DIRECTIONAL DEPENDENCE OF SENSING CHARACTERISTICS TO MECHANICAL STIMULUS IN OSTEOBLASTIC CELLS

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Abstract. *It is widely recognized that living bone tissue can change its structure to adapt to the mechanical environment. Osteoblasts play an important role in this adaptive bone remodeling process to reconstruct a new bone, and are believed to sense mechanical stimuli such as stress/strain. However, the mechanism by which cells sense and transduce mechanical signals to the intracellular biochemical-signaling cascade is still unclear. Present study addresses this issue by investigating the characteristics of the response of a single MC3T3-E1 osteoblastic cell to a mechanical stimulus. First, by applying quantitative perturbation to a single cell by a microneedle, the change in intracellular calcium ion concentration $[Ca^{2+}]_i$ was observed as a primal signaling response to the mechanical stimulus, and the threshold value of the perturbation as the mechanical stimulus was quantitatively evaluated. Second, to study directional dependence of the response to the mechanical stimulus, the effect of actin fiber orientation on the threshold value of the calcium response was investigated at various magnitudes and directions of the stimulus. As a result, directional dependence of the osteoblastic response to the perturbation was found. This result is phenomenological evidence that the cytoskeletal actin fiber structures are involved in the mechanotransduction mechanism.*

1. INTRODUCTION

Bone tissue changes its structure to adapt such prevailing functional requirements as supporting the body weight and protecting organs by remodeling in a changing mechanical environment [1]. Bone remodeling is an adaptation process through complex and coordinated series of cellular events including osteoclastic resorption and osteoblastic formation [2]. In this process, physical signals by mechanical loading/deformation play important regulatory roles in cellular activities [3], and lead to intracellular signaling cascades [4, 5]. Although the effects of mechanical stimuli on bone cells have been investigate by *in vitro* experiments [6-12], little is known about the mechanism by which osteoblasts sense mechanical signals and transfer them into an intracellular signaling response. To clarify how

a single cell detects and transmits a mechanical stimulus, identification and characterization of the pathway have to be carried out focusing on the cellular mechanical components in a single osteoblastic cell.

It has been suggested that the extracellular matrix-integrin-cytoskeletal structure is a candidate for the mechanotransduction pathway [13, 3], and that the cytoskeletal actin fibers influence the cellular response to a mechanical stimulus [12]. The cytoskeleton is one of the major intracellular determinants of cellular morphology and functions. If this cytoskeletal actin fiber structure plays an important role in the mechanotransduction mechanism in osteoblastic cells, the characteristics of the structure may affect the subsequent signaling pathway, which can be observed as a change in the cellular signaling response to a mechanical stimulus.

The purposes of this present study are to investigate the characteristics of the response of a single osteoblastic cell to a mechanical stimulus, and to examine the involvement of the cytoskeletal actin fiber structure in the mechanotransduction pathway. By applying quantitative perturbation to an osteoblastic cell with a microneedle, a change in the intracellular calcium ion concentration, $[Ca^{2+}]_i$, was observed as the primary signaling response to the mechanical stimulus. And to study the directional dependence of the response to the mechanical stimulus, the effect of actin fiber orientation on the threshold value of the calcium signaling response was investigated for various magnitudes and directions of the stimulus.

2. MATERIALS AND METHODS

2.1 CELL CULTURE

Osteoblast-like MC3T3-E1 cells obtained from RIKEN Cell Bank were plated on a glass-bottom dish ($\phi = 35$ mm) at a density of 10^5 cells / dish, cultured in the α-minimum essential medium (α-MEM, ICN Biomedicals) containing 10% fetal bovine serum (FBS, ICN Biomedicals), and maintained in a 95% air and 5% CO_2 humidified environment at 37˚C.

2.2 INTRACELLULAR CALCIUM IMAGING

The cells were incubated for 3 hours after plating and then loaded with 5 μM fluo 3-AM (Dojindo Molecular Technologies), a fluorescent Ca^{2+} indicator, in FBS−free α−MEM for 1 hour. The cells were then rinsed with PBS and returned to FBS-free α−MEM.

The change in the intracellular calcium ion concentration, $[Ca^{2+}]_i$, was observed by a confocal laser scanning microscope (MRC-1024/MP, Bio-Rad) at room temperature (23˚C). The observed region was 222 x 222 μm^2. Each fluorescence image at a resolution of 512 x 512 pixels and height of 2 μm from the bottom of the dish was scanned at a rate of 1.5 sec per image.

2.3 CELL ORIENTATION

The fluorescent and transmitted images of the osteoblastic cell are superimposed and shown in Fig. 1, in which the tip of the microneedle can be seen at the center of the cell. The cytoskeletal actin fibers in osteoblastic cells have an aligned structure whose direction coincides with the major axis of the spindle-shaped cell. The angle between the microneedle and the cell axis is defined as θ°, as illustrated in Fig. 2, in which the cell axis was determined by image processing software (Image Pro Plus, Media Cybernetics) as the major axis of an ellipse fitted to the cell shape.

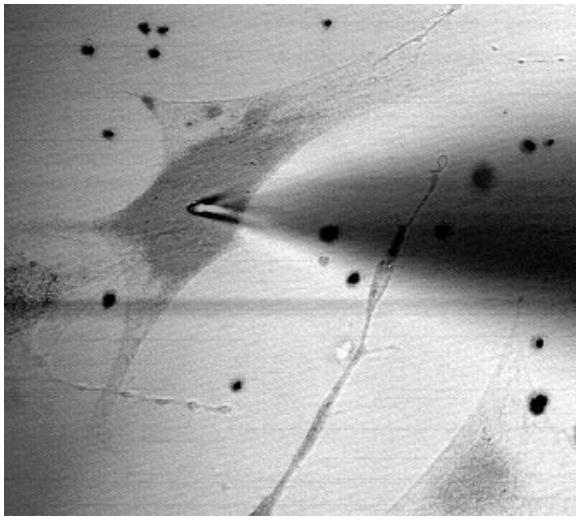


Figure 1. Superimposed fluorescent and transmitted images of an osteoblastic cell and the tip of the microneedle.

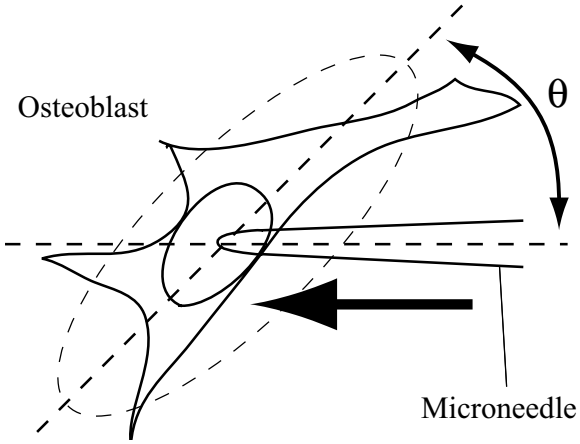
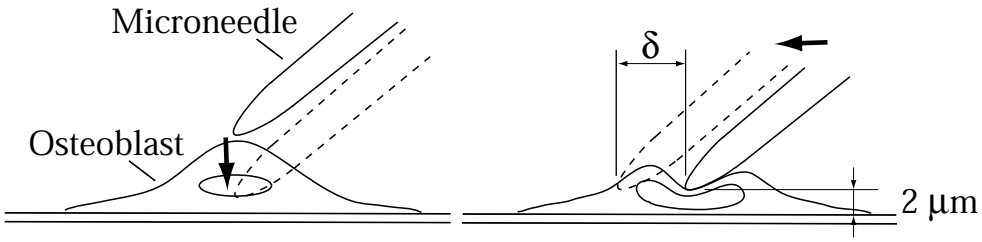


Figure 2. Definition of angle θ between the cell axis and the microneedle (direction of applied deformation).

2.4 MECHANICAL STIMULATION

A mechanical stimulus in the form of perturbation was applied to a single osteoblastic cell by the tip of a glass microneedle of 10μm in diameter. The tip of the microneedle had been heated to make it smooth and round. The microneedle was attached to a three-dimensional hydraulic micromanipulator (MHW-103 , Narishige) at an angle of 40° between the microneedle axis and the dish plane.

A schematic diagram of the deformation applied to a single cell is shown in Fig. 3. First, the tip of the microneedle was moved down vertically to indent the cell surface at a height of 2 μm from the bottom of the dish and held there for a few seconds, as illustrated in Fig. 3(a). Second, after confirming the lack of any cellular response to this indentation, the microneedle was moved horizontally in the direction of angle θ° at a speed of 10 μm/s to deform the cell, as illustrated in Fig. 3(b), in which the displacement of the tip is defined as δ μm.



(a) Indentation (b) Slide

Figure 3. Schematic diagram of the deformation applied to a single osteoblastic cell by the tip of a microneedle.

3. RESULTS

A transient increase in the intracellular calcium ion concentration, $[Ca^{2+}]_i$, in a single osteoblastic cell in response to the applied mechanical stimulus was observed as an increase in the fluorescence intensity, as shown in Fig. 4. Figure 5 plots the time-course change in the average fluorescence intensity in the cell, in which arrows (a) to (d) respectively correspond to the cells in Figs. 4(a) to (d). Figure 4(a) shows the fluorescent image of the cell before stimulation. When the tip of microneedle was indented vertically at the center of the cell, the shadow of the tip was observed as a black spot (white arrow) on the focal plane at a height of 2 μm from the bottom of the dish, as shown in Fig. 4(b). After confirming that this indentation had not induced any significant change in $[Ca^{2+}]_i$, the tip of the microneedle was displaced horizontally by $\delta = 8$ μm to apply deformation to the cell at $t = 0$ s, as shown in Fig. 4(c). Immediately after this stimulation, the fluorescence intensity increased and spread within the cell. Subsequently, at $t = 12$ s, the intensity reached its peak value as shown in Fig. 4(d), and then, gradually decreased toward the basal level before stimulation, as shown in Fig. 5. Once the calcium response was observed, the microneedle was immediately moved away from the cell. This transient increase in $[Ca^{2+}]_i$ was a typical response to the mechanical stimulation using a microneedle.

However, there were cases in which no cellular response was apparent after mechanical stimulation, as shown in Figs. 6 and 7, even though the same magnitude of deformation, $\delta = 8$ μm, was applied. Figure 7 plots the time-course change in the averaged fluorescence intensity in the cell, in which arrows (a) to (e) respectively correspond to the cells in Figs. 6(a) to (e). Figures 6(a) and (b) respectively show the fluorescent images of the cell before stimulation and after indentation. At $t = 0$ s, the tip of microneedle was displaced horizontally, $\delta = 8$ μm, to apply deformation to the cell, as shown in Fig. 6(c), in which movement of the black spot (white arrow) can be seen. However, no change in the fluorescence intensity was apparent at $t = 9$ s, as shown in Figs. 6(d) and 7. To confirm that the cell had the ability to respond to a mechanical stimulus, the magnitude of the displacement was increased to $\delta = +10$ μm, which was applied at $t = 20$ s (marked by * in Fig. 7), and an increase in the fluorescence intensity was observed as shown in Fig. 7.

When the local deformation was applied to a single osteoblastic cell by the microneedle, some cells responded with increase in $[Ca^{2+}]_i$ and some did not, as is shown in a typical case in Figs. 4 and 6. In order to examine the effect of the magnitude of deformation on the cellular response, displacement magnitude δ in the horizontal direction was varied from 2 μm to 12 μm at 2μm intervals. If the cytoskeletal actin fiber structure were to play

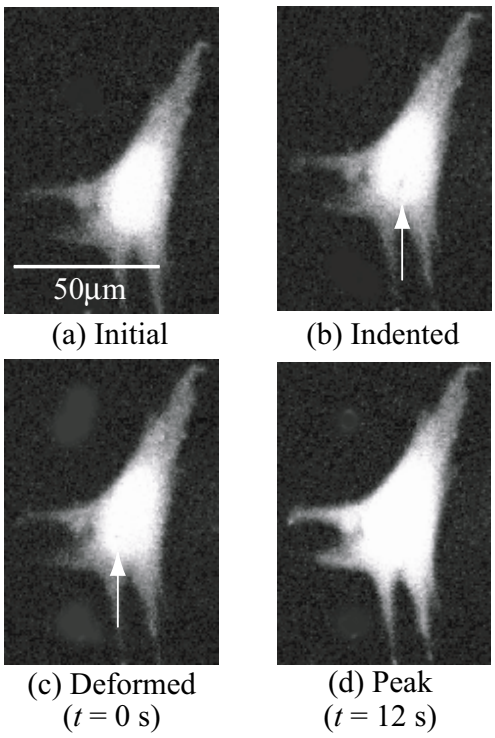


Figure 4. Calcium signaling response to the deformation applied to a single osteoblastic cell (fluo 3 image).

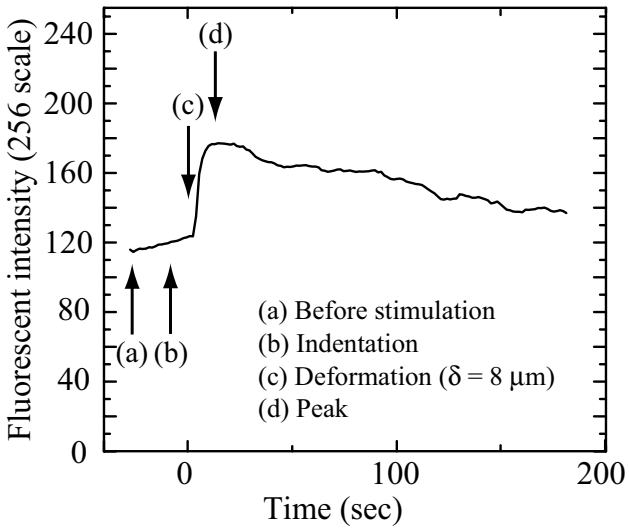


Figure 5. Change in fluorescence intensity with time observed in a single osteoblastic cell due to the deformation applied by a glass microneedle.

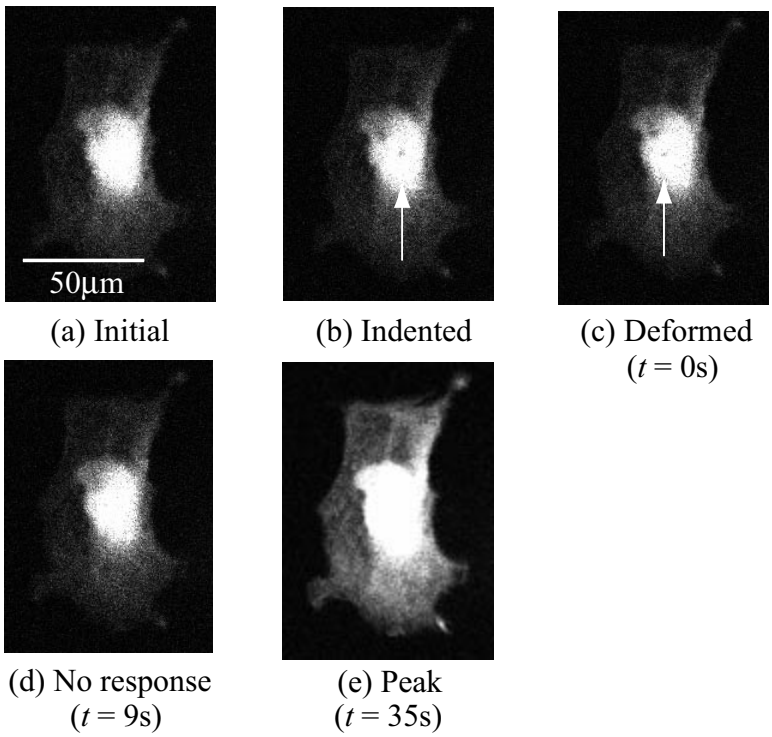


Figure 6. Lack of any calcium signaling response to the deformation applied to a single osteoblastic cell (fluo 3 image).

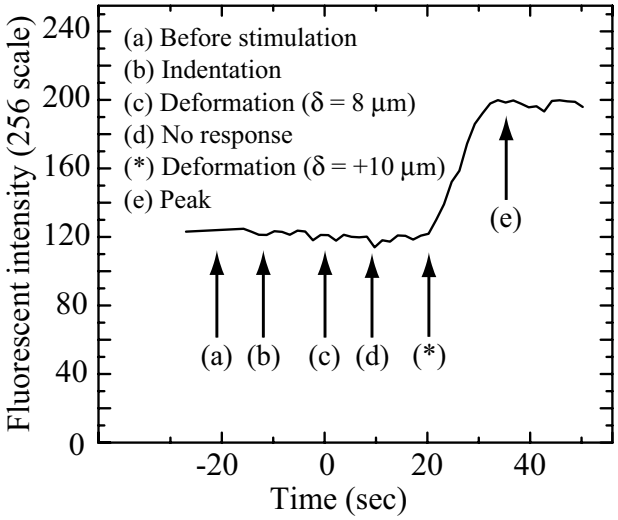


Figure 7. Change in the fluorescence intensity with time observed in a single osteoblastic cell due to the deformation applied by a glass microneedle.

an important role in the mechanotransduction mechanism in osteoblastic cells, the characteristics of the structure may affect the characteristics of the cellular response to a mechanical stimulus. Here, we focus on the orientation of the cytoskeletal actin fiber structure in the osteoblastic cells that may result in directional dependence of the response to a mechanical stimulus. To examine the directional dependence of the cellular response, the above-mentioned cellular calcium response to the mechanical stimulus was studied by applying a deformation at various θ angles.

The cells were divided into three groups based on angle θ: group A (◆), $\theta = 0 - 30$ deg ($n = 35$); group B (■), $\theta = 30 - 60$ deg ($n = 35$); and group C (▲), $\theta = 60 - 90$ deg ($n = 39$). Probability P of the cells that responded is plotted in Fig. 8 against the magnitude of applied displacement δ. As can be seen in this figure, for smaller displacements $\delta = 2 - 6$ μm, no cells responded to the stimulation in any groups. For displacement $\delta = 8$ μm, the cells in group A did not respond, while 25% and 44% of the respective cells in groups B and C responded to the stimulation. For displacement $\delta = 10$ μm, 14%, 50%, and 63% of the respective cells in groups A, B, and C responded. It can be observed for each group, that a larger displacement led to a higher probability of response. In addition, for displacements $\delta = 8$ μm and 10 μm, a larger θ angle seemed to lead to a higher probability of response. These results indicate that the sensitivity of an osteoblastic cell to a mechanical stimulus is affected by the direction of displacement, that is, the sensitivity seems to be related to the angle of the cytoskeletal actin fibers orientation.

To investigate the directional dependence of the osteoblastic response to a mechanical stimulus, the experimental data were analyzed in order to plot probability P of cells that responded against applied deformation angle θ. Figures 9(a) to (c) respectively present the dependence of angle θ of the applied deformation on probability P of the responded cells for applied displacements $\delta = 8$, 10, and 12 μm, in which the probability was plotted as a function of the angle of the applied deformation by taking a moving average within 10° at a 5° interval.

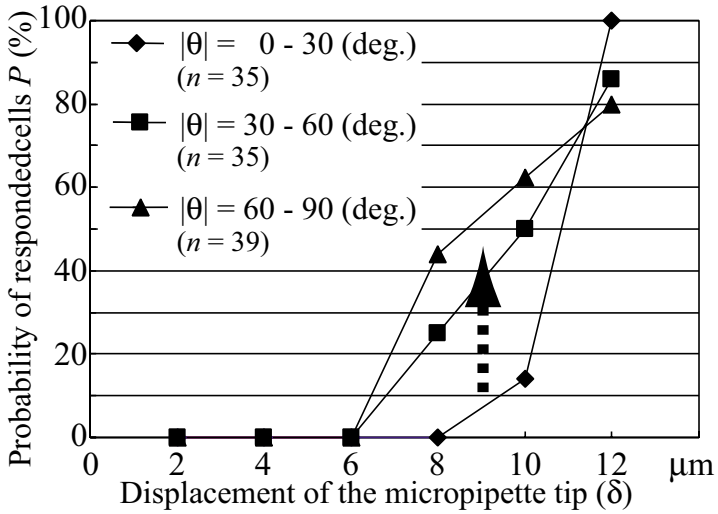
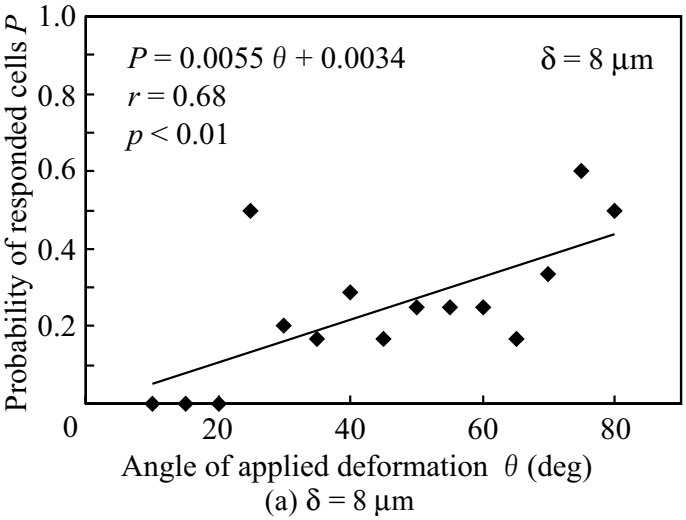
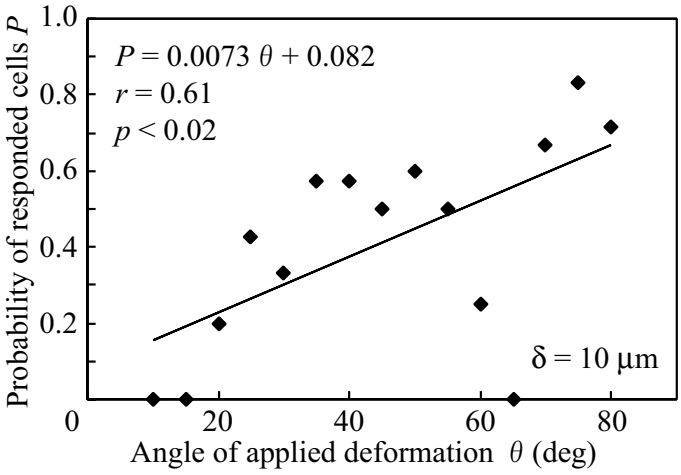


Figure 8. Probability of responding cells in three groups according to the angle of applied deformation.



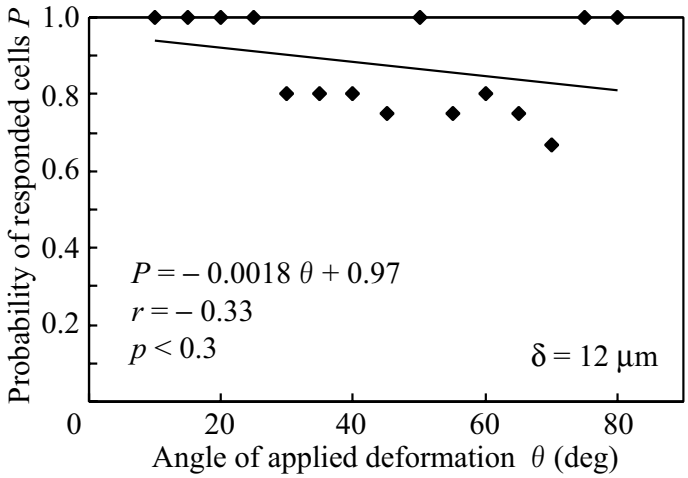
(a) δ = 8 μm

Figure 9. Directional dependence of the osteoblastic response to mechanical stimulation.



(b) δ = 10 μm

Figure 9. Directional dependence of the osteoblastic response to mechanical stimulation.



(c) δ = 12 μm

Figure 9. Directional dependence of the osteoblastic response to mechanical stimulation.

For displacement δ = 8 μm, as shown in Fig. 9(a), probability P of the cellular response increased with increasing applied deformation angle θ. A linear regression analysis showed correlation coefficient r = 0.68, indicating that P and θ are significantly correlated with each other ($p < 0.01$). For stimulation δ = 10 μm, the linear regression analysis showed significant positive correlation ($p < 0.02$) between P and θ with coefficient r = 0.61, as indicated in Fig. 9(b). Comparing these two cases, the increase in P with increasing θ for δ = 10 μm was higher than that for δ = 8.0 μm. In contrast to these cases, a linear regression analysis for δ = 12 μm showed no significant correlation between P and θ because most of the cells responded to the stimulus, as indicated in Fig. 9(c).

4. DISCUSSION AND CONCLUSION

In the present study, no calcium signaling response was observed for a small deformation by a microneedle, and a larger deformation caused a higher probability of the response. This displacement magnitude dependence indicates the existence of a threshold value in sensing a mechanical stimulus in osteoblastic cells, which reminds us of the tissue remodeling response with a threshold value around the remodeling equilibrium [14-15]. However, the applied displacement δ in this *in vitro* experiment cannot be transformed into a strain value. Thus, for quantitative comparison with the *in vivo* experimental data, controlled stress or strain measure should be applied to the cell as a mechanical input.

The results of the present study suggest directional dependence of the threshold value at which osteoblastic cells respond to a mechanical stimulus, which leads to the sensitivity to a mechanical stimulus depending on the direction relative to the alignment of the cytoskeletal actin fiber structure.

The directional dependence of the osteoblastic response to perturbation provides one phenomenological evidence for the cytoskeletal actin fiber structure being involved in the mechanotransduction mechanism which might be related to cellular polarized behavior. Even though the results obtained in this study are still a phenomenological observation of the involvement of the cytoskeletal actin fiber structure in the mechanotransduction process in osteoblastic cells, the approach from a mechanical viewpoints is one step toward gaining an insight into the mechanotransduction mechanism and providing a fundamental basis for studies in the field of bone mechanobiology [16].

5. REFERENCES

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