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Osteoblast alignment, elongation and migration on grooved polystyrene surfaces patterned by Langmuir–Blodgett lithography

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Abstract

Topographically patterned surfaces are known to influence cellular behavior in a controllable manner. However, the relatively large surface areas (several cm^2) required for many biomaterial applications are beyond the practical limits of traditional lithography. Langmuir–Blodgett lithography, a recently developed method, was used to fabricate regularly spaced grooves of different depths (50 and 150 nm) with a periodicity of 500 nm over several square centimeter on silicon surfaces. These topographies were transferred into polystyrene surfaces by means of nanoimprinting. Primary osteoblasts were cultured on the patterned polymer surfaces. They were observed to align, elongate and migrate parallel to the grooves. The combination of Langmuir–Blodgett lithography with nanoimprinting enables the fabrication of large, nanostructured surface areas on a wide spectrum of different biomaterials. Osteoblasts show a significant anisotropic behavior to these surfaces, which can enhance cell settlement on the surface or be used to direct tissue generation on the biomaterial interface.

Keywords: Osteoblast; Bone tissue engineering; Interface; Langmuir–Blodgett lithography; Nanoimprinting; Nanotopography

1. Introduction

The topographical structure of a surface can dictate cellular responses such as orientation, migration and activation [1]. For example, surface microroughening is a clinically useful method to improve the biocompatibility of certain biomaterial interfaces [2]. Osteoblast-like cells have been known for some time to align with topographical features such as the edge of a coverslip [3]. While anisotropic grinding of surfaces using paper with various grain sizes enables the simple fabrication of irregular grooves along which cells can align [4], there is evidence that the symmetry or regularity of microscopic surface topographies also plays an important role in cellular responses to surfaces [5]. The advent of microlithography (especially photolithography and electron beam lithography developed by the microelectronics industry) has allowed the fabrication of surfaces with well-defined chemical and/or topographical patterns on the same (or smaller) scale as individual cells. Topographically patterned surfaces can subsequently be transferred (and mass-produced) into polymeric surfaces, for instance by molding, embossing or solution casting, making them suitable for the study of cell–substrate interactions with topographically-patterned polymers [6]. These methods have been used to quantify the response of a variety of cell types to regularly grooved topographies [7].

However, many biomaterial applications require several square centimeters of surface patterned with nanometer scale features, which is beyond the practical limits of traditional lithography. Langmuir–Blodgett (LB) lithography is a recently developed method in which, chemical patterns are formed by the transfer of self-organizing monolayers from the air–water interface onto solid substrates by dipping the substrate through the interface. For instance LB films of L-α-dipalmitoylphosphatidylcholine (DPPC) can form regularly spaced stripes over several square centimeter with line-widths down to 100 nm [8]. The periodicity and width of the

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stripes can be controlled by the transfer conditions. These molecularly thin patterns can then be converted into topographical features by means of chemical etching (Fig. 1), which can subsequently be used as masters nanoimprinting into polymer surfaces [9]. The major advantage offered by LB lithography is that arbitrarily large surfaces can be patterned in a relatively cheap, simple and high throughput manner. By fabricating large surface areas patterned with grooves smaller than the size of one cell, we were able to use this method to quantify the topography-induced alignment, elongation and anisotropic migration of a proliferation front of a population of confluent cells.

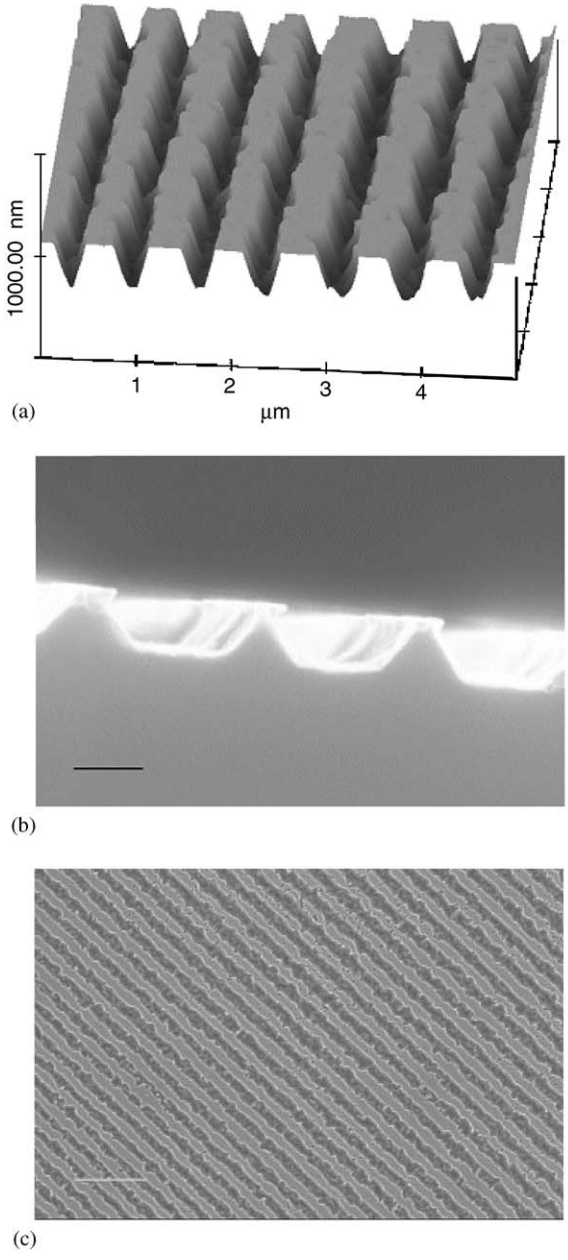

Fig. 1. Anisotropic nanotopography: Groove depth and local periodicity were characterized by SFM (a) and SEM (b) and (c). The images show silicon surfaces used as masters for printing into polystyrene.

Several studies have observed surface directed cell migration by following the motion of individual cells over limited surface areas 1 and 7 [1]. Another approach (that requires larger surface areas) enables the study of a proliferation-driven migration front. By confining a confluent population of cells to a defined region by means of a physical barrier (which we will refer to as a "fence") one can observe how the shape of the population evolves upon removal of the fence [10]. Dalton et al. [11] have used this method to direct the migration of a confluent monolayer of epithelial cells on a microgrooved as well as the migration of cells from intact epithelial tissue. We applied the fencing method to quantify the anisotropic migration of a proliferation front of osteoblasts on grooves of smaller dimensions.

2. Materials and methods

2.1. Fabrication of surfaces

LB lithography was used to prepare two different grooved silicon masters ($2 \times 5\,cm^2$) with a periodicity of 500 ± 100 nm and etch depths of 50 and 150 nm, as measured with scanning force microscopy (SFM—Fig 1a) and confirmed with cross-sectional scanning electron microscopy (SEM—Fig 1b). A smooth piece of silicon was used as a control. The silicon surfaces were used as templates for hot embossing (nanoimprinting) of polystyrene cut from Petri dishes purchased from Greiner. The details of the LB lithography are explained in Ref. [9]. The topographically patterned polymer surface was then made hydrophilic (in order to improve the attachment of the cells) by exposure to a 50 W O_2 plasma (Templa System 100-E plasma system) at 1 mbar for 10 s [20].

2.2. Cell culture

Primary osteoblasts were obtained from calf periost [21]. High growth enhancement medium (ICN Biomedicals GmbH, Eschwege, Germany) supplemented with 10% fetal calf serum, 250 µg/ml amphotericin B, 10 000 IU/ml penicillin, 10 000 µg/ml streptomycin and 200 mM L-glutamine (Biochrom KG seromed, Berlin, Germany) was used as the culture medium. The cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. All cell culture experiments were performed at least 3 times and in triplicate.

For attachment, alignment, elongation and migration assays cells were gently washed 3 times, fixed with glutaraldehyde, and stained with toluidine blue. Immunhistochemical staining was carried in the following way. Cells were cultured for 1 day before fixation in

Methanol at −20° for 20 min, washed 3 times in PBS buffer (pH 7.4) on an agitator for 5 min each. Blocking was then carried out for 5 min with 1% BSA (Sigma) in PBS buffer, incubated for 60 min in an antibody solution (anti-actin or anti-vinculin, Sigma) for 1 h. Samples were washed again 3 times in PBS buffer and blocked with 1% BSA. Samples were then incubated for 60 min in a solution of secondary antibodies (Alexa fluor 488, Sigma) for 1 h. Samples were washed again 3 times in PBS buffer, allowed to air dry for 10 min, then mounted on glass slides using glycergel mounting medium (DAKO) for fluorescence microscopy.

The attachment of the cells was studied as a function of culture time. 10 000 cells/cm^2 were cultured between a period of 1 h and 2 days (1, 2, 4, 8, 24, and 48 h). At least 8 micrographs were taken from random areas of each surface, and the number of cells within each image was counted.

The cellular alignment and elongation was determined by culturing 10 000 cells/cm^2 for 2 days. The cells were stained with toluidine blue as in the attachment assay. At least 8 micrographs were taken from random areas of the surfaces, making sure that any grooved surfaces had the grooves oriented in a vertical fashion. The exact groove orientation was confirmed from images of light diffracted from the gratings. Images were processed using ImageJ (open source image analysis software, downloaded from http://rsb.info.nih.gov/ij/index.html). Each cell was fit to the shape of an ellipse using the "analyze particle" command. The orientations of the ellipses as well their aspect ratios were used to quantify cellular alignment and elongation, respectively.

In order to study the properties of migratory proliferation front on these surfaces 60 000 cells/cm^2 cells were seeded on the surfaces of interest, confined within a circular silicone barrier (5 mm diameter, Flexiperm, In Vitro Systems & Services). After 4 days, the barrier was removed, new media was added, and the cells were cultured 4 days more. During the second 4 days the area of the population roughly doubled as the cells proliferated and migrated at the perimeter of the population. The cells were stained with toluidine blue as in the attachment assay. An ellipse was fit to the shape of the cell population and used to quantify the "anisotropy of migration" which we define as

$$\frac{x_1 - d}{x_2 - d} - 1, \tag{1}$$

where x_1 and x_2 are the major and minor axis of the ellipse fit to the shape of the cell population, respectively, and d is the diameter of the circular barrier.

2.3. Microscopy

SEM was performed with a field emission instrument (FE-SEM, LEO 1530 VP) at an acceleration voltage of 20 KeV and a secondary electron detector. SFM was carried out on a dimension 3000 (Digital Instruments) operating in tapping mode with silicon cantilevers (Nanosensors).

Optical microscopy was carried out on an Zeiss fluorescence microscope (Axioplan). Transmission mode using a halogen source was used for the observation of toluidine blue stained samples. Fluorescence microscopy was carried out using a UV source and the appropriate filters for the particular dye used.

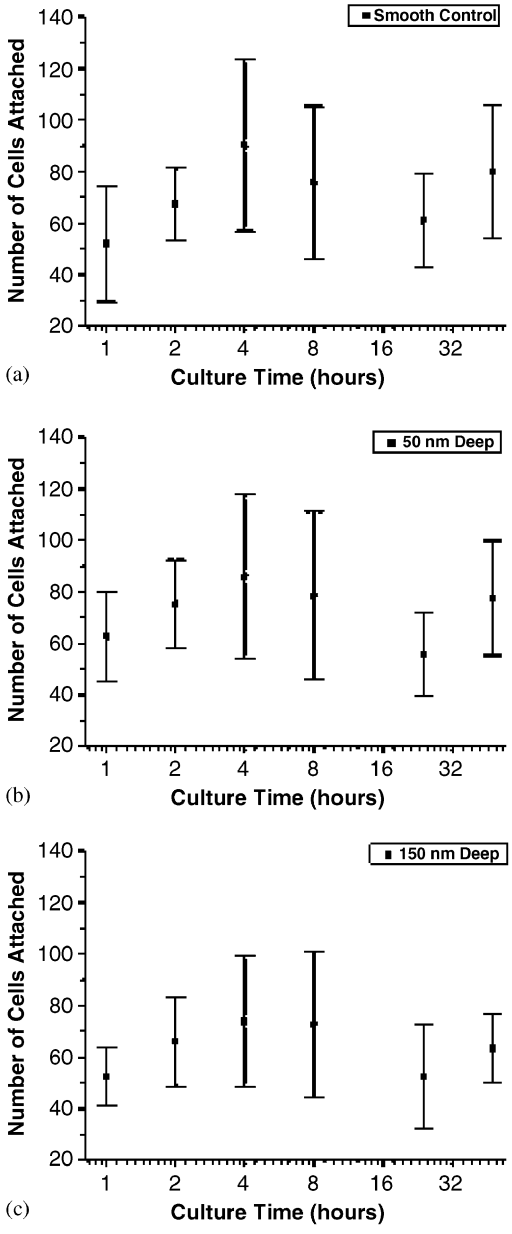

Fig. 2. Cellular attachment was relatively homogeneous on plasma treated polystyrene: (a) A smooth control, (b) 50 nm deep grooves and (c) 150 nm deep grooves. Error bars represent the standard deviation.

2.4. Statistics

For the alignment assay, the total percentage of cells aligned within 30° of the direction of the grooves was compared with that of the control. Likewise, a significant difference in the elongation of oriented cells was determined by comparing the mean aspect ratio of all cells oriented within 30° of the groove direction with the mean aspect ratio of cells in the control. In all cases, means were compared with the control using the Students' *t*-test. Unless otherwise noted, ±values represent the standard deviation of the mean.

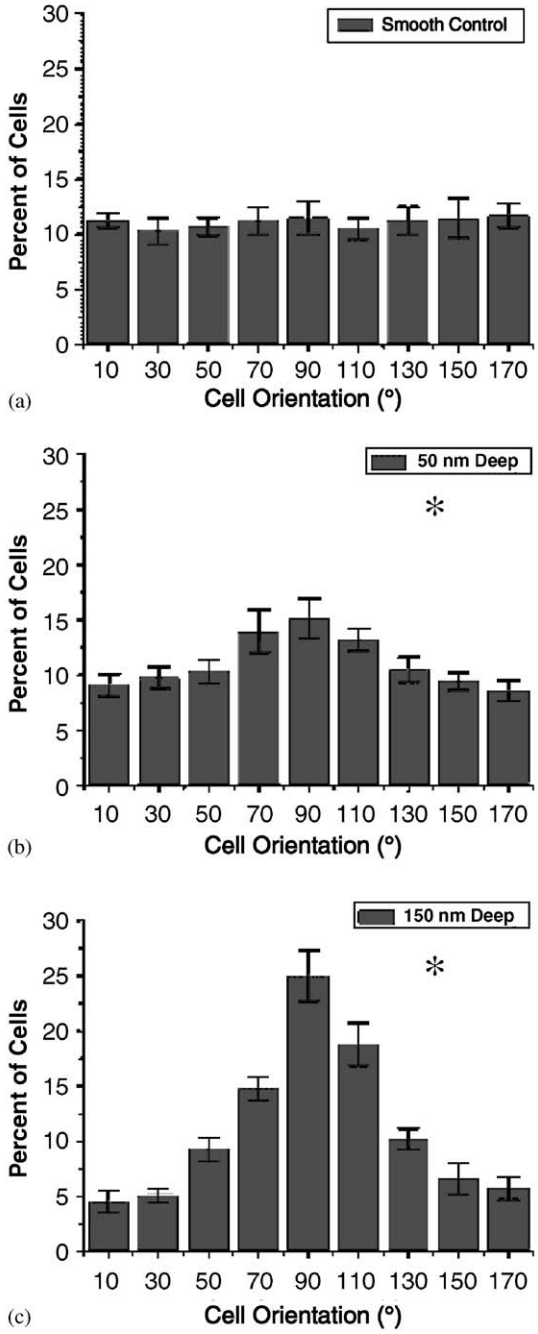

Fig. 3. Cellular alignment: histograms showing the distribution of cellular orientation on (a) a smooth control, (b) 50 nm deep grooves and (c) 150 nm deep grooves. The groove direction is 90°. Error bars show the standard deviation. A significant difference ($P<0.001$) from the control is denoted by *.

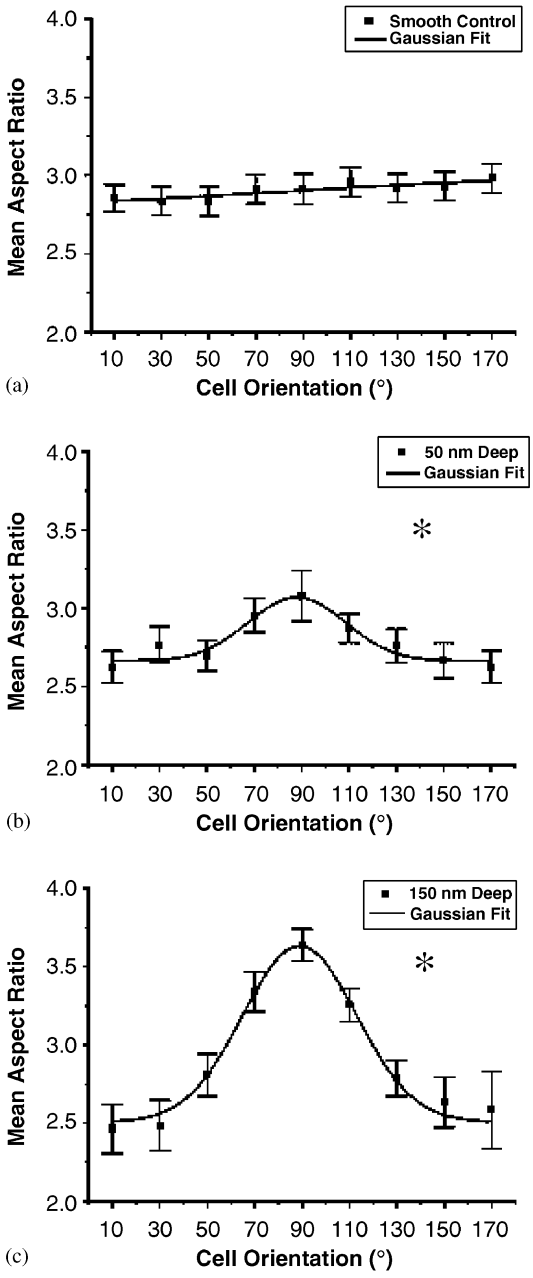

Fig. 4. Cellular elongation is illustrated by plotting the mean aspect ratio of the cells (length/width) as a function of their orientation on (a) a smooth control, (b) 50 nm deep grooves and (c) 150 nm deep grooves. The groove direction is 90°. A Gaussian curve was fit to the data as an aid to the eye. Error bars represent twice the standard error of the mean. A significant difference ($P<0.001$) from the control is denoted by *.

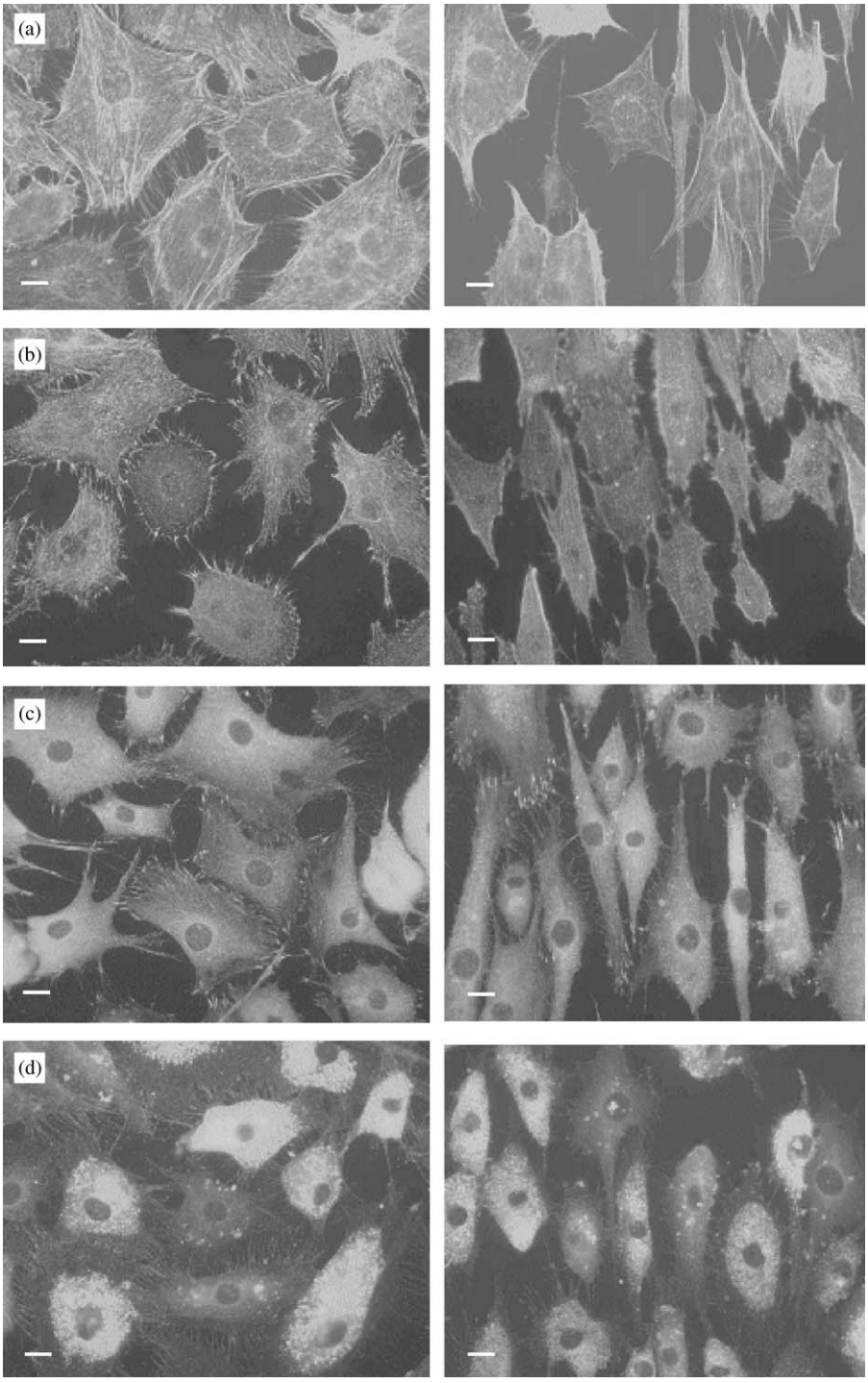

Fig. 5. Cytoskeletal structure: Fluoresence micrographs of immunohistochemically labeled cells. Left colum shows cells on a smooth surface, and the right column shows cells on 150 nm deep grooves. Grooves are oriented vertically. (a) actin, (b) actinin, (c) vinculin, (d) integrin (fibronectin receptor). Bar = 10 μm.

3. Results and discussion

In order to determine the suitability of the embossed polystyrene surfaces for the quantification of cellular responses, the cell attachment to the surface after plasma treatment was measured as a function of culture-time. During the first 2 and 3 days, the number of cells attached per unit area remained relatively constant. After this cell spreading stage, the cells began to proliferate. Cells in the attachment assay were qualitatively observed to begin alignment and elongation after 8 h and appeared more aligned after 2 days.

Without the plasma treatment the reproducibility of the cell attachment assay was very low. While the

surfaces were still in the culture medium, optical microscopy revealed cells homogeneously attached to this surface. However, after the fixation and staining procedure cells detached from various parts of the surface, resulting in inhomogeneous cell densities. Nevertheless, the number of cells attached to the plasma-treated surface was relatively homogeneous at all culture times and over the entire surface (Fig. 2). We thus chose to apply the plasma treatment to surfaces used for alignment, elongation and migration experiments.

Preliminary cell culture experiments on surfaces of various groove depth and periodicity revealed qualitative cell alignment on grooves with a pitch of 500 nm and a depth of 150 nm [9]. Since literature has suggested that groove depth is more important that groove width in inducing alignment [12], we chose to vary the depth in an attempt to find the minimum depth that could induce a detectable alignment.

Cells cultured on the 150 nm deep grooves showed a strong alignment in the direction of the grooves (Fig. 3c), with 59% of the cells oriented within 30° of the groove direction. Although not qualitatively apparent during the experiments, an alignment of the cells cultured on the 50 nm deep grooves became statistically visible (Fig. 3b) with 42% of the cells oriented within 30° of the grooves, as compared with the 33% in the control (Fig. 3a). Analysis of ellipses fit to the shape of each cell showed that cells oriented parallel to the grooves tended to be longer, compared with cells oriented in other directions (Fig. 4). The surface topography therefore not only induces a cellular orientation, but also promotes elongation in the direction of the grooves.

Immunohistochemical staining for actin and actinin showed that the cytoskeleton of oriented cells was also aligned in the direction of the grooves (Figs. 5a and b). Cells labeled for vinculin (a protein associated with focal adhesions) showed that vinculin was concentrated at opposite ends of aligned cells (Fig. 5c). Integrin (and fibronectin—data not shown) appeared to be largely confined to vesicles within the cell at the time of staining (after 1 day of culture).

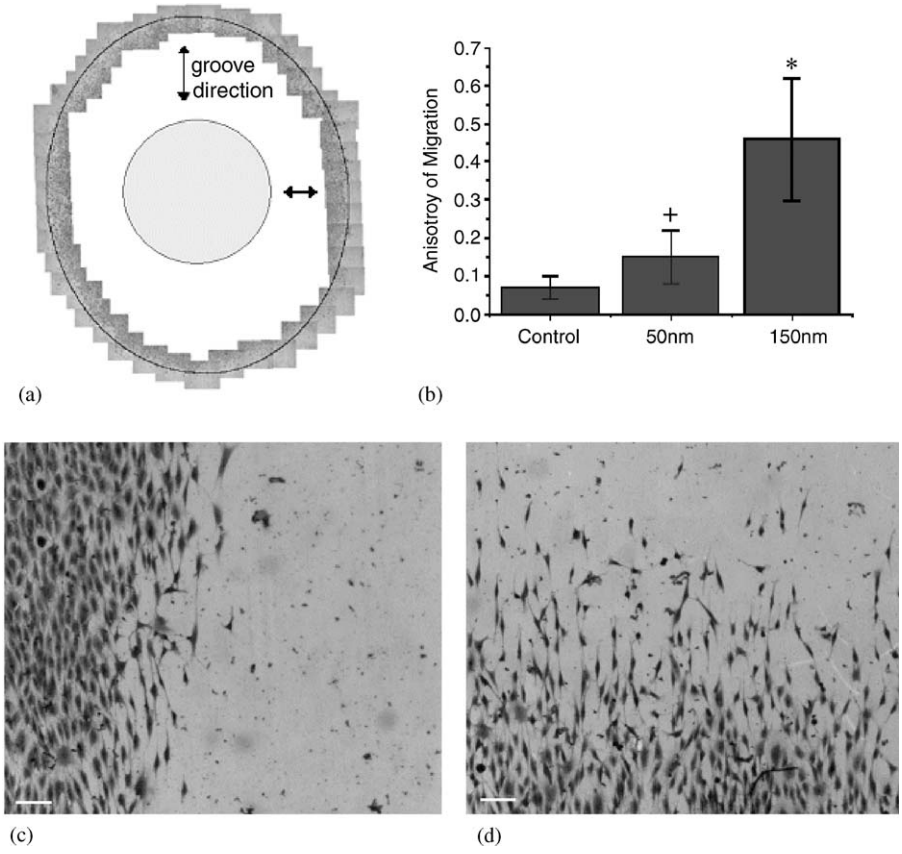

Fig 6. Anisotropic migration: (a) Mosaic of micrographs taken of the proliferation front of a population of cells. The circle in the center shows the 5 mm diameter region where the cells were seeded. An ellipse was fit to the perimeter of the population after the silicone barrier was removed and the cells were allowed to migrate for 4 days. (b) Mean anisotropy of migration calculated using formula 1. Error bars show the standard deviation. A significant difference from the control with is denoted by $^{+}$ ($P<0.05$) or * ($P<0.001$). (c) and (d) show optical micrographs of the perimeter of a cell population on the 150 nm deep grooves taken perpendicular and parallel and to the groove direction (vertical), respectively. More individual, isolated cells can be seen migrating from the front that is extending parallel to the groove direction. Bar = 100 μm.

On the 150 nm deep grooves, the proliferation front of the cell population extended faster in the direction of the grooves in all cases. Analyzing the length and width of an ellipse fit to the shape of the cell population, we deduce that the cells migrate 1.46 ± 0.16 times faster parallel to these grooves than perpendicular (Fig. 6a). In the case of the 50 nm deep grooves, anisotropic migration was also observed (Fig. 6b). However, the orientation of the elliptical shape of these cell populations was not always the same as the groove direction. These grooves therefore appear to be too shallow to consistently control the direction of tissue growth.

Polystyrene was chosen as a model biomaterial for this work because of its well-known properties and ubiquitous use as a cell culture substrate (i.e. Petri dishes). However, a variety of biomaterials could be used to replicate the nano-topographies fabricated using LB lithography. Walboomers et al. noted the possibility that plasma treatment of grooved polystyrene might not completely penetrate the groves, and may produce unwanted chemical heterogeneities on otherwise chemically homogeneous topographies [12]. However, we qualitatively observed a similar alignment on both the treated and untreated surfaces. It is therefore unlikely that a plasma-induced artifact is the main mechanism behind the alignment.

A more likely origin of differences in surface free energy results from the topography itself. Since surface free energy depends on interfacial curvature [13], it is to be expected that grooved surfaces will exhibit heterogeneous surface energies even if the surface is chemically homogeneous. Indeed, topographically induced wettability is a well-known phenomena [14,15]. Macroscopic drops of water have already been reported to detect the anisotropy of the same topographies used in this work [9]. However, unlike a drop of pure water, cellular shape and motility are largely influenced by the cytoskeleton. According to the hypothesis that the cytoskeleton consists of a tensegrity architecture, the cell will tend to form focal adhesions in locations that balance cytoskeletal forces [16]. In this sense, the localization of vinculin at opposite ends of opposite ends of cells aligned with the grooves could be explained as balancing tension generated from the surface anisotropy.

4. Conclusions

Biological materials both sense and generate forces on a variety of scales including cellular, sub-celluar and molecular [17], as well as larger scales. For instance, an unfortunately well-known failure of long-term orthopaedic implants is speculated to be based on a loss of integrity of the bone-implant interface. Topographical structuring of implant interfaces, for instance by laser micromachining [18], has been shown to improve the long-term stability of this interface in vivo. However, traditional lithographic methods are limited either in the minimum feature size or the areal throughput [19]. It is therefore impractical to generate patterns over surface areas large enough for biomaterial applications with nanometer dimensions. LB lithography is unique in that it is able to fabricate complex topographic patterns with controllable nanoscale lateral features, i.e. much smaller than the size of an individual cell, over areas of several square centimeter. For this reason, LB lithography is a promising approach to surface patterning of biomaterial interfaces for implantology or tissue engineering.

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