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Extending neurites sense the depth of the underlying topography during neuronal differentiation and contact guidance

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ABSTRACT

The topography of the extracellular microenvironment influences cell morphology, provides conduct guidance and directs cell differentiation. Aspect ratio and dimension of topography have been shown to affect cell behaviours, but the ability and mechanism of depth-sensing is not clearly understood. We showed that murine neural progenitor cells (mNPCs) can sense the depth of the micro-gratings. Neurite elongation, alignment and neuronal differentiation were observed to increase with grating depth. We proposed a mechanism for depth-sensing by growing neurites: filopodial adhesion in the growth cones favour elongation but the bending rigidity of the neurite cytoskeleton resists it. Thus, perpendicular extension on deeper grooves is unfavourable as neurites need to bend over a larger angle. A quantitative model was developed and its prediction of neurite growth on gratings fit well with the experimental data. The results indicated that mNPC fate can be directed by appropriately designed patterned surfaces.

1. Introduction

Neural progenitor cells (NPCs) and neural stem cells (NSCs) found in the central nervous system (CNS) are known to have multilineage potential and the ability to self-replicate [1]. The ability to differentiate into neurons, oligodendrocytes and astrocytes made these cells promising candidates for cell therapy of CNS injury. Reports of NPCs transplantation to damaged CNS sites in animal models have further demonstrated their therapeutic potential [2,3]. However, the lack of understanding of the signals that guide the proliferation, differentiation and integration of NPCs remain an obstacle for clinical cell based therapies [4,5].

Contact guidance is the phenomenon where extracellular topography induces cells to respond differentially in terms of morphology and behaviour. *In vivo*, during development, the neural tissue is in contact with a dynamic extracellular matrix (ECM), which presents both physical and biochemical cues to guide the morphogenesis and differentiation of the neuronal cells. During the development of the neocortex, neuronal cells are also known to migrate along tracks of radial glial cells [6,7]. Contact guidance is also evident in the response following periphery nervous system (PNS) injury where Schwann cells form longitudinal tracks to support and guide axon regrowth [8]. Therefore, it is postulated that the incorporation of topographical cues in neural tissue engineering scaffold design can be a powerful tool to direct neuronal cell behaviour and aid nerve regeneration.

A number of studies have been carried out to assess the response of neuronal cells to topographical cues. Neuronal type cells have been cultured on isotropic topographies such as micro-sized pillars [9,10] as well as anisotopic topographies such as gratings [11–19], microchannels, electrospun fibres [20,21] and aligned co-cultured cells [22,23]. Topography feature size has been shown to affect neurite length, alignment and branching. In general, on anisotropic patterns, neurites tend to extend in the

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direction of the topography though perpendicular contact guidance has also been reported [12,13,24]. Apart from cell morphology and alignment, topography has also been shown to affect the differentiation rate of neural progenitors into neurons. NPCs showed various differentiation potential into glial and neuronal lineage when cultured on an array of multiple topographies [25]. Higher NSC neuronal differentiation rates were found on aligned nano-fibres compared to micro-fibres [26]. Enhanced neuronal differentiation was also observed on 20 μm wide gratings compared to 5 and 10 μm wide gratings [19]. In a more recent study, anisotropic features of gratings and concentric circles were shown enhance the neuronal differentiation of NSCs which was also upregulated with decreasing feature size [27]. Besides neural cells, mouse embryonic stem cells showed higher cell adhesion and proliferation on 3D zeolite microstructure coatings than on glass surface [28]. Such zeolite coatings have also been shown to promote the differentiation of human foetal osteoblasts into adult osteoblasts [28,29].

In this article, we studied how feature dimension can affect the contact guidance of neuronal cells on anisotropic grooved substrates. We hypothesized that neurites can sense the depth of the grooved substrates and exhibit different morphologies on different depths. A computational model to predict neurite behaviour on grooves with different depths to gain a deeper biophysical understanding of neurite depth-sensing was also presented. We further investigated whether feature depth enhances the neural differentiation of murine NPCs. Understanding the depth-sensing mechanism of the neurites can help the design of instructive scaffolds which can direct cell fate and growth of neural progenitors, thereby, contribute to the study of nerve regeneration. This work complements the above-mentioned works on zeolite coated scaffolds and further highlights the role of anisotropic substrate topography on the proliferation and differentiation of cells.

2. Materials and methods

2.1. Preparation of polydimethylsiloxane substrates

Soft lithography was used to fabricate polydimethylsiloxane (PDMS) substrates of 2 μm wide gratings with 2 μm spacing and differing heights (0.35, 0.8, 2 and 4 μm). The silicon master molds were cleaned with nitrogen gas and treated with (1H, 1H, 2H, 2H)-perfluorodecyltrichlorosilane (Sigma). The molds were then washed with 0.01% Triton X-100 (Biorad) and blown dry with nitrogen gas. PDMS base and curing agent (Sylgard 184 Silicone Elastomer Kit, Dow Corning) were mixed with a 10:1 ratio and degassed in a desiccator for 30 min. The mixture was poured over the master molds, degassed in a desiccator for at least 30 min and cured at 60 °C for a minimum duration of 12 h. Upon cooling to room temperature, the PDMS substrates were gently peeled off from the master molds.

To check the fidelity of replication, PDMS samples were sputter-coated with 11-nm-thick platinum (JEOL JFC 1600 Auto Fine Coater) and imaged with field emission SEM (JEOL) or filamentous SEM (6010 LV, JEOL). Random images of each sample type were captured and feature size was measured with Image J (National Institute of Health, Bethesda, MD, USA). At least 26 gratings were measured for the width and spacing while at least 15 were measured for the gratings height.

Prior to cell seeding, the PDMS substrates were air plasma treated for 60s at 29.6W (Harrick Scientific Corporation PDC-002), cleaned with 70% ethanol and sterilized via UV irradiation for 30 min. Subsequently, the substrates were incubated with 33 μg/ml of poly-L-ornithine (PLO, Sigma–Aldrich) in sterile distilled water at 37 °C overnight before coating with 20 μg/ml of natural mouse laminin (GIBCO, Life Technologies) in DMEM/F12 (Biological Industries) for a minimum of 4 h at 37 °C. The poly-L-ornithine (PLO) and laminin coatings are required for the attachment and growth of the murine neural progenitor cells (mNPCs). Laminin is an extracellular matrix (ECM) protein which was shown to enhance the migration, expansion, differentiation, and neurite outgrowth of NPCs [30]. Previous studies have indicated that the PLO and laminin coating on PDMS were relatively thin and did not significantly affect surface topography [25,31].

2.2. Maintenance of neural progenitor cells

Primary murine neural progenitor cells (mNPCs) were isolated from the hippocampal region of a day-5 postnatal mouse brain in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines as previously described [25]. The mNPCs were expanded on 6-well plates pre-coated with 10 μg/mL of natural mouse laminin (GIBCO, Life Technologies). The maintenance medium is composed of DMEM/F12 (Biological Industries), 1× N2 Supplement (GIBCO, Life Technologies) and 1× Penicillin–Streptomycin solution (Caisson). Basic fibroblast growth factor (bFGF, GIBCO, Life Technologies) and epidermal growth factor (EGF, GIBCO, Life Technologies) were supplemented to the maintenance medium every day at a final concentration of 20 ng/ml.

2.3. Differentiation of neural progenitor cells on polydimethylsiloxane substrates

The primary mNPCs of passage 19 to 22 were used for the differentiation experiments. The mNPCs in the same passage range were previously characterized and shown to express Nestin, Vimentin, Sox2, Brain Lipid-binding protein (BLBP) and ki67. This is indicative of the undifferentiated state of the cells and the capacity for self-renewal [25]. The mNPCs were detached and dissociated into single cells with Accutase (Stem Cell Technologies) and subsequently seeded on the PDMS substrates in maintenance medium. The cells were suspended in 0.5 ml of culture medium on 48-well plates and incubated at 37 °C and 5% CO_2.

For differentiation into the neuronal lineage, a seeding density of 15,000 cells/cm^2 was used. The differentiation period was divided into two phases: induction and maturation phase, both of which lasted for 7 days. Total medium replacement was made one day after seeding with the neural induction media consisting of DMEM/F12, 1 × N2 Supplement, 1 × Penicillin-Streptomycin and 5 ng/ml bFGF. After 7 days, the induction medium was replaced with the neuronal maturation medium consisting of DMEM/F12:Neurobasal media (1:1 ratio, GIBCO, Life Technologies), 1X B27 Supplement (GIBCO, Life Technologies), 0.25× N2 Supplement and 1× Penicillin–Streptomycin. Medium change was carried out on alternate days and the experiment was halted after 14 days of culture.

For astrocyte differentiation, a seeding density of 15,000 cells/cm^2 was used. Total medium replacement was made one day after seeding with medium consisting of DMEM/F12, 1× N2 supplements, 1% foetal bovine serum (FBS, Invitrogen) and 1× Penicillin–Streptomycin. Medium change was carried out on alternate days and the experiment was halted after 3 days of culture.

2.4. Scanning electron microscopy of cell samples on topographical substrates

At the end of the experiment, the samples were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate and 3 mM $CaCl_2$ and dehydrated through a graded ethanol series. Critical point drying was done by transferring the samples to 100% hexamethyldisiloxane (HDMS, Sigma–Aldrich) through a graded series of ethanol and HDMS mixtures. The samples were then sputter-coated with 11-nm-thick platinum (JEOL JFC 1600 Auto Fine Coater) and imaged with field emission SEM (JEOL) or filamentous SEM (6010 LV, JEOL).

2.5. Immunofluorescence staining

After the end of each experiment, the samples were fixed with 4% paraformaldehyde (Sigma–Aldrich) and stained following standard immunofluorescence staining protocol. Following permeabilization with 0.25% Triton X-100, the samples were blocked with 1% bovine serum albumin (BSA) and 10% goat serum for 1 h at room temperature. They were then incubated with primary antibodies at 4 °C overnight, followed by 1 h incubation in secondary antibody solution at room temperature. The primary antibodies and the dilutions used in this study are as follows: rabbit anti-β-tubulin III (Tuj1) antibody (1:600, Sigma–Aldrich) and mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:600, Millipore). The secondary antibodies used were Alexa Fluor 546 and Alexa Fluor 488 (both at 1:750, Invitrogen). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen). Imaging was performed with the Leica SP5 confocal microscope or the Leica epifluorescence microscope equipped with Q imaging camera (QImaging).

2.6. Image analysis

Random images of each sample were captured and processed with Image J (National Institute of Health, Bethesda, MD, USA).

The mNPCs samples differentiated via the 14 day neuronal differentiation protocol were used for the morphological analysis. Neurite length and angle were measured for cells stained positive for TUJ1 and DAPI. Length measurements were performed by manually tracing the entire neurite using Image J and are reported as a mean of all the cells analysed. Average neurite length is defined as the sum of the length of all the neurites from a single cell divided by the total number of neurites the same cell has. Maximum neurite length is defined as the length of the longest neurite of each cell. For branched neurites, neurite length was determined by taking the average length of the tracings from the cell body to each end point. Angle measurements were made by drawing a straight line from one end of the neurite to the other end. The angle made by the drawn straight line with respect to the grating axis is defined as θ, with $(90^\circ - \theta)$ defined as γ (see Fig. 3A). When θ is less than 15°, the neurite is considered aligned or parallel to the gratings. When γ is less than 15°, the neurite is considered perpendicular to the gratings. For unpatterned substrates, a random direction was chosen as the axis. Neurite alignment is reported as a percentage of all the neurites analysed. Measurements were averaged over 3 different experiments, with at least 2 replicas in each experiment. Neurites of at least 48 cells per replica were measured.