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Differential integrin expression regulates cell sensing of the matrix nanoscale geometry

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ABSTRACT

The nanoscale geometry and topography of the extra-cellular matrix (ECM) is an important parameter controlling cell adhesion and phenotype. Similarly, integrin expression and the geometrical maturation of adhesions they regulate have been correlated with important changes in cell spreading and phenotype. However, how integrin expression controls the nanoscale sensing of the ECM geometry is not clearly understood. Here we develop a new nanopatterning technique, electrospun nanofiber lithography (ENL), which allows the production of a quasi-2D fibrous nanopattern with controlled dimensions (250–1000 nm) and densities. ENL relies on electrospun fibres to act as a mask for the controlled growth of protein-resistant polymer brushes. SEM, AFM and immunofluorescence imaging were used to characterise the resulting patterns and the adsorption of the extra-cellular matrix protein fibronectin to the patterned fibres. The control of adhesion formation was studied, as well as the remodelling and deposition of novel matrix. Cell spreading was found to be regulated by the size of fibres, similarly to previous observations made on circular nanopatterns. However, cell shape and polarity were more significantly affected. These changes correlated with important cytoskeleton reorganisation, with a gradual decrease in stress fibre formation as the pattern dimensions decrease. Finally, the differential expression of αvβ3 and α5β1 integrins in engineered cell lines was found to be an important mediator of cell sensing of the nanoscale geometry of the ECM.

Statement of Significance

The novel nanofiber patterns developed in this study, via ENL, mimic the geometry and continuity of natural matrices found in the stroma of tissues, whilst preserving a quasi-2D character (to facilitate imaging and for comparison with other 2D systems such as micropatterned monolayers and circular nanopatches generated by colloidal lithography). These results demonstrate that the nanoscale geometry of the ECM plays an important role in regulating cell adhesion and that this is modulated by integrin expression. This is an important finding as it implies that the knowledge of the biochemical context underlying the integrin-mediated adhesive machinery of specific cell types should allow better design of biomaterials and biointerfaces. Indeed, changes in integrin expression are often associated with the control of cell proliferation and differentiation.

1. Introduction

Cells are not "free-standing" objects but require to adhere to the *extracellular matrix* (ECM) and to other cells in order to survive, carry out their function and form more complex structures (tissues). It has been shown in the last decades that cells feel and respond to the physical properties of the complex environment constituted by the ECM, contact with neighbouring cells and soluble growth factors and cytokines. Focal adhesion (FA) formation and maturation are important processes via which cells sense and adhere to the ECM and impact on signalling pathways eventually controlling cell phenotype [1,2]. Such cellular sensing of the

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adhesive landscape occurs at different length scales, from the microscale, at which cell shape and multi-cellular assemblies are controlled [3–5], to the nanoscale, at which the formation and dynamics of single adhesions are regulated [6–11]. Importantly, underlying these phenomena, the prevention of FA assembly leads to the disruption of the cell cytoskeleton: when cells are forced to adhere on small areas or when adhesion points are too far apart to allow integrin clustering and the interaction of key adapter proteins, cell spreading is impaired and other signalling pathways are disrupted leading, for example, to stem cell differentiation [12,13].

FAs are initiated and regulated by the binding of integrins to the ECM [14]. This phenomenon is followed by their clustering and the recruitment of other molecules, amongst which vinculin, talin, VASP, zyxin, paxillin, p130 Cas, focal adhesion kinase (FAK) and integrin-linked kinase (ILK) play an important role in determining FA stability and cell phenotype. In addition, the precise positioning of these molecules and 3D organisation of the structure of FAs is important to the stability of FAs formed, their ability to sustain mechanical forces and transmit downstream signals [15,16]. The dynamics of such processes are regulated by the transmission of forces bidirectionally, inside-out and outside-in: when cells cannot exert adequate grip on their surrounding environment, FAs are destabilised [17]. In turn, the reorganisation of the cell cytoskeleton and formation of stress fibre (contractile actin bundles) are strongly dependent on transmission of intracellular forces. Adapter proteins such as talin and vinculin play an important role in such processes and regulate adhesion size, shape, as well as cell spreading and shape [18–20]. Upon activation of vinculin, FAs are generally stabilised, increase in size and, as a result, cell migration decreases. Considering the essential role of integrin clustering to the formation and development of FAs, it is clear that integrin expression itself should have a profound impact on cell adhesion and associated mechanotransduction. Indeed, integrin expression level and the type of heterodimers expressed (e.g. $\alpha5\beta1$ vs $\alpha v\beta3$) was found to impact on cell shape, the architecture of the cytoskeleton, as well as cell motility [21–23]. Such changes are associated with marked changes in signalling via Rho GTPases [22,24] and, strikingly, the scattering of cell clusters [21]. The shape, number and size of FAs was also found to be strongly correlated with such changes in phenotype [21]. Such effect may be explained by the differential regulation of Rho GTPases Rac and RhoA [22], as well as the differential binding affinity of $\beta1$ and $\beta3$ integrins for soluble fibronectin (and associated impact on fibrillogenesis). These phenomena also correlate with important changes in the dynamics and nanoscale organisation of $\alpha5\beta1$ and $\alpha v\beta3$ integrins [22,25]. Differential regulation of FA maturation and cell phenotype was also evidenced between different $\beta1$ heterodimers (e.g. $\alpha5$ and $\alpha4$) [26]. In addition, $\alpha5\beta1$ and $\alpha v\beta3$ integrins play important roles in the ability of cells to generate forces, respond to applied mechanical stimulations and associated mechanotransduction [27,28]. Hence, these studies highlight a direct relationship between integrin expression, the regulation of the shape and size of adhesions and the sensing of physical properties of the ECM.

The sensing of the ECM is thus of particular importance to the regulation of cell phenotype. To investigate the role of different microenvironmental cues, engineered biointerfaces presenting controlled chemistry, topography and mechanical properties have been developed. In particular, the modification of surfaces to create micro- to nano-scale features has been exploited to regulate FA maturation and cell phenotype. Techniques to modify the surface range from bottom-up to top-down approaches. Self-assembly methods have allowed the formation of nanofeatures with controlled spatial distributions [6]. Replication methods such as photolithography and soft lithography are also widely used to reproduce features with the use of a mask or stamp. In the latter, a patterned elastomer is used to print molecules on a surface. Micro contact printing (μCP), which normally uses PDMS, has been widely used to produce protein microarrays for cell-based assays [17,29,30]. Combining such μCP with polymer brush growth (after printing of an initiator molecule) allows the generation of particularly stable protein arrays that allow to control cell adhesion over long periods of culture time (beyond 7 days [29]). However, molecular diffusion during the printing process does not allow high resolution patterning (below 1 μm). Direct writing is another approach used to deliver molecules to a surface for chemical patterning: inkjet printing (IJP) and dip-pen nanolithography (DPN) have been applied to the deposition of protein arrays for cell patterning [31,32]. Whilst high resolution (especially with DPN) and flexible, direct writing approaches are generally slow and with low throughput.

We previously showed that the size of adhesions (100 nm to 3 μm circular patches) primarily controls the assembly of the cytoskeleton, and that blocking the geometrical maturation of adhesions does not restrict protein recruitment significantly, nor the phosphorylation of proteins recruited to adhesions or the assembly of ECM proteins at adhesion sites [10]. In contrast, we found that adhesion dynamics (rate of diffusion of vinculin to the adhesions) was altered. This may highlight the role of adhesion dynamics, assembly and disassembly, as important stages involved in nanoscale sensing. In this respect, the continuity of the matrix, its geometry and topography are expected to regulate such dynamic processes. How such nanoscale geometrical cues impact on adhesion size and shape as well as cell spreading and shape is not understood. In addition, how the differential expression of integrins impacts on the sensing of nanoscale geometry is not clear.

Here we developed a novel patterning platform, electrospun nanofiber lithography (ENL), allowing the generation of cell-adhesive nanofibrous substrates on large scale suitable for the detailed investigation of mechanisms underlying cell sensing of the nanoscale geometry of the ECM. This platform is based on the deposition of electrospun fibres with controlled diameters in the range of 150–1500 nm, which are then used as masks for the controlled growth of protein- and cell-resistant polymer brushes via surface initiated atom transfer radical polymerization (Fig. 1). The nanofibers generated using this method mimic better the fibrous structure of some natural matrices, but without introducing complex 3D effects (topography and changes in fibre curvature). In addition, these quasi-2D nanofibers allow comparison with other 2D nanopatterns displaying circular patches of controlled diameters, previously studied [10]. This allows to investigate relationships between adhesion size and geometry and cell shape and spreading in a more realistic scenario, including with respect to the continuity of the matrix. Finally, as ENL allows the patterning of thin glass coverslips and does not introduce structures with strong refractive index mismatch, it is compatible with a broad range of high resolution live imaging microscopy techniques. This platform was then used to investigate the influence of adhesion geometry on cell spreading and shape. We make comparisons between cell response to the size of nanopatterns in the case of circular discrete patches and continuous nanofibers with similar range of sizes and pattern density. Finally, we investigate the role of integrin expression on nanoscale sensing of the geometry of the adhesive landscape.

2. Materials and methods

2.1. Chemicals and materials

Oligo(ethylene glycol methyl ether methacrylate) (OEGMA, M_w 300), CuCl, $CuCl_2$, 2,2′-dipyridyl (bpy), Poly (methyl methacrylate) (PMMA) (average $M_w \sim 350{,}000$ and 996,000), triethylamine