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Organization of mesenchymal stem cells is controlled by micropatterned silicon substrates

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Abstract

Mesenchymal stem cells (MSCs) can differentiate into various cellular lineages, including osteoblasts (that deposit hydroxyapatite, the main mineral constituent of bone), and also exhibit a high morphological plasticity. Here we grew for the first time MSCs on micropatterned silicon chips, in order to induce topography-guided alignment. Light microscopy, scanning electron microscopy and atomic force microscopy were used to characterize the cell response on various length scales. A notable alignment and movement of MSCs along the microgrooves on the chips was revealed. The cells were shown to inhabit the grooves rather than ridges and exhibited an elongated shape, with unusually long processes. On these cells, we revealed rhizome structures arranged along the extensions, which may serve as adhesion centers and participate in elongation and locomotion.

Keywords: Mesenchymal stem cells; Micropatterned substrates; Atomic force microscopy; Cellular bioengineering

1. Introduction

The complex bone architecture is created by osteoblasts that deposit the constituent mineral carbonated hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Mesenchymal stem cells (MSC) are multipotent cell populations that can differentiate, among other lineages, to osteoblasts. Unlike their terminally differentiated progenies, MSCs are mobile. This fact could be exploited for alignment into predefined patterns, prior to differentiation and biomineral creation.

The influence of surfaces on cellular parameters, such as adhesion [1,2], proliferation [3], migration [4,5] and orientation [6,7], has been extensively studied and shown to play an important role in the formation of tissues and organs. Cells may interact with the substratum via chemical, physical and topological surface parameters. Several studies have demonstrated diverse cellular responses to substrates with different surface chemistries [8,9]. Differential gene expression has been shown for several cell types on surfaces of varying hydrophobicity [10]. Other studies demonstrated increased in vivo apoptosis and reduced foreign body giant cell formation on hydrophilic and anionic surfaces, compared to hydrophobic and cationic substrates [11].

The interaction between different types of cells and materials with various surface topography has also been addressed. A "contact guidance" of fibroblasts by polystyrene microgrooved structures has been demonstrated [12,13], and similar substrate topography has been tested on microgrooved silicon for fibroblasts [14] and epithelial cells [15].

While these studies highlight the importance of biomaterial surface properties in modulating cellular behavior, the underlying mechanisms responsible for generating cell responses, adhesion and locomotion are not fully elucidated.

In the case of anchorage dependent cellular systems, surface parameters may play an important role in maintaining phenotypic characteristics and tissue integrity. Such parameters may be of a particular importance in cell types having a high degree of phenotypic plasticity such as MSCs.

MSCs refers to adult mesenchymal cells with the potential to produce progeny that differentiate to a variety of mesenchymal

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cell types such as fibroblasts, adipocytes, chondrocytes and osteoblasts [16–19]. It has been previously demonstrated that a variety of chemical cues, bone promoting factors may affect cellular fate and induce osteoblastic phenotype, which follows by enhanced biomineralization and creation of bone-like organization [20,21].

Since MSCs posses a high degree of phenotypic plasticity, their function may be highly dependent upon the topology and the molecular structure of the substrates, presence of adhesion molecules at the cell surface and the consequent transmembrane transduction of contact induced signals [22]. In addition, their capacity to differentiate to a variety of lineages, during which cells transform, move and change their morphology, may require mechanisms that involve specific recognition by chemical and topology sensitive receptors [23], adhesion [24] and cytoskeletal reorganization. Due to their potential in a variety of cell-based engineering applications, understanding the control of finely fabricated surfaces on MSCs phenotypic transformation is an important step towards the ability to construct complex cellular systems.

In the present work, we used micropatterned silicon substrates to direct MSCs by contact guidance and gain insights into the mechanisms involved in the induced cellular locomotion and alignment. We chose to use silicon substrates because of their frequent use as biocompatible materials and a variety of controlled surface topography that can be produced using this material [2,25–27]. We applied a combination of microscopies, including (real-time) light microscopy, scanning electron microscopy (SEM) and atomic force microscopy (AFM) in order to characterize cellular features from micro to nano scale and study growth dynamics of MSCs.

2. Materials and methods

2.1. Micropatterned silicon chips

Experiments were performed on micropatterned and smooth silicon surfaces as control group. Silicon gratings (TGZ11; Mikromasch, Estonia) are 1-D arrays of rectangular SiO_2 steps on a Si wafer. The structure is coated by Si_3N_4 to prevent Si oxidation. Step height is: 1600±1% nm, with 10 μm pitch (Fig. 1).

2.2. Cell culture and sample preparation

Mesencymal stem cells of mouse origin (Mus-musculus; ATCC/CRL-12424) were used in the experiment. The cells were incubated using Dulbecco modified Eagle's medium (DMEM) supplemented with 4.5 g/l D-glucose, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum 1% L-glutamin (Biological industries, Israel) and 1% Pen-Strep-Nystatin Solution (Biological industries, Israel). Cell cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Approximately 500 cells were seeded on each sample.

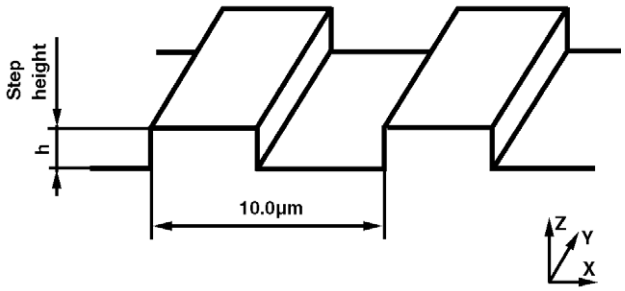

Fig. 1. Diagrammatic representation of the micropatterned silicon chip. Step height is 1.6 μm. Stripes are 5 μm wide.

In preparation for all types of microscopy examination (with the exception of live imaging), the same sample preparation procedure was used. Moreover, the same samples (when possible) were examined with all types of microscopy. The following fixation protocol was proven to prevent collapse of cells upon dehydration, thus prevent artifacts. Two-day-old cultures were fixed in 2% parformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (37 °C) for 30 min, and then washed twice in PBS for 10 min. Samples were rinsed in a series of dehydration solutions (50%, 75%, 90%, 95% v/v ethanol in distilled H_2O) for 15 min, and subsequently in absolute ethanol for 10 min three times. Samples were then washed in a series of hexamethyldisilazane (HMDS) solutions (33.3%, 50% and 66.6% in v/v absolute ethanol) for 1 min, and then washed three times with HMDS 100%.

2.3. Light microscopy

Light microscopy was performed using a Zeiss Axioplan 2 microscope. Images were taken using a Spot 2 (Diagnostic Instruments, MI, USA) CCD camera. Cells were fixed and dried as described above.

2.4. Real-time imaging

Live cells were imaged with a Nikon SMZ1500 stereomicroscope, equipped with a Nikon DX1200 CCD camera. Images were captured at intervals of 5 min, for a total duration of 12 h, in a homemade chamber.

2.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) analysis of the cells was carried out using a Quanta 200 ESEM/SEM, FEI instrument operating with beam energies of 10 to 15 kV. Cells were fixed and dried as described above and coated with sputtered Gold.

2.6. Atomic force microscopy (AFM)

Cells were investigated with an atomic force microscope (AFM) (NSOM/SPM-100, Nanonics Imaging Ltd., Jerusalem,

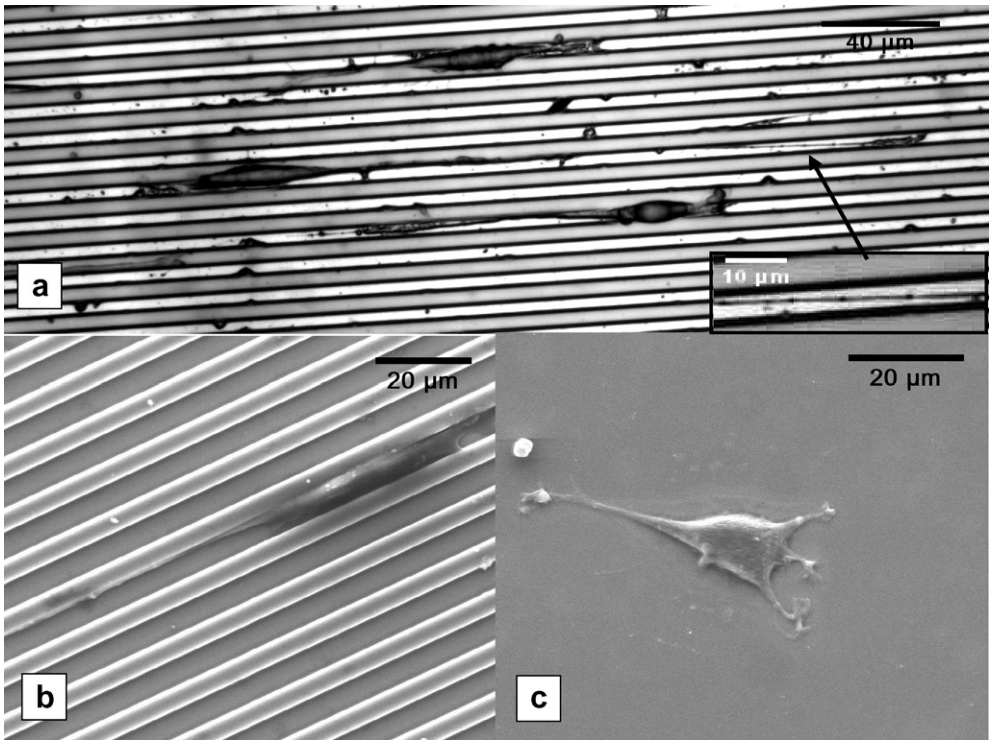

Fig. 2. (a) Light microscopy overview image of cells aligned on a Si grid. Rhizomes are marked with a black arrow and further enlarged in the inset. (b) SEM image of a cell on the micropatterned silicon surface. A short, crescent-shaped extension and a long, thin extension are visible. (c) SEM image of cell on a smooth silicon control surface, exhibiting a common fibroblast appearance.

Israel), integrated with an upright Zeiss Axiotech^{Vario} light microscope. Cells were fixed and dried as described above.

3. Results and discussion

After two days of incubation, MSCs aligned along the micropatterned steps as shown in Fig. 2a (light microscopy) and Fig. 2b (SEM). In contrast, the control group grown on smooth silicon surfaces developed a fibroblast like morphology (Fig. 2c). MSCs on the microgrooved substrate exhibited an exceptional morphological plasticity, forming an asymmetric, needle-like shape, with two distinct ends. One end is closer to the cell body than the other, elongated extension (Fig. 2a,b). The cellular extensions were found to be as long as 200 µm. (Fig. 2a).

Both light and electron microscopies provide two-dimensional information; therefore, it is impossible to judge whether the cells adhere to the grooves or ridges on the substrate. AFM images, however, undoubtedly demonstrate that the cells occupied the grooves rather than the ridges (Fig. 3) and adhered to the lower parts of the patterned surface. With the aid of the AFM, we further characterized the two dissimilar edges. The short extension, observed also in Fig. 2a, appears to have a crescent shaped edge that occupies the whole width of the groove and a thickness of roughly 1 µm (Fig. 4a).

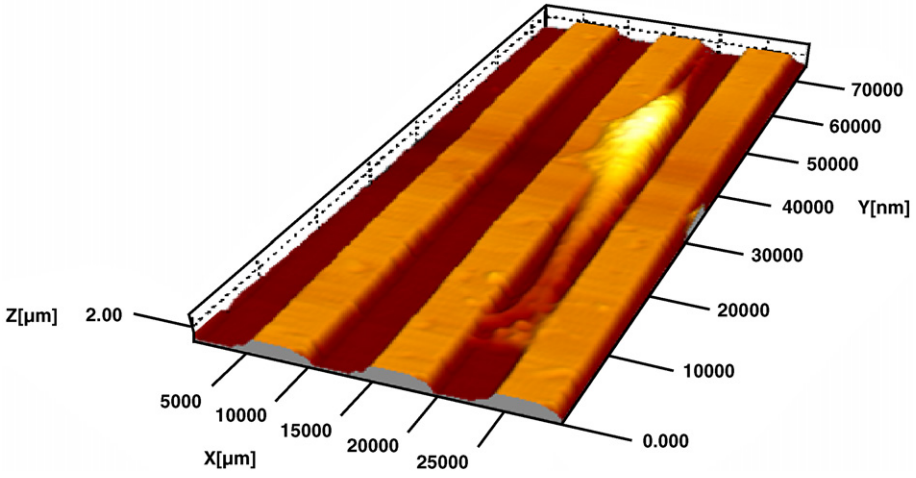

Fig. 3. AFM image of cell on micropatterned silicon surface clearly showing that the cell is occupying the low area, between ridges.

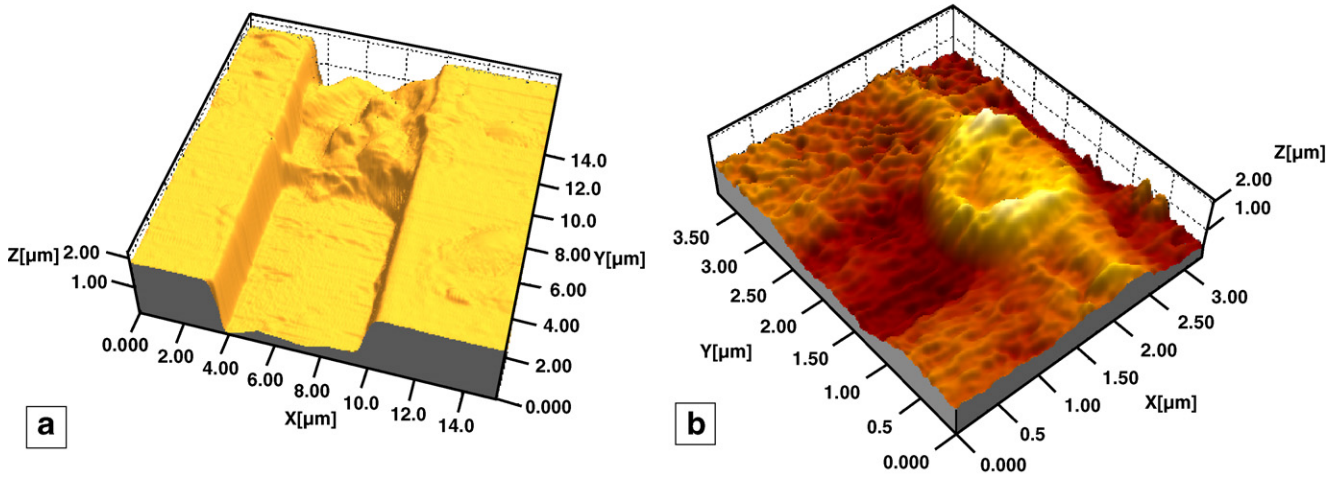

Fig. 4. AFM images of the opposed ends of a cell. (a) Crescent-shaped end and (b) image of a rhizome structure, showing its diameter of ~ 2 μm. Note the thin extension on both sides of the rhizome, of ~ 350 nm width.

The long, thin extensions, also visible in Fig. 2a, consist of a 350 nm tubular rod and are decorated with ~ 2 μm diameter rhizomes, ~ 8 μm apart (Fig. 4b). In order to study the function of these cellular processes, we imaged live cells, as they adhere and move along the micropatterned substrate and on smooth silicon surfaces, as a control. The comparison is presented in Fig. 5. The stripes from the micropatterned surface image (Fig. 5a) were removed by digital image processing (FFT filtering), in order to allow a better comparison with the smooth surface presented in Fig. 5b. The series of images in Fig. 5a revealed that, on the grooved surfaces, a leading edge is extended out the cell body, taking the shape of a long, thin process, subsequently the growth of the long extension stops, and the cell body is displaced in the same direction, while the edge of the long extension is stationary. This is opposite to the direction of crawling of the cells on the smooth surface (Fig. 5b). Here the wide edge is leading, while the thin, long extension is trailing. We suggest

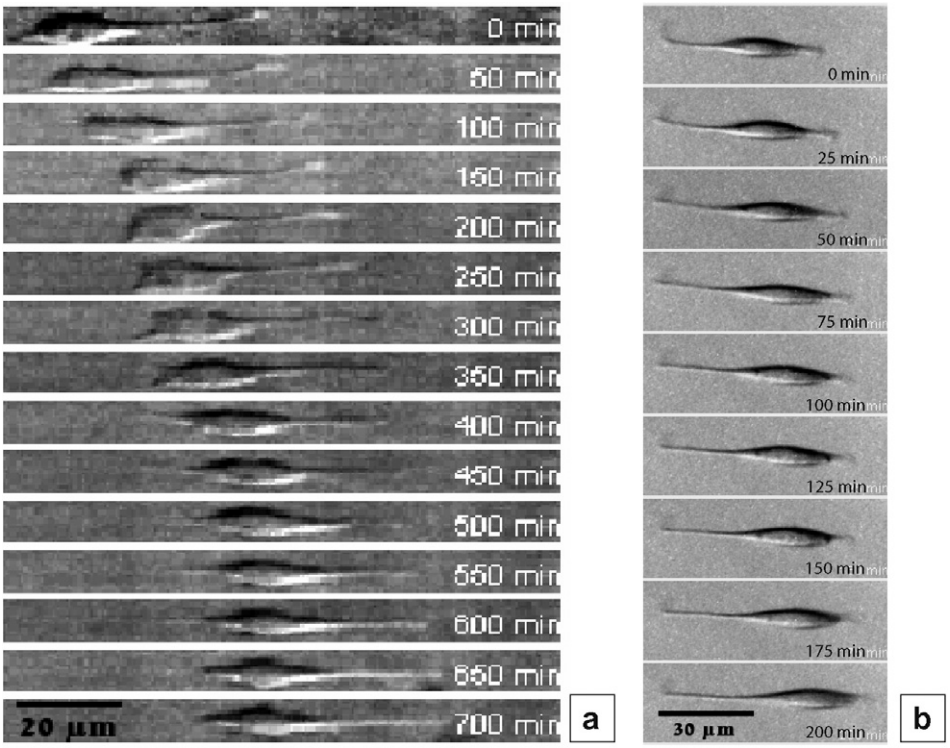

Fig. 5. Time lapse frames from a live imaging movie of MSCs growing on silicon. (a) A cell on micropatterned silicon. The grooves were removed using FFT filtering from the images, in order to allow for better comparison with the smooth surface in (b). One cycle of crawling is shown: the cell is pulling its body and the long extension is used to anchor it to the surface; subsequently, the cell body is stationary and the leading extension is elongated towards a new anchoring site. (b) A cell on a control smooth silicon surface: crawling is performed in a direction opposite to that in (a) the body is leading and the extension is following.

that the two different types of processes described earlier have two distinct functions. The crescent shaped, wide edge, that occupies the whole width of the groove, probably serves as a rear anchorage apparatus, while the dynamic, thin extension is probing the surface ahead and leads the way. The rhizome structures, found along the extensions, may serve as adhesion centers that anchor the thin, fragile extension.

Previously reported experiments suggest that adhesion is stimulated by contact with topographical boundaries [28–31]. In our system, the geometry is such that it clearly limits the ability of cells to stretch in directions perpendicular to the grooves directions; however, they have an equal amount of freedom to move in two directions along the grooves. Indeed, we observe adjacent cells moving in opposite directions, along parallel tracks and moreover, cells that change their movement direction by 180°, several times during the experiment.

4. Conclusions

We have shown that MSCs were strongly affected by a microgrooved substrate, consequently aligning, growing and moving along the patterned trenches. This movement mode was shown to be different than that of cells growing on smooth surfaces, indicating that it is induced solely by topography. AFM revealed that the cells inhabit the lower parts of the grooves, and characterized unusually long and thin processes, with rhizomes along them. We suggest that the revealed rhizomes are constituents of a locomotion mechanism, where they may serve as an anchorage apparatus. We have shown that MSCs can be guided along a predesigned track, without loosing their locomotion capacity. We thus suggest that it should be possible to organize MSCs in even more complex patterns, prior to induction of osteoblastic differentiation. These results may pave the way towards cellular bioengineering of biomimetic materials.

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