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Topographic effect on human induced pluripotent stem cells differentiation towards neuronal lineage

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ABSTRACT

Pluripotent stem cells have the potential to develop into all cell types of the adult body. Besides chemical and mechanical cues, topographical effect of surfaces could also contribute to the development of new therapies in regenerative medicine. In the present study, we tested the effects of nanograting substrates with different widths (width:350 nm/2 μm/5 μm, height: 300 nm) on human induced pluripotent stem cells (hiPSCs), in particular regarding the commitment of stem cell differentiation to desired phenotypes. We found that nuclei of hiPSCs could align and elongate in the direction of the nano/microstructure, whereas they distributed randomly on flat surfaces. The contact guidance significantly increased when the cells were cultured on the surface with smaller pitch. Gene expression profiling by real-time PCR and immunostaining showed significant up-regulation of neuronal markers on nanostructured substrates either with solely topographical cues or combined with pre-neuronal induction. A width of 350 nm, in particular, induced highest neuronal marker expression. This study demonstrates the significance of topography, especially regarding the width of the structures, in directing differentiation of hiPSCs towards the neuronal lineage. Our study suggests the potential applications of surface topography in clinical regenerative medicine for nerve injury repair.

1. Introduction

Customized supports and scaffolds with controlled nano/micro scale topography can readily be generated by state-of-the-art fabrication technologies. Topographic surfaces at micro and nano-scale may play a key role in the development of new therapies, since anchorage-dependent cells require adhesive surfaces to exert forces and consequently spread and develop [1,2]. Cell functions are modulated by complex topographical features *in vivo*, such as extracellular matrix (ECM) geometry [3,4], and they also respond to synthetic topographical cues *in vitro* [5–7]. Topographic surfaces with micro- and nano-scale features induce changes in cell alignment, polarization, elongation, migration, proliferation and gene expression [8–10]. Hence, topographical cues may also be used as a tool to induce stem cell differentiation into different cell types.

Human embryonic stem cells (hESCs) are capable to self-renew and differentiate into all three germ layers. They have been extensively studied due to their promising applications as cell sources for cell therapy, tissue repair and implantation [11–14]. For brain repair and neural regeneration, it is essential to induce differentiation of hESCs efficiently towards specific neuronal lineages rather than any other cell types. Currently, most of such studies are focused on chemical and biological cues. In previous publications, mouse ESCs have been differentiated into neuroectoderm in an adherent monoculture system in serum-free condition. The whole procedure can be done without co-culture with any other cell types or cell aggregation. While up to data, this monolayer differentiation can not be achieved in hESCs yet. Neuronal differentiation methods reported still require generation of neuronal progenitor cells first, involving manual dissection or isolation and re-differentiation to certain specific neuron type due to the complexity and heterogeneity during the neuronal induction. In some regards, these methods are quite time consuming and still lack of consistency and quality of results.

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In our previous study, we have successfully promoted the transition of hESCs into the neuroectoderm lineage using inhibition of three signaling pathways [15,16] in a rapid and efficient way [17]. Further improvement would be neuronal induction as monolayer with higher efficiency and more homogeneity for future screening purpose, especially high-content imaging analysis. For this purpose, topographical effect on hESCs differentiation would be interesting to follow, as such topography induced neuronal differentiation has been studied by others [18,19]. In order to further contribute to research on cell attachment, proliferations, and differentiation, and develop next generation medical devices and implants, cell–substrate interactions should be intensively explored.

In the current study, we tested topographical effects of different sizes (grating structures with ridge width = groove width: 350 nm/2 μm/5 μm, height: 300 nm) on human induced pluripotent stem cells (hiPSCs) growth, in particular to commit stem cell differentiation to desired phenotypes. Our goal is to manipulate the differentiation of hESCs with physical cues, so that a uniform population of precursors can be obtained *in vitro*.

2. Materials and methods

2.1. Surface preparation

The silicon master substrate (ridge width = groove width: 350 nm/2 μm/5 μm, depth = 300 nm) was fabricated via laser inference lithography (NIL Technology ApS, Denmark). The master substrate was used as a mold to fabricate replicas with nano-imprinting lithography into Poly(methyl methacrylate) (PMMA). Then, these PMMA substrates were used for the pattern transfer to polydimethylsiloxane (PDMS) substrates [20]. PDMS (Sylgard 184, Dow Corning, Dortmund, Germany) was polymerized by mixing the pre-polymer with the curing agent in a ratio of 10:1. The mixture was cured on the master substrates at 65 °C for 24 h. The substrates were treated with oxygen plasma (50 W for 15 s) to increase surface wettability and cell adhesion. They were sterilized with 70% ethanol, washed with phosphate buffered saline (PBS) and cut into 1 cm^2 prior to use in the experiments. The surface topography of the PDMS substrates was verified by atomic force microscopy (Digital Instruments, USA) (Fig. 1).

2.2. hiPSCs generation

The hiPSC line used here is line SFS.1, which was produced from foreskin fibroblasts (ATCC #CRL-2097) by following Melton's protocol [21] but using reprogramming factors (OCT4, SOX2, KLF4 and c-Myc) packaged into Sendai virus particles (Invitrogen) [22]. Sendai virus (SeV), also known as murine parainfluenza virus type 1 or hemagglutinating virus of Japan (HVJ), is a negative sense, single-stranded RNA virus. Thus the reprogrammed SFS.1 cells were free from viral genome, and after up to 5 passage, zero footprint can be achieved and no viral RNA were detected in SFS.1 (Supplementary Figure S1). Both cell lines showed typical hESCs morphology and growth characteristics, and further characterized according to standard assays (Supplementary Figures S2, S3).

2.3. Cell culture and differentiation

All cell culture and differentiation, if not specially indicated, were carried on in incubator with 37 °C and 5% CO_2. SFS.1 cells were cultured under feeder-free condition on Matrigel-coated 6-well plates using MEF-conditioned medium [23]. However, other culture systems such as homemade or commercial defined media should also work. At the time of starting a differentiation experiment, SFS.1 cells should be sub-confluent and actively growing.

For neuronal differentiation by directly seeding hiPSCs without any previous induction or modification, single hiPSCs dissociated from colonies were seeded on nano/microstructured and flat PDMS substrates coated with 1:80 diluted Matrigel [24] which placed on 24-well culture plates. Cells were seeded at 10^6 cells/cm^2 (according to gradient initial density test shown in Supplementary Figures S4) in knockout Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), supplemented with 2% fetal calf serum, 1 × penicillin/streptomycin (PAA), incubated for 14 days before.

For improved differentiation, we combined topographical cues with our previous protocol. In our previous protocol, cells were harvested as single cell and seeded in fixed numbers into 96 V-bottom well plates, to induce homogeneously sized embryoid bodies (EBs) over night (day −1 to 0), as detailed elsewhere [17,24]. For neuroectoderm induction, newly formed EBs were transferred to U-bottom ultra-low attachment plates in neural induction media through triple pathway inhibitor treatment. On day 4, EBs were seeded as a whole onto Matrigel coated surfaces for 4 more days (Fig. 2A). Alternatively, with combined method, after 4 days of pathway inhibitor treatment of EBs, EBs were dissociated with Accutase into single cells and seeded as monolayer at a density of 10^6 cells/cm^2 on Matrigel coated nano/microstructured PDMS substrates, and were allowed to differentiate for 4 more days (Fig. 2B).

2.4. Cell direction angles characterization

The cell direction angles, defined as the orientation of the major elliptic axis of individual cell with respect to the direction of gratings, were measured using ImageJ software (http://rsb.info.nih.gov/ij/). For analysis, five separate regions of every sample were photographed, and over 200 cells were measured from every sample separately. Direction angles were subsequently grouped in 10-degree increments ranging from 0 to 90°. Cell direction within less than 10-degree is considered to be aligned.

2.5. Histology and immunohistochemistry

For immunostaining, cells were fixed with 4% paraformaldehyde for 10 min, permeabilized in 0.2–1% Triton X-100 for 10 min. Samples were incubated in anti-Beta-III-tubulin mouse antibody (Sigma, T8660) for 1 h followed by Alexa Fluor 488-conjugated secondary antibody and Hoechst DNA stain for 1 h. A Zeiss AxioVert 200 fluorescence microscope was used to collect and analyze all cell images.

2.6. Real time RT-PCR

Cells were lysed directly on substrate or in culture dishes, using RNeasy mini kits with on-column DNA digestion (Qiagen) for RNA isolation. Reverse transcription, real-time PCR and gene expression analysis were carried on as indicated in previous publication [17]. The sequence of primers of specific genes analyzed in the result are listed in Supplementary Table 1.

3. Results

3.1. Topographic effect of structured PDMS substrates on differentiation of hiPSCs into ectoderm lineage

The topographic effect of nano/microstructured PDMS substrates was investigated first by directly seeding hiPSCs onto nano/microstructured substrates without any previous induction or modification. As clearly indicated in Fig. 3, SFS.1 hiPSCs seeded

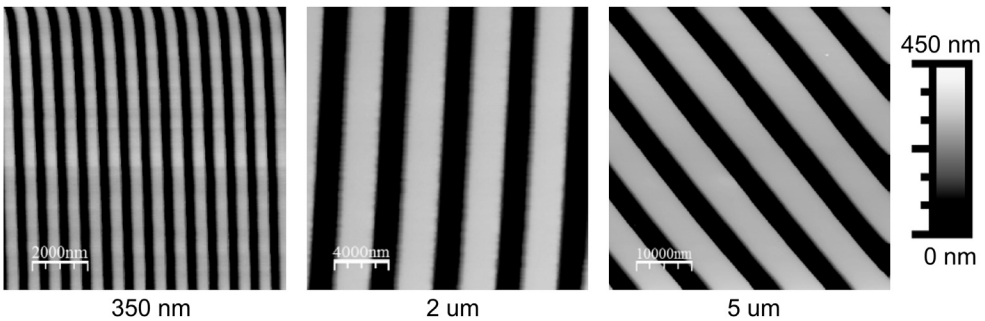

Fig. 1. Atomic force microscopy image of nano/microstructured PDMS substrates. Ridge width = groove width = 350 nm/2 μm/5 μm, height = 300 nm.

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