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Nanotopography promoted neuronal differentiation of human induced pluripotent stem cells

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ABSTRACT

Inefficient neural differentiation of human induced pluripotent stem cells (hiPSCs) motivates recent investigation of the influence of biophysical characteristics of cellular microenvironment, in particular nanotopography, on hiPSC fate decision. However, the roles of geometry and dimensions of nanotopography in neural lineage commitment of hiPSCs have not been well understood. The objective of this study is to delineate the effects of geometry, feature size and height of nanotopography on neuronal differentiation of hiPSCs. HiPSCs were seeded on equally spaced nanogratings (500 and 1000 nm in linewidth) and hexagonally arranged nanopillars (500 nm in diameter), each having a height of 150 or 560 nm, and induced for neuronal differentiation in concert with dual Smad inhibitors. The gratings of 560 nm height reduced cell proliferation, enhanced cytoplasmic localization of Yes-associated protein, and promoted neuronal differentiation (up to 60% βIII-tubulin$^+$ cells) compared with the flat control. Nanograting-induced cell polarity and cytoplasmic YAP localization were shown to be critical to the induced neural differentiation of hiPSCs. The derived neuronal cells express MAP2, Tau, glutamate, GABA and Islet-1, indicating the existence of multiple neuronal subtypes. This study contributes to the delineation of cell-nanotopography interactions and provides the insights into the design of nanotopography configuration for pluripotent stem cell neural lineage commitment.

1. Introduction

Human pluripotent stem cells (hPSCs) hold great promise for the treatment of diseases that are untreatable at present as well as for the discovery of new pharmaceutical and therapeutic targets. The recently described human induced pluripotent stem cells (hiPSCs), in particular, have vast therapeutic and pharmaceutical implications because they can be reprogrammed from a patient's own cells and differentiated into cell types affected by a disease state [1–3]. While current differentiation methods mainly rely on biochemical factors such as cytokines, growth factors, and small molecules [4,5], the potentials of biophysical characteristics (stiffness and topography) of cellular microenvironment in hPSC differentiation into clinically relevant cell types have been increasingly appreciated [6–8]. In order to treat neurological disorders, efficient neural differentiation from hPSCs and mechanistic understanding of biophysical regulation of hPSC neural fate decision are in urgent demand [6].

As an important biophysical factor, the substrate topography has pronounced influence on stem cell fate decision [9–11] as well as cellular reprogramming [12]. In particular, nanoscale topography has recently been shown as a potent regulator of stem cell self-renewal, reprogramming, lineage commitment, and metabolomic pathways [9,13–16]. Nanotopographies such as gratings, grooves and fibers were reported to be capable of enhancing neural differentiation of hPSCs [17–19], which provides a promising approach to generate high-purity functional human neurons and to establish physiologically relevant neural tissue models for potential neurological disorder treatments [2]. These recent advancements underscore the importance of understanding the role of hPSC-nanotopography interactions in neuronal lineage commitment from hPSCs.

Although nanotopography has been demonstrated to be able to facilitate neuronal differentiation of hPSCs [19,20], much more work is needed to advance this technology, in particular with hiPSCs. (1) Evaluation of nanotopographical effects on neural differentiation of hPSCs was usually performed in the absence of neurogenic factors, leading to low neural differentiation efficiency

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(up to 20–25%) [20,21]. The capacity of nanotopography-modulated hPSC neural differentiation in the presence of neurogenic factors needs to be explored. (2) The influence of geometry and dimensions of nanotopography on neural differentiation has not been fully investigated. Although neural differentiation of stem cells on nanotopographies of various dimensions has been studied, how the geometry and dimensions of nanotopography affect cell sensing and responding to nanotopography has not been well understood. (3) The underlying mechanism of nanotopographical modulation of neuronal differentiation from hiPSCs is elusive. A recent study indicated that Yes-associated protein (YAP) played a role in the nanograting modulation of neuronal differentiation of hPSCs [22]. Yet the differential YAP intracellular localization of the cells on the nanotopography of various configuration parameters is unclear. Moreover, there is a lack of study on the role of nanotopography in extracellular matrix (ECM) remodeling during neural differentiation.

To fill the technology and knowledge gaps, this study focused on how nanotopography configuration affected and promoted neuronal differentiation of hiPSCs in concert with dual Smad inhibitors [23]. Nanoscale gratings and pillars of different feature sizes, spacings and heights were fabricated on polydimethylsiloxane (PDMS) substrates. The cell spreading and proliferation, early ectoderm induction and the subsequent differentiation of hiPSCs into neuronal cells were assessed on the nanotopographies in comparison with flat control surfaces. YAP nucleocytoplasmic localization and ECM remodeling were evaluated. This study delineated the contribution of nanotopography configuration (geometry and feature width and height) to the neuronal lineage commitment from hPSCs and provided a useful approach for rapid (~14 days) and highly efficient (~60%) neural differentiation from hiPSCs.

2. Materials and methods

2.1. Undifferentiated human iPSC culture

Human iPSK3 cells, derived from human foreskin fibroblasts transfected with plasmid DNA encoding reprogramming factors OCT4, NANOG, SOX2 and LIN28, were kindly provided by Dr. Stephen Duncan, Medical College of Wisconsin, and Dr. David Gilbert, Department of Biological Sciences of Florida State University [24,25]. Human iPSK3 cells were maintained in mTeSR serum-free medium (StemCell Technologies, Vancouver, Canada) on 6-well plates coated with growth factor reduced Geltrex (Life Technologies, Carlsbad, CA, USA). The cells were passaged by Accutase dissociation every 5–6 days and seeded at 1×10^6 cells per well of 6-well plates in the presence of 10 μM Y27632 (Sigma-Aldrich, St. Louis, MO, USA) for the first 24 h.

2.2. Fabrication of nanotopographies

Nanotopographies were generated by electron beam lithography (EBL) as previously reported [26]. Briefly, designed nanopattern was written onto a poly(methylmethacrylate) (PMMA) thin film spin-coated on a silicon substrate by using a focused electron beam of 20 nm in diameter. The PMMA nanopattern was then deposited with a thin nickel layer. After the lift-off process, a reversed nickel pattern left on the silicon substrate functioned as a mask for the following reaction ion etching (RIE) process, which determined the height of nanotopography.

The PDMS nanotopography replica was made by casting and curing the mixture of PDMS resin and curing agent (Sylgard 184 kit, Dow Corning, MI, USA) in a 10:1.05 w/w ratio on the EBL mold for 4 h at 70 °C. A slightly higher concentration of curing agent was added to reduce the amount of uncured oligomers [27]. To allow seeding enough cells for biological analyses, the PDMS nanotopography was expanded to a large area of nanopatterned substrate by applying a stitch technique developed previously [28]. Briefly, multiple PDMS molds, replicated from an EBL mold were aligned with nanopatterns face-down on a silicon wafer and glued with a PDMS prepolymer layer on the backside. After curing at 80 °C for 2 h, a large nanopatterned surface was created. The stitched mold was then imprinted into a polystyrene (PS) substrate, which served as a master mold to replicate working PDMS nanotopography.

2.3. Scanning electron microscopy (SEM) observations

The PDMS nanotopographies were sputter-coated with a gold layer of ~10 nm thick by using a Denton Vacuum Desk V sputter unit (Denton Vacuum, LLC, Moorestown, NJ, USA), and examined using a field emission SEM (JEOL JSM-7600F, Peabody, MA, USA).

2.4. Neural differentiation of hiPSCs

Prior to cell seeding, the PDMS substrates including the nanotopographies and the flat surfaces were transferred into 24-well plates, sterilized with 70% ethanol followed by UV exposure, each for 30 min, and then incubated with 1% Geltrex for at least 1 h. Human iPSK3 cells were seeded onto the PDMS substrates at 5×10^4 cells/cm^2 in a differentiation medium composed of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) plus 2% B27 serum-free supplement (Life Technologies) based on the protocols modified from literatures [23,29]. Y27632 (10 μM) was added in the seeding step and removed after 24 h. After one day, the cells were treated with dual Smad signaling inhibitors: 10 μM SB431542 (Sigma-Aldrich) and 100 nM LDN193189 (Sigma-Aldrich). After 7 days, the cells were treated with fibroblast growth factor (FGF)-2 (10 ng/mL, Life Technologies) and retinoic acid (RA) (2 μM, Sigma-Aldrich) till Day 14–21. The cells were characterized for 5-bromo-2-deoxyuridine (BrdU) and Oct-4 at Day 2, PAX6 at Day 7, and various neural markers (e.g., βIII-tubulin, Nestin, and MAP2) during day 14–21 cells. The neural differentiation process was illustrated in Scheme S1 (Supplementary materials).

2.5. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in phosphate buffer saline (PBS) for 15 min at room temperature, and permeabilized with 0.2-0.5% Triton X-100 (Sigma-Aldrich) in PBS for 10 min. The samples were then blocked and incubated with various mouse or rabbit primary antibodies (Table S1 in Supplementary materials) for 2 h. After washing, the cells were incubated with the corresponding secondary antibody: Alexa Fluor® 488 goat anti-Mouse IgG or Alexa Fluor® 594 goat anti-Rabbit IgG (Life Technologies). F-actin was stained with Alexa Fluor® 594 Phalloidin (Life Technologies). The nuclei were counterstained with Hoechst 33342. Imaging was performed with a fluorescent microscope (Olympus IX70 or Nikon TS100F) or a Zeiss LSM 880 confocal microscope.

2.6. Proliferation assay

The cells grown on the PDMS substrates were incubated in growth medium containing 10 μM BrdU (Sigma-Aldrich) for 90 min [30]. The cells were then fixed with 70% cold ethanol, followed by a denaturation step using 2 N HCl/0.5% Triton X-100 for 30 min in the dark. The samples were reduced with 1 mg/mL sodium borohydride for 5 min and incubated with mouse anti-BrdU (1:100, Life Technologies) in blocking buffer (0.5% Tween 20/1% bovine serum albumin in PBS), followed by the incubation with Alexa Fluor® 488