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Research Article

Dynamics of Spreading and Alignment of Cells Cultured *In Vitro* on a Grooved Polymer Surface

Thomas Peterbauer,[1,2] Sergii Yakunin,[1] Jakub Siegel,[1] Steffen Hering,[1] Marc Fahrner,[3] Christoph Romanin,[3] and Johannes Heitz[1]

[1] *Institute of Applied Physics, Johannes Kepler University Linz, 4040 Linz, Austria*
[2] *Department of Pharmacology and Toxicology, University of Vienna, 1090 Vienna, Austria*
[3] *Institute of Biophysics, Johannes Kepler University Linz, 4040 Linz, Austria*

Correspondence should be addressed to Johannes Heitz, Johannes.heitz@jku.at

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We used mechanically embossed polyester films to analyze the dynamics of cell alignment and cell-specific factors modulating the response of Chinese hamster ovary (CHO) cells and of a rat myogenic cell line to the surface topography. The films used had grooves with a periodicity of approximately 750 nm and a depth of 150 nm. Both cell lines responded to the topographical feature. On unpatterned control areas, cells of both lines showed a random distribution with orientation angles close to 45°. Both cell types exhibited an elongated morphology on the patterned surface. CHO cells typically showed bipolar spreading. Their contact area increased almost exclusively along the groove direction. Likewise, freshly seeded rat myoblasts displayed protrusions emerging in parallel with the grooves. However, myoblasts frequently had more than two sites with plasma protrusions pulling the cells along different grooves. They could also develop lamellipodia expanding without a preferred direction and long filopodia.

1. Introduction

For mammalian cells, adhesion to the extracellular matrix or to the cell culture substratum when grown *in vitro* plays an essential role for many processes such as proliferation and differentiation. Cells attach to the surface *via* focal adhesions, connecting the surface to the cytoskeleton. Formation of these interfaces is not only affected by surface chemistry (including the presence of ligands), electrostatic charge, wettability, and elastic modulus [1, 2]. Surfaces can provide micro- and nanosized topographical clues to guide alignment, migration of cells or outgrowth of neurites along a specific orientation [3, 4]. To probe the ability of cells to respond to surface topography, grooved substrata are often used, usually resulting in physical guidance along the direction of the grooves and reorganization of the cytoskeleton. Alignment occurs on grooves with periods $<20\,\mu$m [4] and depths down to a threshold around 30–90 nm [5–8]. Despite a large body of literature, however, fundamental questions remain open. The basic mechanism by which cells recognize surface geometry remains obscure. It may be a more or less passive process, whereby adhesion molecules best fitting the local topography simply draw cells or cellular components into the observed shape, or it may involve more elaborate regulatory mechanisms. It is also not clear if there is one uniform mechanism active over the entire range of scales which evoke a response, or if different processes accomplish guidance on the micro- and nanoscale, respectively. The extent of contact guidance depends on cell type used, plating density, and, most import, feature geometry and feature aspect ratio [6, 9–13]. In some instances, growth perpendicular to the groove direction has been observed [14, 15], and the response of cells may depend on soluble factors [15].

We have recently employed laser processing to generate polystyrene films with periodic surface ripple structures with a periodicity of 200 nm–800 nm and a depth of 40 nm–150 nm [16]. We probed a range of cell lines for proliferation and contact guidance. Although the aspect

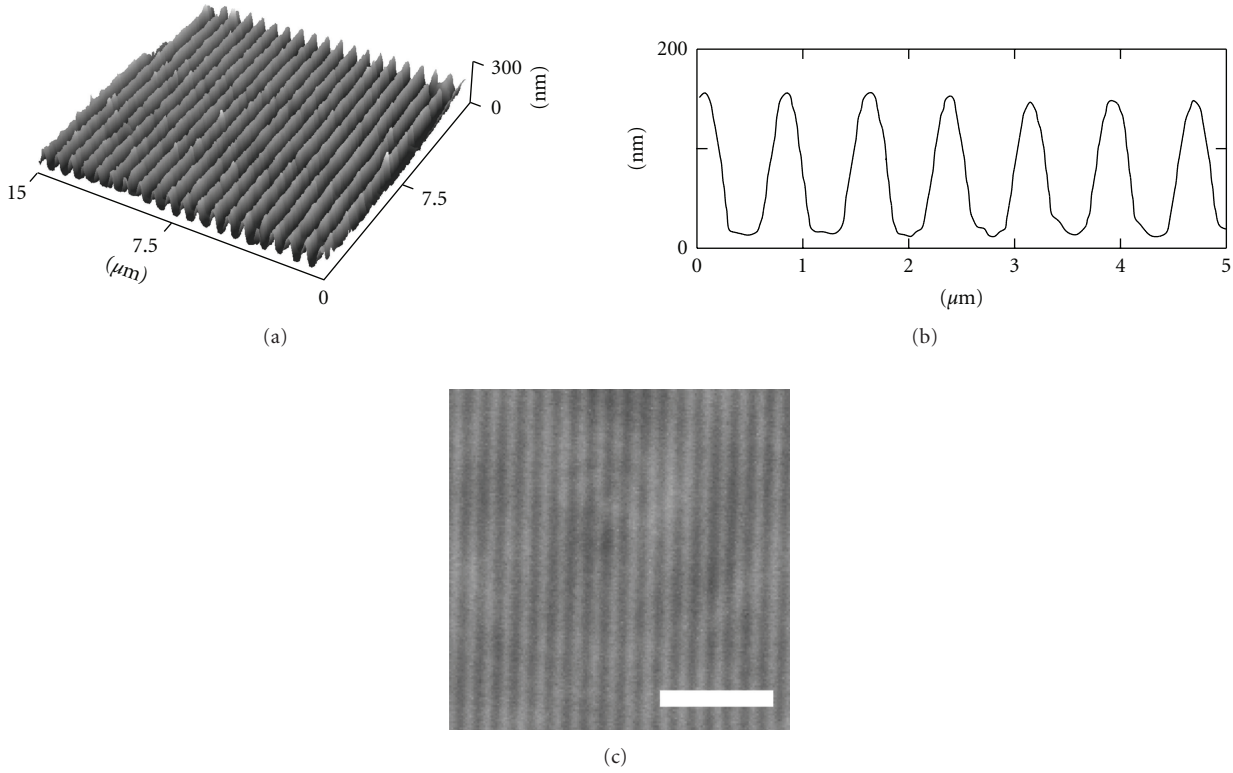


Figure 1: (a) Surface plot of a mechanically embossed polyester film characterized by AFM. (b) Schematic cross-section of an AFM image. (c) Phase-contrast light microscopic image of a patterned film obtained with a 25x oil immersion objective. Scale bar, 5 μm.

ratios of the surfaces were lower than those employed in most other studies (e.g., [6] or [17]), parallel alignment of cells occurred above a cell-specific critical periodicity of 200 nm–400 nm. Here, we focus on the dynamics of cell spreading and alignment, and we attempt to gain some insight in the cell-specific factors responsible for the variability of cell alignment. We employed mechanically embossed polyester films. This replication method, which is frequently used to fabricate diffractive surfaces (e.g., holograms), offers the advantage of low-cost mass production. We selected CHO cells, which are quite sensitive with respect to surface topography [18] and a rat myoblast cell line displaying less pronounced contact guidance [16].

2. Materials and Methods

2.1. Preparation of Patterned Surfaces. The surfaces were fabricated by mechanical embossing of polyester carrier films [19]. The films were coated with an UV-curable polyester resin, imprinted with an embossing machine containing the pattern, and photopolymerized. In some initial experiments, the films were additionally irradiated with far UV light with a wavelength of 172 nm as previously described [20]. The latter treatment, which introduces polar oxidic groups on the surface, enhanced biocompatibility for cell lines weakly attaching to the substratum such as human embryonic kidney (HEK 293) cells, but was found to be unnecessary for other cell types. The surface topography of the films was analysed using a diCP II (Veeco Instruments, Mannheim, Germany) atomic force microscope (AFM). Before use in cell culture, samples were sterilized for 20 minutes with 70% ethanol and washed with phosphate-buffered saline (PBS). The films were placed in 35 mm Petri dishes with a 0.18 mm synthetic bottom permitting oil immersion microscopy (μ-Dishes, ibidi, Martinsried, Germany). Appropriately shaped Teflon rings were inserted to prevent floating of the films.

2.2. Cell Culture and Labelling. CHO-K1 cells and a cell line derived from rat skeletal myoblasts [19] were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; EuroClone, Pero, Italy) supplemented with 10% fetal bovine serum (FBS, EuroClone) and 5 μg mL^{-1} gentamycin (EuroClone) in a humidified atmosphere. Cells were harvested with PBS containing 0.05% trypsin containing 0.02% EDTA and seeded onto the patterned surfaces. Typically, the cells were used in cell passage number between 10 and 30 and seeded onto the substrates at a density of about 1000 cells mm^{-2}. In some experiments, cells were stained after harvesting with the lipophilic fluorescent dye PKH26 (Sigma Aldrich, Vienna, Austria) according to the manufacturer's recommendations. For time-lapse video microscopy (performed at ambient CO_2 concentration), media were supplemented with 50 mM Hepes·NaOH (pH 7.2, Sigma).

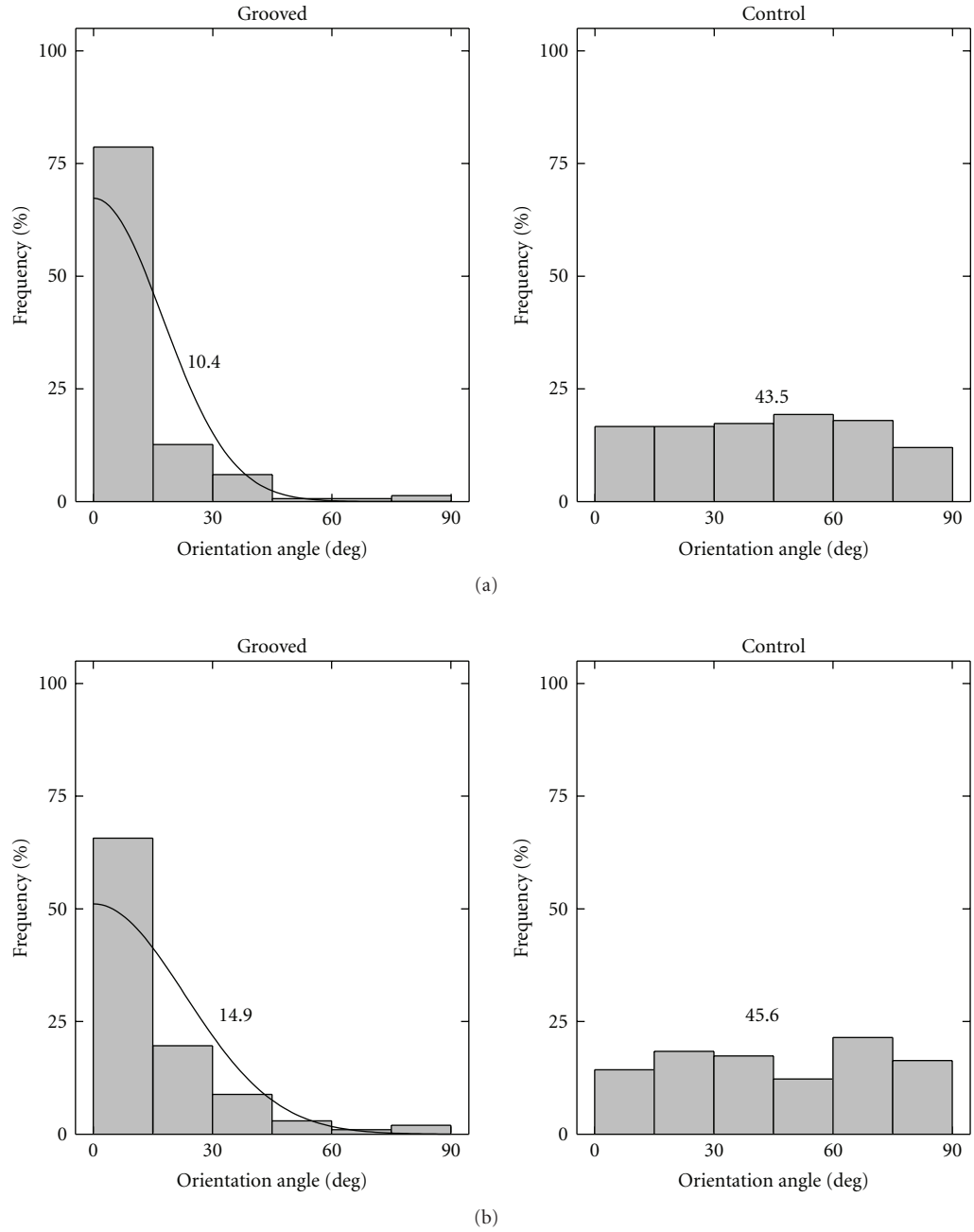


Figure 2: (a) Histograms of orientation angles of CHO cells ($n = 150$) on grooved polyester films (left panel) or on adjacent unpatterned control areas (right panel) one day post seeding. (b) Orientation angles of rat myoblasts ($n = 100$). Curves represent probability density functions of half-normal distributions fitted to the data sets. Inlet values represent average orientation angles.

2.3. Microscopy and Image Analysis. Brightfield, phase contrast and fluorescence microcopic images were captured with an inverted microscope (Axiovert 200, Zeiss, Oberkochen, Germany) equipped with a Zeiss LSM 510 laser scanning module and a transmitted light detector. Image analysis was performed with ImageJ 1.43 (available at http://rsb.info.nih.gov/ij). The outlines of cells were traced using the NeuronJ plugin [21]. After conversion into closed polygons, ellipses were fitted to the outlines. The orientation angle of a cell was defined as the absolute value (in degrees) of the smallest angle between the major axis of the best-fitting ellipse and the direction of the grooves. This approach ignores the sign of the deviation and, thus, gives values which vary between 0° (perfect alignment) and 90° (exactly perpendicular orientation). Cell populations with random orientation should have an average orientation angle of 45°.

The shape of the cells was quantified by measurement of their minimum and maxiumum Feret's diameter (minimum and maximum caliper length, resp.) and by the dimension-

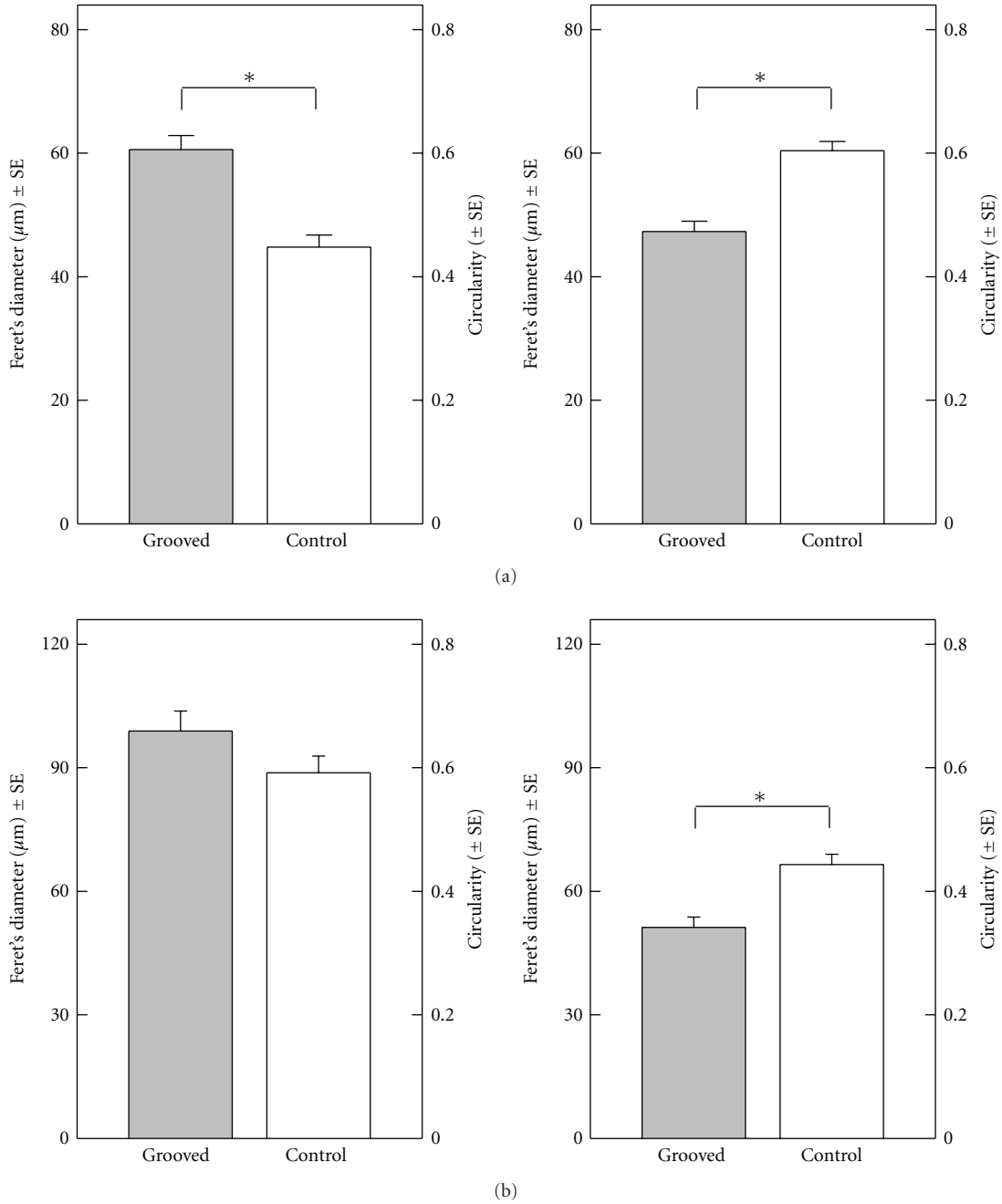


Figure 3: (a) Maximum Feret's diameter and circularity of CHO cells on a grooved substratum (grey bars) or on an adjacent unpatterned control area (open bars) one day post seeding ($n = 150$). (b) Morphology of rat myoblasts. Asterisks denote statistically significant differences ($P < .05$).

less shape index circularity

$$M = \frac{4\pi A}{P^2}, \tag{1}$$

where A is the area of a cell and P is its perimeter. A perfect circle has a circularity of 1, while the values for deformed objects approach zero. The solidity of cells was estimated by the ratio of the area of a cell to the area of its convex hull. Pairwise comparisons for statistical significance were made by Student's t-test. Results were considered as statistically different at $P < .05$.

3. Results

3.1. Characterization of Grooves. The topology of the surfaces generated by mechanical embossing was evaluated by AFM (Figure 1). The average depth of the grooves was 154 ± 11 nm with a periodicity of 758 ± 22 nm. The surface structures were visible by conventional light microscopy,

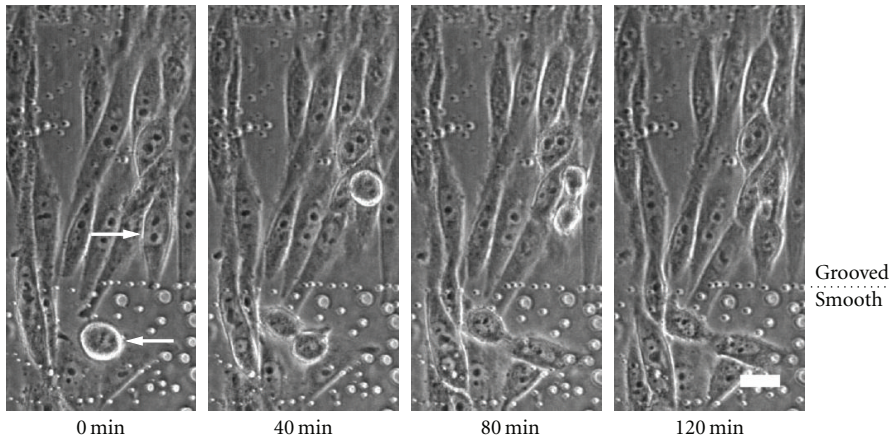


Figure 4: Time-lapse phase contrast images of a near-confluent CHO cell culture growing on a mechanically embossed polyester film. The dashed line denotes the border between an area with grooves (upper part of the images) and smooth control area (lower part of the images). Arrows denote cells which underwent mitosis. Scale bar, 20 μm.

allowing to determine their direction and the orientation angle of cells growing on the surface. Due to refractive properties of the material, however, peak maxima of the line pattern changed with the focus plane. Thus, it was not possible to determine directly whether the lines visible in the light microscopic images correspond to grooves or ridges, respectively. However, we saw in some microscope images features in bright color which were obviously elevated above or on top of the groove structure. From this we conclude that probably the bright lines in the microscope images correspond to the groove ridges, while the valleys between them are more dark.

3.2. Cell Alignment. The two cell lines studied responded to the patterned surface by alignment along the direction of the grooves. The degree of alignment was somewhat variable, depending on the cell culture conditions, passage number and seeding density. When cultured under identical conditions and low to moderate cell densities, CHO cells showed more pronounced alignment than rat myoblasts (Figure 2). This observation is in line with our previous report on the sensitivity of the two cell lines to surface structure periodicity [16]. Cell populations growing on adjacent unpatterned control areas consistently displayed random orientation with average orientation angles close to 45°. The substrate topography not only affected orientation, but also cell morphology (Figure 3). Cell populations growing on grooves were more elongated than control cells and tended to have a higher average maximum Feret's diameters (a statistically significant difference was only found for CHO cells). Both cell lines showed little motility, neither on grooved nor on smooth films (data not shown). Nonetheless, the general alignment on grooved films was maintained even in fairly dense cell layers, in which neighbouring cells can act as physical barriers (Figure 4). Mitotic cells detached from the surface, rounded up, and divided. The daughter cells occupied the space freed by the mother cell by spreading along the direction of grooves. Upon reaching confluency, however, the alignment of rat myoblasts degraded quickly, owing to their tendency to overgrow other cells rather than maintaining a strict monolayer.

3.3. Time-Lapse Microscopy. To study attachment and spreading in more detail, freshly harvested cells were seeded onto patterned films and analyzed by time-lapse microscopy. Under our cell culture conditions, the time required to complete attachment varied considerably. Rat myoblasts could complete attachment within 1-2 hours, while CHO cells required 5 hours or more. No differences between cells seeded onto a grooved surface and control cells on adjacent smooth areas were apparent (data not shown). When seeded onto a grooved surface, many CHO cells displayed strictly bipolar spreading (Figure 5). Short cytoplasmic processes, 3–6 μm in diameter, and bleb-like structures protruded preferentially from two opposing sites of the cells in parallel with the groove direction. The cells elongated along the groove direction with little lateral spreading. The final width of the cells (estimated as the minimum Feret's diameter) as measured one day after seeding was not much above that of freshly trypsinized, round cells (Figure 5(c)).

3.4. Cytoplastic Protrusions. A more complex picture emerged when rat myoblasts were used. Three or more major cytoplasmic protrusions were frequently visible on freshly seeded cells, which emerged not necessarily on opposing poles (Figure 6). Again, initial protrusion of these structures closely followed the direction of the grooves. With increasing diameter during later stages of spreading, the cytoplasmic processes could trespass individual grooves. The cells displaying these features did not necessarily elongate exactly along the groove direction. Being pulled along distinct grooves, they could adopt a triangular or otherwise deformed shape and a skewed alignment with respect to the groove direction. Rat myoblasts could also develop large

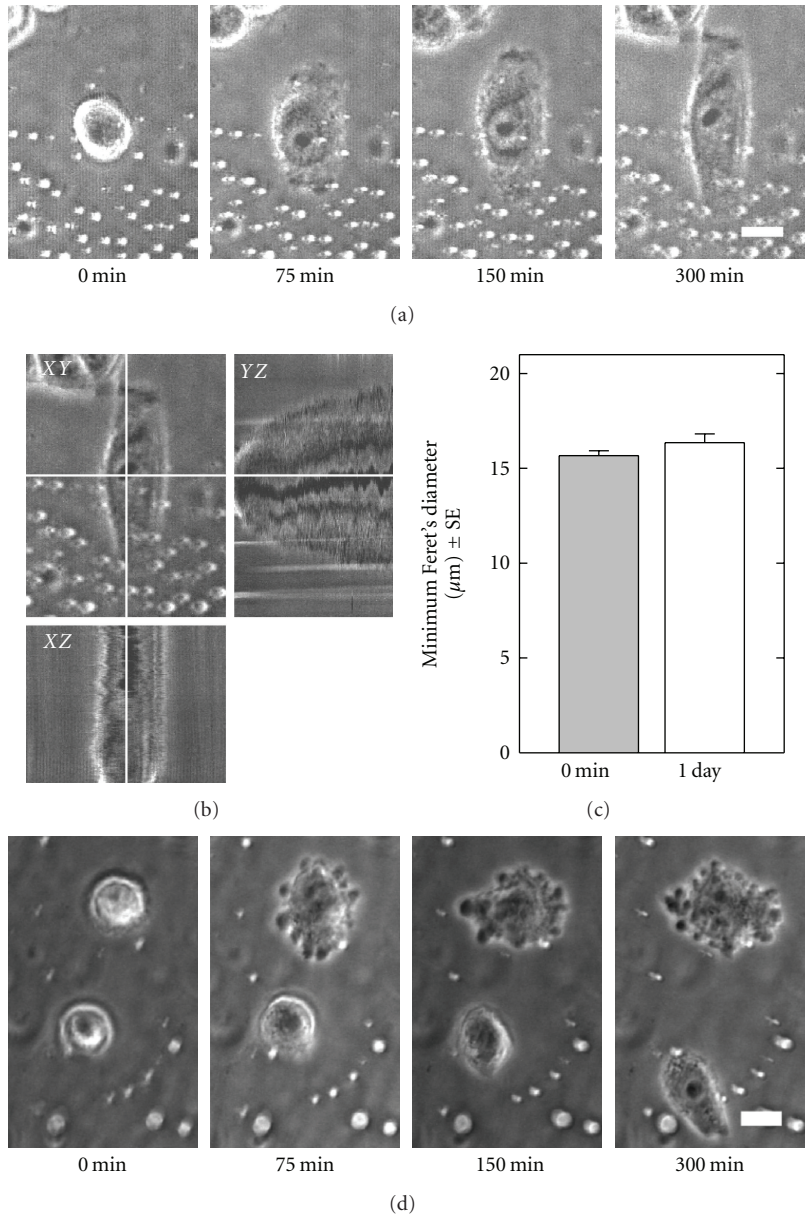


Figure 5: Attachment of CHO cells. (a) Time-lapse phase contrast microcopy of a freshly harvested CHO cell on a grooved substrate. Scale bar, 10 μm. (b) Orthogonal stack slices with time as the z-axis (300 minutes, 150 slices). The cell elongates along the vertically oriented grooves and displays little lateral spreading. (c) Average minimum Feret's diameter of freshly harvested, floating CHO cells (grey bar) and CHO cells cultured for one day on a grooved substratum (open bar). Both evaluations were performed for n = 100 cells. (d) Time-lapse phase contrast microcopy of a freshly harvested CHO cells on a unpatterned smooth substrate.

lamellipodia, which extended not only along the grooves, but also perpendicular to them, resulting in lateral spreading. In addition, freshly harvested rat myoblasts frequently had long filopodia, which could be visualized with the lipophilic membrane dye PKH26 (Figure 7(a)). Most filopodia showed highly dynamic expansion, motions and retractions, but some remained motionless and aligned with the groove direction. We never found CHO cells carrying comparable structures when stained with the dye. Only short cytoplasmic protrusions were observed. A fraction of these structures was aligned with the groove direction (Figure 7(b)).

Overall, our observations indicate that the rat myoblasts developed more complex morphological features (including multiple cytoplasmic protrusions, lamellipodia, and filopodia) than CHO cells. In order to have a simple quantitative measure for these oberservations, we reasoned that the solidity index of a simple oval or rod-shaped cell is equal to one, while more complex shapes will most likely have convex deficiencies along their boundaries. We therefore compared the solidities of rat myoblasts, attached either to the grooved surface or to control areas, with those of CHO cells. Significantly lower values were obtained for rat

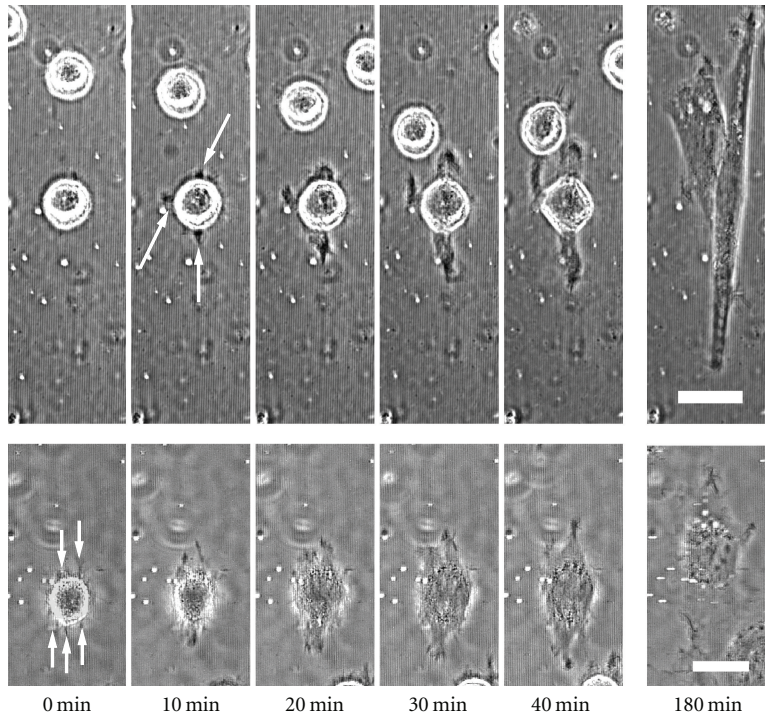


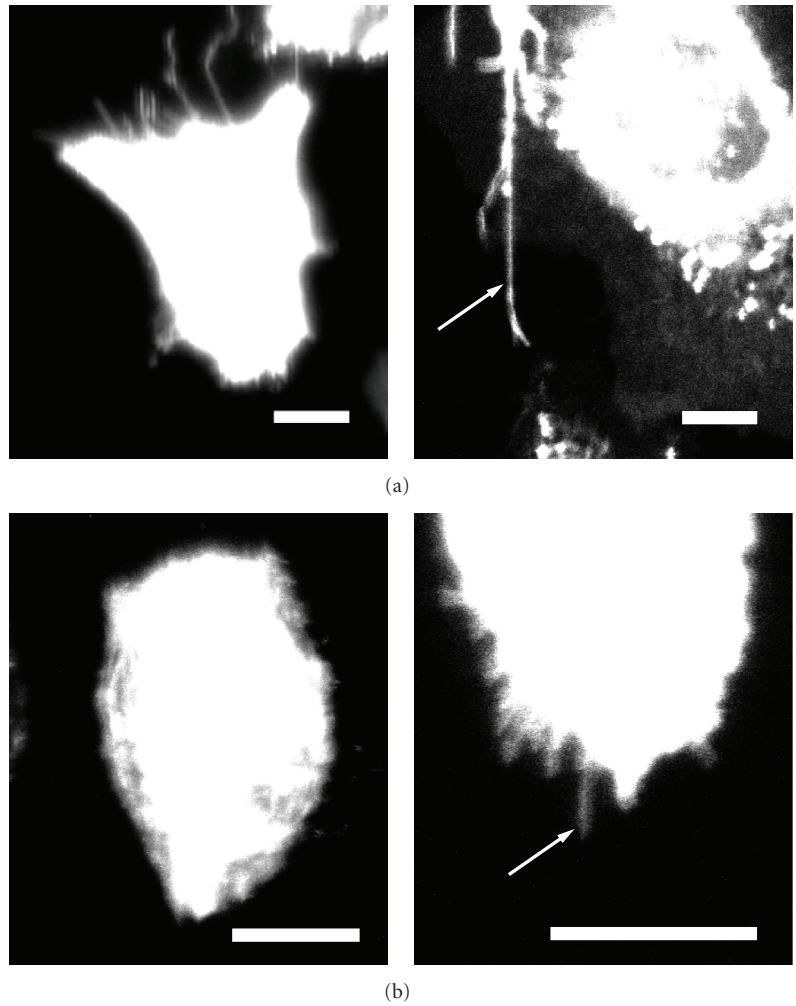
Figure 6: Time-lapse phase contrast images of rat myoblasts freshly seeded onto a grooved polyester film. The grooves were oriented vertically. Arrows indicate the location of cytoplasmic protrusions. The cell in the upper panel shows 3 major initial cytoplasmic protrusions, while multiple protrusions are visible on the cell in the lower panel. Scale bars, 20 μm.

myoblasts (Figure 8). There were no significant differences between cells of one type growing on grooves and control cells on unpatterned areas.

4. Discussion

Mechanical embossing of polyester films provided a convenient method to fabricate a grooved surface to study contact guidance of murine cells. Fairly smooth grooves (with an aspect ratio close to 0.2) were chosen to permit detectable, but not perfect alignment. The two cell lines employed reacted to the surface topology by aligning parallel to the direction of the grooves and by adopting an elongated morphology (Figures 2 and 3). Thus, they followed the general trend observed for a wide range of different cells on comparable surface topologies [4, 22]. The cells aligned primarily during attachment and spreading, regardless whether they were seeded onto the surface after trypsination of whether they originated from mitosis of cells detaching from surface during culture (Figures 4 and 5). While both cell lines displayed orientation angles deviating little from the direction of the grooves, differences became apparent when analyzing the dynamics of cell attachment and spreading. One may distinguish several degrees of complexity of these processes. In the simple case, a cell follows bipolar dilation with little lateral expansion (apart from a prototypical CHO cell, melanocytes would be a good example). In this case, it may be feasible to describe the response to various surface geometries in a dose-response fashion as successfully done by several authors [10, 23]. One has to postulate that these cells possess a kind of "quorum sensing" mechanism which allows the existence of not more than two poles and supresses expansion in other directions. The integrin adhesome network [24] may be a candidate for this task. It must be a dynamic mechanism, since cells can adjust their alignment within a few hours, as indicated by recent work with surfaces with reconfigurable microtopography [25].

Rat myoblasts represented more complex cases. These cells frequently developed protrusions from more sites and/or protrusions with different morphologies (filopodia, lamellipodia), displayed lateral spreading and a deformed shape (Figure 6). These more elaborate morphologies appear to be cell-specific rather than the consequence of the surface topology, at least when quantified with the very basic shape descriptor solidity. However, a common observation was that many of the initial cellular processes emerging from a cell (except lamellipodia) protruded along the groove direction even if the cell as a whole displayed poor alignment with the surface feature after completing attachment. In other words, the protruding organelles of a cell may sense surface geometries at thresholds lower than those determined by measurement of the orientation of entire cells or cell populations. Consequently, it is not clear whether the cell-specific threshold values for contact guidance reported in the literature (which are usually estimated by measuring whole-cell orientations) truly reflect the ability of the cells to sense these geometries. It is quite possible that all mammalian cells share a very similar threshold, which is blurred by cell-specific morphological features affecting the overall cell orientation.



(a)

(b)

Figure 7: Fluorescence images of rat myoblasts (a) and CHO cells (b) growing for one day on grooved substrata. The cells were stained with the membrane dye PKH26 before seeding. The grooves were oriented vertically. Arrows indicate filopodia or cell protrustions aligned with the grooves. The myoblast cell shown in the upper right panel has partially internalized the dye. Scale bars, 10 μm.

Fujita et al. analyzed the dynamics of alignment of mesenchymal stem cells on a surface with similar topographical features as ours [26]. They found that cell protrusions expanded and retracted as if they were probing the surrounding area. Since protrusions perpendicular to the grooves tended to retract more rapidly than those with a parallel orientation, these authors suggested that the retraction phase is an important factor for cell alignment. The cogent idea behind their model is that cell protrusions extending perpendicular to the topographical feature can form focal adhesions only on the ridges, while protrusions extending in parallel can form stable complexes along their entire length. Thus, the latter could be less prone to retraction. The movements of the cell protrusions observed with our cell lines did not allow to distinguish clear cycles of extension and retraction (when viewed at low magnification, they appeared as almost continuous forward movements), but this concept is certainly worth to be further explored.

The role of filopodia in establishing alignment also remains a controversial issue. It is generally believed that filopodia act as sensory organelles probing the environment [27]. They may determine the spatial localization of focal adhesions [28] and they respond to topographical features down to a threshold distance of approximately 35 nm [29]. In addition, filopodia aligned parallel to nanoscaled grooves were found after fixation of the cells (e.g., [15]). However, analysis of the movements of filopodia of mesenchymal stem cells on a grooved substratum did not reveal a specific direction [26]. Our observations also indicate that distinct filopodia are not a prerequisite for contact guidance. Under our cell culture conditions, we only observed short protrusions on CHO cells, although the response of this cell line to the patterned substratum generally outperformed that of rat myoblasts which frequently displayed long filopodia (Figure 7). Our observations do by no means argue against a general sensory role of filopodia. However, long filopodia can easily span the distance of several groove periodicities. During their movements, they often touch the substratum only with their tips. Hence, filopodia may be able to sense topographical features on a very restricted scale, but not

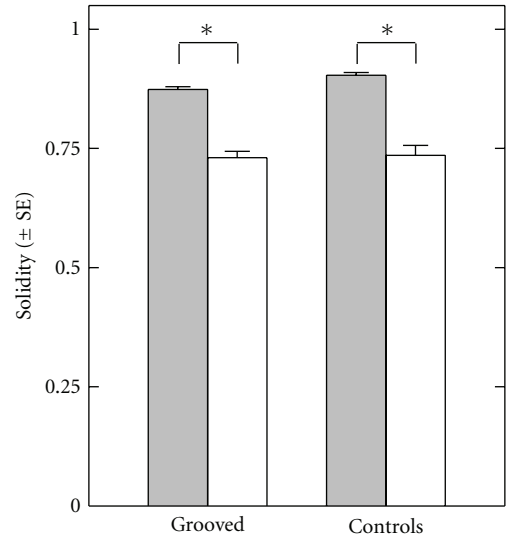


Figure 8: Average solidity of CHO cells (grey bar) and rat myoblasts (open bar) after one day of culture on a grooved polyester film or on adjacent unpatterned control areas ($n > 100$). Asterisks denote a statistically significant difference ($P < .05$).

the direction of grooves which are broader than the average diameter of filopodia. Alternatively, if they can sense the direction, multiple filopodia emerging from one cell with all probing different grooves may still be unable to pull a cell into a specific direction. Clearly, more detailed analyses of the movements of filopodia and the other cellular protrusions and quantification of traction forces applied to the substrate would be helpful to resolve their role in sensing surface topography.

5. Conclusion

We have here demonstrated that mechanical embossing of polymer foils can successfully be utilized to generate large quantities of patterned substrates to study the responses of cells to nanotopographical features. Our results suggest that the oriented spreading of the cell types studied is largely determined by the very first protrusions emerging from a cell, with their location being affected by subtle topographical features. In general, the rat myoblasts developed more complex morphological features (including multiple cytoplasmic protrusions, lamellipodia, and filopodia) than CHO cells. Our studies are still in the stage of basic. However, the findings of this work may be relevant for future applications in cell therapies, tissue engineering, and cell tests for development of novel pharmaceutics.

Acknowledgment

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