Bulletin of the JSME Vol.17, No.4, 2022
Journal of Biomechanical Science and Engineering

【Review Paper】

Investigation of mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes: *in vitro* experiments targeting subcellular components

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Received: 11 July 2022; Revised: 27 July 2022; Accepted: 4 August 2022

Abstract
To understand adaptive bone remodeling in response to external mechanical stimuli, researchers have elucidated the mechanisms of mechanosensing and mechanoresponse through *in vitro* experiments targeting subcellular components from molecules to organelles. Such subcellular experiments have been performed by applying mechanical stimuli to mechanosensitive components and by measuring and observing the dynamic behaviors of the mechanosensitive and mechanoresponsive components. For a better understanding of the importance of the subcellular experiments, this article reviews the recent subcellular experiments for osteoblasts and osteocytes. First, we introduce the tools used for the stimulation and measurement/observation, and we discuss how these tools have contributed to the elucidation of the mechanisms. Second, we shed light on how the findings on the behaviors of the subcellular components have enhanced our basic understanding of the underlying mechanisms. Furthermore, we present future perspectives for subcellular experiments. To do this, we discuss the utilization of microscopes with higher spatial resolution and discuss focus points for a clearer understanding of these mechanisms in osteocytes. Future experiments will reveal how osteoblasts and osteocytes sense and respond to external mechanical stimuli in their surrounding environment in bone, and how cellular behaviors finally lead to the regulation of bone resorption and formation in adaptive bone remodeling.

Keywords: Osteoblasts, Osteocytes, Mechanosensing, Mechanoresponse, Cell biomechanics

1. Introduction

Bone structures continuously adapt to the mechanical stimuli in their surrounding environment (Christen, et al., 2014; Lambers, et al., 2015). Structural adaptations result from bone remodeling caused by cellular activity. On the trabecular and osteonal surfaces in cancellous and cortical bone, mechanically stimulated osteoblasts promote the secretion of extracellular matrix proteins, such as collagen (Kaspar, et al., 2000; Matsugaki, et al., 2013), leading to increased bone formation. In the bone matrix, mechanically stimulated osteocytes are postulated to send signals to the neighboring cells on bone surfaces, osteoclast- (Nakashima, et al., 2011; Kulkarni, et al., 2012; Hao, et al., 2017) and osteoblast- (Taylor, et al., 2007; Bakker, et al., 2014; Hao, et al., 2017) lineage cells, to regulate their activities, leading to changes in bone resorption and formation. Such functional cellular responses to mechanical stimuli are called mechanoresponses and are initiated by mechanosensing (Vogel, et al., 2006). Therefore, elucidation of mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes is essential for a fundamental understanding of bone adaptation through remodeling.

To clarify these mechanisms, it is important to investigate the dynamic behaviors of mechanosensitive and

Paper No.22-00267
J-STAGE Advance Publication date : 26 August, 2022
[DOI: 10.1299/jbse.22-00267]

mechanoresponsive subcellular components, such as molecules, their complexes, and organelles. In the cells under exposure to mechanical stimuli, these components dynamically form and change their structures and shapes, translocate to specific regions, and interact with other subcellular components. For instance, under fluid shear stress, Src molecules translocate from the cytoplasm to the perinulear/nuclear regions in osteocytes (Hum, et al., 2014), focal adhesions are formed in osteoblasts (Serrano, et al., 2018), and the primary cilia deflect at the apical cell surface in osteoblasts and osteocytes (Malone, et al., 2007). Such behaviors of subcellular components have been investigated *in vitro* using subcellular experiments, in which their complex behaviors and the direct links between them have been successfully revealed.

In subcellular experiments, various tools have been used to apply mechanical stimuli to mechanosensitive subcellular components and to measure and observe the behaviors of mechanosensitive and mechanoresponsive subcellular components. In this article, we introduce the tools utilized for stimulation, measurement, and observation, discuss how the tools have contributed to research clarifying the mechanisms, and shed light on how the findings of the behaviors of the components have enhanced our current understanding of the mechanisms in osteoblasts and osteocytes. In addition, to further tackle the unclarified mechanisms, we discuss the utilization of microscopes with higher spatial resolution and discuss future focus points to understand the mechanisms in osteocytes.

2. Tools utilized in subcellular experiments

In subcellular experiments for osteoblasts and osteocytes, various tools (Fig. 1) have been utilized mainly for two purposes: to apply mechanical stimuli to mechanosensitive subcellular components and to measure and observe the subsequent dynamic behaviors of mechanosensitive and mechanoresponsive subcellular components.

For the first purpose, tools for mechanical stimulation have been developed and utilized to target subcellular components throughout the cell (roughly targeted) and those localized in their specific regions (precisely targeted), as reviewed in Section 2.1. Devices to control fluid flow and substrate stretch/rotation have been utilized in roughly targeting of various subcellular components. Magnetic tweezers have been utilized as precise targeting tools for focal adhesions, and the Stokesian fluid stimulus probe (SFSP) has been used for focal adhesions linked to mechanically activated channels.

For the second purpose, tools for measurement and observation have been developed based on the principles of mechanics and optics, as reviewed in Section 2.2. Atomic force microscopy (AFM) has been used as a mechanics-based measurement tool to investigate force transmission from glycocalyx to cytoskeletons and to evaluate the formation of focal adhesions. As observation tools based on optics, fluorescent microscopes have been utilized in combination with a variety of molecular probes for various components, including those that exhibit unique behaviors in osteocytes.

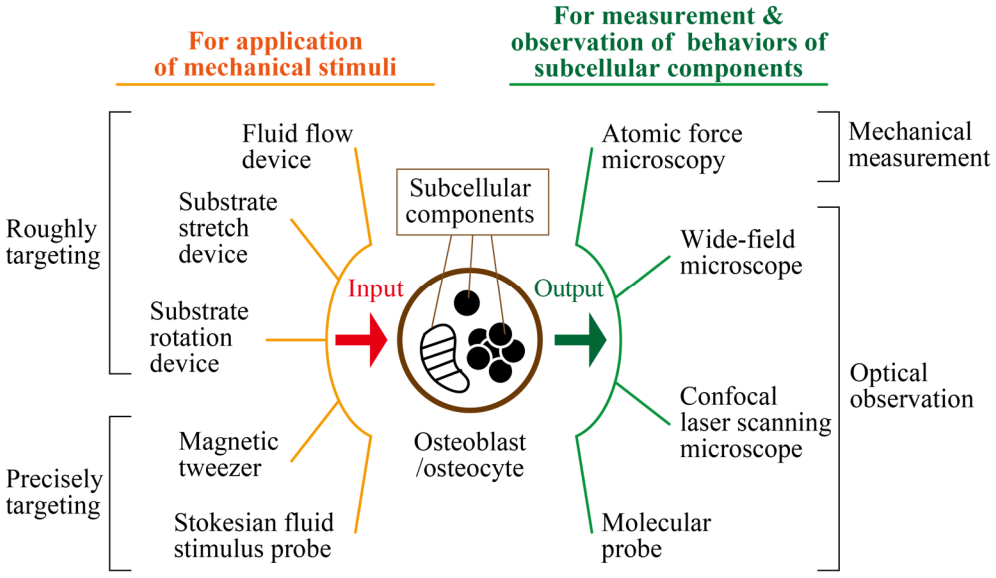

Fig. 1 Tools used in subcellular experiments for osteoblasts and osteocytes.

[DOI: 10.1299/jbse.22-00267]

In this chapter, we explain the tools that have been utilized for stimulation and measurement/observation in subcellular experiments. Moreover, we discuss the contributions of these tools to the research clarifying the mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes.

2.1 Tools for mechanical stimulation

Under mechanical loading, osteoblasts on the bone surface and osteocytes in lacuna-canalicular space in bone matrix encounter fluid shear stress (Wittkowske, et al., 2016). To mimic the fluid flow, the most utilized tool for mechanical stimulation is a type of fluid flow device in which fluid flow driven by a pump or injector is input from the tip of a syringe, pipet, or tube into a chamber. In the chambers of such devices, cells are subjected to fluid shear stress at 0.03-4 Pa in steady (Rangaswami, et al., 2010; Kou, et al., 2011; Batra, et al., 2012, 2014; Baik, et al., 2013; Roy, et al., 2014; Gardinier, et al., 2014; Bin, et al., 2016; Brown, et al., 2016; Morrell, et al., 2018; Yoneda, et al., 2019; Ding, et al., 2019; Sato, et al., 2020; Monteiro, et al., 2021) and cyclic (Baik, et al., 2010, 2013; Kamel, et al., 2010; Young, et al., 2010; Espinha, et al., 2014; Hum, et al., 2014; Roy, et al., 2014; Lee, et al., 2015; Seref-Ferlengez, et al., 2016; Zhang, et al., 2018) manners. Shear stress has succeeded in inducing the behaviors of various subcellular components in osteoblasts and osteocytes, including signaling molecules, molecular channels, cytoskeletons, primary cilia, and vesicles.

Another fluid flow device, referred to as a multifunctional fluid flow device (Lyons, et al., 2016), was designed to expose cells in a single well of cell-culture plate to fluid flow driven by a pump through an inlet port situated in the center of the well. Using this device, cytoplasmic inflow of calcium ions via activated transient receptor potential (TRP) V4 cation channels was stimulated at a shear stress of 0.4 or 1.6 Pa in osteocytic cells, which was required for microtubule-dependent sclerostin downregulation (Lyons, et al., 2017). For the simulation of orbital fluid flow, a shaker was utilized at a shear stress of 0.75 Pa, causing activation of the signaling molecule zinc finger protein of the cerebellum 1 (Zic1) and the consequential induction of Wnt/TCF1 signaling in osteocytic cells (Kalogeropoulos, et al., 2010). The shaker was also capable of simulating oscillating fluid flow, in which primary cilia became shortened in osteoblastic cells (Delaine-Smith, et al., 2014). Shear stress can also be simulated using more basic tools. For example, a pipet was utilized to generate impact on osteocytic cells via dropping fluid through an air-filled environment, in which shear stress with a peak value of 0.1-14 Pa resulted in the opening of connexin hemichannels on the cell bodies (Burra, et al., 2010).

Substrate stretching devices have been utilized to mimic strain of the extracellular bone matrix, in which cells are subjected to stretching at 1-12 % strain driven by a vacuum pump, motor, or weight. Using such devices, cyclic stretching has been applied in a number of studies (Peng, et al., 2012; Matsugaki, et al., 2013; Wang, et al., 2014; Gao, et al., 2016; Li, et al., 2018; Serrano, et al., 2018; Sasaki, et al., 2020; Wang, et al., 2021) to mimic *in vivo* mechanical loading and continuous stretch/compression (Hoshi, et al., 2014; Dalagiorgou, et al., 2017) in a few studies. Stretch-derived mechanical stimuli, which are transmitted via focal adhesions, elicit responses of focal adhesions, molecular channels, collagen, and signaling molecules, such as runt-related transcription factor 2 (Runx2), in osteoblasts and osteocytes. In addition, to mimic changes in gravitational force, such as during space flight, substrate rotation devices have been utilized, in which clinostats (Wei, et al., 2013; Sun, et al., 2015), centrifuges (Zhou, et al., 2015; Woodcock, et al., 2019) and rotators (Shi, et al., 2017, 2020; Xu, et al., 2017; Ding, et al., 2020) have stimulated connexin, actin filaments, microtubules, and primary cilia through exposure to microgravity and hypergravity.

Taken together, fluid flow and substrate stretch/rotation devices have succeeded in inducing the mechanoresponsive behaviors of various subcellular components in a whole cell, which has significantly contributed to clarifying several mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes. However, when the roughly targeting tools are used, experimenters cannot avoid simultaneous mechanical stimulation to multiple subcellular components which initiate multiple downstream signaling cascades, making it difficult to understand in detail the individual mechanisms of cellular mechanosensing and mechanoresponse. To overcome this, we should extensively use precise targeting tools to independently stimulate the components of interest to fully characterize their activated signaling cascades.

To precisely target the subcellular components, magnetic tweezers have been used (Alenghat, et al., 2000; Nakao, et al., 2021), in which an electromagnetic force is generated from the tip of a metal rod coiled with wire. To clarify the mechanism of osteocyte apoptosis caused by mechanical overload, the tweezer generated cyclic mechanical stimuli of 500 pN or 1000 pN to focal adhesions via fibronectin-coated magnetic microbeads (diameter: 4.5 μm) adhered to the cell surface in isolated osteocytes (Nakao, et al., 2021). The stimulation levels of 500 and 1000 pN mimicked normal and

[DOI: 10.1299/jbse.22-00267]

over-loading, respectively. At 1000 pN, produced nitric oxide triggered initial apoptotic cell shrinkage, which is thought to cause bone resorption. These processes did not occur at 500 pN. In addition, a Stokesian fluid stimulus probe (SFSP) has been utilized, in which a physiological level (< 10 pN) of fluid flow is generated from a micropipette with a 0.8 μm tip diameter (Wu, et al., 2011; Thi, et al., 2013). In osteocytic mechanosensitive processes (Adachi, et al., 2009), the fluid flow targeting individual focal adhesions can induce cytoplasmic inflow of calcium ions (Thi, et al., 2013), presumably through the activation of molecular channels.

Tools for precise targeting, such as the magnetic tweezer and SFSP must be utilized at the physiological stimulus magnitude for the target components. In the above studies, the calibration of force-distance relationships and the calculation of quasi-steady Stokes flow-derived forces are important to accurately mimic *in vivo* mechanical stimuli applied to subcellular components.

2.2 Tools for measurement and observation

Atomic force microscopy (AFM) has been used to measure mechanical behaviors of molecules and their complexes on the cell surface. When a force is applied to the components at the AFM probe tip, it is detected by the deflection of the cantilever through changes in the reflection of a laser light beam (Franz, et al., 2008). In mechanical measurements using AFM, the height of the probe, whose tip is modified with antibodies or ligands of the targets, is controlled to pull these targets. This procedure, called tensile-testing, results in the acquisition of force-extension curves, in which molecular force rupture events have been analyzed, as shown in osteoblastic cells (Caneva Soumetz, et al., 2010; Taubenberger, et al., 2010; Marcotti, et al., 2018). Analysis focusing on the slope of the curves preceding the ruptures revealed anchors of glycocalyx to cytoskeletons, which helped clarify the transmission mechanisms of mechanical signals mediated by the glycocalyx (Marcotti, et al., 2018). In addition, by focusing on temporal changes in the slope of the initial curves, the formation of nascent focal adhesions was evaluated, which supports a proposed mechanism of adhesion maturation through enhancement of the force transmission from the adhesions to the actin cytoskeleton (Nakao, et al., 2019). Taken together, the AFM measurements have enabled the investigation of force transmission mechanisms on the bone cell surface through analyses of the force-extension curves.

To optically observe the behaviors of subcellular components, fluorescent microscopes, conventional wide-field and confocal laser scanning microscopes, have been utilized in a number of studies for osteoblasts and osteocytes. In most studies, the observation is performed two-dimensionally in optical sections of a cell, which can lead to an insufficient understanding of how components behave in a three-dimensional/vertical-directional manner. This problem has been overcome in the following studies.

To observe the three-dimensional mechanical behaviors of cytoskeletons in osteocytic cells, a custom-designed dual-microscope system was designed (Baik, et al., 2010, 2013). The microscopes in this system captured the bottom- and lateral-side images of actin filaments and microtubules, leading to the characterization of their complex tensile, compressive, and shear strain under fluid flow for the first time.

In addition, confocal laser scanning microscope has been used to observe how signaling molecules translocate across an optical section of primary cilia in osteocytic cells. This microscope can detect fluorescent light from a thin focal plane by setting a pinhole in the confocal plane (St. Croix, et al., 2005). This made it possible to observe that the ratio of anterograde/retrograde molecular transport in the primary cilia changed under microgravity, which will help reveal the mechanism of reduced osteocyte mechanosensitivity (Ding, et al., 2020).

In combination with fluorescent microscopes, molecular probes have been widely used to label and visualize the dynamic behaviors of subcellular components. In particular, fluorescence resonance energy transfer (FRET)-based probes have been used to visualize conformational changes in molecules. This visualization is realized by the principle in which the excitation energy provided to a fluorescent molecule (donor) excites another fluorescent molecule (acceptor) that is in close proximity to the donor (Pietraszewska-Bogiel, et al., 2011). In osteocytic cells under fluid flow, FRET probes targeting Src revealed nuclear Src activity, which contributed to a better understanding of the mechanism that suppresses the anabolic response of bone subjected to mechanical loading (Hum, et al., 2014). In addition, FRET probes targeting endoplasmic reticulum (ER) molecules have revealed the unique dynamics of calcium ions in ERs (Brown, et al., 2016). Similar FRET probes targeting ciliary molecules have revealed that primary cilia form distinct calcium ion microdomains (Lee, et al., 2015).

In addition to FRET probes, molecular rotors have also been used to measure viscosity. These fluorescent molecule

[DOI: 10.1299/jbse.22-00267]

probes are designed based on the principle that surrounding viscosity affects their level of fluorescence through the rotation rates of meso-phenylene rings (Bahaidarah, et al., 2014). This type of probe revealed that changes in membrane viscosity is an adaptive response to dynamic changes in the gravitational force on osteoblastic cells (Woodcock, et al., 2019).

Notably, the utilization of microscopes in combination with molecular probes has succeeded in revealing various behaviors of subcellular components unique to osteocytes.

3. The behaviors of subcellular components to mechanical stimuli

In subcellular experiments for osteoblasts and osteocytes, the following subcellular components have been investigated: molecular channels and signaling molecules at the molecular scale, focal adhesions and cytoskeletons at the molecular complex scale, and primary cilia and vesicles at the organelle scale (Fig. 2).

Behaviors of these components in the cells to mechanical stimuli are briefly described below. At the molecular level (Sec. 3.1), molecular channels (Pei, et al., 2021) open to allow small signaling molecules to pass through. Downstream signaling molecules localize to the nucleus to control gene expression governing cell signals and fates. At the molecular complex level (Sec. 3.2), focal adhesions (Marie, et al., 2014) form their own structures and interact with other molecules, such as molecular channels. Cytoskeletons (Gould, et al., 2021) undergo changes in their own structures and shapes. At the organelle level (Sec. 3.3), primary cilia (Hoey, et al., 2012) deflect, activate surface molecules, and change cilium length. Vesicles form their structures, translocate from the cytoplasm to the extracellular space, and release signaling molecules.

In this chapter, we shed light on how the findings of such behavior of components at the three scales have enhanced a basic understanding of mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes.

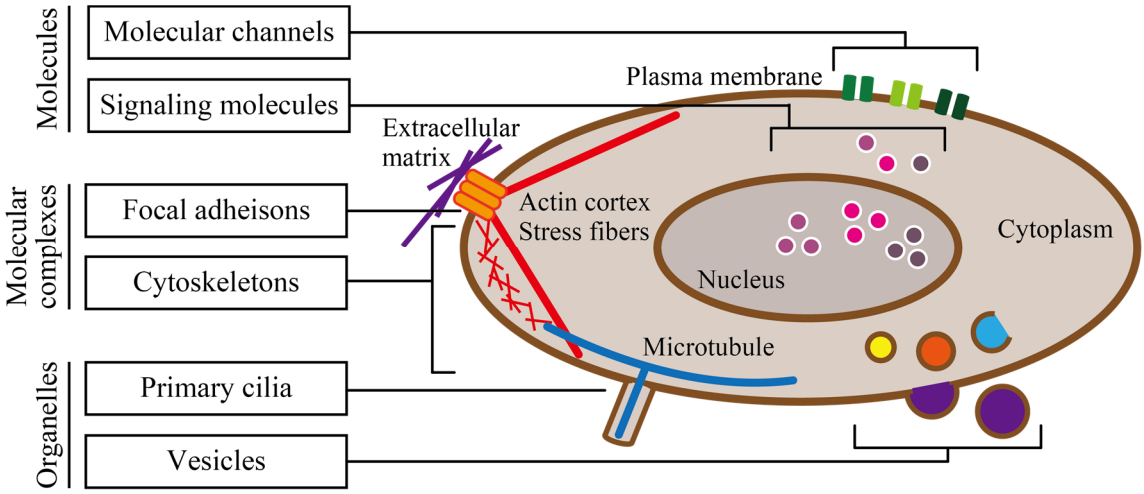

Fig. 2 Subcellular components at scales from molecules to organelles in osteoblasts and osteocytes.

3.1 Molecules

3.1.1 Molecular channels

In osteoblasts, the opening of several types of TRP channels is ensured through the inflow of calcium ions into the cytoplasm. TRPV4 opens in MC3T3-E1 osteoblastic cells in response to fluid flow, which has been suggested to modify osteoblast proliferation (Yoneda, et al., 2019). In osteoblasts, TRPM3 and TRPV4 open in response to hypotonic pressure, which regulates nuclear factor-kappa B ligand (RANKL) and nuclear factor of activated T cells (NFAT) expression leading to the differentiation and proliferation of bone cells (Son, et al., 2018). Furthermore, TRPM7 opens and subsequently induces local calcium oscillations in MG63 osteoblast-like cells in response to fluid flow, and this channel

[DOI: 10.1299/jbse.22-00267]

activity is modulated by the association of TRPM7 with lipid raft domains (Roy, et al., 2014).

In addition to TRP channels, the behaviors of the following channels were investigated in osteoblastic cells. Polycystin1 consisting of an ion channel undergoes cleavage of its C-terminal cytoplasmic tail (CT) in response to substrate stretching. The cleaved CT interacts with JAK2 to induce its activation, resulting in the progression of osteoblast differentiation through Runx2 (Dalagiorgou, et al., 2017). Cav1.2, an L-type calcium channel which is found to be abundant on the cell surface of MC3T3-E1 cells under normal gravity, was found to be decreased by microgravity (Sun, et al., 2015). P2X7R, a purinergic receptor, has been suggested to undergo caveolae-mediated endocytosis in unstimulated MC3T3-E1 cells (Gangadharan, et al., 2015), however, the effects of mechanical stimuli on this endocytosis require further study.

In osteocytes, connexins form a hemichannel for small signaling molecules such as ATP and prostaglandin E to pass through. In MLO-Y4 cells, connexin 43 undergoes reduced translocation from the Golgi apparatus to the cell surface under weightless environment (Xu, et al., 2017). Connexin 43 has also been shown to translocate from the cytoplasm to the cell processes of cells cultured in a gel matrix in response to movement of a titanium plate (Takemura, et al., 2019). This translocation can be important for the modulation of ATP/prostaglandin E signaling levels and for the activation of other signaling pathways. Connexin 43 hemichannels open on the cell body in MLO-Y4 osteocyte-like cells through fluid flow stimulation to both the cell body and dendrite processes (Burra, et al., 2010). This channel opening depends on the presence of glycocalyx on the dendrite processes, providing evidence that the processes are mechanosensitive. Pannexins also form a hemichannel, and a functional complex of the pannexin 1 channel with the P2X7R channel contributes to ATP release from MLO-Y4 cells under fluid flow (Seref-Ferlengez, et al., 2016).

Several calcium channels have also been identified as components of the mechanosensing and mechanotransduction pathways. The opening of TRPV4 results in a decrease in sclerostin, a negative regulator of bone formation, in OCY 454 osteocyte-like cells in response to fluid flow (Lyons, et al., 2017). Similarly, Piezo1 opening is linked to a decrease in sclerostin in IDG-SW3 osteocytic cells under substrate stretching (Sasaki, et al., 2020). T-type voltage-sensitive channels open and regulate ER calcium dynamics in synchronization with intracellular calcium oscillations in MLO-Y4 cells under fluid flow (Brown, et al., 2016). One of the T-type channels, Cav3.2, has been suggested to open to the downstream ATP release in MLO-Y4 cells in response to membrane stretch by hypo-osmotic swelling, leading to bone formation (Thompson, et al., 2011). This ATP release can be enhanced by the association of Cav3.2 with α2δ1 subunits.

In osteoblasts and osteocytes, various types of molecular channels play important roles in regulating the influx/release of signaling molecules. The molecular mechanisms which cause channel opening can be understood through further study of the translocation of these channels and their interaction with other molecules, as shown in the above studies.

3.1.2 Signaling molecules

NF-κB and β-catenin are important mechanoresponsive signaling molecules involved in the regulation of bone formation in osteoblasts and osteocytes. This regulation is mediated by nuclear translocation of these molecules (Kamel, et al., 2010; Young, et al., 2010). Such behaviors of signaling molecules have been reported as follows.

In subcellular experiments with osteoblasts, signaling molecules defined as mechano-growth factors (MGF) localize to the nucleus. MGF receptors may also localize to the nucleus in primary osteoblasts under substrate stretch, which leads to osteoblast proliferation for bone repair (Peng, et al., 2012). A transcription factor called NFATc1 enters the nucleus of MC3T3-E1 cells in response to fluid flow. NFATc1 also induces ERK5 activation, resulting in ERK5 translocation to the nucleus, which promotes osteoblast proliferation (Ding, et al., 2019). Nuclear translocation of ERK5 was also reported in another study (Bin, et al., 2016). According to this study, ERK5 causes the activation of a transcription factor called forkhead box O 3a (FoxO3a) in MC3T3-E1 cells in response to fluid flow, which prevents FoxO3a from translocating to the nucleus and furthermore contributes to a down-regulation of FasL and Bim expression to prevent TNF-α-induced osteoblast apoptosis. Cofilin, which causes the up-regulation of osteogenesis-associated genes, translocates to the nucleus in MG-63 cells under substrate stretch, which may transport cofilin-bound actin into the nucleus (Gao, et al., 2016). An increase in cofilin level in the nucleus of MC3T3-E1 cells under fluid flow (Gardinier, et al., 2014) can regulate the formation of actin stress fibers.

In subcellular experiments with osteocytic cells, histone deacetylases (HDAC) 4 and 5, which are regulators of sclerostin expression, translocate to the nucleus in MLO-Y4 cells under fluid flow (Sato, et al., 2020). This nuclear

[DOI: 10.1299/jbse.22-00267]

translocation is conducted through a signaling pathway involving HDAC phosphorylation by focal adhesion kinase (FAK). In OCY454 cells under fluid flow, Smad2/3 translocate to the nucleus in transforming growth factor (TGF) β signaling pathway, which is associated with bone resorption and formation activities of bone cells (Monteiro, et al., 2021). Zic1, a transcription factor, accumulates in the nuclei of MLO-Y4 cells under fluid flow. Zic1 was suggested to act as a link between mechanosensing and Wnt signaling, perhaps together with ciliary-related proteins Gli1 and Gli3 (Kalogeropoulos, et al., 2010).

The above findings have revealed the importance of signaling molecules in the nucleus in osteoblast and osteocyte mechanoresponses crucial for the regulation of bone remodeling.

3.2 Molecular complexes

3.2.1 Focal adhesions

Subcellular experiments with MC3T3-E1 cells yielded the following findings. Integrins that can bind fibronectin, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$, form nascent focal adhesions in a talin-dependent manner under local compressive force, which could be an important process for adhesion maturation (Nakao, et al., 2019). The signaling molecules PKGII, Src, and SHP-2 are recruited to mechanosomes containing vinculin and integrin β_3 under fluid flow, in which Src activation is promoted for osteoblast proliferation and survival (Rangaswami, et al., 2010).

The behaviors of integrin α_5 and $\alpha_V\beta_3$ have been investigated in MLO-Y4 cells. Under fluid flow, interaction of integrin α_5 with connexin 43 via the scaffold molecule 14-3-3θ is promoted, and this molecular complex is delivered from the Golgi to the plasma membrane for assembly (Batra, et al., 2014). The coupling between integrin α_5 and connexin 43 enhances the hemichannel opening caused by fluid flow, in which integrin $\alpha_5\beta_1$ is activated through PI3K signaling. This process leads to the passage of small bone anabolic factors, such as prostaglandin E_2, which are essential for bone formation and remodeling. (Batra, et al., 2012). Integrin α_5 has been suggested to form strong integrin attachments through coupling with glycocalyx on the cell processes, probably serving as a transmitter of mechanical signals to the cell body, leading to the opening of hemichannels (Burra, et al., 2010). Integrin $\alpha_V\beta_3$ interacts with ion channels in cell processes under fluid flow, inducing calcium ion influx (Thi, et al., 2013). This calcium influx can be caused by P2X7R and Cav3.2, which were reported to co-localize with integrin β_3 (Cabahug-Zuckerman, et al., 2018).

The above studies have enabled us to understand the importance of the interaction of integrins with glycocalyx, calcium channels, hemi-channel molecules, and signaling molecules when considering the mechanoresponse mechanisms of osteoblasts and osteocytes.

3.2.2 Cytoskeletons

In osteoblasts, cytoskeletons in microgravity environments have been reported to undergo a reduction of their structures, as follows. Actin filaments undergo a decrease in the thickness of their cortical structure and internal stress fibers in primary osteoblasts, leading to the acceleration of bone loss through a decrease in osteoblast cellular integrity (Nabavi, et al., 2011). Microtubules undergo a decrease in their curvature and length in primary osteoblasts (Nabavi, et al., 2011), and in branching complexity and maximum branch length in cultured osteoblasts, leading to a reduction in osteoblastic differentiation and mineralization (Shi, et al., 2020). A spindle that normally segregates chromosomes during mitosis is stimulated to form multi-polarized microtubular structures in several types of osteoblastic cells and primary osteoblasts, thereby inhibiting cell proliferation (Wei, et al., 2013).

In osteocytes, actin filaments undergo contractions synchronized with oscillated intracellular calcium dynamics by cyclic compression of the bone (Morrell, et al., 2018). This contraction is observed in MLO-Y4 cells subjected to fluid flow and is thought to be linked to the secretion of extracellular vesicles containing signaling molecules. During the contraction of actin filaments under cyclic fluid flow, their strains are generated heterogeneously in subcellular regions, such as the leading edge to fluid flow (Baik, et al., 2010), indicating the presence of high-sensitivity regions in osteocyte mechanosensing. These strains display an oscillatory strain profile more often than microtubules and have a higher peak-to-trough strain magnitude, suggesting more significant roles of actin filaments in osteocyte mechanosensing (Baik, et al., 2013). However, microtubules have been shown to play a significant role in the regulation of sclerostin expression through downstream NOX2/TRPV4/CaMKII signaling in Ocy454 cells in response to fluid flow (Lyons, et al., 2017).

[DOI: 10.1299/jbse.22-00267]

Actin filaments and microtubules interact closely in generation of mechanical strains. The disruption of microtubules causes actin normal strains to decrease, but actin disruption has little effect on microtubular strain (Baik, et al., 2010).

Short actin filaments are cross-linked by cytoskeletal proteins called as spectrins. This protein is organized as a porous network beneath the plasma membrane of MLO-Y4 cells (Wu, et al., 2017). This spectrin network was suggested to interact with calcium ion channels, nitric oxide synthase (NOS), and connexin 43, which makes it a potential therapeutic target for bone disorders.

Taken together, the above experiments have contributed to the understanding of the detailed structure and shape changes of actin filaments and microtubules in osteoblasts and osteocytes in response to mechanical stimuli. Cytoskeletal proteins, such as spectrin, are thought to be pivotal to understanding the mechanical behaviors of cytoskeletons.

3.3 Organelles

3.3.1 Primary cilia

Primary cilia are required for osteoblastic prostaglandin E_2 release, alkaline phosphatase (ALP) production, and calcification in response to mechanical stimuli (Delaine-Smith, et al., 2014; Shi, et al., 2017). Such mechanoresponses could be regulated by adjusting ciliary mechanosensitivity based on cilia length. Primary cilia become shorter and there were fewer cilia per cell after exposure to fluid flow when compared to static conditions in MLO-A5 cells under fluid flow (Delaine-Smith, et al., 2014) and in isolated osteoblasts under microgravity (Shi, et al., 2017, 2020). This ciliary shortening could be related to cytoplasmic microtubules, because microtubular attachments at the base of the cilium are also reduced under microgravity (Shi, et al., 2020).

The microtubular attachments are enhanced after exposure to fluid flow in MLO-Y4 cells, which was thought to change primary cilia mechanics (Espinha, et al., 2014). The shortening of primary cilia was also observed in MLO-Y4 cells under simulated microgravity (Ding, et al., 2020). Relations of the cilia shortening to the microtubular attachment remain unclarified. However, the cilia shortening was shown to have relation to altering of their internal molecular transport; intraflagellar transport (IFT) particles are transported more frequently from the cilium base towards tip under simulated microgravity than under normal gravity conditions (Ding, et al., 2020). Such changes in the direction of molecular transport could affect osteocyte mechanoresponsiveness through the arrangement of mechanosensitive and mechanoresponsive molecules in cilia.

In osteoblasts and osteocytes, deflection of primary cilia in response to fluid flow (Malone, et al., 2007) can activate mechanosensitive molecules on the surface of the cilia. The following molecules are known to localize in the cilia. Parathyroid hormone 1 receptor (PTH1R), which leads to pro-survival and osteogenic actions, is localized in the cilia of MC3T3-E1 and MLO-Y4 cells (Martín-Guerrero, et al., 2020). PTH1R is activated in response to mechanical stimuli, in both a ligand-independent and -dependent manner to induce osteocytic pro-survival signaling (Maycas, et al., 2015). Adenylyl cyclase (AC) 6, an enzyme that catalyzes the conversion of cAMP from ATP, is also localized and induces up-regulation of Cox-2 gene expression in a cilium- and AC6-dependent manner in MLO-Y4 cells under fluid flow (Kwon, et al., 2010). Furthermore, in MLO-Y4 cells, polycystin-2, TRPV4, and Piezo1, which are ion channels, are localized in cilia. TRPV4 mediates the formation of a Ca^{2+} microdomain dependent on Ca^{2+} entry. This calcium influx leads to an increase in Cox-2 that induces the formation of prostaglandin E_2 (Lee, et al., 2015).

Thus, the following mechanisms can be understood: primary cilia in osteoblasts and osteocytes deflect in response to fluid flow and then presumably activate various mechanosensitive ciliary molecules, leading to downstream molecular signaling. Mechanoresponsive changes in cilia length over time may adjust their mechanosensitivity.

3.3.2 Vesicles

Mechanoresponsive vesicular behaviors in osteoblasts and osteocytes have been investigated as follows. Membrane fusion in vesicular exocytosis processes promotes the repair of mechanically disrupted plasma membranes in a Ca^{2+}/PLC/PKC-dependent manner in osteoblasts (Mikolajewicz, et al., 2018). ATP-containing vesicles are acute mediators of ATP release in MLO-Y4 cells under fluid flow (Kringelbach, et al., 2015), suggesting that the vesicle or its content is released in early mechanoresponsive purinergic signaling pathways in osteocytes.

LAMP1 (lysosomal-associated membrane protein)-positive vesicles were found in osteoblasts and osteocytes in bone

[DOI: 10.1299/jbse.22-00267]

(Solberg, et al., 2015). In these vesicles, RANKL and osteoprotegerin (OPG) are co-localized. RANKL and OPG enhance and suppress osteoclastogenesis, respectively. LAMP1-positive extracellular vesicles in MLO-Y4 cells are released from the cell surface when exposed to fluid flow (Morrell, et al., 2018). These vesicles contain RANKL, OPG, and sclerostin and the total amount of extraction of each molecule increases by the stimulation, suggesting a regulatory mechanism of bone formation and resorption through delivering these vesicles to neighboring osteoblasts and osteoclasts. Furthermore, autophagosomes are formed in MLO-Y4 cells under fluid flow, which enhances ATP release and suppresses non-apoptotic cell death (Zhang, et al., 2018).

As shown in the above studies on osteoblasts and osteocytes, various vesicular behavior (formation, translocation, content release, and membrane fusion) have pivotal roles in cell intactness, fate, and intercellular molecular signaling.

4. Future perspectives

In this chapter, we delineate future perspectives on subcellular experiments for osteoblasts and osteocytes. To do this, we discuss the utilization of microscopes with higher spatial resolutions, which will allow us to observe more detailed behavioral features of subcellular components. In addition, we discuss pertinent focus points for further understanding of the mechanosensing and mechanoresponse mechanisms in osteocytes regulating bone resorption and formation.

4.1 Microscopes with higher spatial resolution

In a number of subcellular experiments for osteoblasts and osteocytes, wide-field and confocal microscopes have been utilized to observe the behaviors of various subcellular components, as described in Sec. 2.2. These microscopes have limited spatial resolutions (approximately several hundred nanometers). For imaging at higher spatial resolution, we propose utilization of the following microscopes in subcellular experiments: total internal reflection fluorescence microscope (TIRFM) and super-resolution microscopes (SRMs).

4.1.1 Total internal reflection fluorescence microscope (TIRFM)

TIRFM is designed to improve the z-resolution based on the principle that an electromagnetic field generated by the total internal reflection of an excitation light penetrates approximately 100 nm into a sample adjacent to its interface with the substrate (Mattheyses, et al., 2010). TIRFM has been utilized to visualize the co-localization of caveolin-1 and Factor XIIIA, which is involved in regulating matrix secretion and deposition, in mature osteoblasts differentiated from MC3T3-E1 cells (Wang, et al., 2015), and the co-localization of plastin 3 with adhesion molecules and actin structure in U2OS and OCY454 cells (Schwebach, et al., 2020). Behavior of such components near the cell membrane in response to mechanical stimuli will be observed with a low fluorescence background by utilizing a TIRFM.

4.1.2 Super-resolution microscopes (SRMs)

SRMs such as the structured illumination microscope (SIM) and the stochastic optical reconstruction microscope (STORM) can overcome the x-y resolution limit of earlier imaging techniques. To increase the resolution, SIM is equipped with illumination that generates light interference patterns (Hirvonen, et al., 2009), and STORM is equipped with illumination that activates only a fraction of fluorophores at a time (Rust, et al., 2006). SIM has been used to observe the co-localization of P2X7R with caveolin-1 within caveolae (60–100 nm membrane invaginations) in MC3T3-E1 cells (Gangadharan, et al., 2015), the association of mitochondria with microtubules along with interconnected cell processes in MLO-Y4 cells (Gao, et al., 2019), and clustering of channel molecules within less than 90 nm of integrin in osteocytes in bone sections (Cabahug-Zuckerman, et al., 2018). This channel clustering was also observed in MLO-Y4 cells by STORM (Cabahug-Zuckerman, et al., 2018). The ability to discern such behavior features of subcellular components in close proximity to one another using these microscopes will be instrumental in the future subcellular experiments on osteoblasts and osteocytes.

[DOI: 10.1299/jbse.22-00267]

4.2 Focus points for further understanding of mechanosensing and mechanoresponse mechanisms in osteocytes

Osteocytes play a pivotal role in mechanically triggered bone resorption and formation by regulating bone cellular activities through mechanosensing and mechanoresponse. The detailed mechanisms of mechanosensing and mechanoresponse have not been fully clarified, especially because of their unique and complex extracellular environments and intracellular structures (Fig. 3). Therefore, here, we focus on the extracellular environments and intracellular structures and discuss these two points for further understanding of the mechanisms.

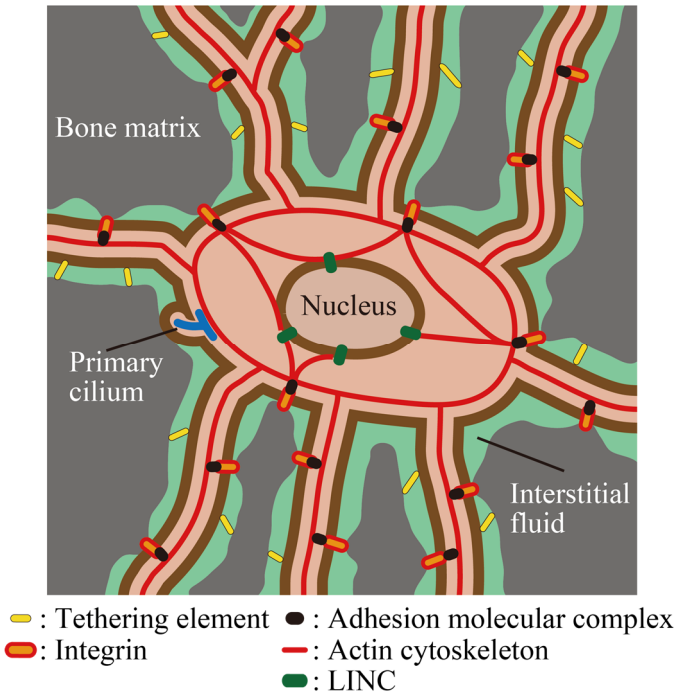

Fig. 3 Extracellular environments and intracellular structures of osteocytes.

4.2.1 Extracellular environments

Osteocytes are embedded in a rigid bone matrix (Fig. 3). The gap size between the osteocyte cell membrane and the surrounding extracellular bone matrix is thought to be shorter than the *in vitro* typical length of primary cilia (approximately 4 μm) (Spasic, et al., 2017). Deflection of the primary cilium produces its membrane strain that triggers the opening of ion channels. However, the above discussion suggests that *in vivo* deflection is physically constrained by the surrounding bone matrix. Thus, ciliary mechanosensing in osteocytes in the bone matrix should be investigated. The constraint of deflection of primary cilium can be mimicked by manipulating microneedles, which will enable us to investigate physiological effects of the cilium on cellular mechanoresponses in response to fluid flow.

The osteocytes cell processes are located in the canaliculi. The canalicular wall and osteocyte process membranes are locally connected by tethering elements (TEs) (Fig. 3), possibly consisting of proteoglycan (You, et al., 2004). The TEs have been suggested to cause local strain concentration in the osteocyte processes via interstitial fluid flow (Yokoyama, et al., 2021), which can trigger the opening of ion channels. Thus, to investigate the contribution of TEs to mechanosensing and mechanoresponse in osteocytes, it is necessary to identify the precise location of TEs along with the osteocyte processes and the molecular entity between the TEs and the channels. The local strain concentration caused by TEs can be mimicked by pulling membrane receptors using AFM cantilever tip that is coated with the ligands. Besides the mimicking, such usage of AFM will enable us to measure force applied to the receptors during local calcium response.

[DOI: 10.1299/jbse.22-00267]

4.2.2 Intracellular structures

Throughout the cell interior, the actin cytoskeletal structure that connects focal adhesions on the cell membrane to the LINCs on the nuclear envelope plays an essential role in transmitting force to the cell nucleus (Donnaloja, et al., 2019) (Fig. 3). The transmitted force can enhance nuclear translocation of signaling molecules by expanding the nuclear pores and furthermore regulate the successive expression of bone resorption/formation-related molecules. Thus, to clarify the mechanism of force transmission from the cell membrane to the nuclear envelope, it is important to reveal the cytoskeletal structure which is three-dimensionally formed, by using microscopes with high x-y and z resolutions. Furthermore, it is important to observe cytoskeletal mechanical behaviors under mechanical force.

The cell processes in osteocytes are known to be highly sensitive to mechanical stimuli (Adachi, et al., 2009). In the cell process, the distance between the cell membrane and bundled actin filaments is relatively small (approximately 25 nm) (Han, et al., 2004; You, et al., 2004). This distance is shorter than the length of adhesion molecular complexes that connect integrins to actin filaments (approximately 100 nm) (Liu, et al., 2015). This suggests that the adhesion molecular complexes in the osteocyte cell processes have a unique structure, which may contribute to the enhanced sensitivity to external mechanical forces. Thus, identifying this unique structure will help us to understand the mechanism of intracellular structure-dependent mechanosensing and mechanoresponse in osteocytic processes. To observe the structure of adhesion molecular complexes in the cell processes, microscopes with nm-scale spatial resolution can be used in combination with FRET sensors that visualize the binding states of adhesion molecules.

5. Conclusions

Mechanosensing and mechanoresponse mechanisms in osteoblasts and osteocytes are key to understanding adaptive bone remodeling. These mechanisms have been elucidated through subcellular experiments. In this article, we reviewed the experimental tools used in recent subcellular experiments on osteoblasts and osteocytes. Tools to apply mechanical stimulation to subcellular components have been utilized with mimicking interstitial fluid flow, bone matrix deformation, and gravitational changes. In addition, tools for the measurement and observation of the dynamic behaviors of the subcellular components have been utilized at scales ranging from molecules to organelles. We also reviewed the measured and observed data to enhance the fundamental understanding of the mechanisms by focusing on the behaviors of molecular channels, signaling molecules, focal adhesions, cytoskeletons, primary cilia, and vesicles. In future experiments, microscopes with higher spatial resolution will enable us to observe more detailed behaviors of the subcellular components in osteoblasts and osteocytes. In addition, investigations focusing on the extracellular environments and intracellular structures of osteocytes will lead to a more comprehensive understanding of their mechanisms.

Future experiments should provide answers to the following questions on the mechanisms in osteoblasts and osteocytes:1) how the components sense the external mechanical stimuli exerted by the bone environment, 2) how these stimuli cause downstream mechanoresponses through the coupling among the intracellular components, and thereby 3) how the components contribute to regulating bone resorption/formation in response to the stimuli.

Acknowledgments

This work was partially supported by a Grant-in-Aid for JSPS fellows (18J23041) and a Grant-in-Aid for Scientific Research (A) (20H00659) from JSPS, Japan. We are grateful to Yoshitaka Kameo and Koichiro Maki for giving us constructive discussion.

Conflict of Interest

There is no conflict of interest.

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[DOI: 10.1299/jbse.22-00267]