Cellular and Molecular Bioengineering, Vol. 1, Nos. 2–3, September 2008 *(© 2008)* pp. 182–188
DOI: 10.1007/s12195-008-0017-7

Bone Cells Grown on Micropatterned Surfaces are More Sensitive to Fluid Shear Stress

LIDAN YOU,[1,2,3] SARA TEMIYASATHIT,[2,3] SEAN R. COYER,[4,5] ANDRÉS J. GARCÍA,[4,5] and CHRISTOPHER R. JACOBS[2,3]

[1]Department of Mechanical and Industrial Engineering, Institute of Biomaterials and Biomedical Engineering, University of Toronto, 5 King's College Road, Toronto, ON, Canada M5S 3G8; [2]Department of Veteran's Affairs, Bone and Joint Rehabilitation R&D Center, Palo Alto, CA, USA; [3]Department of Mechanical Engineering, Stanford University, Stanford, CA, USA; [4]Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA, USA; and [5]Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Altanta, GA, USA

(Received 28 March 2008; accepted 28 July 2008; published online 21 August 2008)

Abstract—Bone has the ability to adapt its structure to meet its mechanical environment. In spite of great efforts to understand the mechanisms underlying this phenomenon, little is known about bone mechanotransduction at the cellular level. In this work, we propose that there is an interaction between the architecture of the cell and mechanosensitivity. Specifically, we hypothesized that cell spreading plays an important role in bone cell mechanotransduction. To test our hypothesis, we utilized micropatterned surfaces that allowed us to control the degree of cell spreading. Focal adhesion area is also known to depend on cell spreading. Thus patterns were utilized that maintained a constant total adhesive contact area, but permitted differing degrees of spreading. MC3T3-E1 osteoblasts cultured on these patterned surfaces were exposed to dynamic fluid shear stress. The fluid flow-induced changes in intracellular calcium $[Ca^{2+}]_i$ were determined as a function of the degree of spreading. Cells grown on micropatterned surfaces were more responsive in general than those grown on traditional unpatterned surfaces. Interestingly, however, we found that cells with a higher degree of spreading were less responsive to mechanical loading. This work provides new insights into the interplay of cellular structure and mechanotransduction.

Keywords—Fluid flow, Focal adhesion, Cytoskeletal tension, Mechanotransduction, Bone remodeling.

INTRODUCTION

Bone implants have been shown to achieve better tissue integration and higher interfacial strength when they are engineered to have micropatterned surfaces.[10] It has been well-documented that these surfaces can influence cellular behavior directly.[21,27] However, it is also possible that the patterns modulate cellular mechanosensitivity and contribute to enhanced implant integration with load application. Bone has the ability to alter its structure in response to the mechanical demands it experiences. Dynamic fluid flow occurs when bone is subjected to habitual mechanical loading, and has been shown to be a potent regulator of bone cell metabolism.[20,32] Nevertheless, the molecular mechanisms whereby loading induced fluid flow is sensed at a cellular level have not been elucidated. One possibility is that application of flow results in deformation and re-organization of cell's cytoskeleton that then triggers downstream signaling events. Both cytoskeletal deformation and the remodeling that follows are greatly influenced by cellular adhesion to the extracellular matrix (ECM) and internal cytoskeletal tension.[6,13]

Cells attach to ECM via transmembrane proteins, primarily integrins.[3,17] Integrin binding of ECM ligands initiates recruitment of an array of structural and signaling proteins to the binding sites to form focal adhesions. Due to this colocalization of structural and signaling molecules, focal adhesions and the cytoskeletal elements associated with them have significant potential as sites of mechanosensing.

Cytoskeletal tension directly affects cellular stiffness, and thus deformation, in response to mechanical loading. Furthermore, cytoskeletal tension may also affect mechanosensation at the adhesion site. Recent studies have shown a close correlation between contractile force and focal adhesion area.[2] It has been shown that increased cell spreading, even while maintaining a constant total cell contact area, leads to greater focal adhesion activity.[7] Further, it has been suggested that higher focal adhesion activity is indicative of greater cytoskeletal tension.[2] Taken together these results suggest that cells that are more spread tend to have a higher cytoskeletal tension.

Address correspondence to Lidan You, Department of Mechanical and Industrial Engineering, Institute of Biomaterials and Biomedical Engineering, University of Toronto, 5 King's College Road, Toronto, ON, Canada M5S 3G8. Electronic mail: youlidan@mie.utoronto.ca

Both cellular adhesion and cytoskeletal tension are critical factors in determining cell deformation and subsequent biochemical signaling. However, it is not clear which factor, adhesion or tension, plays the dominant role. In this study our goal was to manipulate adhesion and cell spreading independently and to determine whether cell spreading modulates bone cell mechanotransduction independent of focal adhesion area. We employed a micropatterened adhesive surface to control cell spreading while maintaining a constant cell contact area. We also examined the effect of growth on a micropatterned surface on cellular mechanoresponsiveness.

METHODS

Cell Culture

MC3T3-E1 osteoblastic cells were cultured in minimal essential alpha medium (MEM-α; Invitrogen (Gibco), Calsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 1% penicillin and streptomycin (Invitrogen) and maintained at 37 °C and 5% CO_2 in a humidified incubator.

Micropatterned Surface

Using standard photolithography, a PDMS stamp was made with arrays of islands of circular posts (3 μm in diameter, 5 μm or 7 μm center to center post spacing, 75 μm center to center island spacing) (Fig. 1) on its surface. Each island was designed for one cell to occupy. As the post (or spot) spacing was increased from 5 μm to 7 μm while maintaining the number of dots, hence keeping the total adhesive area constant, the projected cell area is almost doubled (increased from 529 μm^2 to 961 μm^2). The 75 μm spacing between islands was selected to ensure that a single cell could not span more than one set of spots. Microcontact printing was then used to pattern adhesive islands within a non-fouling background.[13] The PDMS stamp was used to print self-assembled monolayers of CH_3-terminated alkanethiols onto glass slides sequentially coated with titanium (10 nm) and gold (20 nm). Slides were then exposed to tri(ethylene glycol)-terminated alkanethiols to generate non-fouling background.[13] The slides were then coated with 10 μg/mL fibronectin (FN) for 1 h to form adhesive domains. The substrates were flat for both patterned and non-patterned surfaces.

Quantification of Cell Spreading

Cell spreading (cell projected area) was measured using image-processing software (Image J, NIH) from images captured by the CCD camera that connected to the microscope. The cell project areas were determined by manually marking the cell edges from the images.

Oscillatory Fluid Flow

A previously described fluid flow device was used to deliver laminar OFF to MC3T3-E1 osteoblastic cells.[20]

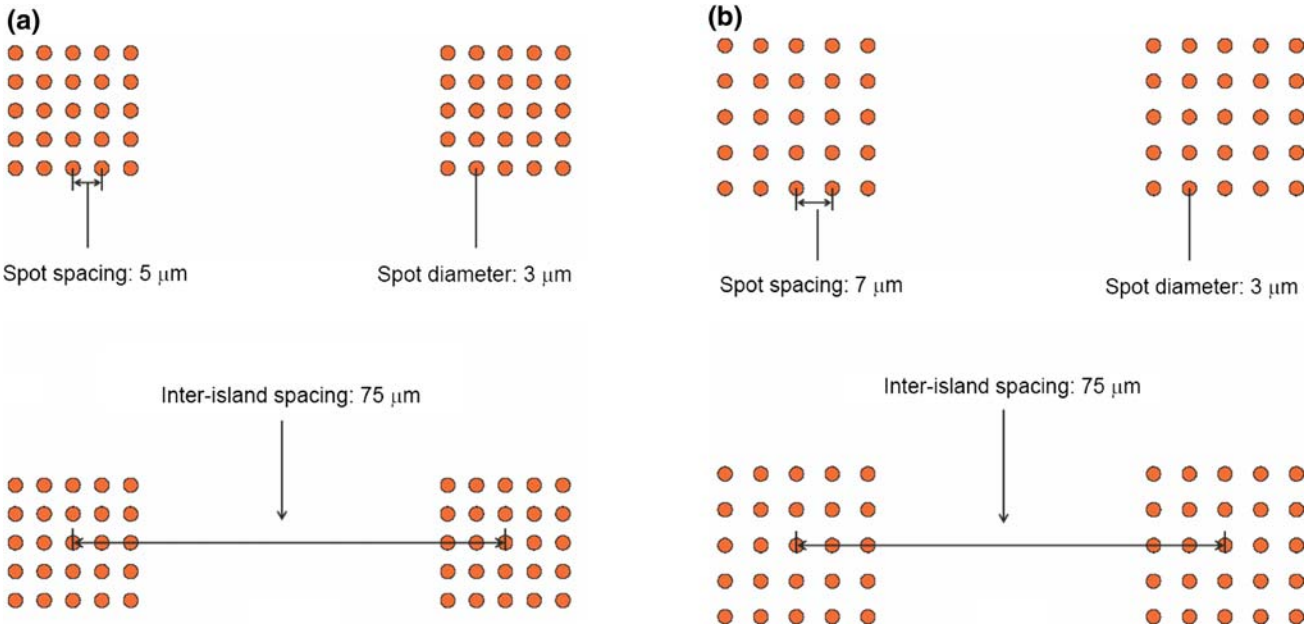

FIGURE 1. Micropattern design for 5 μm (a) and 7 μm (b) spot spacing group. Twenty-five spots form one island for one cell attachment. A total of 50,000 islands were made on one stamp for patterning on one glass slide. The 7 μm spot spacing group use exactly the same design except the spot spacing is changed to 7 μm.

Flow was driven by a Hamilton glass syringe in series with rigid walled tubing, and a parallel plate flow chamber. The syringe was mounted in and driven by an electromechanical loading device (EnduraTec, Eden Prarie, MN). The flow rate was monitored with an ultrasonic flow meter (Transonic Systems Inc., Ithaca, NY). The flow rate was selected to yield peak sinusoidal shear stresses of 1 Pa at 1 Hz. During the calcium imaging experiments, cells were exposed to a total of 3 min of oscillating fluid flow at 1 Pa.

Calcium Mobilization

MC3T3-E1 cells were seeded onto FN-coated micropatterned glass slides (50,000 cells/slide) and FN coated non-patterned glass slides (200,000 cells/slide) 24 h before experimentation. Real-time intracellular levels of calcium were quantified using a ratiometric imaging technique. Prior to exposure to OFF, cells were incubated with 10 μM Fura-2AM (Molecular Probes, Eugene OR) for 30 min at 31 °C (to reduce dye compartmentalization), then washed with fresh MEM-α and 2% FBS. Following Fura-2 loading, the cell-seeded slides were mounted in a parallel plate flow chamber and fixed to the stage of a fluorescent microscope. For 30 min, the cells were left undisturbed. The flow media consisted of MEM-α and 2% FBS. All experiments were performed at room temperature to reduce Fura-2AM dye compartmentalization. Ratio values, which are proportional to the intracellular Ca^{2+} concentration, were obtained for each cell using image analysis software (Metafluor, Universal Imaging, West Chester, PA). A cell response was defined as a transient increase in fluorescent ratio of at least four times the maximum oscillation recorded during the 3 min pre-flow baseline period (the region to the left of the 'flow on' arrow in Fig. 7). The percentage of cells responding is calculated by dividing the number of cells having Ca^{2+} response by the total number of cells being measured.

Statistical Analysis

To conduct multiple comparisons, a one-way ANOVA and Turkey's post hoc test were used. A significance level of 0.05 was employed for all statistical analyses. Data were reported as mean ± SD.

RESULTS

Microcontact Printing and Cell Seeding

Immunofluorescent staining of the fibronectin coated on the patterned surface (Fig. 2) showed that the designed pattern was transferred from the stamp to the slide surface with high fidelity and resolution. Cells adhered to and remained constrained by the micropatterned domains for at least 48 h (Fig. 3). It was observed that occasionally two cells occupied one island (data not shown). Moreover, there were occasional cases when cells were found to occupy only part of the island (data not shown). Nevertheless, most cells adhere to and fully occupy one adhesion island as desired.

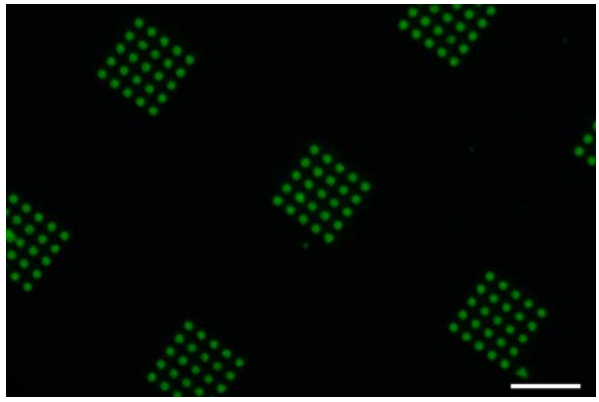

FIGURE 2. Immunofluorescent staining image of fibronectin coated on the patterned Ti–Au coated glass surface. Scale bar, 20 μm.

Quantification of Cell Spreading

Cell spreading was quantified by measuring projected cell areas on non-patterned surface and micropatterned surfaces with 5 or 7 μm center-to-center spot spacing between neighboring spots. Non-patterned cells have significant higher cell spreading (841.2 ± 273.9 μm^2) compared to 5 μm-spacing-patterned cells (530.2 ± 28.5 μm^2) ($p < 0.05$). However, the difference between non-patterned cells and 7 μm-spacing-patterned cells (957.3 ± 97.0 μm^2) is not statistically significant ($p = 0.51$) (Fig. 4).

Intracellular Calcium Mobilization

For intracellular Ca^{2+} response experiments, only cells that fully occupied the entire square shaped island were selected and their intracellular Ca^{2+} responses to fluid flow quantified. At the onset of flow, for both the patterned group and non-patterned group, cells responded with a transient increase in intracellular calcium concentration indicated by ratio value.

Percentage of Cells Responding to Fluid Flow (1 Hz, 1 Pa)

There was a significantly higher percentage of cells responding to fluid flow with increases in $[Ca^{2+}]_i$ on the

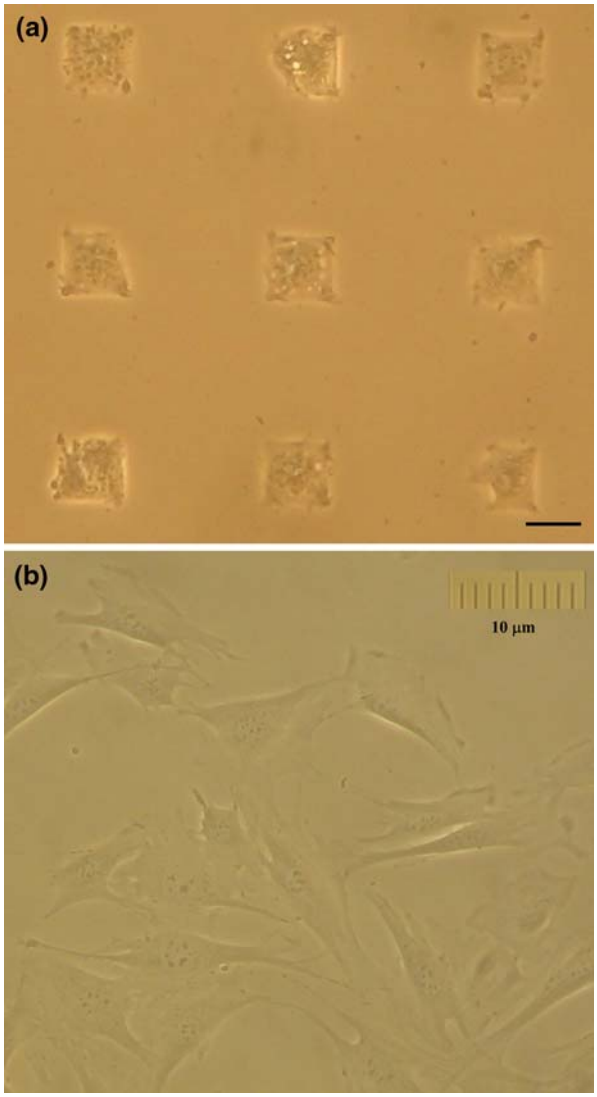

FIGURE 3. (a) Bright field light microscope image of cells that adhered to and remained constrained to the micropatterned domains. Scale bar, 20 μm. (b) Bright field light microscope image of cells that adhered to non-patterned surfaces. Scale bar 10 μm.

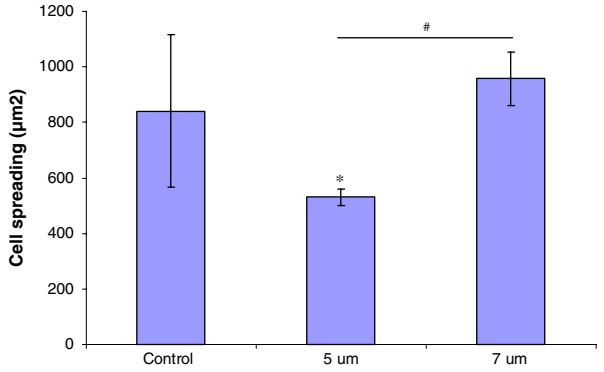

FIGURE 4. Quantification of cell spreading (projected cell area) per cell on non-patterned (control) surface and two different micropatterned surfaces with 5 or 7 μm center-to-center spot spacing between neighboring spots. Bars represents means ± SD ($n = 6$ for all groups; * significant difference between the non-patterned group and the patterned group, $p < 0.05$). # Significant difference between 5-μm-spot-spacing group and 7-μm-spot-spacing group, $p < 0.05$).

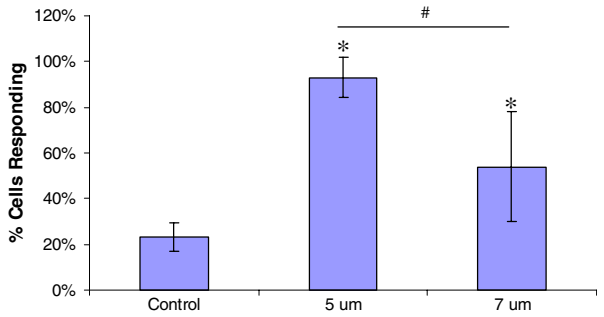

FIGURE 5. Percentage of cells responding with increases in $[Ca^{2+}]_i$. The percentage of cells responding is determined by dividing the number of cells having Ca^{2+} response by the total number of cells being measured. Bars represents means ± SD ($n = 4$ for all groups; * significant difference between the non-patterned group and the patterned group, $p < 0.05$). # Significant difference between 5-μm-spot-spacing group and 7-μm-spot-spacing group, $p < 0.05$).

patterned surfaces (92% for 5 μm spacing, 53% for 7 μm spacing) compared to cells seeded on the non-patterned surface (22%) (Fig. 5). Interestingly, more spread cells grown on the 7 μm spaced pattern were less responsive to dynamic fluid shear stress than cells grown on the 5 μm pattern in terms of the percentage of cells exhibiting at least one calcium transient ($p < 0.05$).

Percentage of Cells Exhibiting Multiple Calcium Transients in Responding to Fluid Flow (1 Hz, 1 Pa)

Cells grown on the patterned surfaces were much more likely to exhibit multiple calcium transients (61% for 5 μm spacing, 23% for 7 μm spacing) compared to cells grown on the unpatterned surface (9%) (Figs. 6 and 7). Similar to what we observed in percentage of cells responding to fluid flow, more spread cells grown on the 7 μm spaced pattern were less responsive to dynamic fluid shear stress than cells grown on the 5 μm pattern in terms of the number exhibiting multiple transients ($p < 0.05$).

DISCUSSION

To date the majority of investigations of cellular mechanisms of bone mechanotransduction have focused on determining the biochemical signaling pathways activated by mechanical stimulation. A relatively small number of studies have considered the mechanical behavior of the cell as an integrated part of the response although this is likely to be critical to

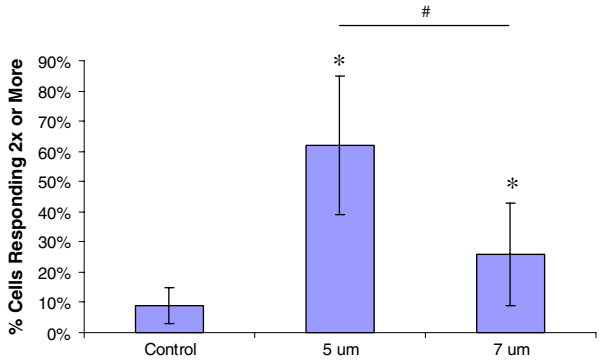

FIGURE 6. Percentage of cells responding with two or more Ca^{2+} responses. Two or more Ca^{2+} responses means that cells respond to fluid flow with two or more times of transient increases in fluorescent ratio which is at least four times the maximum oscillation recorded during the 3 min pre-flow baseline period. Bars represents means ± SD ($n = 4$ for all groups; * significant difference between the non-patterned group and the patterned group, $p < 0.05$). # Significant difference between 5-μm-spot-spacing group and 7-μm-spot-spacing group, $p < 0.05$).

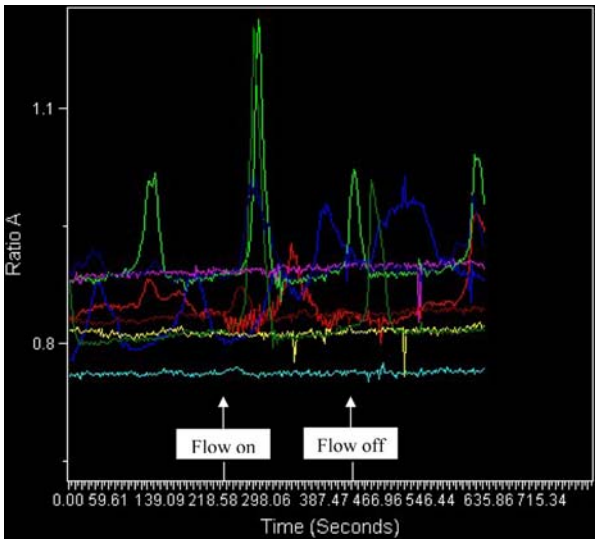

FIGURE 7. Patterned cells exhibit multiple Ca^{2+} responses to oscillatory fluid flow. Each curve represents the ratio value of fluorescence at 340/380, which is proportional to the concentration of free intracellular Ca^{2+}, in one individual cell as a function of time.

understanding how cellular mechanotransduction occurs.[19,23] Other studies have suggested that in addition to maintaining the cell's mechanical integrity, the cytoskeleton plays a central role in sensing mechanical stimuli.[26,28,29] Nevertheless, the role of mechanical tension in the cytoskeleton in bone cell mechanotransduction, as distinguished from the existence of an intact cytoskeleton itself. In this study we utilized micropatterning to modulate cell spreading without the use of pharmacological agents that could potentially affect cell functions.

In this study intracellular calcium $(Ca^{2+})_i$ served as our primary outcome variable. Intracellular Ca^{2+} is an important second messenger molecule in a wide variety of cellular transduction processes. In bone cells specifically it has been shown that many types of mechanical loading[9,14,16,24,31,34] produce transient increases in $[Ca^{2+}]_i$, and this process can occur on a time scale of a few seconds. This transient mobilization of intracellular calcium has been linked to a variety of downstream signaling processes including MAP kinase activity, NFkB translocation to the nucleus, activation of the PI3K-Akt signaling cascade, transcription factor activation, and ultimately expression of osteogenic genes.[8,9,18,22,33] Interestingly, intracellular calcium mobilization due to local mechanical deformation has also been linked to local cytoskeletal actin orientation.[1] Due to its ubiquitous role as a second messenger and involvement in regulation of bone-specific gene transcription, it is an excellent choice for characterizing responsiveness in this study.

Previously it has been reported that surface patterning and topography can have a dramatic effect on bone cell proliferation, motility, and adhesion as well as the interfacial strength of titanium implants.[4,5,11,12,15,30] Given the dramatic effects surface pattern can have on so many aspects of cell metabolism, it is surprising that in the context of mechanotransduction only limited investigations have been conducted. Chen *et al.*[7] have shown that decreased contractility and reorganization of the actin cytoskeleton can occur when a single adhesive spot is displaced in a micropatterned surface similar to the ones used here. Fibroblasts were found to be more sensitive to stretch when grown on grooved surfaces when the grooves were perpendicular to the stretch direction.[25] However, our work represents the first examination of bone cell mechanosensitivity with micropatterned surfaces and the first examination of the response to fluid flow in any cell type. We found that bone cells grown on micropatterned surfaces were dramatically more responsive than cells grown on unpatterned surfaces (Figs. 5 and 6). This was a consistent finding for both the 5 μm and 7 μm spacing. The exact mechanism whereby micropatterned surfaces increase cellular responsiveness to mechanical stimulation is unclear at this point. One possibility is that on the patterned surface adhesive contact area was limited when compared to the unpatterned surface. This would result in a larger force per unit area being transmitted across the focal contacts when the cells were exposed to shear stress, and hence, individual integrins experiencing larger molecular forces. In any case further investigation will be required to more completely characterize the fluid-flow response of cells grown on micropatterned surfaces and the molecular mechanism behind this enhanced sensitivity.

When the results for the 5 μm and 7 μm patterns are compared directly, we observed a decrease in responsiveness with increased spot spacing (Fig. 5). This was unexpected as it actually suggests that cellular sensitivity to mechanical stimulation decreased with increased spreading. It is important to note that the total cell–substrate contact areas for these two patterns are identical. Results from Chen and colleagues[7] suggested that the increase in cell spreading leads to increases in focal adhesion size and activity. This would suggest an increase in cytoskeletal tension when considered along with the Balaban *et al*. study.[2] Thus, our finding is surprising in light of prior studies of the role of the cytoskeleton, and presumably cytoskeletal tension, in mechanotransduction.[26,28,29] One interpretation is that when cells are more spread and cytoskeletal tension increases, the resulting pre-stress makes them stiffer in response to subsequent loading, much as a string or rope under tension becomes stiff with respect to transverse deformation. Thus, it is possible that cells in the 7 μm spacing group undergo less deformation when exposed to shear stress and molecular mechanotransduction mechanisms dependent on deformation are less likely to be activated. However, currently there are no published data that directly demonstrate decreased deformation of cells exposed to shear stress with spreading.

Figure 6 shows that increased cell spreading results in a decreased percentage of cells exhibiting multiple Ca^{2+} responses. It has been established that the patterns of multiple Ca^{2+} response in other cell types has an impact on downstream effects such as gene expression or protein modification. Therefore, the change in Ca^{2+} response patterns observed here reflects the potential role of cell spreading in bone cellular response to mechanical loading.

In summary, in this study we have demonstrated for the first time that cells grown on micropatterned surfaces are dramatically more sensitive to mechanical stimuli than cells grown on smooth surfaces. Interestingly, however, mechanosensitivity actually decreased when the cells were further spread by increasing spot separation while maintaining total adhesive area. This may be a result of decreased deformation in spread cells when exposed to fluid shear stress, although this has not been confirmed.

ACKNOWLEDGMENT

This work was funded by NIH R01AR45989 and R01GM065918.

REFERENCES

[1]Adachi, T., K. Sato, and Y. Tomita. Directional dependence of osteoblastic calcium response to mechanical stimuli. *Biomech. Model. Mechanobiol.* 2(2):73–82, 2003.
[2]Balaban, N. Q., *et al*. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat. Cell Biol.* 3(5):466–472, 2001.
[3]Berrier, A. L., and K. M. Yamada. Cell-matrix adhesion. *J. Cell. Physiol.* 213(3):565–573, 2007.
[4]Briggs, E. P., *et al*. Formation of highly adherent nanoporous alumina on Ti-based substrates: a novel bone implant coating. *J. Mater. Sci. Mater. Med.* 15(9):1021–1029, 2004.
[5]Charest, J. L., *et al*. Combined microscale mechanical topography and chemical patterns on polymer cell culture substrates. *Biomaterials* 27(11):2487–2494, 2006.
[6]Chen, C. S., *et al*. Geometric control of cell life and death. *Science* 276(5317):1425–1428, 1997.
[7]Chen, C. S., *et al*. Cell shape provides global control of focal adhesion assembly. *Biochem. Biophys. Res. Commun.* 307(2):355–361, 2003.
[8]Chen, N. X., *et al*. Fluid shear-induced NFkappaB translocation in osteoblasts is mediated by intracellular calcium release. *Bone* 33(3):399–410, 2003.
[9]Danciu, T. E., *et al*. Calcium regulates the PI3K-Akt pathway in stretched osteoblasts. *FEBS Lett.* 536(1–3): 193–197, 2003.
[10]Deyneka-Dupriez, N., *et al*. Interfacial shear strength of titanium implants in bone is significantly improved by surface topographies with high pit density and microroughness. *J. Biomed. Mater. Res. B Appl. Biomater.* 82(2): 305–312, 2007.
[11]Diniz, M. G., *et al*. Surface topography modulates the osteogenesis in human bone marrow cell cultures grown on titanium samples prepared by a combination of mechanical and acid treatments. *J. Mater. Sci. Mater. Med.* 13(4):421–432, 2002.
[12]Gallant, N. D., *et al*. Micro- and nano-patterned substrates to manipulate cell adhesion. *J. Nanosci. Nanotechnol.* 7(3):803–807, 2007.
[13]Gallant, N. D., K. E. Michael, and A. J. Garcia. Cell adhesion strengthening: contributions of adhesive area, integrin binding, and focal adhesion assembly. *Mol. Biol. Cell* 16(9):4329–4340, 2005.
[14]Genetos, D. C., *et al*. Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. *J. Cell. Physiol.* 212(1):207–214, 2007.
[15]Gray, D. S., J. Tien, and C. S. Chen. Repositioning of cells by mechanotaxis on surfaces with micropatterned Young's modulus. *J. Biomed. Mater. Res. A* 66(3):605–614, 2003.
[16]Guo, X. E., *et al*. Intracellular calcium waves in bone cell networks under single cell nanoindentation. *Mol. Cell. Biomech.* 3(3):95–107, 2006.
[17]Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* 110(6):673–687, 2002.
[18]Inoue, D., S. Kido, and T. Matsumoto. Transcriptional induction of FosB/DeltaFosB gene by mechanical stress in osteoblasts. *J. Biol. Chem.* 279(48):49795–49803, 2004.
[19]Jaasma, M. J., *et al*. Adaptation of cellular mechanical behavior to mechanical loading for osteoblastic cells. *J. Biomech.* 40(9):1938–1945, 2007.

[20]Jacobs, C. R., *et al*. Differential effect of steady versus oscillating flow on bone cells. *J. Biomech*. 31(11):969–976, 1998.
[21]Jager, M., *et al*. Osteoblast differentiation onto different biometals with an endoprosthetic surface topography in vitro. *J. Biomed. Mater. Res. A* 86(1):61–75, 2008.
[22]Katz, S., R. Boland, and G. Santillan. Modulation of ERK 1/2 and p38 MAPK signaling pathways by ATP in osteoblasts: involvement of mechanical stress-activated calcium influx, PKC and Src activation. *Int. J. Biochem. Cell. Biol*. 38(12):2082–2091, 2006.
[23]Kwon, R. Y., and C. R. Jacobs. Time-dependent deformations in bone cells exposed to fluid flow in vitro: investigating the role of cellular deformation in fluid flow-induced signaling. *J. Biomech*. 40(14):3162–3168, 2007.
[24]Li, Y. J., *et al*. Oscillatory fluid flow affects human marrow stromal cell proliferation and differentiation. *J. Orthop. Res*. 22(6):1283–1289, 2004.
[25]Loesberg, W. A., *et al*. The effect of combined cyclic mechanical stretching and microgrooved surface topography on the behavior of fibroblasts. *J. Biomed. Mater. Res. A* 75(3):723–732, 2005.
[26]Malone, A. M., *et al*. The role of actin cytoskeleton in oscillatory fluid flow-induced signaling in MC3T3-E1 osteoblasts. *Am. J. Physiol. Cell. Physiol*. 292(5):C1830–C1836, 2007.
[27]Meredith, D. O., *et al*. Microtopography of metal surfaces influence fibroblast growth by modifying cell shape, cytoskeleton, and adhesion. *J. Orthop. Res*. 25(11):1523–1533, 2007.
[28]Myers, K. A., *et al*. Osteoblast-like cells and fluid flow: cytoskeleton-dependent shear sensitivity. *Biochem. Biophys. Res. Commun*. 364(2):214–219, 2007.
[29]Norvell, S. M., *et al*. Fluid shear stress induction of COX-2 protein and prostaglandin release in cultured MC3T3-E1 osteoblasts does not require intact microfilaments or microtubules. *J. Appl. Physiol*. 96(3):957–966, 2004.
[30]Ogawa, T., C. Sukotjo, and I. Nishimura. Modulated bone matrix-related gene expression is associated with differences in interfacial strength of different implant surface roughness. *J. Prosthodont*. 11(4):241–247, 2002.
[31]Sato, K., *et al*. Measurement of local strain on cell membrane at initiation point of calcium signaling response to applied mechanical stimulus in osteoblastic cells. *J. Biomech*. 40(6): 1246–1255, 2007.
[32]You, J., *et al*. Substrate deformation levels associated with routine physical activity are less stimulatory to bone cells relative to loading-induced oscillatory fluid flow. *J. Biomech. Eng*. 122(4):387–393, 2000.
[33]You, J., *et al*. Osteopontin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of mitogen-activated protein kinase in MC3T3-E1 osteoblasts. *J. Biol. Chem*. 276(16):13365–13371, 2001.
[34]Zhang, M., J. J. Wang, and Y. J. Chen. Effects of mechanical pressure on intracellular calcium release channel and cytoskeletal structure in rabbit mandibular condylar chondrocytes. *Life Sci*. 78(21):2480–2487, 2006.