

Evaluation of initiating characteristics of osteoblastic calcium signaling responses to stretch by video rate time-course observation

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Abstract

Osteoblasts change their intracellular calcium ion concentration in response to mechanical stimuli. Although it has been reported that osteoblasts sense and respond to stretching of a substrate on which osteoblastic cells have adhered, the details of the dynamic characteristics of their calcium signaling response remain unclear. Motion artifacts such as loss of focus during stretch application make it difficult to conduct precise time-course observations of calcium signaling responses. Therefore, in this study, we observed intracellular calcium signaling responses to stretch in a single osteoblastic cell by video rate temporal resolution. Our originally developed cell-stretching microdevice enables *in situ* observation of a stretched cell without excessive motion artifacts such as focus drift. Residual minor effects of motion artifacts were corrected by the fluorescence ratiometric method with fluorescent calcium indicator Fluo 8H and fluorescent cytoplasm dye calcein red-orange. We succeeded to detect the intracellular calcium signaling response to stretch by video rate temporal resolution. The results revealed a time lag from stretch application to initiation of the intracellular calcium signaling response. We compared two time lags measured at two different cell areas: central and peripheral regions of the cell. The time lag in the central region of the cell was shorter than that in the peripheral region. This result suggests that the osteoblastic calcium signaling response to stretching stimuli initiates around the central region of the cell.

Key words: Cell biomechanics, Mechanotransduction, Osteoblasts, Calcium signaling, Stretch stimuli

1. Introduction

It is widely accepted that bone tissue remodels adaptively to the surrounding mechanical environment (Duncan and Turner, 1995). This adaptive remodeling is based on the activities of bone cells including osteoblasts, osteoclasts, and osteocytes. They sense mechanical stimuli and change their bone metabolic activities. Osteocytes are considered as a mechanosensor, and many studies have reported that they sense and respond to mechanical stimuli (Genetos et al., 2007, Adachi et al., 2009a, Adachi et al., 2009b). In addition, osteoblasts are considered to sense bone surface strain caused by external mechanical loading. It has been reported that osteoblasts show a variety of responses to mechanical stimuli such as fluid shear flow (Jacobs, et al., 1998, Genetos, et al., 2005, Jing, et al., 2013), hydrostatic pressure (Roelofsen, et al., 1995, Rath, et al., 2008), direct indentation by a microneedle (Adachi, et al., 2003, Sato, et al., 2007), and substrate stretch strain (Kaspar, et al., 2002, Matsugaki, et al., 2013).

Intracellular calcium ions play an important role as second messengers in signaling cascades (Berridge, et al., 1998). Dynamic spatiotemporal patterns of intracellular calcium signals regulate various cellular functions (Bootman, et al., 2001, Berridge, et al., 2003). Although many studies have reported that osteoblasts increase the intracellular calcium ion

concentration ($[Ca^{2+}]_i$) in response to mechanical stimuli (Jacobs, et al., 1998, Chen, et al., 2000, You, et al., 2001, Adachi, et al., 2003, Tanaka, 2012, Sato, et al., 2015), the details of the initiating dynamic characteristics of the intracellular calcium signaling response remains unclear. In particular, few studies have conducted precise time course observations and evaluation of osteoblastic responses to substrate stretching stimuli. Substrate stretching causes non-negligible motion artifacts during observation, such as loss of focus and moving out of the field of view. Therefore, these motion artifacts impede precise *in situ* time course observations of $[Ca^{2+}]_i$ dynamics under stretch application.

We previously developed an original cell-stretching microdevice to reduce motion artifacts during stretch application (Sato, et al., 2010). In this study, we combined our cell-stretching microdevice with the fluorescence ratiometric method (Lipp and Niggli, 1993) to conduct *in situ* time course observations of $[Ca^{2+}]_i$ under substrate stretch by video rate temporal resolution. The objective of this study was to evaluate the initiating dynamic characteristics of the osteoblastic intracellular calcium signaling response to substrate stretching stimuli through *in situ* video rate time-course observation.

2. Materials and Methods

2.1 Cell culture and fluorescence labeling

The mouse osteoblastic cell line MC3T3-E1 was provided by RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. Cells were cultured in α -minimum essential medium containing 10% fetal bovine serum and maintained in at 37°C with 5% CO₂. Prior to the experiment, cells were seeded on a fibronectin-coated, 35-mm glass bottom dish with built-in cell-stretching microdevices at a density of approximately 5×10^4 cells/dish. After 18 hours of pre-incubation for sufficient cell adhesion, fluorescent Ca²⁺ indicator Fluo 8H and fluorescent cell-permeant dye calcein red-orange were loaded into the cells. Hank's balanced salt solution (HBSS) containing 3 μ M Fluo 8H-AM (AAT bioquest), 1 μ M calcein red-orange (Thermo Fisher), and 0.1% Pluronic F-127 (Life Technologies) was used as a loading buffer. Fluo 8H increases its fluorescent intensity as the Ca²⁺ concentration increases. Conversely, the fluorescent intensity of calcein red-orange is independent of Ca²⁺ concentration. The fluorescent intensity of Fluo 8H is changed by both the influence of $[Ca^{2+}]_i$ and motion artifacts during stretch application. In contrast, the fluorescent intensity of calcein red-orange is changed by the influence of motion artifacts only. Therefore, to calculate the ratio of fluorescent intensities (Fluo 8H:calcein red-orange), we evaluated the change in Ca²⁺ concentration without the influence of motion artifacts due to rigid displacement during stretch application. Normal culture medium was replaced with the loading buffer, and the cells were cultured for 30 minutes in the CO₂ incubator to load the fluorescent indicator dyes into the cells. After dye loading, the cells were rinsed twice with Dulbecco's phosphate-buffered saline and used for fluorescence image acquisition in HBSS (recording buffer).

2.2 Cell-stretching microdevice and stretch conditions

In the present study, the cell-stretching microdevice was slightly different from the previously reported device (Sato, et al., 2010). Figure 1 shows the arrangement of the microdevices on a glass coverslip. Six microdevices were fabricated on a 22-mm² glass coverslip, and the glass coverslip was attached to a 35-mm dish with a ϕ 18 mm hole at the bottom. Each microdevice consisted of one pair of arms fabricated from photoresist SU-8 and a cell-stretching sheet fabricated from a transparent silicone elastomer. Figure 2 shows a microscopic image of the cell-stretching microdevice. Two metal needles were set on the arms. The needle on right side was held by a micromanipulator mounted on the microscope stage to fix one end of the stretching sheet. The needle on the left side was held by the micromanipulator via a piezo electric actuator (MC-140L, Mess-tek) to apply stretch to the other end of the stretching sheet. In the present study, the target strain magnitude was set to 10% and the strain rate was a constant 5% / second. Although we precisely controlled displacement of the metal needle to apply stretch to the stretching sheet using the piezo actuator, the actual strain magnitude on the stretching sheet did not match the target magnitude because of backlash between the needle tip and the device arm. Therefore, we measured the actual nominal strain on the stretching sheet in the time-lapse transmitted image in which we could observe deformation behavior of the stretching sheet.

2.3 Image acquisition and analysis

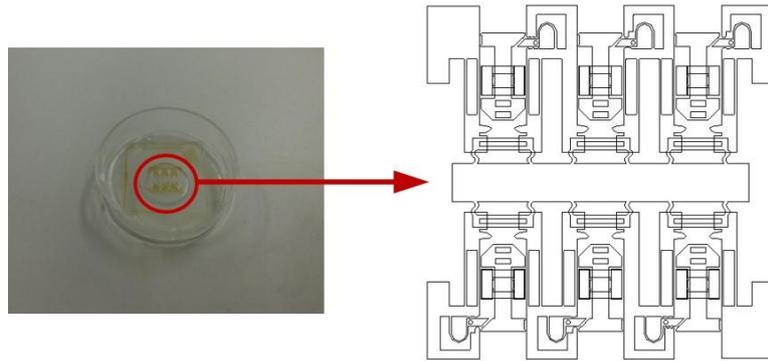


Fig. 1 Cell-stretching microdevice. Six microdevices were fabricated on a glass coverslip. The glass coverslip was attached to a 35-mm dish with a $\phi 18$ mm hole at the bottom. The microdevice consisted of a pair of arms and a cell-stretching sheet.

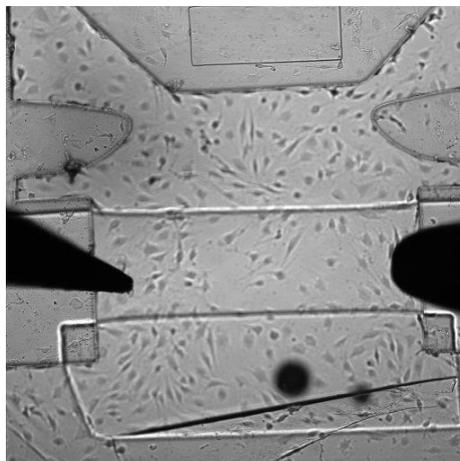


Fig. 2 Microscopic image of the stretching setup of the microdevice. Two metal needles mounted on the micromanipulator were set on the arms of the device. The needle on the left side was connected to a piezo electric actuator and used to apply stretch to the sheet.

Time course fluorescence images of the Ca^{2+} indicators were obtained using an inverted confocal laser scanning microscope (A1R, Nikon) with a $\times 60$ oil immersion objective lens. Fluo 8H and calcein red-orange were excited by 488 and 561 nm lasers, respectively, and their fluorescence was separated using a 525 nm band-pass filter with a 50 nm band width and 595 nm band-pass filter with a 50 nm band width. Images of Fluo 8H and calcein red-orange fluorescence and a transmitted image were acquired simultaneously using three independent photomultiplier tubes. The recorded image size was 512×128 pixels with a 12 bit resolution. The image acquisition rate was approximately 120 frames/second. We applied an averaging filter every four frames. Therefore, the recorded time course image was obtained at a video rate of 30 frames/second. The focal plane was set to the bottom of the adhered cell on the stretch sheet at which the confocal fluorescence image had the largest and most clear shape of the target cell. All experiments were conducted in a normal atmosphere at 24°C . To prevent influences on the cell condition, image acquisitions were completed within 1 hour. Obtained time lapse images were analyzed using ImageJ software (National Institutes of Health). To analyze the time course change in fluorescent intensity of the cell, the target cell had to have been fixed at the same position in the time series images during stretch. Alignment of the sequential time lapse images was manually adjusted to fix the position of the target cell using ImageJ plug-in Align slice according to the position of the cell nucleus and outline of the fluorescence image of the cell. The average value of the fluorescent intensity was calculated in the region of interest (ROI). Although the cell shape deforms because of stretch application, the average value of the fluorescent intensity was calculated using the ROI defined by the initial state (before stretch). In the present study, we defined two ROIs in each cell. The first ROI was set at the central region of the cell. This ROI was smaller than the planer outline of the cell nucleus. The position and shape of the cell nucleus was defined using a transmitted image that showed a contrast image of the cell and the

stretching sheet together. The second ROI was set at the peripheral region of the cell. This ROI was set to surround the cell nucleus. The actual stretch strain magnitude in each experiment was confirmed by measuring deformation of the sheet using the transmitted image. The change in the length between two noticeable spot marks on the sheet was measured and used to calculate the nominal strain on the sheet. Comparisons of the initiating characteristics of the intracellular calcium signaling response were statistically analyzed by the Student's *t*-test.

3. Results

3.1 Time course observation of the calcium signaling response to stretch

In the present study, we observed 26 cells, and eight cells showed a calcium signaling response to stretch application. The percentage of responding cells was 30.8%. Figure 3 shows representative time-course fluorescence images of stretched cells. Fluorescence images are shown with pseudocolor using a lookup table to improve visibility. $t = 0$ was defined as the time point at which image acquisition started. Nominal strain on the stretching sheet (ε) was measured using transmitted images obtained simultaneously with the fluorescence images (images are not shown). Stretch was applied to the sheet in the left direction (shown as a white arrow in the figure). There were three cells in the field of view. Figure 4 shows definition of the ROIs in the target cell. One ROI was set at the central region of the cell and another ROI was set at the peripheral region. The cell nucleus and peripheral region were distinguished by using the transmitted image with brightness and contrast adjustment to improve visibility. Figure 5 shows the time course change in the average

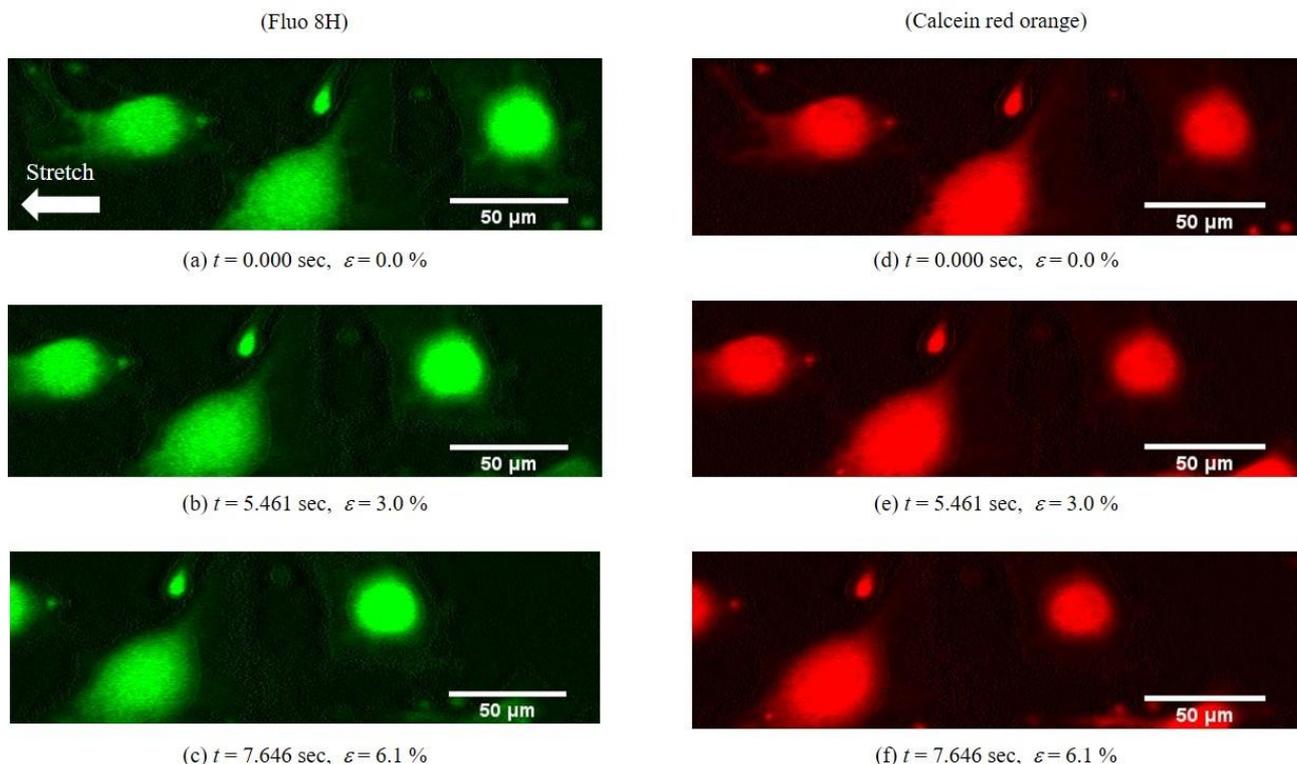


Fig. 3 Time-course fluorescence images of stretched cells. All images are shown with pseudocolor to improve visibility using a lookup table. Left column shows fluorescence images of Fluo 8H and right column shows calcein red-orange. Pairs of images in the same row were recorded at the same time point. The white arrow indicates the direction of stretch. Nominal strain (ε) on the stretching sheet was measured using transmitted images of the sheet obtained simultaneously with the fluorescent images (data not shown). Time t = seconds from the starting time point of image acquisition.

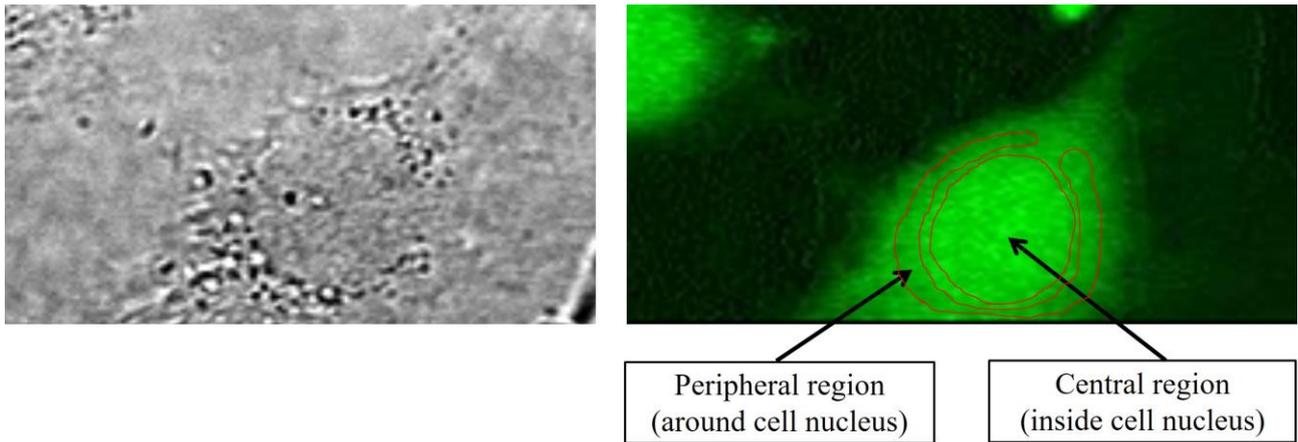


Fig. 4 Definition of the two ROIs in the target cell. One ROI was set at the central region of the cell. This ROI was smaller than the outline of the cell nucleus obtained from the transmitted image (left side, brightness and contrast were adjusted to improve visibility). Another ROI was set at the peripheral region of the cell. This ROI surrounded the cell nucleus.

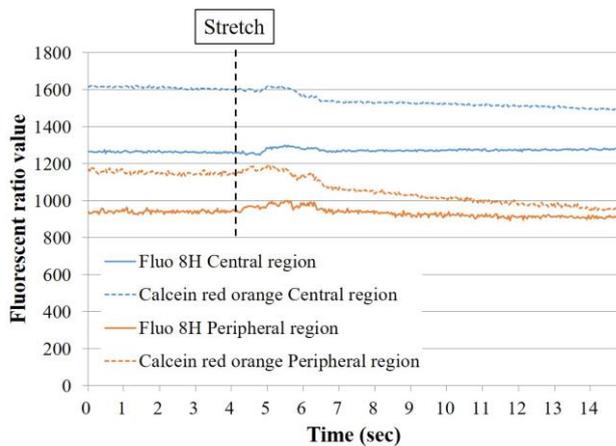


Fig. 5 Time course change in fluorescent intensities of Fluo 8H and calcein red-orange in the two ROIs. The dotted line indicates the starting time point of stretch application.

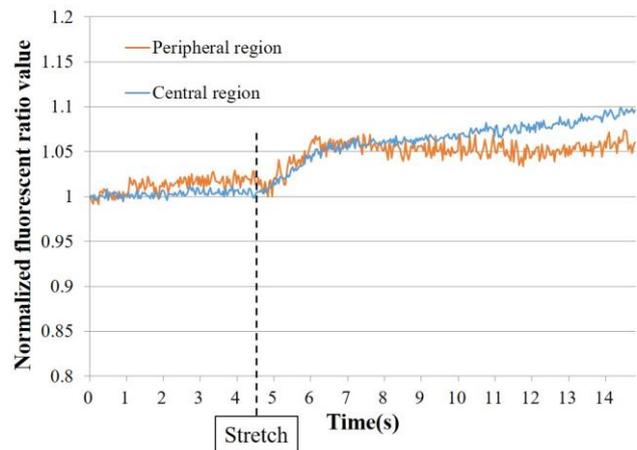


Fig. 6 Time course change in normalized fluorescence ratio values of the two ROIs. The dotted line indicates the starting time point of stretch application.

fluorescent intensities of Fluo 8H and calcein red-orange in the two ROIs. After stretch application, fluorescent intensities showed transient changes in both ROIs. To reduce the influence of motion artifacts on fluorescent intensities during stretch application, we evaluated the fluorescence ratio value. The time course changes in the fluorescence ratio values of the two ROIs are plotted in Figure 6. In this graph, fluorescent ratio values were normalized by the initial value. In both ROIs, the fluorescence ratio value showed a transient increase after stretch application. To estimate the initiation time point of the calcium signaling response, we calculated the approximate lines. One approximate line was calculated from the fluorescence ratio value at the basal phase (before stretch). The other line was calculated from the increasing phase of the fluorescence ratio value after stretch. The initiating time point of the calcium signaling response was defined as the cross point of these two approximate lines. Figure 7 shows calculated approximate lines and defined initiating time points of the calcium signaling response to stretch in the central and peripheral ROIs. As shown in the graph, there were time lags between the time point of stretch application and the initiating time point of the calcium signaling response. Moreover, the time lag in the central ROI was shorter than that in the peripheral ROI. A summary of time points of the intracellular calcium signaling response to stretch is shown in

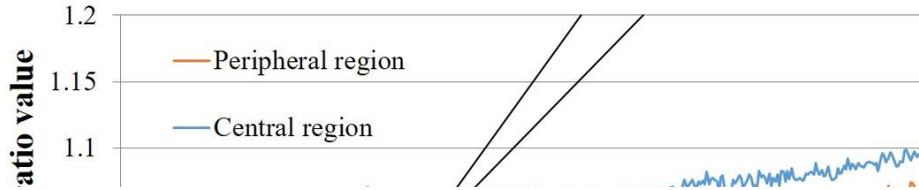


Table 1 Summary of time points of the intracellular calcium signaling response to stretch. There were milliseconds order time lags between the start of stretching and initiation of the cellular response. Time lag of the central region was shorter than that of the peripheral region.

Normalized flu	Time (sec.)
0.95	
0.9	Time point of stretch application
0.85	Initiating time point of Ca ²⁺ response in the central region ROI
0.8	Initiating time point of Ca ²⁺ response in the peripheral region ROI
0	Time-lag at the central region ROI
	Time-lag at the peripheral region ROI

Fig. 7 Definition of the initiating time point of the intracellular calcium signaling response. The initiating time point was defined as the cross point of two approximate lines. One approximate line was calculated from the basal phase of the fluorescence ratio value (before stretch). Another approximate line was calculated from the increasing phase of the fluorescence ratio value after stretch application.

Table 1. In this observation, stretch stimulus was applied at $t = 4.48$ seconds from the image acquisition start. Initiating time points of calcium signaling responses were $t = 4.95$ and 5.40 seconds in central and peripheral ROIs, respectively. Therefore, time lag in the central ROI was 0.47 seconds, and 0.92 seconds in the peripheral ROI. Figure 8 shows a comparison of average time lags in central and peripheral ROIs. Eight cells showed a calcium signaling response. The time lag in the central ROI was significantly shorter than that in the peripheral ROI ($P < 0.05$).

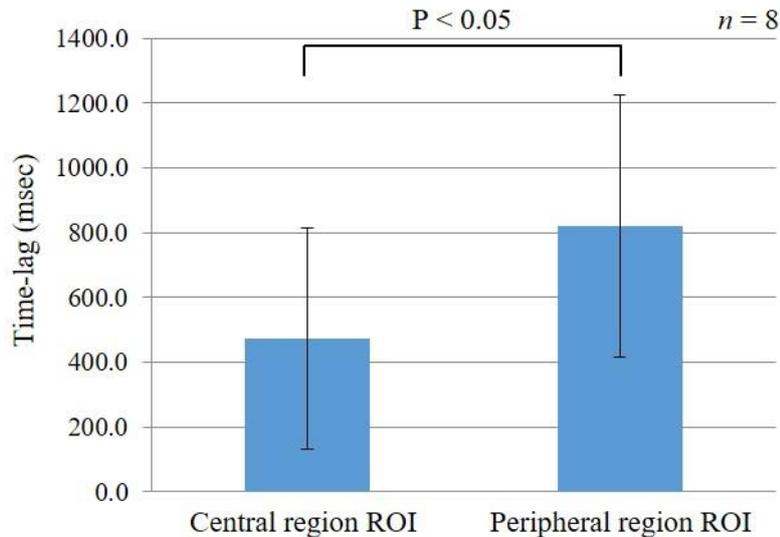


Fig. 8 Comparison of averaged time lags in central ROI and peripheral ROIs. There was a significant difference between the time lags of the two ROIs. The time lag in the central ROI was shorter than that in the peripheral ROI.

4. Discussion

As we have already reported in another literature, not all of cells respond to the stretch application (Sato, et al., 2015). There were 20% to 35% cells which showed response to stretch application. And also in this study, approximately 30% cells showed response which agreed with our previous report. Stretch strain was certainly applied to the cells confirmed by the analysis of transmitted images. The shape of cell was changed during stretch application and the deformation of cell shape corresponded to that of stretching sheet. Although we cannot discuss about the underlying mechanism by which not all of the cell respond to the stretch application with same magnitude only from the results obtained in this study. Authors would like to clarify this issue in the future work.

The stretching stimuli applied in this study is not physiological mechanical stimuli to osteoblastic cells. Substrate stretch deformation of extracellular matrix with the magnitude of 10% is much larger than that of expected in native bone tissue. Although we agree with that there is some limitation to interpret our obtained results into physiological meaning of osteoblastic response in the native tissue, a simplified and well stated experimental setup can bring us a useful illustrations of cellular responses. The substrate stretching stimuli has been used and discussed in the many previously reported literatures. We can compare our results with such previous studies. And our simplified experimental setup brought us a novel findings which suggest a glimpse of mechano-sensing mechanism of osteoblastic cells. An experimental setup which mimics native tissue environment of osteoblasts is probably complex and difficult to conduct precise observations with high spatial and temporal resolutions as conducted in this study.

Based on the observations of our study, there was a time lag between stretch application and the initiating time point of the intracellular calcium signaling response to stretch. There are two possibilities to explain this time lag. One possibility is a threshold strain magnitude at which the osteoblastic cell can sense and respond. The length of the time lag depends on the elapsed time from the initial state (0 strain) to reaching the threshold strain magnitude. Another possibility is that the osteoblastic mechanotransduction mechanism itself has a time lag to transduce mechanical stimuli into intracellular calcium signals. To examine these hypotheses, further studies must be conducted to evaluate the influence on the time lag by changing the stretch strain rate. If the time lag arises from a threshold strain magnitude, a shorter time lag would be observed at a higher strain rate. However, cellular structures such as the cell membrane, cytoskeleton, and nucleus, which are involved in mechanotransduction pathways, have viscoelastic properties. A difference in the stretch strain rate might change the deformation behavior of these cellular structures. As a result, the characteristics of the intracellular calcium signaling response could be changed in osteoblasts. Therefore, we have to estimate the magnitude of the threshold strain by evaluating the percentage of responding cells with a varied stretch strain

magnitude at a constant strain rate. Then, we have to carefully evaluate the relationship between the length of the time lag and the stretch strain rate.

By comparing time lags in central and peripheral ROIs, we found that the time lag in the central region was shorter than that in the peripheral region. In the present study, the confocal focal plane was set to the bottom of the cell. Therefore, we did not evaluate changes in the concentration of calcium ions inside of the cell nucleus, but in the cytoplasm underneath the nucleus. Recent studies have proposed the importance of the cell nucleus in the mechanotransduction mechanism (Wang, et al., 2009, Nagayama, et al., 2015). Our results might also suggest that stretch strain of the substrate is transmitted into the cell via focal adhesions and cytoskeletons, and transduced into biochemical signals around the cell nucleus. We speculate two possibilities, one is that rigid displacement of the cell nucleus, which has a higher elastic modulus compared with other cellular components, might activate a mechano-chemical transducer such as stretch-activate channels. Another possibility is that a complex strain field at a boundary between the cell nucleus and other surrounding cellular components might form a stress concentration. This concentrated higher stress could activate mechano-chemical transducers. To examine this hypothesis, we have to clarify the detailed deformation behavior of subcellular components under stretch application. Furthermore, an inhibition experiment of nesprin family proteins, which link the nucleus to the cytoskeleton, might be useful.

5. Conclusions

In this study, we conducted *in situ* observations of the osteoblastic calcium signaling response to stretch by video rate temporal resolution. Our observations revealed dynamic characteristics of calcium signal initiation as the response to stretch application. We found a time lag between the time point of stretch application and the initiating time point of the calcium signaling response, and the time lag in the central region of the cell was shorter than that in the peripheral region. Our device and experimental system enabled us to assess the cellular responses that arise immediately after stretch application in milliseconds order and at the subcellular level, which are useful to further investigate the mechanotransduction mechanisms of cells.

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