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Dynamic Fluid Flow Induced Mechanobiological Modulation of *In Situ* Osteocytic Calcium Oscillations

Minyi Hu, PhD¹, Guowei Tian, PhD², Daniel E. Gibbons¹, Jian Jiao, MS¹, and Yi-Xian Qin, PhD^{1,*}

¹Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY 11794-5281

²CMIC-Two Photon Imaging Center, Stony Brook University, Stony Brook, NY 11794-5281

Abstract

Distribution of intramedullary pressure (ImP) induced bone fluid flow (BFF) has been suggested to influence the magnitude of mechanotransductory signals within bone. As osteocytes have been suggested as major mechanosensors in bone network, it is still unclear how osteocytes embedded within a mineralized bone matrix respond to the external mechanical stimuli derived from direct coupling of dynamic fluid flow stimulation (DFFS). While *in vitro* osteocytes show unique Ca²⁺ oscillations to fluid shear, the objective of this study was to use a confocal imaging technique to visualize and quantify Ca²⁺ responses in osteocytes *in situ* under DFFS into the marrow cavity of an intact *ex vivo* mouse femur. This study provided significant technical development for evaluating mechanotransduction mechanism in bone cell response by separation of mechanical strain and fluid flow factors using intramedullary pressure stimulation, provides the ability for true real-time imaging and monitoring of bone cell activities during stimulation. Loading frequency dependent Ca²⁺ oscillations in osteocytes indicated the optimized loading at 10Hz, where such induced response was significantly diminished via blockage of the Wnt/β-catenin signaling pathway. The results provided a pilot finding of the potential crosstalk or interaction between Wnt/β-catenin signaling and Ca²⁺ influx signaling of *in situ* osteocytes in response to mechanical signals. Findings from the present study make a valuable tool to investigate how *in situ* osteocytes respond and transduce mechanical signals, e.g. DFFS, as a central mechanosensor.

Keywords

osteocyte; mechanotransduction; calcium signaling; Wnt signaling; bone fluid flow; calcium homeostasis

*Corresponding Author: Yi-Xian Qin, Ph.D., Dept. of Biomedical Engineering, Stony Brook University, Bioengineering Bldg., Rm 215, Stony Brook, NY 11794-5281, Phone: 631-632-1481, Fax: 631-632-8577, Yi-Xian.Qin@stonybrook.edu.

Disclosure

All authors claim no conflicts of interest.

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Introduction

Biophysical stimuli derived from mechanical loading are proven essentials for bone tissue modeling, remodeling and regeneration. Changes in the pressure or velocity of bone fluid flow (BFF) act as a communication medium that connects external loading signals and internal cellular activities in bone, which ultimately regulate the bone remodeling process [1–6]. On the other hand, discontinuation of BFF can result in higher bone loss, leading to conditions such as osteopenia [7–10]. Intramedullary pressure (ImP) is one of the influential factors for BFF and osteogenic signals within bone, which eventually influences bone growth [8].

In vivo studies on turkey ulna and mouse femur models have demonstrated the independent effect of ImP on inductions of potent osteogenic and adaptive responses in bone [8, 11]. Demonstrating in a functional disuse rat model, oscillatory electrical muscle stimulation (MS) was able to induce non-linear ImP and bone strain to mitigate disuse bone loss [12–14]. Adaptation of skeletal nutrient vasculature was also found to be interrelated to ImP alteration [15]. More recently, our group has developed a dynamic hydraulic stimulation (DHS) that meant to directly couple an externally compressive load with internal BFF, which was able to non-invasively distinguish the anabolic role of the ImP factor and the bone deformation factor of BFF in an *in vivo* setting, as well as to establish the translational potential of ImP. As shown in a 4-week hindlimb suspension (HLS) rat study, DHS was able to mitigate disuse trabecular [16] and cortical bone loss [17]. In addition, direct measurements of ImP and bone strain via an operated *in vivo* study showed that DHS generated local ImP that acted independently from simultaneous bone strain. Moreover, the generated ImP was found to fall in a non-linear interrelationship with DHS loading frequencies, and yet in a directly proportional interrelationship with DHS loading magnitudes. Altogether, DHS was suggested as a novel and non-invasive method to isolate the ImP and bone strain factors in an *in vivo* rodent model [18, 19].

Elucidating downstream cellular and molecular effects of BFF and its potential underlying mechanisms to enhance bone quality has gained strong research interests. Subsequent *in vivo* studies of DHS further demonstrated the potential functional process of DHS-derived mechanical signals in bone metabolism. A longitudinal *in vivo* study using HLS rats was designed to evaluate mesenchymal stem cell (MSC) populations within the bone marrow in response to daily DHS loading over a course of 21-day [20]. In addition, alterations in gene expressions of osteogenic growth factors and transcription factors in response to DHS as a function of time was also evaluated [17]. A strong time-dependent manner of inductions of bone marrow MSCs as well as osteogenic gene expressions was observed in both studies.

As the most abundant cells in bone, osteocytes' critical role has been shown by targeted ablation of them, which led to altered bone modeling/remodeling with defective mechanotransduction [21]. Therefore, osteocyte mechanotransduction has been gaining significant amount of research attention for its great clinical potential in diseases involving dysfunctional bone remodeling, such as osteoporosis. While their cell bodies embedded within the fluid-filled mineralized bone matrix, the cell processes of osteocytes contact each other and possibly other cell types, allowing small signaling molecules to be transported

between cells. BFF into the osteocyte canaliculi also triggers this cell-cell communication. This essential network acts as the central mechanosensor and aids in regulating bone modeling/remodeling and coordinating the adaptation of bone to the mechanical stimuli applied to the skeleton through BFF [22–24].

External mechanical stimuli promote bone formation via activation of intracellular signaling pathways that converge with growth factors to express osteogenic transcription factors. Bone cells perceive such external signals that trigger numerous intracellular responses including the release of prostaglandin E2 (PGE2) into the lacunar-canalicular fluid, where binding of PGE2 to its receptors (EP2 and/or EP4) leads to inhibition of GSK-3 β and intracellular accumulation of free β -catenin. Nuclear translocation of β -catenin alters the expressions of a number of key target genes including the reduced expressions of sclerostin (Sost) and Dickkopf-related protein 1 (Dkk-1) and the increased expression of Wnt. After all, these changes facilitate the binding of Wnt to LRP5-Fz and amplify the load signal [25]. Recognized as an important regulator of bone mass and bone cell functions, Wnt/ β -catenin signaling pathway may transmit the mechanical signals sensed by osteocytes to bone surface [26, 27]. Moreover, potential crosstalk between Wnt/ β -catenin and prostaglandin signaling pathways in response to loading may also reduce the expressions of Sost and Dkk-1 [27, 28].

In response to external physical signals, escalation of intracellular Ca²⁺ has been observed as one of the earliest biochemical events in bone cells [29]. In change of mechanical environment, cellular functions may be regulated by triggered biochemical signaling cascades in response to changes in the upstream intracellular Ca²⁺ concentration [30]. Furthermore, in response to *in vitro* and *in situ* mechanical stimulations, osteocytes seem to be more sensitive than osteoblasts, in terms of Ca²⁺ oscillations. Recent studies have observed the real-time Ca²⁺ oscillations in response to fluid shear on *ex vivo* bone segments, as well as a direct mechanical stimulation on an intact *ex vivo* mouse tibia [31–33]. However, these studies either investigated the effect of fluid shear on osteocytic Ca²⁺ oscillations only on *ex vivo* bone segment surfaces that were not yet adapted for dynamic mechanical events within bone, or studied the effect of osteocytic Ca²⁺ oscillations of intact mouse long bones deformable bone loading that the recording was done only at the resting period with time delay. In addition, the Ca²⁺ oscillation pattern of an intact *ex vivo* mouse long bone has not been studied with a direct dynamic fluid flow stimulation (DFFS) into the marrow cavity that is highly related to the ImP/BFF mechanism. Therefore, the objective of the study was to provide a novel, non-bone-deforming experimental approach to real-time monitor the effect of DFFS into the bone marrow cavity of a fresh, intact mouse long bone on *in situ* osteocytic Ca²⁺ oscillations. Furthermore, the role of Wnt/ β -catenin signaling pathway in the induced Ca²⁺ oscillations was also evaluated.

Materials and Methods

Preparation of mouse femur samples

All animal protocols were approved by Stony Brook University IACUC. Female C57BL/6J mice of 3-month-old (Jackson Laboratory, Bar Harbor, ME, United States) were used to obtain fresh, intact femur samples immediately after euthanasia by CO₂ inhalation. The experimental groups were 1) 1Hz DFFS, 2) 5Hz DFFS, 3) 10Hz DFFS, 4) 20Hz DFFS, 5)

Wnt/ β -catenin inhibition + 10Hz DFFS (n=5 per group). The surrounding soft tissues of the femur samples were gently removed, maintained with periosteum. The bone samples were then incubated in DMEM with 5% FBS and 1% penicillin/streptomycin. A hole was drilled into the marrow cavity of each bone sample from the distal end using a 24-gauge micro drill bit.

Inhibition of Wnt/ β -catenin signaling pathway and Ca^{2+} fluorescent staining

ICG-001 (Selleck Chemicals, United States and Europe) was used to antagonize Wnt/ β -catenin-TCF-mediated transcription. For Group 5 (Wnt/ β -catenin inhibition + 10Hz), the femur samples were incubated in 25 μM of ICG-001 in culture medium for 1 hour at room temperature. Meanwhile, the samples of the other groups (1Hz, 5Hz, 10 Hz and 20Hz of DFFS) were incubated in culture medium for 1 hour at room temperature.

For Ca^{2+} fluorescent labeling, the bone samples were incubated for 2 hours in 15 μM Fluo-8 AM (Santa Cruz Biotechnology, Inc., Dallas, Texas, U.S.A.) dissolved in dimethyl sulfoxide (DMSO) and culture medium before confocal imaging [31].

DFFS Loading

A custom-made experimental setup was built for imaging *in situ* osteocyte Ca^{2+} response in mouse femurs (Figure 1). For DFFS, the drilled hole of each femur sample was tightly sealed with a 24-gauge catheter that was connected to a water-filled syringe pump. The syringe pump was controlled by a function generator that set the loading frequency and amplitude. DFFS with loading frequencies of 1 Hz, 5 Hz, 10 Hz, and 20 Hz and a constant loading magnitude of 1 V was applied to the bone samples for 10 sec of baseline - 30 sec of loading - 10 sec of post-loading. Real-time confocal imaging (Zeiss LSM 510 META NLO Two-Photon Laser Scanning Confocal Microscope System) with 40X objective, 488-nm laser excitation, and 2 frames/sec (512x512 pixel images) was performed to capture the Ca^{2+} signals of the osteocytes within each bone that was subjected to DFFS.

Intramedullary pressure (ImP) measurements

To quantify DFFS-induced ImP within the marrow cavity in response to various loading frequencies, a separate set of experiments were performed on five long bone samples from 3-month-old female C57BL/6J mice to directly measure the ImP values during DFFS. A 24-gauge micro drill bit was used to drill a hole into the marrow cavity from the distal end of each bone sample. The drilled hole of each femur sample was then tightly sealed with a 24-gauge catheter that was linked to a water-filled 3-way catheter connecting to a syringe pump loader and a micro-cardiovascular pressure transducer (Millar Instruments, SPR-524, Houston, TX). The loading parameters of the syringe pump was controlled by a function generator that set a constant voltage of 1 V and a spectrum of frequencies of 0 Hz (baseline), 0.1 Hz, 0.5 Hz, 1 Hz, 2 Hz, 3 Hz, 5 Hz, 7 Hz, 10 Hz, 15 Hz, 20 Hz, and 25 Hz for 1 min per frequency point. Real-time measurements of the DFFS-induced ImP values over the range of loading frequencies were recorded.

Data Analysis

Data are shown as mean $\pm$ SD. To analyze the confocal imaging data, the fluorescent intensity of the cell bodies was extracted as a function of time using the microscope software (LSM Image Browser). By normalization to the baseline intensity, the percentage of response cells, first Ca^{2+} spike magnitude, and the time to initiate the first spike were quantified. GraphPad Prism 4 Software (GraphPad Software Inc., La Jolla, CA) was used to perform one-way ANOVA with Tukey's *post hoc* test to determine the statistical significance. The effect of ICG-001 was compared against the 10 Hz group; therefore, Bonferroni's *post hoc* test was performed.

To analyze the ImP measurement data, each run of the recorded data was separated into the respective frequency steps; and then each step was analyzed individually through a Fast Fourier Transform (FFT) using MatLab. Each frequency step was divided into twenty intervals, where maximum and minimum values were averaged and identified as the peak-to-peak ImP values for the corresponding loading frequency. The calculated peak-to-peak ImP values were then normalized to the corresponding baseline values and reported as mean $\pm$ SD. The effects of treatments were evaluated using a one-way ANOVA with Tukey's *post-hoc* test using GraphPad Prism 4 Software.

Results

The *ex vivo* Ca^{2+} imaging model at baseline

At baseline level, *in situ* osteocytes stained with Fluo-8 AM Ca^{2+} indicator were imaged using a confocal microscope at a focal plane of $\sim 40\mu\text{m}$ below the periosteal surface. The layer of mineralized bone matrix enclosing many lacunae-like osteocytes was observed in the differential interference contrast (DIC) images. Fluorescent stained cell bodies of the embedded osteocytes were also observed (Figure 2).

In situ osteocytes displayed Ca^{2+} oscillations in response to DFFS

Real-time fluorescent images of *in situ* osteocytes were captured to record the Ca^{2+} oscillations in response to DFFS. Representative traces of Ca^{2+} responses of single osteocytes from each experimental group are shown in Figure 3. The Ca^{2+} traces from each measurement were calibrated by normalization of the fluorescent intensity to the corresponded baseline. DFFS at 1Hz did not induce any Ca^{2+} response within the osteocytes. However, osteocytes responded to DFFS at other tested loading frequencies with Ca^{2+} signaling in the form of multiple Ca^{2+} spikes. DFFS loading at 5 Hz, 10 Hz, and 20 Hz of loading led to 25% ($p < 0.001$ vs. 10Hz), 93% ($p < 0.001$ vs. 1Hz; $p < 0.001$ vs. 5Hz), and 52% ($p < 0.05$ vs. 1Hz) of responsive cells, respectively (Figure 4). Results of the number of Ca^{2+} spikes showed that DFFS loading at 5 Hz, 10 Hz, and 20 Hz led to 1.4 ($p < 0.05$ vs. 10 Hz; $p < 0.01$ vs. 20 Hz), 2.6 ($p < 0.05$ vs. 5 Hz), and 3.0 ($p < 0.01$ vs. 5 Hz) spikes, respectively (Figure 5). Results of normalized spike magnitude showed that DFFS loading at 10 Hz led to 5% and 7% greater Ca^{2+} spike magnitudes compared to 5 Hz ($p < 0.05$ vs. 10Hz) and 20 Hz ($p < 0.01$ vs. 5Hz) of the loading, respectively (Figure 6). Although not statistically significant, DFFS loading at 10 Hz exhibited about 27% shorter and 3.9% longer Ca^{2+} initiation time compared to 5Hz and 20Hz of the loading, respectively (Figure 7).

Wnt/ β -catenin signaling pathway contributed to DFFS induced *in situ* osteocyte Ca^{2+} oscillations

Data showed significantly reduced osteocyte Ca^{2+} responses when Wnt/ β -catenin signaling pathway was blocked by ICG-001 (Figure 4 – 7). The ICG-001 group was subjected to 10Hz DFFS loading, to be compared against the 10Hz group as control. Upon Wnt/ β -catenin signaling blockage, the percentage of responsive cells was reduced by 83% compared to control ($p < 0.001$; Figure 4). Obstruction of this pathway also reduced the number of Ca^{2+} spikes by 12% compared to the control, although it was not statistically significant (Figure 5). A significant decrease in Ca^{2+} spike magnitude ($p < 0.05$; Figure 6) and an increase in the time needed to elicit the first observed Ca^{2+} spike by 19% ($p > 0.05$; Figure 7), compared to the control, were also observed upon the ICG-001 treatment.

ImP was unchanged over a range of DFFS loading frequencies

Oscillatory DFFS into the *ex vivo* mouse long bones, loaded at various frequencies, generated minimal fluid pressures in the marrow cavities. The peak-to-peak ImP values measured at each frequency point during the DFFS loading was normalized to the ImP baseline level; and then the normalized ImP values were plotted and shown in Figure 8. The data of the normalized ImP against frequency was observed as a linear trend with values in the order of 1.0 ± 0.02 at 0.1 Hz, 0.98 ± 0.02 at 0.5 Hz, 0.99 ± 0.06 at 1 Hz, 1.01 ± 0.07 at 2 Hz, 1.02 ± 0.08 at 3 Hz, 1.03 ± 0.11 at 5 Hz, 1.04 ± 0.13 at 7 Hz, 1.05 ± 0.14 at 10 Hz, 1.01 ± 0.15 at 15 Hz, 1.01 ± 0.17 at 20 Hz, and 1.03 ± 0.15 at 25 Hz. No difference among any of the paired groups was detected ($p > 0.05$), indicating that the ImP within the loaded long bones was unchanged over the range of DFFS loading frequencies from 0.1 Hz to 25 Hz.

Discussion

Advanced experimental designs and imaging techniques in the present study allowed us to visualize and measure, for the first time, how *in situ* osteocytes responded to DFFS in terms of intracellular Ca^{2+} oscillations at various loading frequencies. An interesting trend of non-linear frequency dependent manner in such responses was observed during the real-time image recording. Specifically, DFFS at 10 Hz elicited unique and robust Ca^{2+} spikes in the osteocytes of an intact mouse long bone, which meant to work upstream of signaling cascades that may ultimately regulate specific cellular functions. Moreover, blockage of Wnt/ β -catenin signaling pathway significantly diminished the load-induced Ca^{2+} oscillations, which suggested the crucial role of Wnt/ β -catenin signaling pathway in osteocytes' responses to mechanical signals as well as its potential crosstalk with the Ca^{2+} signaling. Findings from the present study make a valuable tool to investigate how *in situ* osteocytes in the lacunar canaliculi network respond and transduce mechanical signals, e.g. DFFS, as a central mechanosensor.

Mechanotransduction of osteocytes in calvarial bone fragments via a micro-needle displacement or surface fluid shear have been investigated [32, 33]. Recent research has also shown the real-time Ca^{2+} oscillations in response to a direct mechanical stimulation on an intact *ex vivo* mouse tibia [31]. However, the Ca^{2+} oscillation pattern has not been identified through fluid flow alone mechanism, in the absence of mechanical strain, until this study,

which can be achieved with a direct DFFS into the marrow cavity of an intact *ex vivo* mouse long bone to generate fluid shear. Although mechanobiological signals have been recognized as the key regulators for bone cell and tissue responses to loading, it is remained with a challenging research question whether the response is primarily generated by bone strain and/or induced fluid flow. Under normal loading condition, these two factors cannot be separated, which impacts the design of potential clinical interventions. Compared to the existing studies in the literature on *ex vivo* osteocyte Ca^{2+} oscillation response to mechanical loading, our study provides an innovative and unique method to directly deliver a dynamic fluid loading into an intact *ex vivo* mouse long bone in the absence of bone deformation and strain, which is closely related to BFF and fluid shear stress mechanism. This provides a novel approach to completely separate the mechanical strain and fluid flow factors for bone adaptation in response to mechanical loading. The method in this study, via regulation of ImP, overcomes a major challenge in the field, in which using a non-deformative method to elicit osteocyte Ca^{2+} oscillation response in an intact *ex vivo* bone. This developed method can be used for the *in vivo* regulation as well, which opens a new way to further advance the field by this specific loading mechanism. In addition, one may recognize that current method to monitor osteocytic Ca^{2+} oscillations requires a delayed time for deformed bone to go back to resting status, otherwise it would lose focal point of the imaged area. The recent study done by Jing et al. observed osteocyte Ca^{2+} oscillations in response to an axial loading on an intact *ex vivo* mouse tibia [31]. Unfortunately, the osteocytic Ca^{2+} oscillations of the intact mouse long bone subjected to this direct mechanical stimulations were not able to be observed at real-time due to the recovery of the bone deformation caused by the loading. Because the axial loading actually caused bone deformation, which led to the microscope focus shifting, the imaging process had to stop periodically during the loading to be able to capture clear images at a delayed time. Using a direct fluid flow loading, our loading method completely eliminated the error generated by the bone strain/deformation factor. Thus, we were able to capture the images at true real-time continuously. This allowed us to observe the spontaneous Ca^{2+} oscillation response of osteocytes to mechanical loading. Furthermore, this *in situ* experimental model provides a unique platform to apply mechanical signals to the embedded osteocytes within clinically relevant load-bearing skeletal sites, e.g. femur, which may provide great insights into the underlying mechanisms of skeletal mechanotransduction.

In the present study, we discovered direct evidences that, in response to DFFS, the number of responsive osteocytes, Ca^{2+} spike magnitudes and initiation time were highly dependent on the loading frequencies. Direct measurements of ImP during DFFS over a range of loading frequencies (0.1 Hz to 25 Hz) clearly indicated that the fluid pressure within the marrow cavity was unchanged and independent in correspondence to the DFFS loading frequencies. Therefore, the osteocyte Ca^{2+} oscillations upon DFFS was closely related to the loading rate. The *in situ* osteocytes were not only sensitive to DFFS, but also presumably modulated their immediate biochemical responses according to the loading frequencies that ultimately regulated the downstream cellular functions and bone phenotypic responses. Osteocytes then are suggested to be the first gating guard in regulating bone remodeling/modeling via their responses to different mechanical signaling parameters, such as frequency in the present study.

Intense research on Wnt signaling pathways in bone biology have gained strong interests over a decade, which include their critical role in skeletal development, bone quantity maintenance, and potential therapeutic development for regenerative medicine. Wnt signaling pathways with subtle alterations in the intensity, amplitude, location, and duration would significantly affect skeletal development, as well as bone remodeling and regeneration [46]. Central focuses of the related research fall on how Wnt/Lrp5 signaling regulates osteoblasts and osteocytes, the roles of Wnt signaling players in bone development and osteoclastogenesis, translational studies for clinical therapeutics, and diagnostics inhibition of Wnt pathway antagonists [46]. However, the crosstalk between Wnt/ β -catenin signaling with other mechanically induced osteogenic signaling events remains unknown. Specifically, the role of Wnt/ β -catenin signaling on osteocyte Ca^{2+} oscillations, which may ultimately explain the underlying mechanism of how Wnt/ β -catenin affects osteoblastogenesis, is worth the investigation (Figure 9). The present study was the first time to observe reduced osteocyte Ca^{2+} oscillation responses when Wnt/ β -catenin signaling pathway was blocked, which led to a suggestion that Wnt/ β -catenin signaling may play a major role in osteocyte Ca^{2+} oscillations in response to DFFS. The role of Wnt/ β -catenin signaling on osteocyte Ca^{2+} oscillations may ultimately explain the underlying mechanism of how Wnt/ β -catenin affects osteoblastogenesis. Although the exact mechanism has not been fully discovered, the impressive results from our present study provided a pilot finding of the potential crosstalk or inter-dependence between Wnt/ β -catenin signaling and Ca^{2+} influx signaling of *in situ* osteocytes in response to mechanical signals.

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long bone. Using an advanced approach in skeletal mechanobiology, real-time observation of the Ca^{2+} signals clearly indicated the instant response of *in situ* osteocytes to the mechanical signals derived from DFFS. This study provides advances for our better understanding on bone mechanotransduction, which grants great insights into future clinical applications.

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Abbreviations List

BFF	Bone Fluid Flow
DFFS	Dynamic Fluid Flow Stimulation
DHS	Dynamic Hydraulic Stimulation
Dkk-1	Dickkopf-related protein 1
DMSO	Dimethyl Sulfoxide
FFT	Fast Fourier Transform
HLS	Hindlimb Suspension
ImP	Intramedullary Pressure
MSC	Mesenchymal Stem Cell
PGE2	Prostaglandin E2
Sost	Sclerostin

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Highlights

- Dynamic fluid flow stimulation induced Ca^{2+} oscillations in osteocytes *in situ*.
- Induced Ca^{2+} oscillations were loading frequency dependent and optimized at 10Hz.
- Blockage of Wnt/ β -catenin signaling diminished the load induced Ca^{2+} oscillations.

Figure 1.

Schematic representation of the experimental setup of an *ex vivo* mouse femur under DFFS and Ca^{2+} imaging. A drilled hole into the marrow cavity was tightly sealed with a catheter that was connected to a water-filled syringe pump. The syringe pump was controlled by a function generator that set the loading frequency and amplitude. Real-time confocal imaging was performed to capture the Ca^{2+} signals of the embedded osteocytes within the bone that was subjected to DFFS.

Figure 2.

Confocal fluorescence (left) and DIC (right) images of osteocytes within an *ex vivo* mouse femur stained with Fluo-8 AM Ca^{2+} indicator. At baseline level, stained osteocytes were imaged at a focal plane $\sim 40\mu\text{m}$ below the periosteal surface. The layer of mineralized bone matrix enclosing many osteocytes was observed.

Figure 3.

Representative Ca^{2+} traces of osteocytes in an intact mouse femur in response to DFFS at various loading frequencies. DFFS with loading frequencies of 1Hz, 5Hz, 10Hz, and 20Hz was applied to *ex vivo* bone samples for 10sec of baseline - 30sec of loading - 10sec of post-loading.

Figure 4.

Responsive percentage (%) of osteocyte Ca^{2+} responses to DFFS at various frequencies. DFFS at 1Hz did not induce any Ca^{2+} response within the osteocytes. However, 5Hz, 10Hz, and 20Hz of loading lead to 25%, 93%, and 52% of responsive cells, respectively. The responsive percentage of the ICG-001 treated group was 83% lower compared to the 10Hz group as control. * $p < 0.001$ vs. 1Hz; # $p < 0.05$ vs. 5Hz; ^a $p < 0.001$ vs. ICG-001; ^b $p < 0.05$ vs. 1Hz.

Figure 5.

Number of Ca^{2+} spikes of osteocytes' responses to DFFS at various frequencies. Loading at 20Hz elicited the highest number of Ca^{2+} spikes, compared to other loading frequencies. ICG-001 treated group showed 12% less Ca^{2+} spikes compared to the 10Hz group as control. # $p < 0.05$ vs. 5Hz; * $p < 0.01$ vs. 5Hz.

Figure 6. Peak Ca^{2+} magnitude normalized to the baseline value of osteocytes in response to DFFS at various loading frequencies. DFFS at 10 Hz led to a higher Ca^{2+} spike magnitude compared to 5Hz and 20Hz of loading. ICG-001 treated group decreased the peak magnitude normalized to the baseline value by 7% compared to 10 Hz DFFS loading as control. * $p < 0.05$ vs. 10Hz.

Figure 7.

Ca^{2+} spike initiation time (sec) of osteocytes in response to DFFS at various loading frequencies. DFFS loading at 10 Hz exhibited shorter and longer Ca^{2+} spike initiation time compared to 5Hz and 20Hz of loading, respectively. ICG-001 treated group showed 19% longer Ca^{2+} spike initiation time compared to 10 Hz DFFS loading as control.

Figure 8.

Graph shows mean $\pm$ SD values of the normalized ImP measurements to the baseline level.

In the loading frequency spectrum from 0.1Hz to 25Hz, the normalized ImP against frequency was observed as a linear trend with minimum changes. No difference was detected among any of the paired groups ($p > 0.05$).

Figure 9.

Bone cells respond to mechanical stimuli that trigger downstream molecular responses including the release of PGE2. Binding of PGE2 to its receptors (EP2 and/or EP4) inhibits GSK-3 β and accumulates intracellular β -catenin. Nuclear translocation of β -catenin reduces gene expressions in Sost and Dkk-1 and increased gene expression of Wnt. These net changes facilitate the binding of Wnt to LRP5-Fz and amplify the load signal.