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Boning up on Wolff's Law: Mechanical regulation of the cells that make and maintain bone

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ABSTRACT

Bone tissue forms and is remodeled in response to the mechanical forces that it experiences, a phenomenon described by Wolff's Law. Mechanically induced formation and adaptation of bone tissue is mediated by bone cells that sense and respond to local mechanical cues. In this review, the forces experienced by bone cells, the mechanotransduction pathways involved, and the responses elicited are considered. Particular attention is given to two cell types that have emerged as key players in bone mechanobiology: osteocytes, the putative primary mechanosensors in intact bone; and osteoprogenitors, the cells responsible for bone formation and recently implicated in ectopic calcification of cardiovascular tissues. Mechanoregulation of bone involves a complex interplay between these cells, their microenvironments, and other cell types. Thus, dissection of the role of mechanics in regulating bone cell fate and function, and translation of that knowledge to improved therapies, requires identification of relevant cues, multifactorial experimental approaches, and advanced model systems that mimic the mechanobiological environment.

1. Introduction

Bone is a dynamic tissue that is normally renewed through balanced bone resorption and formation processes that are choreographed in space and time. Tight coupling of these processes is required to maintain the skeleton, and loss of coupling results in skeletal pathologies that represent some of the most significant public health threats faced by the growing and aging population.

Bone is remodeled to meet its mechanical demands, suggesting that mechanical forces are among the most potent factors that influence bone formation and resorption. Beginning with the pioneering work of Julius Wolff (translated to English in Wolff (1986)) and Wilhem Roux in the 19th century, much of our understanding of bone mechanobiology has been limited to functional adaptation of the structure and mass of bone at the *tissue* level. But bone tissue remodeling is ultimately mediated by the *cells* in bone: osteocytes, the putative mechanosensors; osteoblasts that deposit bone matrix; osteoclasts that resorb bone; and their progenitors. Definition and predictive control of bone mechanobiological responses therefore requires an improved understanding of the cellular and molecular bases of bone functional adaptation.

In this review, we summarize recent advances and outstanding issues in bone cell mechanobiology, with focus on osteoprogenitor cells and osteocytes. Osteocytes are of particular interest because increasing evidence supports the idea that they are the primary mechanosensors that regulate other cells to choreograph mechanical load-induced bone remodeling. Osteoprogenitor cells are of interest not only because of their vital role in bone (patho)physiology, but also because of their utility as a cell source for regenerative medicine and because recent evidence implicates them in clinically important pathologies involving ectopic bone formation, such as vascular and valvular calcification. The reader is also referred to recent excellent reviews that focus on the mechanobiology of bone tissue and other cell types (Klein-Nulend et al., 2005; Robling et al., 2006; Rubin et al., 2006).

2. Forces experienced by bone cells

Determining the effects of mechanical forces on bone cell fate and function in vivo requires an understanding of the mechanical forces experienced by bone cells in situ (Fig. 1). In mature bone

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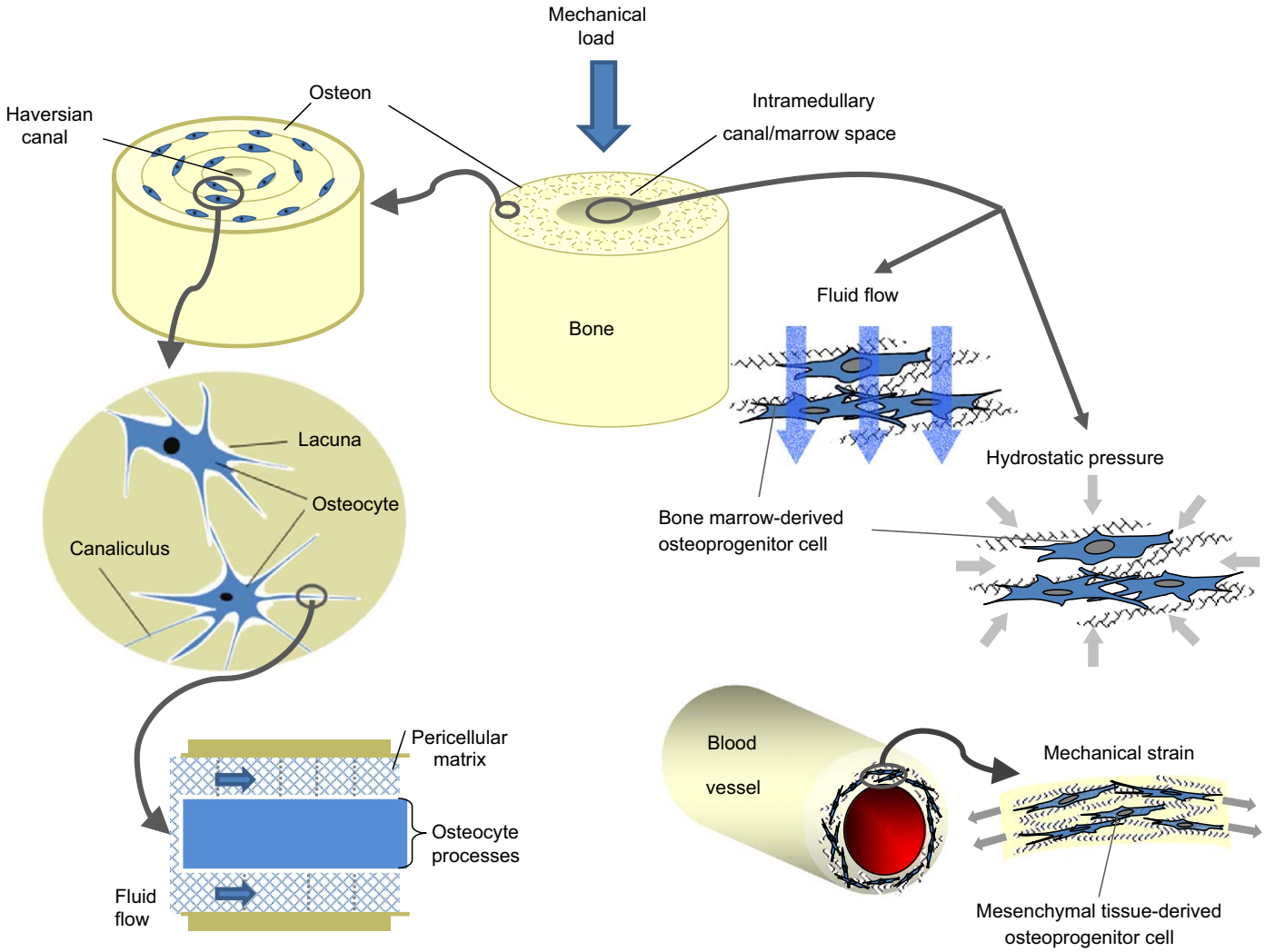

Fig. 1. Schematic of the anatomical locations of and mechanical loads experienced by osteocytes and osteoprogenitor cells. The left side of the figure illustrates the hierarchical structure of bone and the structural organization of osteocytes within bone tissue. Osteocytes are embedded in osteons arranged concentrically around the Haversian canal. An osteocyte resides in a lacuna and connects to other osteocytes through its processes that extend through the cannaliculi. Mechanical loads on bone induce fluid flow in the canalicular space. The fluid-induced drag forces on the pericellular matrix strain the cell processes, eliciting biochemical responses in osteocytes. The right side of the figure illustrates the mechanical loads experienced by osteoprogenitor cells in bone and in other mesenchymal tissues (lower right corner). Osteoprogenitors in the bone marrow likely experience fluid flow and hydrostatic pressure resulting from intramedullary pressure generated during bone deformation. Osteoprogenitors resident postnatally in soft mesenchymal tissues, such as blood vessels and heart valves, are subjected to sizeable mechanical strains.

tissue undergoing remodeling, recent evidence indicates that interstitial fluid flow is a potent regulator of bone cell metabolism, and particularly that of osteocytes, the putative mechanosensor cells in bone (Jacobs et al., 1998; You et al., 2000, 2008b; Tan et al., 2006) (also reviewed in Fritton and Weinbaum, 2009). Osteocytes inhabit a fluid-filled network made up of widely spaced lacunae and are interconnected via cellular processes contained within thin channels known as canaliculi. Mechanical loading on bone induces pressure gradients within bone matrix that lead to interstitial fluid flow in the lacunar–canalicular system (Piekarski and Munro, 1977), which has been demonstrated experimentally through tracer studies (Knothe Tate et al., 2000; Mak et al., 2000; Tami et al., 2003; Wang et al., 2004, 2005). To estimate the forces applied to osteocytes, a structural model of the osteocyte processes and their pericellular matrix was developed in 1994 (Weinbaum et al., 1994) and later refined (You et al., 2001b; Han et al., 2004; Wang et al., 2007). These models predict oscillatory fluid flow (OFF) patterns, resulting in shear stresses of 0.8–3 Pa on osteocytes (Weinbaum et al., 1994; Mi et al., 2005a, b). The current model incorporates conical structures that protrude periodically from the canalicular wall, creating a more accurate asymmetric model of the osteocytic process and its surrounding pericellular space (Anderson and Tate, 2008). This model was the first to predict that high frequency (>30 Hz), low magnitude loads (<1 MPa) loads are sufficient to elicit cellular response, consistent with in vivo observations (Xie et al., 2006).

Physiological loading of bone also induces hydrostatic pressure in the lacunar–canalicular porosity, which is estimated to be 40 times higher than that in the vasculature (Zhang et al., 1998). A change in body position from supine to standing elicits a hydrostatic pressure that is considered static, as the period of loading (>12 h) is long enough for cells to equilibrate. Hydrostatic pressure in the lacunar–canalicular porosity induced by a step load is estimated to be 12% of the applied axial stress (Zhang et al., 1998), but its effects on bone cells are not well studied. Under physiological conditions such as walking, hydrostatic compression is cyclic. Oscillatory loading of 0–18 MPa at 1 Hz was predicted to induce 0.27 MPa fluid pressure in the lacunar–canalicular porosity (Zhang et al., 1998), suggesting that cyclic hydrostatic pressure could be a significant stimulus for bone

remodeling. The effects of hydrostatic pressure on individual cells are complicated by spatial heterogeneity in local cell stiffness; for example, the cell body will deform more than cellular processes under hydrostatic loads, since the cell's body is more compliant than its processes (Docheva et al., 2008). Poroelastic cell models with idealized geometries have been developed to estimate local deformation of a cell under cyclic hydrostatic pressure (Zhang, 2005).

Physiological loading also causes strains in whole bone in vivo which are typically in the range of 0.04–0.3% for animal and human locomotion, but seldom exceed 0.1% (Rubin and Lanyon, 1984; Fritton et al., 2000). It has been shown that this level of strain is likely non-stimulatory to osteocytes and osteoblasts (You et al., 2000). However, recent evidence showed that osteocytes may experience amplified local strains due to either the ultrastructural features inside and around osteocyte processes (You et al., 2001b, 2004; Han et al., 2004; Wang et al., 2007) or the strain concentrations that occur at lacunae (Nicolella et al., 2006) or in bone remodeling units (McNamara et al., 2006).

While interstitial fluid flow and hydrostatic pressure are likely the primary stimuli for osteocytes, the mechanical forces experienced by osteoprogenitors in situ are not well-defined. Similar to osteocytes, progenitor cells in the bone marrow may experience hydrostatic pressures and fluid flow resulting from intramedullary pressure generated by bone deformation (Qin et al., 2003) or they may be subjected to fluid flow as they migrate in the Haversian system, where shear stresses are predicted to be similar to those in the lacunar–canicular network (Swan et al., 2003). However, osteoprogenitors reside not only in the bone marrow, but also in several soft collagenous tissues, many of which deform significantly under physiological loading. Thus, matrix strain may be an important biomechanical stimulus for osteoprogenitors that are resident in soft tissues or that migrate to pre-mineralized tissues in healing fractures and around bone-interfacing implants. While knowledge of the mechanical environment in vivo is important for understanding mechanoregulation of osteoprogenitor cells in (patho)physiological processes, the response of osteoprogenitors to a wider range of mechanical stimuli may be relevant for tissue engineering applications (Bancroft et al., 2002) in which mechanical conditioning can be tuned to direct cell fate and function, regardless of its physiological relevance.

3. Osteoprogenitor mechanobiology

Bone formation occurs not only during embryonic development, and bone modeling, remodeling, and repair, but also in cardiovascular calcification (Johnson et al., 2006; Steiner et al., 2007). This suggests that there remains a large reservoir of osteoprogenitors throughout the body, postnatally. Osteoprogenitors can be defined as cells that have the ability to differentiate from a more primitive state, or transdifferentiate from other functional cell types, to osteoblast-like cells that express characteristic bone transcripts and proteins such as Runx2, alkaline phosphatase (ALP), osteonectin, and osteocalcin, and can form bone matrix (Aubin, 1998). Currently there is no definitive cell surface marker to identify osteoprogenitors and the precise anatomical locations of their niche remain unknown. The only reliable way to identify and measure the frequency of the osteoprogenitor subpopulation in a mesenchymal tissue is using retrospective colony forming unit-osteoprogenitor assays (Bianco et al., 2008). Osteoprogenitor subpopulations have been identified not only in bone marrow, but also with strikingly high frequencies in cardiovascular tissues, including the aortic heart valve (Chen et al., 2009), vascular smooth muscle (Tintut et al., 2003), and capillary beds (Schor et al., 1990; Doherty et al., 1998). Thus, mechanical cues may play an important role in regulating the function and lineage commitment of progenitors in tissues other than bone. For example, heart valves and blood vessels are constantly mechanically stimulated due to blood flow, and calcification patterns in these tissues often coincide spatially with their mechanical environments (Simmons et al., 2005; Johnson et al., 2006).

Here, we summarize recent studies on the effects of various mechanical stimuli on osteoprogenitors and the mechanotransduction mechanisms involved. While a variety of cell types have been used to study osteogenic differentiation, we focus here on studies that used primary progenitor cells isolated from various mesenchymal tissues or immortalized cell lines (including pre-osteoblast MC3T3-E1 cells (Sudo et al., 1983) as osteoprogenitor models.

3.1. Substrate deformation effects on osteoprogenitors

Mechanical strain significantly induces osteogenic differentiation and suppresses adipogenic differentiation in osteoprogenitors (David et al., 2007). Within 24 h of stretching, the mRNA expression of ALP (Tanno et al., 2003; Fan et al., 2007; Qi et al., 2008), Runx2 (Haasper et al., 2008; Qi et al., 2008), BMP2 (Tanno et al., 2003), BMP4 (Tanno et al., 2003) and collagen I (Haasper et al., 2008) are significantly increased. After 1–2 weeks of cyclic stretching, octeocalcin and Runx2 protein levels are significantly increased (David et al., 2007) and matrix mineralization occurs (Simmons et al., 2004). The magnitude of the osteogenic response is dependent on the magnitude of strain mediated through FosB activation (Haasper et al., 2008). Notably, the cellular response to mechanical strain is also dependent on the differentiation of stage of osteoprogenitors. Mechanical strain applied at earlier stages of differentiation elicits higher Runx2 and type I collagen mRNA expression, suppresses proliferation, and induces more apoptosis relative to more committed osteoprogenitors (Weyts et al., 2003; Jansen et al., 2004).

Applying forces to the cell focal adhesions by stretching the extracellular matrix (ECM) triggers focal adhesion kinase activity, which in turn activates downstream mitogen-activated protein kinase (MAPK) signaling pathways (Ward et al., 2007). Mechanical strain induces an initial rapid phosphorylation of MAPKs (Simmons et al., 2003, 2004; Jansen et al., 2004; Fan et al., 2007). Interestingly, although mechanical strain activates MAPK activity and inhibition of the MAPKs in static culture significantly decreases mineralization, inhibition of these pathways had little or no effect on strain-induced osteogenic differentiation in vascular osteoprogenitors (Simmons et al., 2004). This suggests that mechanical strain also activates other signaling pathways to promote osteogenic differentiation. Mechanosensitive calcium channels are activated under mechanical strain. When calcium channels are blocked pharmacologically or when cells are cultured in calcium free media, strain-induced upregulation of ALP is inhibited (Tanno et al., 2003). Limited data suggest that the Wnt signaling pathway can be regulated mechanically to promote osteogenesis. In particular, mechanical strain has been shown to upregulate mRNA expression of Wnt10B and the Frizzled 2 receptor (both are important regulators for bone formation) with an increased level of active β-catenin nuclear translocation in osteoprogenitors (Robinson et al., 2006; Armstrong et al., 2007).

3.2. Fluid flow effects on osteoprogenitors

Fluid flow is predicted in the intramedullary space and Haversian system in response to bone loading, suggesting that

fluid flow is a relevant stimulus for osteoprogenitors in these locations. Additionally, osteoprogenitors are more responsive to shear stress than substrate deformation, when both are applied in the physiological ranges expected in hard tissue.

Although most studies have reported that fluid flow promotes osteogenic differentiation, many inconsistencies exist. In many parallel plate flow chamber studies, flow-induced shear stress upregulated mRNA expression of some osteogenic markers, but had no effect or even inhibited other important osteogenic markers such as ALP, Runx2, and osteocalcin (Li et al., 2004; Kreke et al., 2008; Scaglione et al., 2008; Jekir and Donahue, 2009). These inconsistent results might be explained by differences in substrate surface chemistry and the flow patterns used in each study. ECM proteins and substrate materials play an important role in regulating osteogenic differentiation (Chastain et al., 2006), but their effects have not been thoroughly studied in combination with mechanical stimuli. Scaglione et al. (2008) showed that calcium phosphate coating on glass slides surprisingly inhibited flow-induced upregulation of osteopontin (OPN) and bone sialoprotein mRNA expression, demonstrating the critical role of substrate surface chemistry in flow-induced mechanosensing. Limited studies have shown that some cellular responses, including osteopontin mRNA expression and ERK1/2 phosphorylation (Batra et al., 2005; Kreke et al., 2008), are dependent on the specific flow pattern (i.e., continuous, intermittent, and oscillatory flow; shear stress magnitude; and flow frequency). The sensitivity of osteoprogenitor response to the flow pattern might partially account for discrepant observations among various studies.

Three-dimensional cell culture systems in which cells are seeded in hydrogels or scaffolds and cultured in spinner flasks or flow perfusion chambers have also been used to study the effect of flow. These systems are relevant for bone tissue engineering bioreactors, in which perfusion is used both for media exchange and to simulate growth. Similar to many 2D systems, fluid flow promotes osteogenic differentiation in 3D systems (Meinel et al., 2004; Datta et al., 2006; Stiehler et al., 2009). The fluid-induced osteogenic response was also dependent on substrate material (Datta et al., 2006). Although 3D systems may better mimic the in vivo cellular environment, it is difficult to uncouple the effects of shear stress and mass transport. Further because of the heterogeneity in pore sizes and interconnectivity in the scaffolds, it is difficult to determine the magnitude of shear stress experienced by the cells, except as estimated by computational fluid dynamics (Porter et al., 2005).

Primary cilia, gap junctions, and the cytoskeleton have been identified as participating in osteoprogenitor mechanosensing of fluid flow. Primary cilia are non-motile organelles that project from the cell surface into the extracellular space and deflect under flow. Mechanosensing through primary cilia in osteoprogenitors was independent of calcium flux and stretch-activated channels, and was required for a flow-induced osteogenic response, as flow-induced upregulation of OPN mRNA and prostaglandin E_2 (PGE_2) secretion were absent when primary cilia were removed pharmacologically (Malone et al., 2007a). The same effects were observed when gap junctions in osteoprogenitors were blocked pharmacologically (Jekir and Donahue, 2009). Steady fluid flow (SFF) accelerated actin stress fiber formation in osteoprogenitors (Malone et al., 2007b) compared with OFF (Ponik et al., 2007). The responsiveness of osteoprogenitors to flow may depend on cytoskeletal tension: using micropatterning to control cell spreading while maintaining constant total adhesive contact area, You et al. (2008a) showed that more spread osteoprogenitors were less responsive to mechanical loading.

Fluid flow also increases intracellular calcium concentration in osteoprogenitors by releasing intracellularly stored calcium ions, independent of activation of mechanosensitive calcium channels (You et al., 2001a; Riddle et al., 2006). This is in contrast to what was reported in mature osteoblasts (Li et al., 2002) in which mechanosensitive calcium channels were recruited. ERK1/2 and p38 MAPK were also activated due to fluid flow to promote osteogenesis, as inhibition of either kinase attenuated flow-induced osteogenic response (You et al., 2001a). Notably, depleting intracellular calcium stores pharmacologically led to decreased osteogenic response (You et al., 2001a) but had no effect on ERK1/2 activity (Riddle et al., 2006). This suggests that fluid flow activates ERK1/2 independently of calcium signaling and p38 MAPK is a potential target of downstream flow-induced calcium signaling. Additionally, disruption of the actin cytoskeleton and its polymerization did not inhibit intracellular calcium mobilization in osteoprogenitors subjected to OFF (Malone et al., 2007b). Instead, cells with compromised actin cytoskeleton showed greater calcium responses and increased PGE_2 release compared with untreated cells. The results of this study are counterintuitive in that PGE_2 release and mobilization of intracellular calcium does not depend on an intact actin cytoskeleton. It is possible that the compromised cytoskeleton may lead to increased cell deformation, thereby indirectly contributing to increased intracellular calcium and PGE_2 release.

3.3. Hydrostatic pressure and compression effects on osteoprogenitors

Osteoprogenitors can sense (likely in part through integrins (Schwartz and DeSimone, 2008)) and respond to mechanical stress due to hydrostatic pressure and compressive loading. Mechanical stress significantly induced ALP activity and mRNA expression of Runx2, BMP2, osterix, collagen I, osteocalcin, osteonectin, and OPN in osteoprogenitors (Kim et al., 2007; Sim et al., 2007; Yanagisawa et al., 2007; Rath et al., 2008; Wagner et al., 2008; Liu et al., 2009a, b). After 1–2 weeks of mechanical loading, mineralization and osteocalcin and OPN protein expression were also significantly increased compare to static controls (Duty et al., 2007; Kim et al., 2007; Rath et al., 2008; Yanagisawa et al., 2008). Notably, the osteogenic response is dependent on the magnitude of mechanical stress in a biphasic manner, indicating that there exists an optimal range of stress for inducing osteogenic differentiation (Sim et al., 2007; Yanagisawa et al., 2007, 2008; Rath et al., 2008). However, the optimal range of stress has yet to be defined, as reported optimal stresses vary from 49 Pa to 11.8 kPa. Osteogenic response to hydrostatic pressure is also dependent on the differentiation stage of osteoprogenitors, with less committed cells being more sensitive (Liu et al., 2009a, b).

ERK1/2 and p38 MAPK play a positive but non-essential role in stress mechanotransduction. Mechanical stress significantly induced phosphorylation of ERK1/2 and p38 MAPK, but pharmacological inhibition of these two kinases only partially inhibited stress-induced osteogenic differentiation (Kim et al., 2007; Yanagisawa et al., 2007; Liu et al., 2009a), suggesting other signaling pathways are involved. Limited data showed that the Wnt signaling pathway is activated when hydrostatic pressure is applied, with Wnt10B and Wnt4 mRNA expression upregulated under mechanical loading. Upregulation of Wnt10B was partially dependent on ERK1/2 phosphorylation but Wnt4 expression was independent of ERK1/2 activity (Liu et al., 2009b).

3.4. Substrate stiffness effects on osteoprogenitors

As is the case with many cell types (Yip et al., 2008), osteoprogenitors sense the stiffness of the extracellular matrix and respond with altered cellular function including proliferation

(Kong et al., 2005), differentiation (Engler et al., 2006; Khatiwala et al., 2006), and apoptosis (Kong et al., 2005). Although the mechanism is still unknown, the cellular response to matrix stiffness is dependent on the differentiation stage of osteoprogenitors (Hsiong et al., 2008). When osteoprogenitors are in the later stage of lineage commitment, they appear to be more responsive to mechanical cues from the ECM. Some studies have shown that stiffer matrices promote more significant osteogenic differentiation and mineralization (Khatiwala et al., 2006). However, Engler et al (2006) showed a biphasic dependence of osteogenic differentiation and matrix stiffness, where the physiologically relevant stiffness range (25–40 kPa) was optimal for osteogenic differentiation. Yip et al. (2009) also observed more significant osteogenic differentiation in this optimal stiffness range compared to stiffer matrices. The discrepancies might be due to the differences in experimental setup including ECM surface chemistry and protein, and differentiation stage of osteoprogenitors. Additionally, differences in lineage differentiation potential between model cell systems may account for the biphasic response observed in primary osteoprogenitors, which tend to differentiate to myofibroblasts on much stiffer substrates (Yip et al., 2009).

Non-muscle myosin II plays a critical role in stiffness-directed lineage commitment in osteoprogenitors, as its inhibition hindered osteogenic differentiation (Engler et al., 2006). Another mechanism by which matrix stiffness regulates osteogenic differentiation involves MAPK activation downstream of the RhoA-ROCK signaling pathway. Runx2 mRNA expression of MC3T3-E1 pre-osteoblasts was increased on stiffer substrates and correlated with elevated ERK1/2 activity. Inhibition of RhoA, ROCK, and MAPK diminished Runx2 activity, and delayed the onset of osteogenesis as shown by altered osteocalcin, bone sialoprotein, gene expression, ALP activity, and mineralization (Khatiwala et al., 2008).

4. Osteocyte mechanobiology

Osteocytes are terminally differentiated osteoblasts defined as mature osteocytes based on their location in the lacunae (Noble, 2008). Isolation of primary osteocytes for study in vitro is challenging and it is not clear that primary osteocytes retain their osteocyte properties when removed from the location that defines them. While studies on osteocyte mechanotransduction have used a variety of cell models (including MC3T3-E1 pre-osteoblasts), the most definitive models available today, and the focus of this section, are primary osteocytes and the MLO-Y4 osteocyte-like cell line (Bonewald, 1999).

The critical role of osteocytes in normal bone remodeling is evident from mouse models in which osteocyte malfunction (Phillips et al., 2008) or absence (Tatsumi et al., 2007) leads to bone fragility consistent with that seen in aging and osteoporosis. Notably, while osteocyte ablation increased bone resorptive activities in mice under physiological loading, the same mice were resistant to disuse-induced bone loss when subjected to hind limb unloading (Tatsumi et al., 2007). These observations suggest that osteocytes sense mechanical stimuli applied to the skeleton and regulate load-induced remodeling responses, an ideal role for them due to their abundance and location. Because osteocytes experience fluid flow and hydrostatic pressure in their local mechanical environment, most studies have focused on osteocyte responses to these stimuli. Also of significant interest is definition of the mechanisms by which osteocytes communicate with so-called effector cells (e.g., osteoclasts, osteoblasts, and their precursors) to regulate bone remodeling. Recent findings related to osteocyte mechanotransduction and regulation of remodeling are reviewed below.

4.1. Osteocyte mechanosensing and mechanotransduction

Osteocytes, like other cells, are able to respond to mechanical forces using a variety of sensor and signaling mechanisms, including ion channels, integrins and the cytoskeleton, gap junctions and hemichannels, and primary cilia.

4.1.1. Ion channels

The identities and roles of ion channels involved in osteoblast mechanotransduction have been well-studied (Hung et al., 1996; Chen et al., 2000, 2005; You et al., 2001a; Bergh et al., 2003, 2004; Brauer et al., 2003; Zanello and Norman, 2003; Charras et al., 2004; Hughes et al., 2006), but less is known about the role of ion channels in osteocyte mechanotransduction. Osteocytes from rat ulna and chicken calvaria express gadolinium-sensitive cation channels that mediate cellular response to strain (Rawlinson et al., 1996) and flow (Ajubi et al., 1999). Osteocytes from rat calvaria (Miyauchi et al., 2000) additionally express nifedipine-sensitive (L-type voltage-dependent) channels and the α, β, and γ units of epithelium sodium channels (ENaC) (Mikuni-Takagaki, 1999), all of which are strain-responsive. The difference in ion channel expression in rat calvarial versus limb osteocytes is intriguing and may relate to their differential mechanosensitivity. The expression of three potassium channels (Gu et al., 2001a) and L-type channels (Gu et al., 2001b) was identified in MLO-Y4 cells, but their role in mechanotransduction is not yet defined.

4.1.2. Integrins and the cytoskeleton

Osteocyte integrins are required for load-induced bone remodeling in vivo. In conditional knockout mice whose cortical osteocytes were depleted of $\beta1$ integrin, disuse by hindlimb unloading did not reduce cortical bone size and strength in contrast to wild type mice (Phillips et al., 2008).

A variety of intracellular signaling pathways are activated in osteocytes in an integrin-dependent manner. Ligation of the $\alpha_V\beta_3$ integrin with echistatin (a soluble ligand) increased calcium signaling in osteocytes subjected to membrane stretch by hypotonic swelling compared with non-ligated cells (Miyauchi et al., 2006). Strain-induced ERK1/2 activation prevented osteocyte apoptosis, but required integrin engagement, intact actin filaments and microtubules, FAK activation, Src kinase activity, and phosphorylation of the adaptor protein Shc (Plotkin et al., 2005).

You et al. (2001b) suggested that cytoskeleton-mediated signaling in response to fluid flow could occur due to drag forces on the pericellular matrix, as tethering elements (presumably composed of integrins) connect the canalicular wall and pericellular matrix to the osteocyte cytoskeleton (You et al., 2004). Degradation of the glycosaminoglycan component of the pericellular matrix has been show to diminish responsiveness to fluid flow in MLOY4 osteocytes (Reilly et al., 2003). Also fluid flow applied to MLO-Y4 cells elicited stress fiber formation and increased both the number (Ponik et al., 2007) and elongation of dendrites, a process that was dependent on E11, an early osteocyte-selective protein (Zhang et al., 2006).

Notably, integrin and cytoskeleton-mediated mechanosensing of fluid flow may differ between osteocytes and osteoblasts. For example, fluid flow induced recruitment of fimbrin, an actin-binding protein, to the end of osteoblast, but not osteocyte, stress fibers (Kamioka et al., 2004) and increased calcium signaling in more osteoblasts than osteocytes (Kamioka et al., 2006). In contrast to osteoblasts, calcium responses in osteocytes were immune to disruption of focal adhesions by Arg-Gly-Asp-containing peptides,

most likely because of low expression of vinculin (Kamioka et al., 2006). Additionally, flow-induced production of PGE_2 in MLO-Y4 cells was inhibited by cytoskeletal disruption, whereas it was enhanced in MC3T3-E1 pre-osteoblasts (McGarry et al., 2005). The contrasting PGE_2 responses may occur via cytoskeletal modulation of stretch-sensitive ion channels, which are dominant in osteocytes (Rawlinson et al., 1996).

4.1.3. Gap junctions and hemichannels

Gap junctions and hemichannels provide direct cell–cell communication between osteocytes and between osteocytes and osteoblasts (Yellowley et al., 2000; Guo et al., 2006). Gap junctions are also present in osteoclasts, possibly facilitating similar cell–cell interaction (Ilvesaro et al., 2000). Conventional gap junctions are formed when connexons from adjacent membranes join to form an intercellular channel. Connexons are composed of connexin (Cx) subunits, with Cx43 being the most abundant connexin in MLO-Y4 cells and primary osteocytes (Kato et al., 1997). Connexons also form unapposed hemichannels that function independent of gap junction channels in single plasma membranes to facilitate communication between the intracellular and extracellular spaces (Goodenough and Paul, 2003). Mechanical loading applied to mouse incisors in vivo increased Cx43 protein expression in osteocytes (more so than in osteoblasts) (Gluhak-Heinrich et al., 2006). Fluid flow-induced release of ATP (Genetos et al., 2007) and PGE_2 (Jiang and Cherian, 2003; Cherian et al., 2005; Genetos et al., 2007; Siller-Jackson et al., 2008) is mediated in part by hemichannels in MLO-Y4 cells, but not in MC3T3-E1 cells exposed to OFF (Genetos et al., 2007). Flow-induced ATP release mediated hemichannel opening and PGE_2 release in one study (Li et al., 2005) but not another (Genetos et al., 2007), perhaps due to differences in flow pattern (SFF vs. OFF) and flow duration.

4.1.4. Primary cilia

Primary cilia and the associated polycystin 1 and 2 (PC1/PC2) mechanosensory complex are known to sense mechanical strain and regulate differentiation in kidney epithelial cells (Nauli et al., 2003). MC3T3-E1 and MLO-Y4 cells express the PC1 gene, and its inactivation delayed endochondral and intramembranous bone formation in mice, whereas constitutive expression of PC1 increased expression of Runx2 and other osteoblast markers in MC3T3-E1 cells (Xiao et al., 2006). This finding suggests a possible role for cilia in anabolic signaling in osteoblasts and osteocytes. It was later confirmed that both MC3T3-E1 and MLO-Y4 cells possess primary cilia, which are deflected by fluid flow (Malone et al., 2007a). Osteogenic and bone resorptive responses to fluid flow required primary cilia, and these responses were independent of Ca^{2+} flux and stretch-activated ion channels.

4.2. Effects of mechanical stimuli on osteocyte regulation of bone remodeling

Osteocytes are believed to regulate bone remodeling by signaling to other cells via soluble autocrine and paracrine signals, and direct cell–cell contact. Several candidate molecules have been identified and shown to be mechanically regulated. For example, receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) are two key molecules that mediate bone resorption. RANKL stimulates osteoclast precursors to commit to the osteoclastic phenotype while OPG blocks osteoclastogenesis and decreases the survival of pre-existing osteoclasts. Therefore, the relative abundance of RANKL to OPG determines propensity to bone resorption. In osteocytes subjected to OFF, the RANKL/OPG ratio decreased, and osteoclast formation from RAW264.7 cells in direct cell–cell contact with the osteocytes was repressed as well (You et al., 2008b). Additionally, conditioned media from both MLO-Y4 cells (You et al., 2008b) and chicken calvaria osteocytes (Tan et al., 2007) exposed to flow inhibited osteoclastogenesis, suggesting that osteocytes under physiological mechanical stimuli may prevent bone resorption via soluble factors like RANKL and OPG.

Recent in vivo data implicate sclerostin as another important mechanically regulated protein in bone remodeling. Sclerostin, encoded by the Sost gene, inhibits bone formation by blocking Wnt signaling, and is expressed primarily by osteocytes in adult bone. Cyclic ulnar loading in mice and rats in vivo reduced Sost and sclerostin levels proportional to the level of loading (Robling et al., 2008). From the same study, Sost expression was increased in the tibiae of tail-suspended mice, but there was no change in the number of sclerostin positive cells. During tail suspension of transgenic mice ablated of osteocytes, sclerostin mRNA did not increase, in contrast to large increases in wild type mice (Tatsumi et al., 2007). These observations, along with recent data demonstrating fluid flow upregulation of Wnt3a gene expression in osteocytes (Santos et al., 2009), suggest a Wnt-dependent mechanism by which osteocytes may regulate load-induced bone adaptation.

Knockout of OPN in mice prevented bone loss in response to tail suspension, suggesting a vital role for OPN in bone remodeling (Ishijima et al., 2002). Osteocytes appear to play a role in OPN-mediated remodeling, as expression of OPN is triggered in response to bone disuse in vivo and in hypoxic MLO-Y4 cells (as an in vitro model of disuse) (Gross et al., 2005). Paradoxically, fluid flow decreased OPN protein expression but increased OPN release in MLO-Y4 cells (Ponik et al., 2007).

PGE_2 is also implicated in both modeling and remodeling of bone (Tian et al., 2008). Fluid flow applied to MLO-Y4 cells upregulated Cox-2, the gene that encodes prostaglandin-endoperoxide synthase 2, a key enzyme in the production of PGE_2 (Ponik et al., 2007) and increased PGE_2 release through an ATP-mediated pathway, but independent of hemichannel activation (Genetos et al., 2007). The flow-induced PGE_2 response required an intact cytoskeleton in MLO-Y4 cells, but not in MC3T3-E1 pre-osteoblasts (McGarry et al., 2005).

Nitric oxide (NO) is a small, short-lived second messenger, the production of which is mechanically regulated. For example, PFF-induced NO release in MLO-Y4 cells that did not require an intact actin network and was enhanced with microtubule disruption (McGarry et al., 2005). Force applied to integrins on suspended MLO-Y4 cells induced increased NO release (Bacabac et al., 2008). Poking of a single MLO-Y4 with a microneedle increased intracellular NO production by 94%, and NO in the surrounding osteocytes by 31–150% (Vatsa et al., 2007). Intercellular communication via mechanically induced osteocyte NO likely plays a key role in bone remodeling, as inhibition of NO attenuated both the inhibitory effect of osteocyte flow-conditioned medium on osteoclast formation (Tan et al., 2007) and the pro-survival effect of fluid flow on osteocytes (Tan et al., 2008a).

The relationship between mechanical loading, osteocyte apoptosis and bone remodeling is intriguing. In vivo, there is a significant correlation between remodeling indices and the density of apoptotic osteocytes (Hedgecock et al., 2007), supporting the theory that apoptotic osteocytes are associated with, and may influence, bone remodeling. Osteocyte apoptosis may be mechanically regulated as PFF prevented TNF-α-induced apoptosis in osteocytes, but not in osteoblasts (Tan et al., 2006). Emerging data from our labs shows a similar response to OFF (Cheung et al., 2009). Cyclic hydraulic pressure also has been

shown to better maintain osteocyte viability in calf bone explants relative to unloaded controls (Takai et al., 2004).

5. Summary and future directions

The evolution of the field of bone mechanobiology from Wolff's Law and tissue-level studies to investigations at the cellular level has improved our fundamental knowledge and provided new perspectives on how mechanical forces regulate bone formation and resorption (Fig. 2). However, with this knowledge has come increased awareness of the complexities of the systems and processes involved in mechanoregulation of bone, and the recognition that our understanding of the cellular and molecular mechanisms is nascent. Broadly, an improved understanding of cellular mechanobiology requires an approach that moves beyond reductionism to consider cell response to mechanical stimuli in the context of other microenvironmental cues. This "systems biology" approach will require better definition of relevant cues, multifactorial experimental approaches, and enabling technologies. In the context of bone cell mechanobiology, we view the following unresolved issues as being of particular importance:

- *Mechanosensory and mechanotransduction mechanisms*: Knowledge of mechanosensory mechanisms involved in osteoprogenitor differentiation and osteocyte function remains limited. In particular, it is unclear how mechanical cues translate to chemical signals and integrate with other signals to elicit downstream responses in time- and rate-of-force-dependent manners (Vogel and Sheetz, 2006). The roles of other mechanosensory organelles, such as the plasma membrane (Apodaca, 2002), caveolea (Frank and Lisanti, 2006), membrane proteins, and the cytoskeleton (Hahn and Schwartz, 2009) remain poorly understood. Osteocyte responses to fluid flow are reasonably well-studied, but the effects of hydrostatic pressure are largely unknown. Further, the signaling pathways that link early mechanically induced responses in osteocytes to

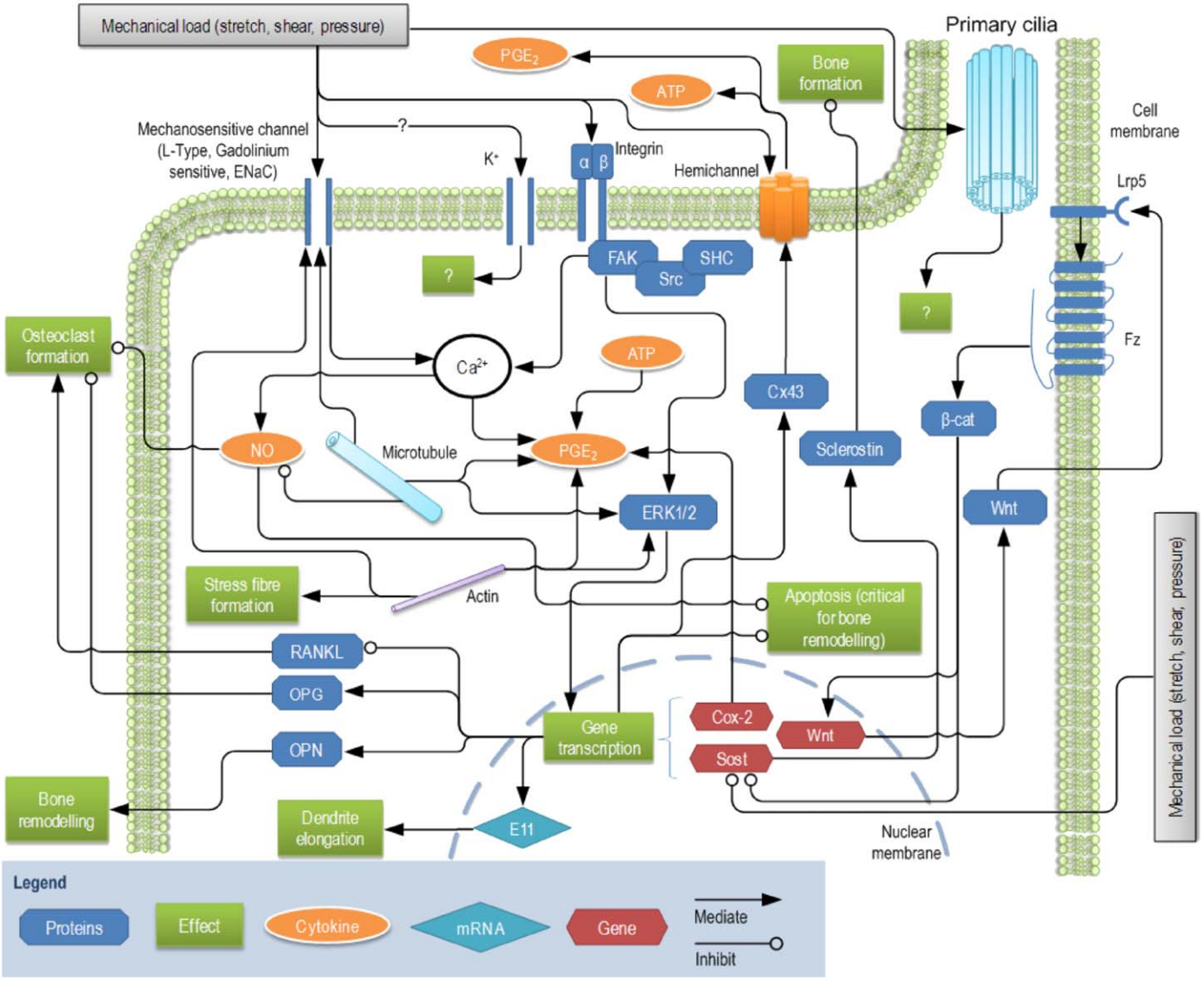

Fig. 2. Schematic of sensors, signaling pathways, and responses involved in osteocyte mechanobiology. Much of the current state of knowledge regarding osteocyte mechanobiology is represented in the schematic, although details are lacking in many cases (see text). The detailed signaling mechanisms involved in osteoprogenitor mechanobiology are poorly defined. While osteoprogenitor cells appear to use many of the same sensors (e.g., integrins, ion channels, gap junctions, and primary cilia) and pathways (e.g., ERK1/2 and other MAPKs, Ca^{2+}, and Wnt) as osteocytes, their responses to distinct mechanical stimuli often differ and are not as well defined (see text for details).

the production of factors that regulate effector cells need to be elucidated. Broadly, additional insight into mechanosensory mechanisms and their physiological relevance may come from experimental and mathematical model-based characterization of the deformation of cellular organelles and changes in mechanosensory protein conformation in response to various types of mechanical stimuli.

- *Coordinated cell–cell interactions*: The effect of direct cell–cell contact on mechanosensing in bone cells is not defined. Cell–cell contact is a dynamic process that affects cytoskeletal assembly (Yeung et al., 2005) and its linkages to transmembrane proteins, which is likely to alter cellular response to external mechanical cues. Additionally, bone cells in vivo are in constant communication through paracrine signals, with important implications for effector cell function and perhaps even modulation of cellular response to mechanical stimuli. Beyond communication between bone cells, mechanical regulation of paracrine signaling between bone cells and other cell types (e.g., endothelial cells) is likely a vital component of the bone formation and remodeling processes.
- *Integrated effects to multiple microenvironmental cues*: Bone cells are sensitive to microenvironmental cues beyond mechanical stimuli, including ECM proteins, substrate surface chemistry, substrate stiffness, chemotransport, and soluble signals (Donahue et al., 2003). These factors can significantly alter the mechanically induced cellular response, and therefore care must be taken in experimental design and interpretation of results. The combinatorial effects of multiple microenvironmental cues with mechanical stimuli remain poorly understood.
- *Advanced model systems*: To facilitate multivariate analyses, it will be necessary to develop efficient and high-throughput cell culture platforms that permit cell culture surface patterning with various ECM proteins, multiple and variable culture media compositions, tunable substrate stiffness, and application of mechanical stimuli. This may be accomplished through advances in biomaterial design, microelectromechanical systems, and microfluidic devices. Gomez-Sjoberg et al. (2007) built a fully-automated cell culture screening system based on a microfluidic chip to perform 96 independent experiments simultaneously with varying cell seeding density, composition of culture medium, feeding schedule, and length of shear stress application. For mechanobiology research, microfabricated high-throughput devices that are capable of applying various mechanical stimuli are required and are emerging (Kamotani et al., 2008; Tan et al., 2008b; Moraes et al., in press). Additional complexity is required to mimic the 3D microenvironment surrounding bone cells, which in the case of osteocytes has a significant effect on the fluid flow-induced local strain on the cell body and processes (You et al., 2001b; Han et al., 2004; Anderson and Tate, 2008). Again, microfabrication methods may be used to construct more physiologically relevant culture conditions that also enable observation of single cell response to mechanical loads, such as that developed by You et al. (2008c) to study osteocyte mechanotransduction.

Addressing these issues will provide fundamental knowledge and new insights that ultimately will translate to improved pharmacological and regenerative therapies to not only treat or prevent bone loss, but also prevent ectopic mineralization in the cardiovascular system.

Conflict of interest

None.

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