 DOI: 10.1002/masy.201051038

Dynamics of the Alignment of Mammalian Cells on a Nano-Structured Polymer Surface

Thomas Peterbauer,[1,2] *Sergii Yakunin,*[1] *Jakub Siegel,*[1] *Johannes Heitz**[1]

Summary: *In vitro* cultured mammalian cells are able to sense micro- and nanotopographical features of their substratum, resulting in contact guidance of the cells along topographical features, altered motility, proliferation and differentiation. We here used embossed polyester films with nanogrooves to study the dynamics of cell spreading and alignment of chinese hamster ovary (CHO) cells and cells of a rat myogenic cell line. Our results suggest that the response of the cells studied is driven by initial cytoplasmic protrusions, which emerge along the nanogrooves and pull the cells along the direction of the nanogrooves.

Keywords: cell attachment; cell orientation; embossing; filopodia; nanotopography

Introduction

Surfaces with nano-scale topographical features are frequently used to probe the ability of mammalian cells to respond to environmental clues and their impact on cell adhesion, morphology, migration, differentiation and signalling.[1] The results of these studies form a basis for the development of materials with improved properties for cell culture, medical devices and artificial organs.

A range of factors affects the behaviour of cells on structured surfaces, including surface chemistry, feature geometry, feature aspect ratio and differences in the cell types used.[2] In a recent communication, we have employed polystyrene films with periodic surface ripple structures generated by laser processing to determine the threshold at which alignment of a range of cell types occurs.[3] The ripples had a periodicity of 200 - 800 nm and a depth of 40 - 150 nm. The aspect ratios of the surfaces were much lower than those employed in other studies,[4–5] yet we could demonstrate that even on these smooth substrates contact guidance occurs above a cell-specific critical periodicity of 200 – 400 nm. We here present data on the dynamics of cell alignment and attempt to gain insight into the nature of the cell-specific component(s) responsible for the observed differences in cell behaviour. We used foils mechanically embossed with nanogrooves. This technique is frequently employed to generate diffractive surface structures like holograms and allows production of surfaces on a large scale.

Generation of Patterned Surfaces

Substrata were generated by ultraviolet (UV) embossing of polyester films.[6] Carrier films were coated with an UV-curable polyester resin, imprinted with an embossing tool containing the nanopattern, and photopolymerized. The topography of the films was examined by atomic force microscopy (AFM, diCP-II, Veeco). Before use, samples were surface-sterilized with 70% ethanol and washed with phosphate-buffered saline (PBS). The films were placed in Petri dishes with a thin (0.18 mm) plastic bottom permitting microscopy with immersion oil (μ-Dishes, ibidi) and were fixed to the

[1] Institute of Applied Physics, Johannes Kepler University Linz, A-4040 Linz, Austria
Fax: (+43) 732 2468 9242.;
E-mail: johannes.heitz@jku.at
[2] Department of Pharmacology and Toxicology, University of Vienna, A-1090 Vienna, Austria

bottom with appropriately shaped teflon inserts.

Cell Culture and Labelling

CHO cells and a cell line derived from rat skeletal myoblasts[7] were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM, EuroClone) supplemented with 10% fetal bovine serum (FBS, EuroClone) and 5 $\mu g\,ml^{-1}$ gentamycin (EuroClone) in a humidified atmosphere containing 5% CO_2. Cells were dissociated with PBS containing 0.05% trypsin and 0.02% EDTA and seeded onto the patternded surfaces. Staining of cells with the lipophilic fluorescent dye PKH26 (Sigma Aldrich) was performed according to the manufacturer's recommendations. For time-lapse video microscopy (performed at ambient CO_2 concentration), the medium was supplemented with 50 mM HEPES.NaOH (pH 7.2).

Microscopy and Image Analysis

Phase contrast and fluorescence microscopic images were acquired using an Axiovert 200M inverted microscope (Zeiss) equipped with a Zeiss LSM 510 META laser scanning module and a transmitted light detector. Images were analysed with ImageJ v. 1.43 software (http://rsb.info.nih.gov/ij). Cell outlines were digitized using the NeuronJ plugin.[8] The deviation of the cell orientation from the groove direction (orientation angle, OA) was defined as the absolute value of the smallest angle between the major axis of the ellipse best fitting the perimeter of an individual cell and the nanogroove direction, yielding values between 0 and 90°. An OA of 0° denotes perfect alignment, while a population of randomly oriented cells should have a mean OA of 45°. Note that the cells used were more or less symmetrical and were seeded onto a symmetrical pattern. Thus, it is meaningless whether deviations of cells from the groove direction occur clockwise or counter-clockwise. Assuming that imperfect alignments follow a normal distribution with mean zero (groove direction) and variance σ^2, OA values follow a half-normal distribution[9,10] with mean μ given by

$$\mu = \sigma\sqrt{\frac{2}{\pi}} \tag{1}$$

To compare distributions for statistically significant differences, F-tests (rather than the usual t-tests) were performed,[11] using σ calculated from μ.

The dimensionless index circularity was defined as

$$M = \frac{4\pi\,\text{Area}}{\text{Perimeter}^2} \tag{2}$$

Comparisons for statistical significance were made here by t-tests.

Results were considered significant at an error probability $p < 0.05$ for F-tests as well as for t-tests.

Results

The foils used had grooves with a periodicity of approximately 750 nm and a depth of 150 nm as resolved by AFM (Fig. 1). Chinese hamster ovary (CHO) cells as well as cells from a rat myogenic cell line responded to the nanotopographical feature. One day post seeding onto the patterned surface, the average orientation angle of CHO cells was 8.3° (Fig. 2). As expected, randomly orientated cells were

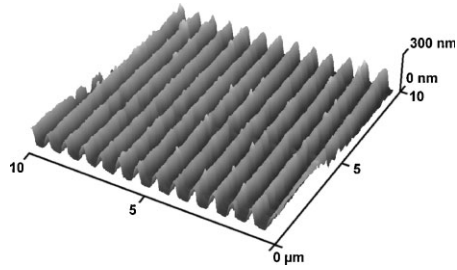

Figure 1.
AFM image a polyester film with embossed nanogrooves.

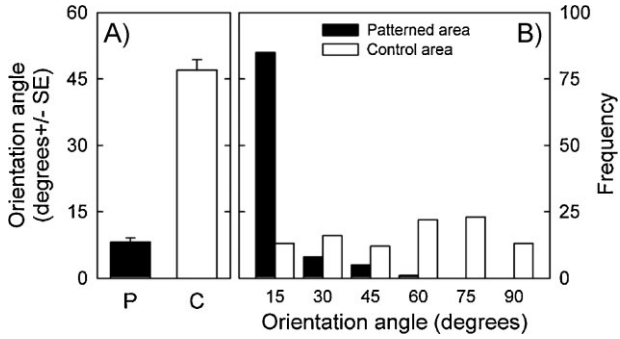

Figure 2.
Alignment of CHO cells on nanogrooves (*closed bars*) and unpatterned control substratum (*open bars*). Average orientation angle (A) and histogram (B) one day post seeding ($n = 100$).

observed on an adjacent unpatterned control area. Their average orientation angle (46.9°) was significantly larger compared with aligned cells.

The effect of the nanotopography was not merely restricted to cell orientation. When expressing cell shape in terms of shape descriptors, differences in morphology were also observed. CHOs showed a circularity of 0.43 ± 0.19 on the patterned surface, significantly lower compared with a value of 0.59 ± 0.18 on unpatterned area (i.e., the cells on nanogrooves were more elongated than control cells).

We have previously noted that a myoblast cell line derived from rat skeletal muscle responds somewhat weaker to smooth nanotopographical features than other cell types tested.[3] In line with this observation, OA values for this myoblast line were higher than those of CHO cells (14.9° in the experiment shown in Fig. 3), with a higher proportion of cells not aligned with the nanogrooves. This distribution was significantly different compared with control myoblasts as well as with CHOs seeded on nanogrooves. The circularity of myoblasts was 0.34 ± 0.17 on patterned areas and 0.44 ± 0.16 on unpatterned contol areas. Myoblasts as well as CHOs were found to robustly align along the nanogrooves as judged by visual inspection in at least 3 independent experiments on different batches of patterned films.

Time-lapse video microscopy of attached cells revealed very little motility of both cell lines (not shown), suggesting that the cell orientation is not the conse-

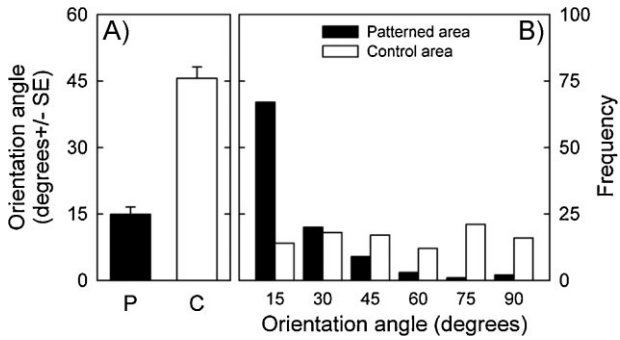

Figure 3.
Alignment of rat myoblasts on nanogrooves (*closed bars*) and unpatterned control substratum (*open bars*). Average orientation angle (A) and histogram (B) one day post seeding ($n = 100$).

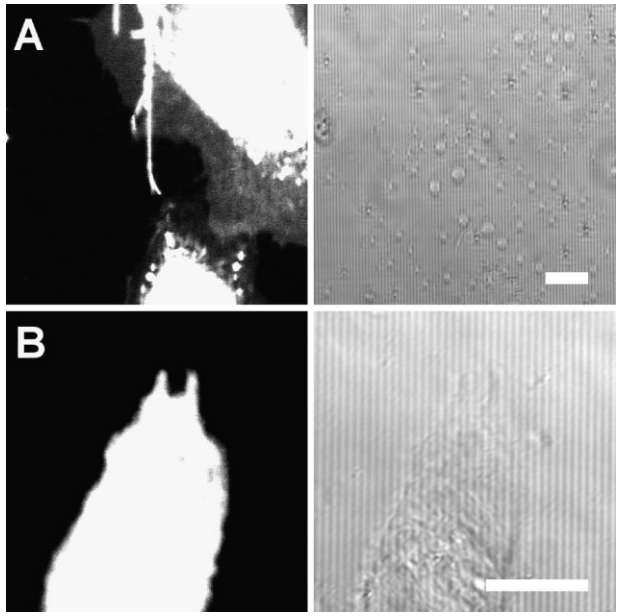

Figure 4.
Fuorescence images (*left panel*) of rat myoblasts (A) and CHO cells (B) stained PKH26. The nanogrooves of the substrate are visible as vertical lines in the corresponding phase contrast images (*right panel*). Scale bar, 10 μm.

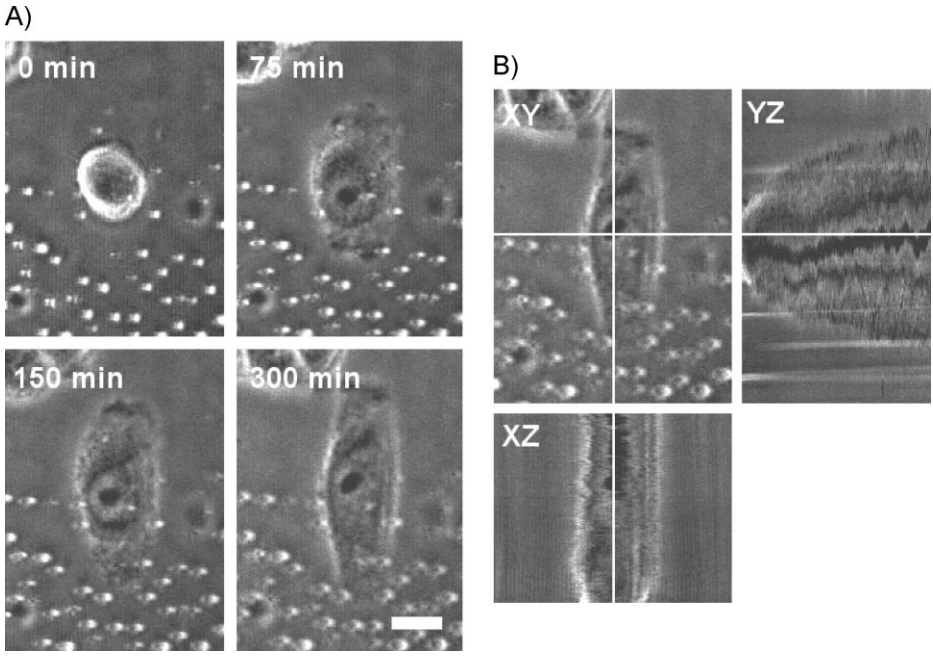

Figure 5.
Time-lapse phase contrast images of a CHO cell seeded onto a patterned substrate (A). Orthogonal stack slices (B) with time as the z-axis (300 min, 150 slices). The cell spreads almost exclusively along the vertically oriented nanogrooves. Scale bar, 10 μm.

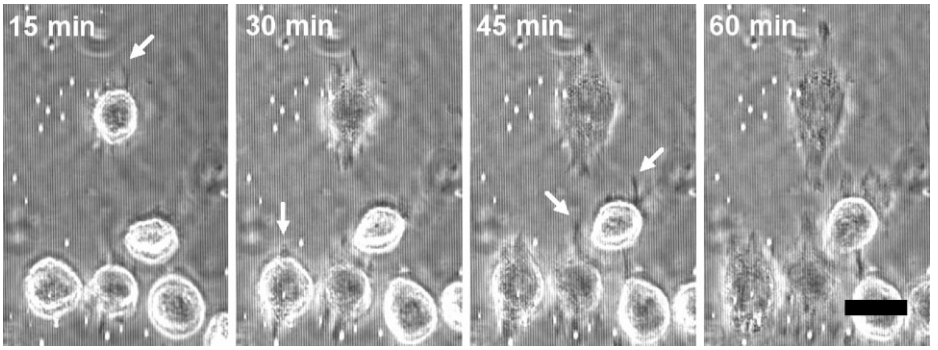

Figure 6.
Time-lapse phase contrast images of rat myoblasts seeded onto a patterned substrate. Arrows denote cytoplasmic protrusions emerging along nanogrooves (visible as vertical lines). Scale bar, 20 μm.

quence of guided migration. Hence, it appears that it is already established during cell attachment and/or spreading.

The time required to complete attachment varied considerably. Rat myoblasts were able to attach within 1 – 2 h to the substratum, while CHO cells required 5 h or more. During attachment, rat myoblasts frequently showed long filopodia as visualized with the fluorescent membrane dye PKH26. These filopodia showed rapid movements, but some remained aligned along the nanogrooves (Fig. 4). In contrast, CHO cells displayed only short cytoplasmic protrusions.

The majority of the CHO cells showed bipolar spreading. Their contact area increased almost exclusively along the groove direction (Fig. 5). Myoblasts, however, frequently had more than two sites with plasma protrusions pulling the cells along different grooves, resulting in a less-defined shape and cell spreading along the axis perpendicular to the orientation of the nanogrooves (Fig. 6).

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. However, cells with a strictly bipolar spreading of cytoplasmic protrusions from two opposing poles (CHOs) show better alignment than myoblasts with a less restricted locations of initial spreading. Filopodia do not appear to be a prerequisite for alignment.

Acknowledgements: This work was supported by the Austrian NANO initiative (project NSI_NBPF).

[1] B. Geiger, J. P. Spatz, A. D. Bershadsky, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 21.
[2] E. Martínez, E. Engel, J. A. Planell, J. Samitier, *Ann. Anat. -Anat. Anz.* **2009**, *191*, 126.
[3] E. Rebollar, I. Frischauf, M. Olbrich, T. Peterbauer, S. Hering, J. Preiner, C. Romanin, J. Heitz, *Biomaterials* **2008**, *29*, 1796.
[4] W. A. Loesberg, J. te Riet, F. C. M. J. M. van Delft, P. Schon, C. G. Figdor, S. Speller, J. J. W. A. van Loon, X. F. Walboomers, J. A. Jansen, *Biomaterials* **2007**, *28*, 3944.
[5] F. C. M. J. M. van Delft, F. C. van den Heuvel, W. A. Loesberg, J. te Riet, P. Schön, C. G. Figdor, S. Speller, J. J. W. A. van Loon, X. F. Walboomers, J. A. Jansen, *Microelectron. Eng.* **2008**, *85*, 1362.
[6] A. del Campo, E. Arzt, *Chem. Rev.* **2008**, *108*, 911.
[7] H. C. Ott, S. Berjukow, R. Marksteiner, E. Margreiter, G. Bock, G. Laufer, S. Hering, *J. Physiol.* **2004**, *558*, 793.

[8] E. Meijering, M. Jacob, J.-C. F. Sarria, P. Steiner, H. Hirling, M. Unser, *Cytom. Part A* **2004**, *58*, 167.
[9] J. M. Bland, http://www-users.york.ac.uk/~mb55/talks/halfnor.pdf, accessed May 2010.
[10] J. M. Bland, D. G. Altman, DG *Stat. Methods Med. Res.* **1999**, *8*, 135.
[11] I. N. Bronstein, K. A. Semendjajew, "*Taschenbuch der Mathematik*", Verlag Harri Deutsch, Thun 1985, p. 686ff.

