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Temporal application of topography to increase the rate of neural differentiation from human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells (hPSCs) are a promising cell source for tissue engineering and regenerative medicine, especially in the field of neurobiology. Neural differentiation protocols have been developed to differentiate hPSCs into specific neural cells, but these predominantly rely on biochemical cues. Recently, differentiation protocols have incorporated topographical cues to increase the total neuronal yield. However, the means by which these topographical cues improve neuronal yield remains unknown. In this study, we explored the effect of topography on the neural differentiation of hPSC by quantitatively studying the changes in marker expression at a transcript and protein level. We found that 2 μm gratings increase the rate of neural differentiation, and that an additional culture period of 2 μm gratings in the absence of neurotrophic signals can improve the neural differentiation of hPSCs. We envisage that this work can be incorporated into future differentiation protocols to decrease the differentiation period as well as the biochemical signals added, thus generating hPSC-derived neural cells in a more cost effective and efficient manner.

1. Introduction

Human embryonic stem cells (hESCs), and more recently induced pluripotent stem cells (iPSCs), have been cited as great potential cell sources for tissue engineering and regenerative medicine, due to their ability to self-renew indefinitely or differentiate into any somatic cell type [1,2]. There has been particular interest in deriving neural cells for the treatment of stroke [3], spinal cord injuries [4] and neurodegenerative diseases, such as Parkinson's disease [5,6]. Multiple protocols have been developed for the generation of neurons, oligodendrocytes, and astrocytes [4,6,7], with many protocols generating neural stem cells (NSCs) or neuroprogenitors (NPs) in an intermediate stage [8–11]. This intermediate stage has elicited particular interest, as these NSCs and NPs can be expanded further before differentiating them into any of the three desired neural cell types [6,12–15]. There are two common approaches to differentiate hESCs or iPSCs into NSC/NPs *in vitro*. The first requires 14–28 days of differentiation beginning with the formation of embryoid bodies followed by neuroectodermal and neural precursor cell induction by stage specific cues, such as fibroblast growth factor-2, retinoic acid, and/or noggin [6,8,13,14,16]. The second approach is completed in 11 days using adherent cultures and inhibition of SMAD signaling by Noggin and SB431542, thereby inducing ectodermal differentiation through the prevention of mesendodermal differentiation [11,17].

Recently, a third approach has been proposed – to use topography to induce and augment neural differentiation [18–21]. Carlberg et al. first demonstrated that topography could be used to differentiate hESC into dopaminergic neurons using an electrospun polyurethane nanofiber scaffold and neurotrophic factors [18]. Lee et al. then demonstrated the efficaciousness of using a 350 nm ridge/groove topography to induce hESC neuronal differentiation

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without additional neurotrophic factors [19]. Lee and co-workers were able to generate mature neurons from hESC in 5 days in unsupplemented hESC maintenance media. However, this study was predominantly qualitative and did not quantify the neuronal yield, leaving the efficiency of this approach undetermined. Shahbazi et al. demonstrated that a nanofibrillar surface with a mean fiber diameter of 400 nm was able to induce a higher number of NSC/NPs within 12–18 days, and mature motor neurons within 36 days [20]. This work is complemented by a study, published earlier in the same year, that demonstrated that aligned nano- and microfibers (mean diameter of 250 nm and 1 μm, respectively) were able to improve the neuronal differentiation of hESC-derived NP, as compared to random fibers [21]. This did not report a significant difference between the induction capabilities of nano- and microfibers. Thus, it has been shown that the use of grating or fiber topographies can improve the neuronal yield of hESC. However, the method by which the topographies improved neuronal yield remains unknown.

Here, we probe the effect of gratings on early human pluripotent stem cell (hPSC) to NSC/NP differentiation, focusing on whether topography increases the rate of neural differentiation or preferentially differentiated the cells into the neural lineage. We also explored the use of an additional pre-exposure to topography to improve neural differentiation. We chose to use gratings, rather than fibers as our topography, as they offer a constant feature size and orientation, unlike fibers. Also, as the gratings could be heat embossed onto tissue-culture polystyrene (TCPS), it would be possible to make comparisons to the published neural differentiation studies, which were done on TCPS. To determine the method by which topography improves early neural differentiation, we tracked the progression of hPSCs to NSC/NPs with and without the use of gratings by monitoring the changes in expression of key surface proteins and transcripts with flow cytometry and reverse transcription – quantitative polymerase chain reaction, respectively. Morphological changes due to culture on the gratings were also monitored using scanning electron microscopy and immunostaining.

2. Materials & methods

2.1. Fabrication of patterned samples with soft lithography and heat embossing

All topographies were reproduced on poly-(dimethylsiloxane) (PDMS) using soft lithography on an imprinted poly(methyl methacrylate) (PMMA)-coated Si master mold, as previously described [22]. Briefly, the PMMA master molds were fluorinated with trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (Sigma, Cat# 44893), washed with 0.01% Triton X-100 and cleaned with nitrogen gas, prior to soft lithography. The PMMA master mold was placed on the bottom of a small-size weight boat and 5 g of degassed PDMS (Dow Corning, SYLGARD® 184 Elastomer Kit) was poured onto the wafer. The PDMS was prepared at a ratio of 9:1 of the base and curing agent. The PDMS-covered mold was degassed for an additional 30 min, baked at 70 °C overnight, cooled to room temperature and then separated from the master mold. The pre-patterned PDMS molds were used to fabricate patterned tissue-culture polystyrene (TCPS) by heat embossing. Pieces of TCPS were embossed at ~0.3–0.5 MPa and 175 °C for 90 s. Patterned TCPS samples were then plasma oxidized (Triple P, Duratek, Inc.) using a 110 W, 2.45 GHz radio frequency discharge applied to an oxygen gas pressure of 2.7×10^{-1} Torr for 1 min, UV sterilized in a biological safety cabinet for 1 h, and coated with the appropriate extracellular matrix. A list of topographies used in this study can be found in Supplementary Table 1.

2.2. Scanning electron microscopy (SEM)

To confirm the fidelity of pattern replication and to observe cell morphology on topography, SEM was performed. Uncoated patterned TCPS samples were sputter-coated (JEOL Ltd., JFC-1600 auto fine coater) with 10 nm coating of platinum and viewed with a JEOL high vacuum SEM (JEOL Ltd., JSM_6010LV) at 10 kV. Samples with extracellular matrices or cells were fixed for 1 h in 4% paraformaldehyde and 1% glutaraldehyde (Sigma, Cat#G5882) in 0.1 M sodium cacodylate (Sigma, Cat#C0250) and 3 mM $CaCl_2$ buffer, rinsed 3 times for 5 min each in buffer and washed twice for 5 min in deionized water. The samples were then dehydrated in a graded ethanol series and critical point-dried in a graded series of ethanol-hexamethyldisilazane (Sigma, Cat#440191) mixtures. Critical point-dried samples were then sputter-coated with 10 nm coating of platinum and viewed with a JEOL high vacuum SEM at 10 kV. All steps were performed at room temperature.

2.3. Atomic force microscopy (AFM)

To confirm that the patterns were not masked by Matrigel, liquid AFM was performed in tapping mode using the Dimension Icon AFM with ScanAsyst (Bruker AXS, http://www.bruker-axs.com) with the super sharp improved super cone tip (Team Nanotec, Cat #SS-ISC (450C0.2-R)).

2.4. Cell culture

The hESC line, HES-3, was obtained from ES Cell International (http://www.escellinternational.com). The cells were cultured at 37°C/5% CO_2 on TCPS dishes, coated with 283.3 μg/ml of Matrigel (BD Biosciences, Cat#354234). The cells were fed every day with hESC maintenance media (KO-medium) conditioned by mitomycin-C inactivated immortalized mouse embryonic fibroblasts (2.5×10^5 cells/ml) and supplemented by 10 ng/ml of fibroblast growth factor-2 (Invitrogen, Cat#PHG0261), as previously described [23]. Mechanical passaging was done every 7 days using the STEMPRO EZPassage Tool (Invitrogen, Cat#23181).

The H7 hESC line, obtained from the WiCell Research Institute (http://www.wicell.org/), was cultured using the mTeSR1 media system (Stem Cell Technologies) on hESC-qualified Matrigel-coated dishes (101.4 μg/ml, BD Biosciences, Cat#354277), which were passaged enzymatically as clumps with dispase, as previously described [24,25].

Induced pluripotent stem cells derived from lung fibroblasts (iPS-IMR90), were obtained from James Thomson [1]. iPSCs were cultured on hESC-qualified Matrigel-coated dishes in CM supplemented with 100 ng/ml of fibroblast growth factor-2, as previously described [26].

2.5. Neurogenic differentiation with topography

hPSC differentiation on topography followed a 4-step process, modified from a protocol by Wu et al. [6], in a half-adherent (Fig. 1Ai) or fully-adherent (Fig. 1Aii) procedure. Briefly, hPSC colonies were cut into uniform sized pieces using the EZPassage Tool and seeded onto Matrigel-coated patterned or unpatterned TCPS samples and cultured in maintenance conditions (see Section 2.4) for 7 days. In the half-adherent protocol, cells were scraped off samples and cultured as embryoid bodies for 4 days in unconditioned and unsupplemented KO-medium on non-adherent suspension culture dishes. In the fully-adherent protocol, cells on the samples were cultured in unconditioned and unsupplemented KO-medium for 4 days. After 4 days, the embryoid bodies or the colonies on the samples were plated onto laminin-1-coated patterned or unpatterned TCPS samples in N2B27 medium supplemented with 500 ng/ml of Noggin. The media was changed every other day. After 10 days, compact clumps had formed and were cut manually using a pipette in the half-adherent protocol. These clumps were cultured as neurospheres in non-adherent suspension culture dishes in N2B27 media supplemented with 20 ng/ml of EGF and 20 ng/ml of FGF-2. In the fully-adherent protocol, cells on the samples were cultured in N2B27 media supplemented with 20 ng/ml of EGF and 20 ng/ml of FGF-2. In both protocols, the media was exchanged every 3–4 days.

2.6. Flow cytometry

Cells were harvested as a single cell suspension using TrypLE™ Express and washed in 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). Cells were incubated with the primary antibody for 30 min at 4 °C. The cells were then washed with and resuspended in cold 1% BSA/PBS, and incubated with the secondary antibody for 20 min at 4 °C. The cells were then washed with and resuspended in cold 1% BSA/PBS, and incubated with the APC-conjugated primary antibodies for 30 min at 4 °C. A final wash with cold 1% BSA/PBS was performed before data acquisition using BD CellQuest Pro on the BD FACSCalibur flow cytometer (BD Biosciences) or using Guava Express Pro on the Guava easyCyte 8HT System (Millipore). Data was analyzed using FlowJo (v7.6.3, Tree Star Inc.).

A list of primary and secondary antibodies can be found in Supplementary Table 2. As a negative control, cells were only stained with the appropriate secondary antibody.

Flow cytometry data is presented as a percentage of mid- and high-expressing cells, as defined by the 3rd and 4th partition when the logarithmic scale is partitioned into 5 equal parts (Fig. 1B).

2.7. Immunostaining

Cells were fixed with 4% paraformaldehyde in PBS (Affymetrix, Cat#19943) for 20 min at room temperature, and then washed twice with PBS for 5 min each. Samples were then permeabilized and blocked with 0.1% Triton X-100 and 10% normal goat serum (Dako, Cat# X0907) in PBS for 45 min at room temperature. Following incubation with primary antibodies in a 10× dilution of blocking buffer