Nanotechnology for guided cell differentiation

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The morphogenesis of mammalian organs and tissues relies on the ability of individual cells to respond to a vast range of extracellular signals. Among these are gradients of soluble molecules such as growth factor and cell-secreted mitogens encoding for preferential directions over long distances. Much of the exsisting literature concerning guided cell differentiation focused on the identification and biological characterization of these molecules. In recent years an increasing body of evidence revealed that a second determinant of cell activity is provided by the chemical and physical properties of the extracellular matrix (ECM) in the cell proximity. These guidance cues are read by cells in a process that requires the activity of specific cell adhesion machineries and the remodeling of the cell shape. Several cellular responses such as polarization, migration, proliferation, and apoptosis are elicited by a direct cell-ECM interaction in virtually all cell types. A pivotal role in this process is played by the substrate nanotopography. In order to unravel the mechanisms by which differentiating cells read the local topography it is crucial to decouple its effects from those stemming from other chemical and physical stimuli. Thanks to recent advances in biomaterial nanofabrication, selected morphological aspects of the ECM are now reproducible in vitro through the realization of artificial scaffolds with controlled topography (Fig. 1). The related research activity at NEST lab follows two strategies: (i) improving the chemical and morphological properties of biomimetic two-dimensional (2D) scaffolds with nanostructured surface modifications (ii) understanding the cellular mechanisms regulating topographical guidance. Concerning line (i), during 2007-2008 we introduced original, biocompatible, nanostructured scaffolds for high-resolution time-lapse experiments made of standard tissue-culture polystyrene (TCPS) or cyclic olephine copolymer (COC), which were shown to induce efficient neurite guidance during neuronal differentiation. The effect of variation in the geometry of the topographical features was also investigated. Concerning line (ii) we studied the influence of anisotropic nanotopographies on neuronal differentiation and migration and revealed that neurite alignment and polarization are obtained via a geometrical constraint of cellular adhesions resulting in an angular modulation of neurite elongation and persistence. The involvement of cell-generated contractility and cytoskeletal remodeling in this process was also demonstrated.

Nanostructured biomimetic scaffolds and applications

During 2007-2008 the possibility of imprinting anisotropic nano-textures (i.e. nanogratings, alternating lines of grooves and ridges of submicron size) on thermoplastic materials was successfully explored. Particular attention was paid to the physiological suitability of the resulting cell scaffold. To this aim tissue-culture polystyrene (TCPS) was initially chosen [1]. The desired topography could be induced by nanoimprint lithography (NIL) [1,2]. In these conditions no further surface chemical functionalization was added (Fig. 1A). The resulting imprinted substrates proved optimal for the growth of PC12 cells, a well-established model of neuronal differentiation [3]. The net effect of cell-nanogratings interaction on TCPS substrates was investigated by means of life cell microscopy. Specifically two aspects were analysed; the efficiency of neurite alignment (Fig. 1B) and the cell polarity state (Fig. 1C-E) induced by nanogratings with varying geometries [1,2]. The tested geometries were the following: linewidths of 5 µm, 2 µm, 1 µm, 750 nm and 500 nm and periodicities of 10 µm, 4 µm, 2 µm, 1.5 µm and 1 µm respectively (Fig. 1B). As previously reported for other cell types, PC12 cells were only marginally affected by larger structures (>1 µm linewidth) whereas sub-micron topography led to highly increased non-isotropic differentiation. Figure 1 displays the degree of axon outgrowth control determined by 750 and 500 nm

linewidth gratings. The latter was found to be more effective, leading to over 90% of the axons aligning within 3° to the gratings, and almost 70% of axons aligned within 10°. For what concerns 750 nm linewidth substrates, 75% of axons were aligned within 30° and the 36% within 10°. For comparison, the same analysis was performed on cells differentiating on flat, unpatterned TCPS: no preferential directionality was observed in this case (Fig. 1B).

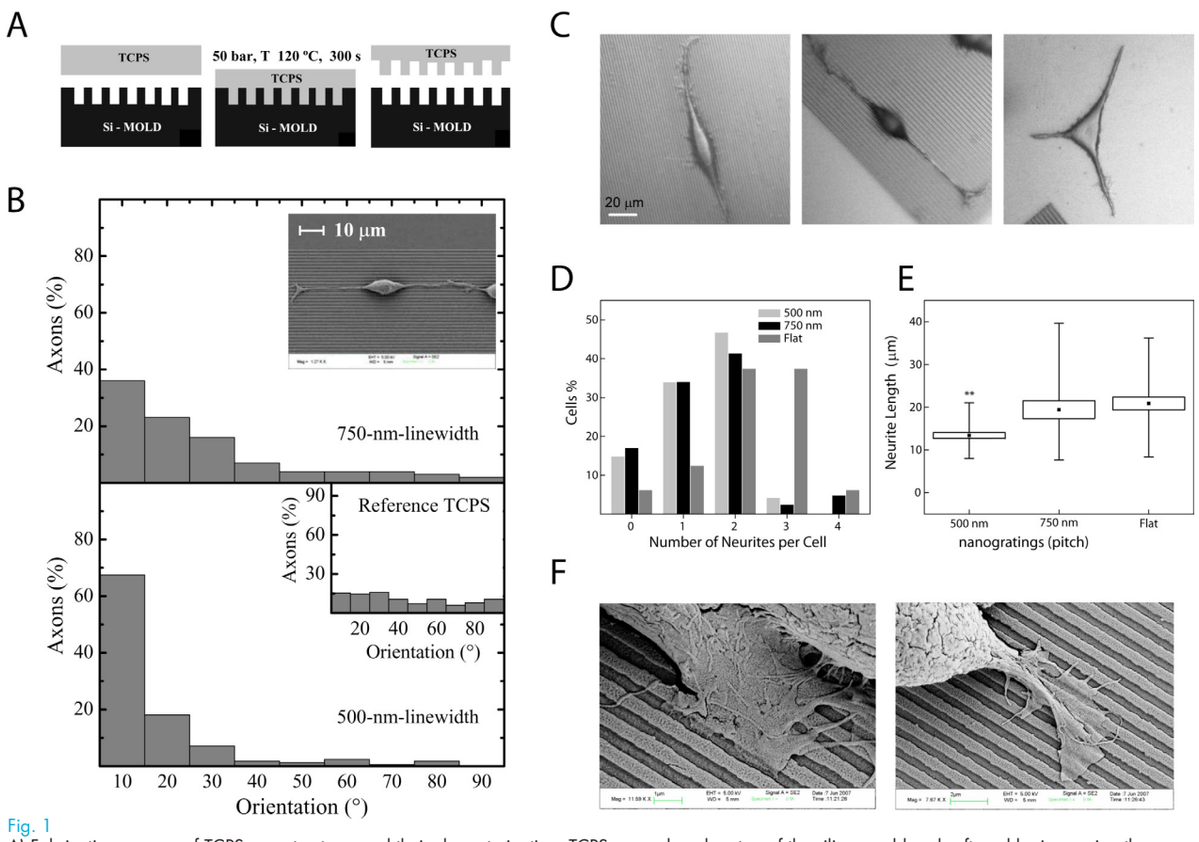

Fig. 1
A) Fabrication process of TCPS-nanostructures and their characterization. TCPS were placed on top of the silicon mold and softened by increasing the temperature up to 120 °C (left). 20 bar pressure was applied for 5 min before cooling down to 70 °C (middle). The pressure was finally released and mold and TCPS detached (right). B) Quantitative analysis of guided axon outgrowth on TCPS nanogratings: percentage of axons as a function of the angle between the axon and grating-line directions for 750 nm (upper panel) and 500 nm (lower panel) linewidth gratings. The same analysis is reported in the inset of the lower panel for cell differentiation on flat TCPS. Inset of upper panel: scanning electron microscope image of a PC12 cell on a nanograting showing bipolar conformation, enhanced body elongation and guided axon outgrowth. C) Typical cell phenotype on 500 nm grating (left), 750 nm grating (center), and flat surface (right). D) Distribution of the number of neurites per cell. E) Average neurite length. Neurite lengths are reported as 10-90 percentile distribution. F) SEM images of PC12 cells differentiating on nanopatterned substrates: details of sprouting filopodia and lamellipodia.

Nanogratings reduced to two the number of neurites produced by PC12 cells upon treatment with NGF (Fig. 1D). Neuronal bipolarity was correlated with an increased stretching of the cell body and a reduced length of the cell protrusions (Fig. 1E).

We next analysed the mechanisms governing the interaction between differentiating neuronal cells and our nanoimprinted topographies. Our work aimed at defining a biological link between the topographical configuration of the substrate and the resulting cell polarity. For this activity we evaluated the use of new biocompatible materials with enhanced optical properties allowing the use of high-resolution live-cell microscopy in physiological conditions [4]. In this direction the nanoimprint protocol defined for TCPS could be further developed to obtain similar nanogratings imprinted on COC. Using these improved substrates we revealed the role played by integrin-

based adhesion complexes, the focal adhesions, during neuronal differentiation on anisotropic nanotopographies. First, we showed that during neuronal differentiation, topographical anisotropies control focal adhesion maturation. Modulation of adhesions was the driving mechanism that selected the properly aligned neurites. Notably, polarity selection via focal adhesion control was sufficient to induce a set of developmentally relevant changes, such as the transition from a multipolar to a bipolar cell shape and the alignment of opposing neurites. Second, we demonstrated that a guiding topography can induce and consolidate a single polarity state by a patterned regulation of the cell differentiation machinery. Hence, differentiating neurons not only can 'read' the topographical guidance cues, but also can re-localize specific cellular activities along preferential directions and thus 'learn' achieving a committed differentiation.

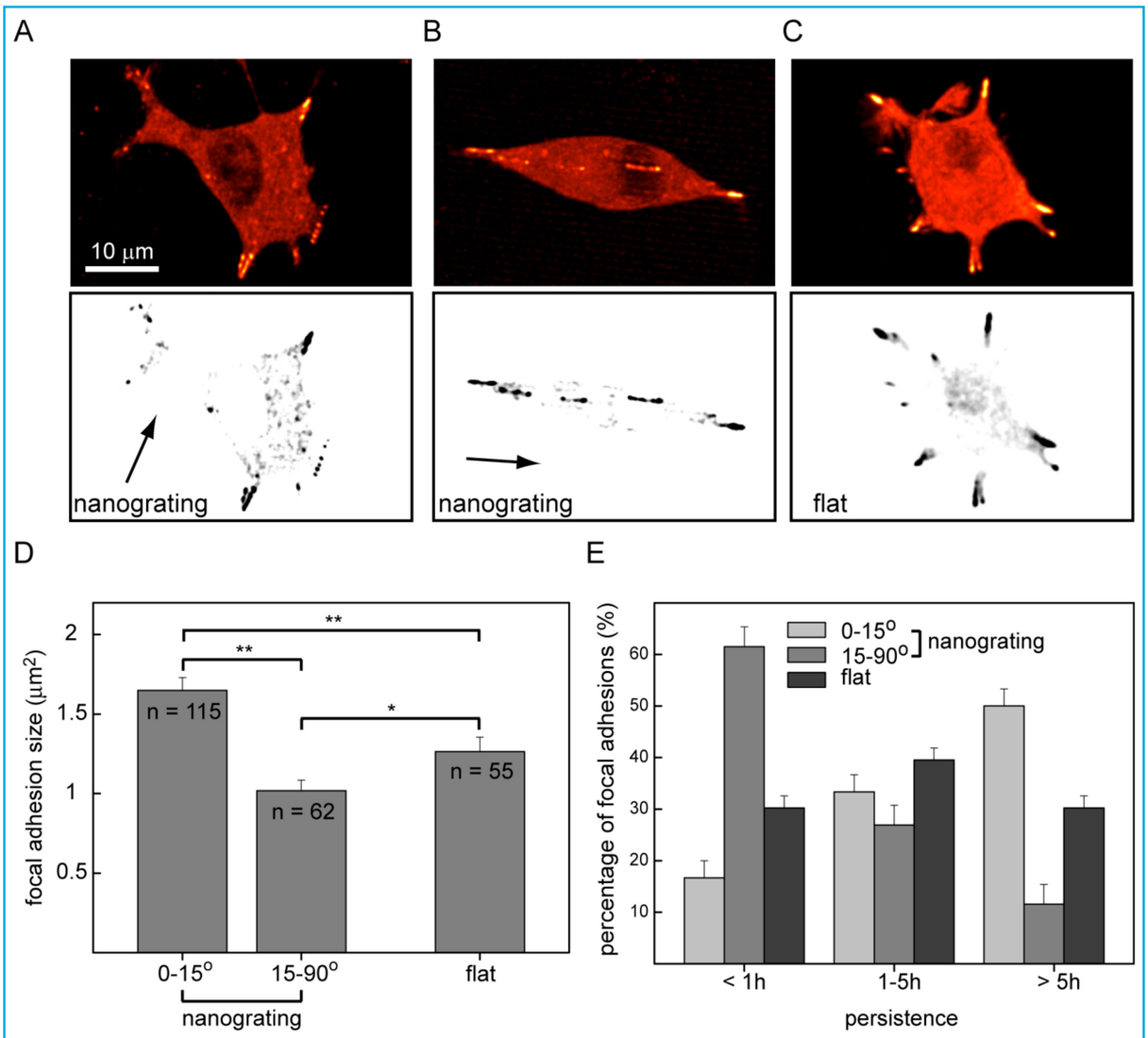

Fig. 2
Angular modulation of focal adhesion size and persistence. A) and B) Distribution of paxillin-EGFP fluorescent signal in PC12 cells differentiating on nanogratings, and [C)] on flat substrates. Statistical comparison of focal adhesion size D) and persistence E) on nanogratings and on flat substrates.

Moreover, we addressed the effect of nanogratings on the migration properties of differentiating PC12 cells and correlate their behavior with the polarity state induced by the substrate. During neuronal differentiation, cell-substrate interaction is sufficient to induce directional migration along the nanogratings (Fig. 3). Control cells contacting flat substrates migrated freely in all directions while cells differentiating on nanogratings showed slower migration characterized by an angular restriction that confined cell movements. Finally, we showed that directional migration on nanogratings is linked to a specific organization of the cell cytoskeleton reflecting the nanograting directionality.

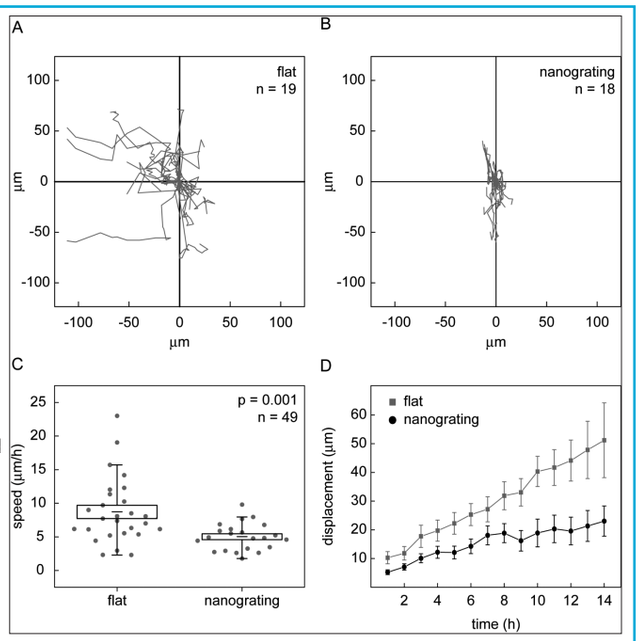

Fig. 3
Migration of PC12 cells differentiating on nanogratings or on flat substrates. A) Individual trajectories of PC12 cells (n=19) migrating on flat substrate. B) Individual trajectories of PC12 cells (n=18) migrating on nanogratings. For each trajectory (gray lines in panels A and B) the initial cell position was translated into the origin of the graph. C) Statistical analysis of cell speed during migration on nanogratings and flat substrates. The 5 to 95 percentile distribution (vertical line) was derived from individual data points (gray circles). The population mean is reported as a horizontal black line enclosed in a rectangular box whose length represents the standard error of the mean. The reported p-value was obtained performing a Mann-Whitney test. For this analysis 49 cells were considered. D) Cell displacement during migration. The average distance traveled from the origin is reported for cells migrating on flat substrates (gray squares) and on nanogratings (black circles) for each time of measure (_t = 1 h), for a total of 14 hours.

References

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