

# Alignment and proliferation of MC3T3-E1 osteoblasts in microgrooved silicone substrata subjected to cyclic stretching

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## Abstract

Previous studies have shown that many types of cells align in microgrooves in static cultures. However, whether cells remain aligned and also proliferate in microgrooves under stretching conditions has not been determined. We grew MC3T3-E1 osteoblasts in deformable silicone dishes containing microgrooves oriented in the stretch direction. We found that with or without 4% stretching, cells aligned in microgrooves of all sizes, with the groove and ridge widths ranged from 1 to 6  $\mu\text{m}$ , but the same groove depth of about 1.6  $\mu\text{m}$ . In addition, actin cytoskeleton and nuclei became highly aligned in the microgrooves with and without 4% cyclic stretching. To further examine whether MC3T3-E1 osteoblasts proliferate in microgrooves with cyclic stretching, we grew the cells in six-well silicone dishes containing microgrooves in three wells and smooth surfaces in other three wells. After 4% cyclic stretching for 3, 4, and 7 days, we found that cell numbers in the microgrooves were not significantly different ( $p > 0.05$ ) from those on the smooth surface ( $p > 0.05$ ). Taken together, these results show that MC3T3-E1 osteoblasts can align and proliferate in microgrooves with 4% cyclic stretching. We suggest that the silicone microgrooves can be a useful tool to study the phenotype of MC3T3-E1 osteoblasts under controlled substrate strains. The silicone microgrooves can also be useful for delivering defined substrate strains to other adherent cells in cultures. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Contact guidance; Microgrooves; Mechanical stretching; Alignment; Proliferation; Cell mechanics

## 1. Introduction

First introduced by Weiss (1945), the term contact guidance describes the orientation of cell locomotion as a response to surface micropatterns. Using a variety of substrata, including the internal collagenous layer of fish scale, plasma clots, and glass fibers, Weiss (1959) extensively studied the cell behavior of contact guidance. Subsequently, cell contact guidance was more closely studied using parallel surface microgrooves made on a variety of materials, because the microgrooves have the advantage of a defined and uniform topography. Previous studies have shown that many types of cells can be aligned in the

microgrooves (Dunn and Heath, 1976; Brunette, 1986; Dow et al., 1987; Clark et al., 1990). For example, Brunette (1986) examined the spreading and orientation of epithelial cells on titanium-coated substrata with vertical-walled grooves and v-shaped grooves, 3–60  $\mu\text{m}$  deep, by light, transmission and scanning electron microscopy. It was found that cells were markedly oriented in all the grooved substrata examined. The cell contact guidance not only occurs *in vitro*, but also occurs on microgrooved artificial substrata *in vivo*. In fact, in a study designed to test the hypothesis that contact guidance occurred *in vivo*, Chehroudi et al. (1990) showed that the down-growth of epithelium on a percutaneous implant can be enhanced by microgrooves (3  $\mu\text{m}$  deep, 10  $\mu\text{m}$  pitch) oriented along the long axis of the implant, or inhibited by the same microgrooves oriented perpendicular to the long axis. It is now recognized that surface topography affects cell shape and cell locomotion, mRNA expression (Chou et al., 1995), and more complex cellular responses such as tissue organization and mineralization *in vivo* (see review by Brunette and Chehroudi, 1999). Recently,

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the examination of cell behavior in microgrooves has been extended to cell proliferation (den Braber et al., 1996).

Although previous studies have provided important information on the effect of microgrooves on cell alignment and proliferation, most, if not all, of these studies were conducted in static cultures. That is, the microgrooves for studying the cell contact guidance were not subjected to mechanical deformation. However, mechanical loading, e.g., on microgrooved implants to bone, is an essential part of cellular environment *in vivo*. Hence, it is important to know the interaction between the cell contact guidance and mechanical loading, about which information has been lacking. Furthermore, the combined morphological effect of microgrooves with cyclic stretching on actin cytoskeleton and nuclei has not been adequately examined. Both actin cytoskeleton and nucleus are the intracellular structures that play a vital role in various cellular functions such as proliferation and differentiation (Ingber, 1991).

The purpose of this study was to test the hypothesis that MC3T3-E1 osteoblasts align and proliferate in microgrooved substrata with and without cyclic stretching. To test the hypothesis, we grew MC3T3-E1 osteoblasts in silicone microgrooves and examined cell alignment, proliferation, and re-arrangement of actin cytoskeleton and nuclei under cyclic stretching conditions. MC3T3 cells were chosen, because they are a model cell line that has been widely used to study phenotype expression of the osteoblastic cells in static culture (Stein and Lian, 1993). And we wanted to explore the possibility of using the silicone microgrooves to study phenotype expression of the MC3T3-E1 osteoblasts under controlled dynamic culture conditions.

## 2. Materials and methods

### 2.1. Cell culture

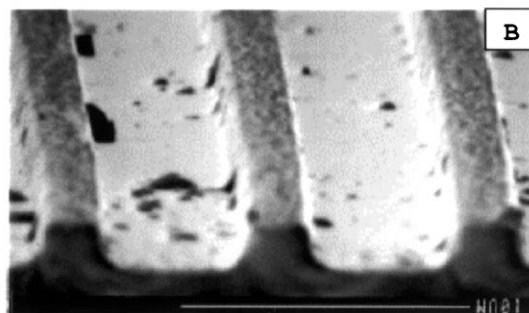
MC3T3-E1 osteoblasts, originally derived from mouse calvaria, were grown in 35 mm diameter multi-well plastic dishes containing  $\alpha$ -MEM supplemented with 10% fetal bovine serum (FBS, Gibco Labs), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml),  $\beta$ -glycerol phosphate (5 mM) and 25  $\mu$ g/ml ascorbic acid (Sigma). The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.

### 2.2. Cell alignment experiments

Because silicone is highly transparent, elastic, and non-toxic to cultured cells (Wang et al., 1995), custom-made silicone dishes were used for growing MC3T3-E1 osteoblasts and applying cyclic stretch to the cells. Silicone dishes with smooth culture surfaces were made by

2/3	3/3	1/1		5/3	6/3
2/2	3/2	1/2		5/2	6/2
2/1	3/1	1/3		5/1	6/1

(A)



B

Fig. 1. (A) Microgroove patches and smooth surfaces used for cell alignment experiments. Note that there are a total of 18 patches, each about 1 cm<sup>2</sup>. Fifteen of the patches had microgrooves, and the remaining three had smooth surfaces. The numbers in each patch represent the groove/ridge widths. The depth of microgrooves was about 1.6  $\mu$ m. These patches of microgrooves and smooth surfaces were bonded to the bottom of a silicone dish, with the groove direction along the dish long axis, in which cyclic stretching was applied. (B) A representative scanning electron microphotograph of the microgrooves. The grooves shown here are 5  $\mu$ m in the groove width and 2  $\mu$ m in the ridge width. Note that the walls of the grooves are close to vertical.

a molding process. Briefly, two silicone fluid components, 601A and 601B (Wacker Chemie, Munich, Germany), in a ratio of 10 : 1, were mixed in a glass container. Entrapped air in the silicone mixture was extracted by vacuum. The silicone was then slowly poured into a Plexiglas dish mold, where the area corresponding to each dish culture surface was covered with a piece of smooth glass. After de-molding, silicone dishes having smooth culture surfaces were obtained. The culture surface of the dish was rectangular, with an area of 3 cm  $\times$  6 cm.

Silicone dishes with microgrooves were obtained by replacing the smooth surfaces with microgrooved silicone membranes. The silicone membranes were made by molding silicone against a microgrooved silicon wafer, which was fabricated using standard lithographic and reactive ion etching techniques. The resulting silicone membrane, about 2-mm thick, contained 18 patches, 1 cm<sup>2</sup> each. Fifteen patches had microgrooves, with the same depth of 1.6  $\mu$ m, but varying groove/ridge widths that ranged from 1 to 6  $\mu$ m. The remaining three patches had smooth surfaces. Each microgrooved membrane was

cut to a size of 3.5 cm × 6.5 cm. Then, it was bonded, using silicone glue (Dow Corning, MI), to the bottom of a silicone dish whose smooth culture surface had been removed. The direction of the microgrooves was along the dish's long axis, which was also the stretching direction (Fig. 1).

The dishes were thoroughly washed with 70% ethanol and rinsed with double distilled water. To promote cell attachment, culture surfaces of the dishes were coated with 10 µg/ml ProNectin-F (Protein Polymer Technologies, Inc., San Diego, CA) for 5 min, and washed twice with phosphate buffered saline (PBS). MC3T3-E1 osteoblasts from passages 12 to 15 were plated to the dishes at a density of 5000 cells/cm<sup>2</sup> and incubated at 37°C. After incubation for 24 h, cyclic stretches of 0 (i.e., without stretch for control) and 4%, at 1 Hz, were applied to the dishes for up to 20 days. The stretching was only interrupted when medium was changed every 3 days, or when phase contrast microphotographs of the cells were taken (Kodak 100 slide films). The stretching was applied using a custom-made stretching apparatus previously described (Neidlinger-Wilke et al., 1994). Briefly, the apparatus consists of six stations, on which silicone dishes were mounted and held at their two ends by six stationary clamps and six moving clamps. The stationary clamps held the dishes in place, and the moving clamps, which, through the connection to six drive shafts that transmit the linear motion from the cam-follower mechanism, stretched the dishes cyclically. The stretching magnitude was controlled by the eccentricity of circular cams, which were rotated by a DC motor.

### 2.3. Cell proliferation experiments

To examine cell proliferation, six-well silicone dishes were used. The dishes were the same as those used in the cell alignment experiments above, except that the dish well was divided into six small wells, each having a culture surface of about 3 cm<sup>2</sup>. Along one side of the dish, three wells contained microgrooves with the same depth of 2 µm, but varying groove/ridge widths of 12/12, 18/18, and 24/24 µm. The direction of the microgrooves was along the dish long axis, that is, the stretching direction. On the opposite side of the three wells containing microgrooves, there were also three wells, which had only smooth surfaces.

MC3T3-E1 osteoblasts were plated to four six-well dishes, coated with ProNectin-F (10 µg/ml), at a density of 10<sup>4</sup> cells/cm<sup>2</sup>. The cells were incubated overnight (12–16 h), and then cyclic stretches of 0 (control) and 4%, at 1 Hz, were applied to the dishes for 3, 4, and 7 days. At the end of each stretching period, medium was removed from the wells. After washing twice with PBS, cells in each well were treated with 0.5 ml of 0.05% trypsin for 5 min, followed by addition of 0.5 ml of α-MEM to neutralize the trypsin. The cell suspension was then

transferred to a 15-ml conical tube (Falcon). Following the same procedure, a cell suspension from another six-well dish was transferred to the same conical tube. After centrifugation at 1000 rpm for 5 min, medium was extracted and 0.5 ml of fresh α-MEM was added and mixed well by pipetting up and down. Finally, using a hemocytometer, cell numbers were counted on a Nikon microscope.

### 2.4. Staining actin filaments and nuclei

Actin filaments and nuclei were stained for fluorescent microscopy using the following procedures. The cells were washed twice with PBS, fixed in a solution of 3% para-formaldehyde and 2% sucrose in PBS for 30 min, and then extracted with a permeabilizer (10 g of sucrose, 0.5 g of HEPES, 0.4 ml of 5 M NaCl, 3 ml of 1 M MgCl<sub>2</sub>, and 0.1 ml of Triton-X100 in 75 ml of distilled water) for 5 min. Next, the cells were washed twice with ice-cold PBS, and incubated with propidium iodide (1 µl propidium iodide dissolved in 3.3 ml of PBS) at room temperature for 15 min. This was followed by two washes in PBS and staining with 0.165 µM rhodamine phalloidin or the same concentration of fluorescein phalloidin at room temperature for 30 min. Finally, stained cells were viewed and photographed on a fluorescence microscope (Nikon, Microphot-FX). In addition, actin cytoskeletons and nuclei of the cells in microgrooves were examined on a confocal microscope, consisting of a Nikon inverted microscope, and a desktop computer with Bio-Rad MRC-600 software installed for the three-dimensional reconstruction of sectional confocal images.

### 2.5. Statistics

The Kruskal-Wallis test (Rosner, 1990) was used to determine whether there was an overall significant difference in proliferation among cells grown in the three sizes of microgrooves, which were 12/12, 18/18, and 24/24 µm in the groove/ridge widths. The Wilcoxon signed rank test was used to determine whether cell proliferations in microgrooves and on smooth surfaces were significantly different. The difference was considered to be significant if  $p < 0.05$ .

## 3. Results

MC3T3-E1 osteoblasts grown on smooth surfaces were randomly oriented without stretching. In contrast, the same cells were oriented along the groove direction of all sizes of microgrooves. With 4% stretch for 3 days, the cells in the microgrooves remained aligned, but the cells on the smooth surface had no apparent specific orientation (Fig. 2). Furthermore, with 4% stretch for 20 days, the cells were still aligned in the groove direction (Fig. 3).

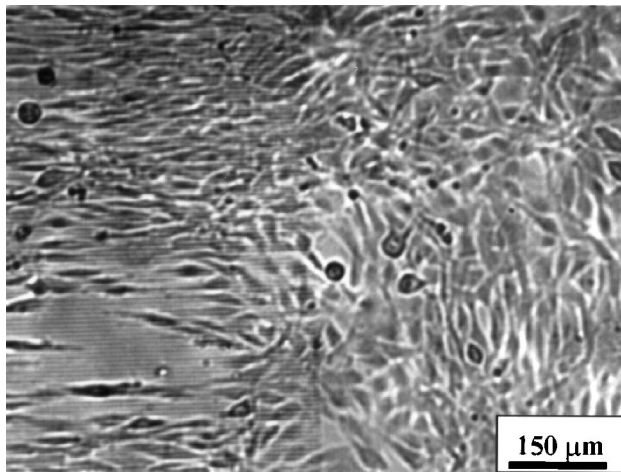


Fig. 2. The alignment of MC3T3-E1 osteoblasts with 4% stretch (horizontal) for 3 days. It is seen that after the stretching, the cells on the smooth surface (right part of the photograph) randomly oriented, whereas the cells in microgrooves ( $2/3 \mu\text{m}$  in the groove/ridge width) remained aligned in the groove direction, which is horizontal. Note that before stretching, cells were aligned in microgrooves (not shown).

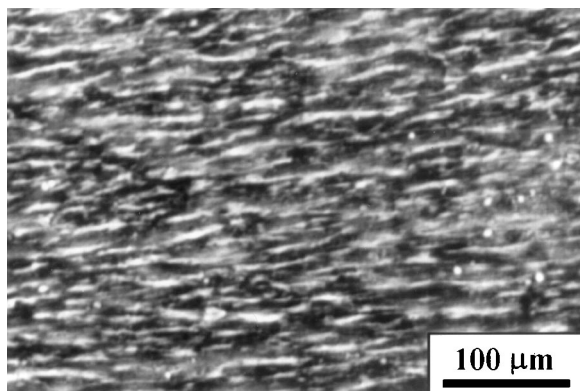


Fig. 3. MC3T3-E1 osteoblasts remained aligned in microgrooves ( $5/1 \mu\text{m}$  in the groove/ridge widths) after 4% stretch for 20 days. Note that the groove direction, which was also the stretching direction, is horizontal. But because cells formed dense layers, the underlying microgrooves are not visible.

Additionally, in the absence of stretching, actin filaments and nuclei of MC3T3-E1 osteoblasts were aligned in the direction of all sizes of the grooves. Confocal microscopy further revealed that the aligned actin filaments and nuclei actually lie in different levels, indicating that the cells formed multi-layers but still aligned in the groove direction (Fig. 4). In contrast, actin filaments and nuclei of the cells on smooth surfaces formed a curved pattern (Fig. 5). With 4% stretch for 20 days, similar patterns of actin filaments and nuclei of the cells in the microgrooves and on the smooth surface were observed (data not shown).

In separate experiments, proliferation of MC3T3-E1 cells both in microgrooves and on smooth surfaces was

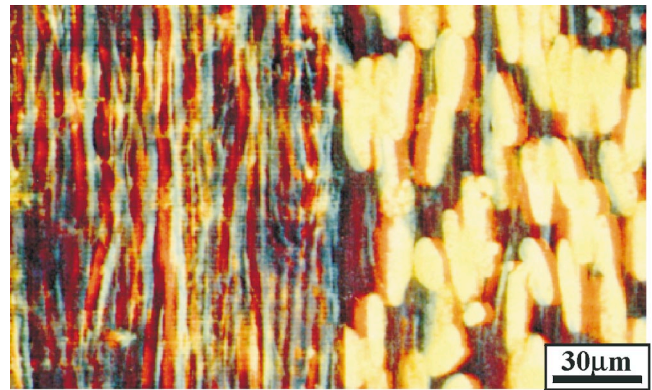


Fig. 4. A representative confocal microphotograph of actin filament bundles and nuclei of MC3T3-E1 osteoblasts in microgrooves ( $5/2 \mu\text{m}$  in the groove/ridge width) without stretching. It is evident that actin filaments were aligned in the groove direction (vertical), as did the nuclei which had an elongated shape. Because the cells formed multi-layers, the actin filament bundles and nuclei also had a three-dimensional structure, but they all aligned in the groove direction.

examined. Cell numbers (means  $\pm$  SDs) in the microgrooves and on the smooth surfaces at 3, 4 and 7 days were shown in Figs. 6 and 7. It was found that with or without 4% stretch, cell numbers in the three microgrooves ( $12/12$ ,  $18/18$ , and  $24/24 \mu\text{m}$  in groove/ridge widths) were not significantly different ( $p > 0.05$ ). Furthermore, with or without 4% stretch, cell numbers in the microgrooves and on the smooth surfaces were not significantly different either ( $p > 0.05$ ).

#### 4. Discussion

There are three major findings in this study. First, MC3T3-E1 osteoblasts were aligned in silicone microgrooves without stretch, and remained aligned with 4% cyclic stretch. Second, cell proliferation in the microgrooves, with the same depth of  $2 \mu\text{m}$ , and the groove/ridge widths from  $12$  to  $24 \mu\text{m}$ , was not significantly different from that on the smooth surfaces with or without 4% stretch. Third, actin cytoskeletons of the cells in the microgrooves were remodeled: They became highly elongated bundles aligned in the groove direction. In addition, nuclei appeared to have an elliptic shape and orient toward the groove direction. The 4% cyclic stretching appeared not to change the alignment of actin cytoskeleton and nuclei.

How do MC3T3-E1 cells align in microgrooves? Although the exact mechanism is not clear, the cell alignment may be due to mechanical restriction on the formation of certain linear bundles of microfilaments involved in cell locomotion (Dunn and Heath, 1976). Cell alignment in microgrooves may also involve focal adhesions (Ohara and Buck, 1979), which line in rows within the grooves (Dunn and Brown, 1986). In

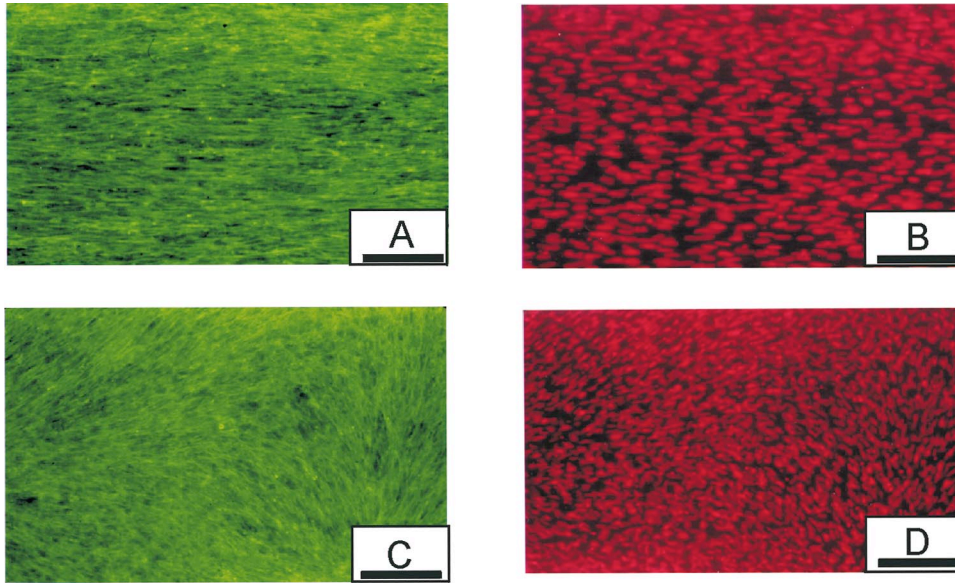


Fig. 5. Patterns of actin filaments and nuclei of the MC3T3-E1 osteoblasts in the microgrooves (A, B), with 5/3  $\mu\text{m}$  in the groove/ridge width, and on the smooth surface (C, D). It is evident that in the microgrooves, these cells had thick bundles of actin filaments, and their nuclei aligned in the groove direction. In contrast, on the smooth surface, the cells had curved actin filament bundles and non-aligned nuclei. Similar orientation patterns of actin filaments and nuclei were found when the cells were subjected to 4% cyclic stretch. (Bar: 150  $\mu\text{m}$ ).

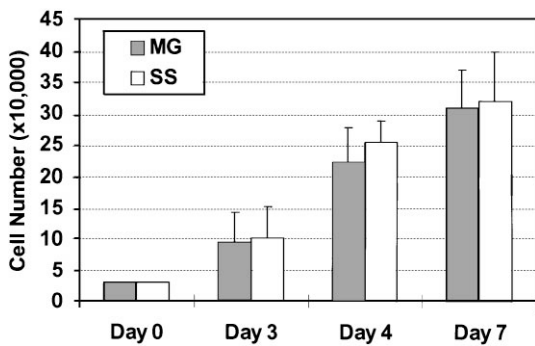


Fig. 6. Proliferations of MC3T3-E1 osteoblasts grown in microgrooves (MG) and smooth surface (SS) without stretch. No significant difference in proliferation between MG and SS was found ( $p > 0.05$ , Wilcoxon signed rank test). Note that data were obtained from four cultures, and represented by mean  $\pm$  SD.

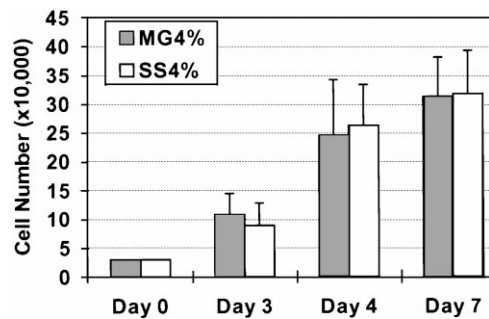


Fig. 7. Proliferations of MC3T3-E1 osteoblasts grown in 4% stretched microgrooves (MG4%) and 4% stretched smooth surface (SS4%). No significant difference in proliferation between MG4% and SS4% was found ( $p > 0.05$ , Wilcoxon signed rank test). Note that data were obtained from four cultures, and represented by mean  $\pm$  SD.

addition to the actin cytoskeleton and focal adhesions, microtubules may play a major role in cell alignment in microgrooves, since they were the first structures to become aligned when cells spread in microgrooves (Oakley and Brunette, 1993).

This study showed that without stretch, proliferation of MC3T3-E1 cells in the three sizes of microgrooves (12/12, 18/18 and 24/24  $\mu\text{m}$  in groove/ridge widths) was not different from that on the smooth surfaces. In a recent study, den Braber et al. (1996) grew rabbit dermal fibroblasts in silicon microgrooves with groove and ridge widths of 2, 5 and 10  $\mu\text{m}$ , and a depth of 0.5  $\mu\text{m}$ . They

showed that cell proliferation was not affected by either the presence of the microgrooves or the dimension of the grooves. Thus, proliferation of cells in microgrooves appears to be cell-type dependent (Ricci et al., 1993; den Braber et al., 1996): Different types of cells may have a different proliferative response to similar dimensions of microgrooves. Indeed, Ricci et al. (1994) reported that overall growth of rat bone marrow colonies in microgrooves was changed. In addition, other factors can also affect proliferation of cells in microgrooves. These include surface wettability and free energy (den Braber et al., 1995). In addition, surface coating with extracellular

matrix proteins, such as the ProNectin-F used in this study, may also influence proliferation of MC3T3-E1 cells.

Interestingly, this study showed that with 4% cyclic stretch, proliferation of the MC3T3-E1 osteoblasts in the microgrooves and on the smooth surface was not significantly different. This result appears to be surprising since, if cells attached in the microgrooves and on smooth surface equally well, cells in the microgrooves were subjected to, on average, larger strains than cells on the smooth surface. This is because all cells in the microgrooves were aligned in the stretching direction and were therefore subjected to the same substrate deformation, which was the maximal substrate strain applied (4%). In contrast, many cells on the smooth surface were subjected to smaller substrate deformations ( $< 4\%$ ), because they were oriented in directions where, according to the strain theory (Fung, 1994), smaller strain than that in the stretching direction acted on the cells. Thus, the lack of difference between cell proliferation in the microgrooves and on the smooth surface under 4% cyclic stretch implies that larger substrate strains may not induce greater proliferation in the MC3T3-E1 osteoblasts. This seems to be consistent with a previous finding that under large uniaxial stretches ( $> 1.0\%$ ), osteoblast proliferations in the stimulated cultures and unstretched controls were not significantly different (Neidlinger-Wilke et al., 1994). It should be noted, however, that the difference in cell proliferation in response to substrate strains may depend on many factors, including the type (uniaxial or biaxial) of stretching applied, the source (e.g., calvaria or limb) from which osteoblasts are derived (Rawlinson et al., 1995), and possibly the culture conditions (Stanford et al., 1995), such as the type of growth medium used.

Furthermore, it should be pointed out that under normal loading conditions *in vivo*, only small strains ( $< 4\%$ ) are believed to act on osteoblasts (Neidlinger-Wilke et al., 1994; Rawlinson et al., 1995; Stanford et al., 1995), although the exact magnitude of strains that act on osteoblasts *in vivo* is not known. In sites of bone fracture healing, however, osteoblasts are likely subjected to much higher strains and 4% strain may not be unreasonable. Therefore, the results of this study with 4% cyclic strain should be interpreted to reflect the behavior of osteoblasts in response to mechanical strains during bone fracture healing.

This study showed that MC3T3-E1 cells contained actin filaments and nuclei that were aligned markedly in the groove direction. This was in contrast to the cells on the smooth surface, which had randomly oriented actin filaments and nuclei. However, the mechanism of the actin filament and nucleus alignment is unclear. One possibility is that microgrooves impose mechanical restrictions on the formation of actin filaments, which are known to be involved in cell spreading and locomotion

(Dunn and Brown, 1986), so that only actin filaments along the groove direction can be assembled and extended. Furthermore, the change in the pattern of the actin cytoskeleton, an inherent tensile structure, may also affect the nucleus orientation, since the actin cytoskeleton is connected to the intermediate filaments, which are linked with the nucleus (Ingber, 1993). Thus, the tension in the actin filament bundles in the groove direction may pull the nucleus in the same direction as the bundles. Evidence that supports this notion of pulling the nucleus through actin filaments comes from a previous study, which showed that pulling integrins that link with actin filaments can instantly distort the nucleus (Maniotis et al., 1997).

The results of this study make it possible to use silicone microgrooves to deliver defined substrate strains to MC3T3-E1 osteoblasts, since the cells in the grooves maintained the alignment under cyclic substrate stretching. Moreover, the cells still proliferated in silicone microgrooves just as well as those on smooth surfaces, either without or with 4% stretch. The unaltered cell proliferation is important to study the phenotype of MC3T3-E1 cells, since the cellular phenotype development follows three principal sequences: proliferation, extracellular matrix maturation, and mineralization (Stein and Lian, 1993). In other words, without the phase of proliferation, the phenotype of MC3T3-E1 osteoblasts will not be developed. Using the silicone microgrooves, one can study the phenotype of MC3T3-E1 cells in both static and dynamic culture conditions.

The silicone microgrooves may be also used for other types of cells as well for delivering controlled substrate strains. It is known that there are three strain components, namely, axial strain, transverse strain, and shear strain, on a deformed substrate surface. These three strains act on individual cells adherent to the substrate (Tran-Son-Tay, 1993). In general, these strains vary from one direction to the other. Therefore, by use of microgrooves with various orientations, various combinations of substrate strains can be applied to cells aligned in the grooves. Compared with biaxial stretching techniques (Hung and Williams, 1994; Schaffer et al., 1994), the use of microgrooves may produce substrate strains closer to those *in vivo*. For example, ligament and tendon fibroblasts, which align with collagen fibers *in vivo*, are subjected to one-directional stretching. For these cells, the use of microgrooves to grow and stretch cells may provide a better experimental model for studying mechanically induced cellular responses, such as DNA and protein synthesis.

In summary, this study showed that MC3T3-E1 osteoblasts aligned in silicone microgrooves without stretch and remained so with 4% cyclic stretch. Furthermore, the cell proliferation was not altered in the presence of silicone microgrooves with or without 4% cyclic stretch. The silicone microgroove system can be used for

studying the phenotype of MC3T3-E1 osteoblasts under dynamic culture conditions. It can also be used to deliver controlled substrate strains to other types of adherent cells in culture.

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