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Three dimensional spatial separation of cells in response to microtopography

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ABSTRACT

Cellular organization, migration and proliferation in three-dimensions play a critical role in numerous physiological and pathological processes. Nano- and micro-fabrication approaches have demonstrated that nano- and micro-scale topographies of the cellular microenvironment directly impact organization, migration and proliferation. In this study, we investigated these dynamics of two cell types (NIH3T3 fibroblast and MDCK epithelial cells) in response to microscale grooves whose dimensions exceed typical cell sizes. Our results demonstrate that fibroblasts display a clear preference for proliferating along groove ridges whereas epithelial cells preferentially proliferate in the grooves. Importantly, these cell-type dependent behaviours were also maintained when in co-culture. We show that it is possible to spatially separate a mixed suspension of two cell types by allowing them to migrate and proliferate on a substrate with engineered microtopographies. This ability may have important implications for investigating the mechanisms that facilitate cellular topographic sensing. Moreover, our results may provide insights towards the controlled development of complex three-dimensional multi-cellular constructs.

1. Introduction

The physical properties of the cellular microenvironment play a crucial role in governing numerous critical physiological and pathological pathways [1–5]. In vivo, cells are exposed to and reside in an intricate mesh of proteins known as the extracellular matrix (ECM) [6]. It is well known that complex physical and biochemical interactions between cells and their ECM regulate differentiation, proliferation and migration [1–5]. Moreover, the physical properties of the ECM, such as matrix topography and mechanical properties, also play a major role in modulating cell biology [7–14]. In the laboratory, cells are traditionally cultured on flat two-dimensional surfaces. In comparison to the in vivo matrix microenvironment, these surfaces often lack the complex nano- and micro-scale topographies found in vivo. Indeed, engineered substrates with tunable nano- and micro-scale topographies are now becoming extensively employed in many studies [7–28]. Substrate topography can be modulated in numerous ways, including altering surface roughness through chemical or plasma treatments to creating long-range ordered features with micro- and nano-scale fabrication approaches [28].

Cellular responses to nanoscale topographies have been extensively studied and may have the potential in aiding elucidation of complex control mechanisms involved in many biological pathways [18]. Fabricated nanoscale grooves, holes and pillars arranged in ordered patterns or in spatial gradients have all been employed to study cellular responses to topography [7,8,13]. Importantly, as these structures are far smaller than typical cell size, an individual cell will be exposed to many features at any given time. It has been observed that cells display an exquisite sensitivity to nanoscale changes in aspect ratio, density and spacing of these features, often in a cell-type dependent manner [7–14]. For example, nanoscale grooves will affect the alignment and migration dynamics of many cell types (such as fibroblasts, neurons and smooth muscle cells) [7,8,13]. This is a process known as contact guidance [29] and has been observed in response to both micro- and nano-scale topographic features. In vivo, migration is extremely important in many physiological and pathological processes (such as cancer metastasis, wound healing and embryogenesis) and is highly sensitive to the nanotopography of the ECM [7,8,13]. In addition to cell

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morphology and migrations, nanoscale grooves also modulate subcellular organization of the cytoarchitecture as well as numerous signalling pathways [19,22,27]. In several cases, cell proliferation has also been observed to display a sensitivity to substrate topography in many cell types [15,30–32]. Finally, during very complex processes, such as stem cell differentiation, it is becoming clear that stem cell fate is influenced by the integration of a multitude of nanotopographical, physical and biochemical cues [4,15,17,22,23].

In contrast to previous work investigating the role of topographical cues smaller than the typical length scale of a cell, a large number of studies have employed substrates with microscale topographies [11,13,16,20,24–26,28]. In many studies, surfaces containing grooves whose geometries (depth, width and ridge width) can vary in a range of less than 10 μm to greater than 100 μm have been employed to demonstrate effects on cell alignment, migration and organization. Importantly, as the groove sizes become larger than a typical cell, this allows for the appearance of several phenomena. Cells not only align with the direction of the grooves, but have also display bridging behaviour. Bridging behaviour occurs when fibroblast preferentially migrate and proliferate along groove ridges to form bridges from one ridge to another or between the bottom of the groove and the top of a ridge [20,24]. Moreover, bridging also occurs when cells form a connection between the bottom of the groove and the top of a ridge [24]. This type of behaviour is very much dependent on the geometric properties of the grooves and in the case of fibroblasts, at least three distinct geometric regimes of behaviour have been characterized [24].

Here, we microfabricated a series of 50 μm deep grooves with increasing widths (25, 50, 100 and 500 μm) and 100 μm wide ridges. In contrast to previous studies, we compared the influence of these substrates on two distinct cell types (NIH3T3 fibroblast and MDCK epithelial cells). NIH3T3 cells are highly motile and lack the strong cell–cell coupling and tight junctions found in MDCK cells. Therefore, we hypothesized that the two cell types may display distinct responses to substrate topography. Cells were cultured on the grooved substrates and were examined after 4, 24, 48 and 72 h of culture. At each time point we quantified the three-dimensional cellular alignment and organization for each cell type on each substrate. Finally, as multiple cell types are found in close contact in vivo, we also grew co-cultures of cells on the substrates. This approach allowed us to examine the influence of contact guidance in a mixture of fibroblast and epithelial cells.

2. Materials and methods

2.1. Substrate fabrication

Master substrates were created with standard soft photolithography techniques on silicon wafers (Universitywafers.com, USA). The wafers were cleaned with a Piranha wet etch solution (3:1 sulphuric acid:hydrogen peroxide), followed by immersion in de-ionized water and subsequent dehydration by baking at 200 °C for 30 min. SU-8 2015 photoresist (Microchem, USA) was then spin coated to a uniform film thickness of 50 μm. A master mould was created by transferring photomask patterns to the photoresist according to the photoresist manufacturer's protocol. The photomask consisted of separate 2.25 cm^2 square regions each containing 1.5 cm long black lines, spaced every 100 μm. The widths of the lines varied in each region and were either 25, 50 or 100 μm. Polydimethylsiloxane (PDMS) substrates with defined topographies were created by pouring a 1:10 solution of curing agent:elastomer (Sylgard 184, Ellsworth Adhesives) over the photoresist master. The PDMS was allowed to crosslink in a convection oven at 80 °C for 3 h. A schematic of the substrate fabrication process is shown in Fig. 1. To functionalize the PDMS substrates, they were air plasma treated at 30 W for 30 s to generate hydroxyl groups. The substrates were then immediately coated with 5 $\mu g/cm^2$ rat-tail collagen I (Gibco), incubated at room temperature for 1 h and then rinsed in PBS. Scanning electron microscopy (SEM) of gold-coated PDMS substrates were acquired with a JEOL JSM-7500F FESEM.

2.2. Cell culture

NIH3T3 mouse fibroblast cells and Madin Darby Canine Kidney (MDCK) epithelial cells were cultured in high glucose DMEM containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin antibiotics (all from Hyclone). The cells were cultured at 37 °C and 5% CO_2 in 100 mm dishes. For experiments, functionalized PDMS substrates were placed into 35 mm diameter dishes and the cells were seeded at a density of 20,000 cells/cm^2. Cells were grown for either 4, 24, 48 or 72 h before inspection. For co-culture experiments, an equal number of NIH3T3 and MDCK cells were thoroughly mixed and then seeded and imaged in the same manner as mono-culture experiments.

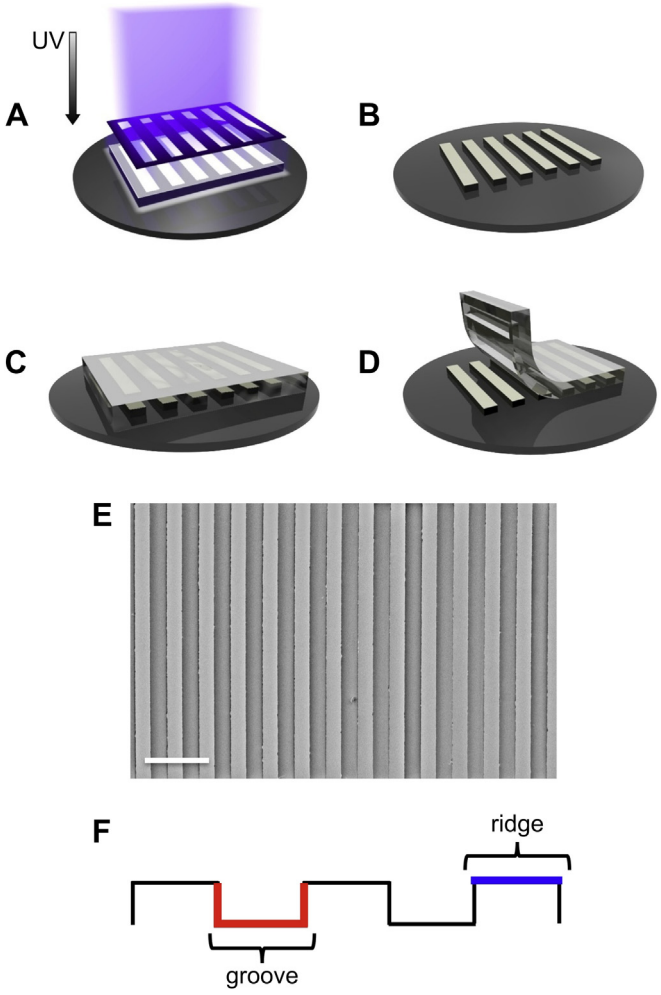

Fig. 1. Microfabrication of microscale grooves in PDMS. (A) After spin coating a 50 μm thick SU8 photoresist onto a silicon wafer, UV light is shone through a mask to crosslink exposed areas. (B) After developing the wafer, un-crosslinked SU8 is removed leaving behind rectangular features. (C) PDMS is poured over the features and cured. (D) The PDMS is then peeled from the substrate and the substrate microtopography is then functionalized with collagen. (E) A top-down SEM image of the PDMS substrate reveals the structure of a typical microtopography with 100 μm grooves and ridges. (F) For the purposes of this study we defined cells as in a 'groove' (red region) or on a 'ridge (blue region), as shown in the schematic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Immunofluorescence staining, live cell staining and microscopy

Cells cultured on PDMS substrates were fixed with 3.5% paraformaldehyde and permeabilized with Triton X-100 at 37 °C. Cells were stained for actin using phalloidin conjugated to Alexa Fluor 546 (Invitrogen) and DNA was stained using DAPI (Invitrogen). A full protocol has been published previously [33]. Samples were then mounted using Vectashield (Vector Labs) and a #1 coverslip placed on top of the PDMS substrate. The sample was inverted and then imaged with confocal microscopy. In co-culture NIH3T3 cells were pre-loaded with the live cell dye CellTracker Green CMFDA (Invitrogen) following manufacturer protocols and cultured with MDCK cells for 4 or 48 h. After the allotted time in culture, all cells were loaded with live cell nuclear stain, Hoechst 33342 (Invitrogen). In some cases co-cultures were imaged live with a Nikon Ti-E inverted phase contrast and fluorescence microscope with a long working distance 40× objective or they were fixed (but not