the response of bone cells to loading is related to cytoskeletal properties. The same group developed a novel application of two-particle microrheology, for which a 3D in vitro system was devised to quantify the forces induced by cells on attached fibronectin-coated probes ($4 \mu\text{m}$). The frequency at which the cells generate forces on the beads is related to the metabolic activity of the cell [33]. With this device and using NO production as a read-out, the material properties of round suspended MLO-Y4 osteocytes and flat adherent MLO-Y4 osteocytes were characterized. Osteocytes with round suspended morphology required lower force stimulation in order to show an increase in NO production, even though they were an order-of-magnitude more elastic compared to flat adherent cells [34]. Apparently, elastic osteocytes seem to require less mechanical forces in order to respond than stiffer cells [34]. In contrast, flat adherent MLO-Y4 osteocytes, primary chicken osteocytes, MC3T3-E1 osteoblasts, and primary chicken osteoblasts all showed a similar elastic modulus of less than 1 kPa [33]. This indicates that differences in mechanosensitivity between osteocytes and osteoblasts might not only be directly related to the elasticity of the cell, but also to other cell-specific properties, i.e., the presence of receptors or ion channels in the membrane, or how cells change their material properties in relation to deformation.

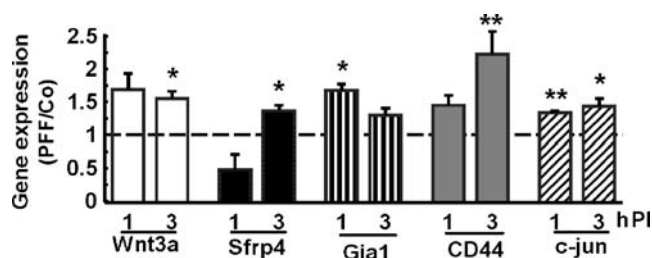


Fig. 1 Mechanical loading by pulsating fluid flow up-regulates gene expression of Wnts, Wnt antagonist, and Wnt target genes in MLO-Y4 osteocytes. One hour of PFF followed by 3 h of post-incubation without PFF (PI) up-regulated mRNA expression levels of Wnt3a and the antagonist SFRP4. One hour of PFF followed by 1 to 3 h of post-incubation without PFF increased mRNA expression of the target genes connexin-43, c-jun, and CD44. Values were normalized for GAPDH, PBGD, HPRT, and 18s and expressed as mean±SEM of PFF-treated-over-control ratios of three to six independent cultures. *PFF* pulsating fluid flow, *Co* control, *SFRP4* secreted frizzled related protein 4, *Gja1* connexin-43, *CD44* CD44 antigen, *PI* post-incubation without PFF. Significant effect of PFF, * $p < 0.05$; ** $p < 0.01$

Key signaling molecules in mechanotransduction: NO, prostaglandins, and Wnt

An important step in the chain of events leading to adaption of bone to mechanical loading is the transduction of physical stimuli into biochemical factors that can alter the activity of the osteoblasts and osteoclasts. An important early response to mechanical loading is the influx of calcium ions. The calcium release may occur directly via mechanosensitive ion channels in the plasma membrane which induce release of calcium from internal stores [18, 35–39]. Calcium release can also occur indirectly via the opening of hemichannels (un-apposed halves of gap junctions) that result in release of ATP and NAD^+ , which in turn raise the intracellular calcium levels amplifying the wave propagation of calcium [40, 41]. The rise in intracellular calcium concentration is necessary for activation of calcium/calmodulin-dependent proteins such as NOS. The activation of phospholipase A_2 results a.o. in the stimulation of arachidonic acid production and prostaglandin E_2 (PGE_2) release mediated by the enzyme cyclooxygenase (COX) [37]. It has been shown in vitro that pulsating fluid flow (PFF) stimulates within minutes the release of NO and prostaglandins PGE_2 and PGI_2 from osteocytes, while osteoblasts were less responsive and osteoprogenitor cells were the least responsive [42–44]. Moreover, COX-2, one of the known isoforms of COX, can be induced by mechanical loading in vitro [45]. Again, osteocytes were much more responsive than osteoblasts and osteoprogenitor cells. After a 15-min treatment with PFF, osteocytes exhibited a three-fold increase of COX-2 messenger RNA (mRNA) expression while the other two cell populations showed no increase [46]. Moreover, in osteocytes, the

induction of COX-2 was sustained up to 1 h after mechanical loading was ceased. These results suggest that as bone cells mature, they increase their capacity to produce prostaglandins in response to fluid flow [47], either by direct response to load or by increased expression of COX-2 after cessation of the mechanical stimuli. Because induction of COX-2 is a crucial step in the induction of bone formation by mechanical loading in vivo [47], these results provide direct experimental support for the concept that osteocytes, the long-living terminal differentiation stage of osteoblasts, function as the “professional” mechanosensors in bone tissue.

Another family of molecules that very recently has been identified as mediator of the adaptive response of bone to mechanical loading is the Wnt family of proteins. Wnts belong to a family of secreted glycoproteins and have been associated with the adaptative response of bone to mechanical loading [48–50]. Inactivating mutations in the human low-density lipoprotein receptor-related protein 5 (LRP5) were shown to cause osteoporosis, while gain-of-function mutations in the LRP5 co-receptor increased Wnt signaling resulting in higher bone mass [48–50]. Although evidence is accumulating that Wnts are involved in the regulation of bone mechanical adaptation, it is unknown which cells produce Wnts in response to mechanical loading. Santos and colleagues [51] have shown that 1 h of pulsating fluid flow (0.7 ± 0.3 Pa, 5 Hz) up-regulated mRNA expression of Wnt3a as well as the Wnt antagonist SFRP4 in MLO-Y4 osteocytes at 1 to 3 h after cessation of the fluid flow stimulus (Fig. 1). These results suggest that osteocytes in vitro are able to respond to fluid shear stress by modulation of mRNA expression of molecules involved in Wnt signaling. Importantly, PFF also up-regulated gene expression of known Wnt target genes such as connexin 43, c-jun, and CD44 in MLO-Y4 osteocytes indicating that

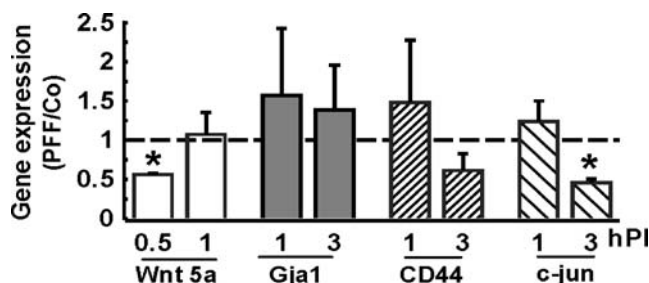


Fig. 2 Mechanical loading by pulsating fluid flow down-regulates gene expression of Wnts and Wnt target genes in MC3T3-E1 osteoblasts. One hour of PFF followed by 0.5 h of post-incubation without PFF (PI) down-regulated mRNA expression levels of Wnt5a and the target gene c-jun. Values were normalized for GAPDH, PBGD, HPRT, and 18s and expressed as mean±SEM of PFF-treated-over-control ratios of three to six independent cultures. *PFF* pulsating fluid flow, *Co* control, *SFRP4* secreted frizzled related protein 4, *Gja1* connexin-43, *CD44* CD44 antigen, *PI* post-incubation without PFF. Significant effect of PFF, * $p < 0.05$

mechanical loading activated the canonical Wnt signaling pathway (Fig. 1). The response to PFF was different in MC3T3-E1 osteoblasts (Fig. 2), i.e., the expression of most Wnt-related genes, including Wnt5a and c-jun, was down-regulated in response to PFF which underscores the specificity of the mechano-response of osteocytes in terms of Wnt expression. Mechanical loading might thus lead to Wnt production by osteocytes thereby driving the mechanical adaptation of bone [51].

Osteocytes as conductors of bone remodeling and orchestrators of osteoblast and osteoclast activity

The final step of the mechanical signal transduction pathway towards bone remodeling is the transmission of molecules produced by osteocytes to the effector cells, i.e., osteoblasts and osteoclasts. Considering the close physical proximity of osteocytes to local osteoblasts and periosteal fibroblasts, it is highly plausible that soluble factors produced by osteocytes act in a paracrine manner to affect these cells. Thus, soluble mediators may regulate the properties of neighboring bone cell populations including their proliferation and differentiation. It has been shown that treatment of osteocytes with mechanical loading by PFF produce the most potent conditioned medium that inhibits osteoblast proliferation and stimulates alkaline phosphatase activity as compared to conditioned medium produced by osteoblasts and periosteal fibroblasts [52]. In addition, the fact that the osteocyte-conditioned medium regulates the properties of both osteoblasts and periosteal fibroblasts in a conserved NO-dependent mechanism lends support to the hypothesis that the osteocyte is an orchestrator of different cell populations in bone in response to mechanical loading [52]. Tan and colleagues [53] have shown that osteocytes subjected to mechanical loading by PFF inhibit osteoclast formation and resorption via soluble factors. The release of these factors was at least partially dependent on activation of an NO pathway in osteocytes as a response to fluid flow. The osteocyte appeared to be more responsive to fluid flow than the osteoblast and periosteal fibroblast regarding the production of soluble factors affecting osteoclast formation and bone resorption. This suggests a regulatory role for osteocytes in osteoclast formation and bone resorption during bone remodeling such as occurs after application of a mechanical load [53].

Conclusions

Understanding the role of osteocytes in bone mechanosensation and the consequence for bone metabolism and turnover is of vital importance. During the last decade,

molecular mechanisms and pathways involved in osteocyte mechanosensation have been identified and expanded significantly. It remains to be determined what makes osteocytes more responsive to shear stress than osteoblasts and what role the cell body, cell processes, and even cilia may play in this response.

The osteocyte likely orchestrates bone remodeling in the adult skeleton by directing both osteoblast and osteoclast function. New discoveries with regards to the cellular mechanisms underlying the process of mechanical adaptation of bone may lead to potential therapeutic targets in the treatment of diseases involving impaired bone turnover, e.g., osteoporosis or osteopetrosis.

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Conflicts of interest None.

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