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Enhanced differentiation of neural progenitor cells into neurons of the mesencephalic dopaminergic subtype on topographical patterns

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease attributed to the loss of midbrain dopaminergic (DA) neurons. The current lack of predictive models for this disease has been hampered by the acquirement of robust cells, posing a major barrier to drug development. Differentiation of stem cells into subtype specific cells may be guided by appropriate topographical cues but the role of topography has hitherto not been well understood. We used a Multi-Architecture (MARC) chip with various topographical structures and identified three topographies, which generate DA neurons from murine hippocampal neural progenitor cells with the highest percentage of neuronal (β-III-tubulin positive) and dopaminergic (tyrosine hydroxylase positive) populations. Analysis on single pattern structures showed that 2 μm gratings with 2 μm spacing and 2 μm height (2 μm gratings) and 2 μm gratings with hierarchical structure produced cells with the highest gene expression of TH and PITX3, with the longest neurite and highest percentage of alignment. Quantitative image analysis showed the 2 μm gratings produced cells with the highest expression of pituitary homeobox 3 (PITX3), LIM homeobox transcription factor 1 alpha (LMX1a), aldehyde dehydrogenase 1 family member A1 (ALDH1a1) and microtubule associated protein 2 (MAP2), as compared to nano-gratings and unpatterned controls. These patterns also enhance DA neuron differentiation on different substrate rigidities, as seen on both poly-dimethylsiloxane (PDMS) and tissue culture polystyrene (TCPS) substrates. These results show the use of topographical influence for neuronal subtype specification, which could be translated into a wide range of clinical applications for PD.
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1. Introduction

Stem cell research could potentially impact the development of disease-modifying therapies, such as those designed to treat Parkinson's disease (PD), which has been hampered by the lack of predictive and progressive cellular models. Parkinson's disease, which affects more than 2% of the population over 65 years of age, and is associated with motor and cognitive deficits, has been attributed to the loss of midbrain dopaminergic (DA) neurons within the pars compacta of the substantia nigra [1]. The development of new *in vitro* models of the disease, such as using patient-derived pluripotent stem cells, would enable drugs against disease pathology to be screened more efficiently. These methods can then be used to evaluate environmental and genetic factors implicated in PD, as well as elucidate the underlying biological mechanisms associated with this disease.

Although considerable progress has been made in deriving DA neuronal cells from stem cells, more work must be done to derive DA cells that are robust and reproducible in quality and quantity, and that can be enriched for selective subtypes of DA neurons. The desired cell population must be clearly defined and stringently purified before stem cell-derived DA neurons can be used for future

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clinical applications. Deriving midbrain DA neurons *in vitro* requires several biochemical cues including a variety of growth factors and patterning neurotrophic factors to increase derivation efficiency [2,3]. However, this can be impractical and expensive for large-scale production of neurons. Although methods have been devised using fewer growth factors [4], such methods yield lower subtype specific neurons, requiring purification of the cell population, which further reduces the cell yield.

Interactions between cells and their mechanical microenvironment play important roles in determining cell fate and cell behaviour. Cell survival, proliferation and their propensity to differentiate into specific cell types are affected by extracellular biochemical and biophysical signals [5]. Accumulating evidence demonstrates that the topography of substrates that cells adhere to influence their response [6–14]. In our previous work, we have shown that patterned nanostructures are able to induce neuronal marker expression in human mesenchymal stem cells [15]. Neural stem cells are also influenced by nanoscale patterned substrates, which for instance, cause them to elongate along poly (L-lactic acid) fibres with significant outgrowth of neurites in the direction of the fibres [16]. Similarly, other groups have shown that aligned topographical structures are able to promote differentiation and neurite alignment as well as influence cell maturation [17–19].

By taking into account biophysical influences in neuronal differentiation, other studies have shown that certain topographies can enhance differentiation of neural progenitors toward neural lineages [20,21]. Additional studies have also shown that topography can enhance neuronal differentiation of other stem cell types [21–25]. However, differentiation of these stem cells into particular neuronal subtypes remains unexplored for specific applications, such as the treatment of PD.

Here, we hypothesize that certain topographies can improve the efficiency of DA neuron derivation. We used an 18-pattern Multi-Architecture (MARC) chip with a combination of micro- or nano-patterned substrates to optimize and identify patterns or structures that can best improve the efficiency of neural progenitor cell differentiation into the midbrain DA neuronal subtype. We showed that gratings with specific dimensions improve derivation as well as maturation of the DA neuron subtype, with an associated alignment of these neurons along the grating patterns. The results of this study may thus reveal how topographical cues contribute to the efficiency of DA neuron derivation for cellular models of PD.

Table 1
List of multi-architectural patterns on the MARC chip replicas used in the murine neural progenitor cell (mNPC) culture.

Category	No.	Pattern description
Gratings	1	2 μm gratings × 2 μm spacing × 2 μm height
	2	1 μm gratings × 2 μm spacing × 2 μm height
	3	2 μm gratings × 1 μm spacing × 1 μm height
	4	250 nm gratings × 250 nm spacing × 150 nm height
	5	250 nm gratings × 250 nm spacing × 250 nm height
Hierarchical gratings	6	2 μm gratings × 2 μm space & perpendicular 250 nm grating/250 nm spacing
	7	2 μm gratings × 2 μm space & parallel 250 nm grating/250 nm spacing
	8	2 μm gratings × 2 μm space & 250 nm dimple overlay
Wells	9	2 μm diameter wells with 10 μm pitch
	10	500 nm diameter wells with 10 μm pitch
	11	130 nm diameter wells with 400 nm pitch
	12	250 nm diameter wells with 500 nm pitch
Pillars	13	1 μm diameter pillars with 8 μm pitch
Microlens	14	1 μm pitch microlens (concave)
	15	1 μm pitch microlens (convex)
	16	1.8 μm diameter with 2 μm pitch microlens (concave)
	17	1.8 μm diameter with 2 μm pitch microlens (convex)
Motheye	18	Motheye with 270 nm pitch

2. Materials and methods

2.1. Fabrication of MARC chip

The Multi-Architecture (MARC) chip is a versatile customizable topography microarray with a size of 2.2 cm × 2.2 cm which can incorporate different topographies [20]. Each of the different topographies has a field area of approximately 4 mm^2. The MARC chip used in this study consists of 18 distinct surface topographies of 6 × 6 array in duplicates and an unpatterned polycarbonate film, serving as the unpatterned control field. The dimensions, construction and arrangement of the MARC chip are presented in Supplementary Fig. 1. Table 1 lists the multi-architectural patterns on the MARC chip replicas on polymethylsiloxane (PDMS, section 2.2) used in the experiments. In the texts henceforth, we would use the codes labelled in Table 1 when referring to the topographies.

2.2. Fabrication and replication of PDMS and TCPS substrates

Polydimethylsiloxane (PDMS) substrates were fabricated using the Sylgard 184 Silicone Elastomer kit (Dow Corning, Michigan, USA) via soft lithography. The silicon molds used consist of the 18-pattern MARC chip with the patterns as shown in Supplementary Fig. 1. The Sylgard 184 polymer (PDMS) was mixed with the accompanying curing agent in a 10:1 ratio and desiccated under vacuum for 30 min. The mixture was then poured into the silicon molds or unpatterned culture dishes, desiccated for another 30 min and placed into a 70 °C oven to cure for 2 h before demolding. On the other hand, the tissue culture polystyrene (TCPS) replicas were prepared by heat embossing.

2.3. Preparation of substrates for murine neural progenitor cell (mNPC) culture

The PDMS or TCPS substrates were cut to fit into the culture plates and air-plasma treated (Harrick Plasma, Ithaca, NY, USA) for 120 s at 29.6 W. After washing in 100% ethanol, the substrates were placed under ultraviolet light for 40 min and coated with 33 μg/ml poly-L-ornithine solution (Sigma–Aldrich, Missouri, USA) overnight. The substrates were then washed twice with sterile water and coated with 20 μg/ml laminin (Life Technologies, California, USA) overnight for controlled differentiation of mNPC. After each coating, vacuum degassing was applied for a short period to force the extracellular matrix proteins into the patterns.

2.4. Culture of murine neural progenitor cells (mNPCs)

The mNPCs (passages 12–19) were isolated from the hippocampus of 5-day old mice [20] and maintained on 8 μg/ml of laminin (Life Technologies) in a neural progenitor expansion medium. This medium contained Dulbecco's modified Eagle medium (DMEM)/nutrient mixture F12 (Biological Industries, Israel) in a 1:1 ratio, 1× N2 supplements (Life Technologies) and 1× penicillin-streptomycin (Caisson Biotech, Texas, USA). 20 ng/ml basic fibroblast growth factor (bFGF) (Life Technologies) and 20 ng/ml epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA) were supplemented daily, with a complete medium change every two days. Upon achieving 80–90% confluence, cell passaging was performed by detaching with Accutase (Innovative Cell Technologies Inc, California, USA) and incubating at 37 °C for 3 min. The cell suspension was centrifuged at 1100 rpm for 5 min and seeded onto laminin-coated plates at an approximately 1:3 ratio. Primary mNPCs were characterized by their immune-reactivity against nestin and SOX2, and their ability to differentiate into neurons and astrocytes (Supplementary Fig. 2). All procedures and care of animals were in accordance with the local Institutional Animal Care and Use Committee (IACUC) guidelines.

2.5. Controlled differentiation of mNPCs into midbrain dopaminergic neurons

The mNPCs were seeded at 15,000 cells per cm^2 on the PDMS or TCPS substrates in the neural progenitor expansion medium. After allowing attachment of cells for 24 h, the growth factors were withdrawn to induce differentiation into neuronal lineage. The medium was then changed to neural induction medium containing DMEM/F12, 1× N2 supplement, 1× penicillin-streptomycin solution, 5 ng/ml bFGF (Life Technologies) and 1 μg/ml laminin. Half of the medium was replaced every two days. On the 7th day of culture, the medium was changed to neural patterning medium containing Neurobasal (Life Technologies) and DMEM/F12 in 1:1 ratio, 1× B27 supplement (Life Technologies), 0.25× N2 supplement and 1× penicillin-streptomycin. The medium was supplemented with 100 ng/ml fibroblast growth factor 8 (FGF-8) (Sigma–Aldrich), 200 ng/ml recombinant Sonic Hedgehog (SHH) (R&D Systems) as well as 1 μg/ml laminin, and was replaced every two days. Differentiation was halted on the 11th or 19th day of culture. A graphical representation of the protocol is shown in Fig. 1A.

2.6. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 15 min and subsequently blocked with 10% goat serum (Life Technologies) or donkey serum (Merck Millipore, Germany) and permeabilized with 0.1% Triton-X for 60 min. Primary antibodies were diluted in 1% goat or donkey serum and incubated overnight at 4°C (Supplementary Table 1), followed by overnight incubation with the appropriate secondary antibodies at 4 °C. The secondary antibodies used were goat anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 488, goat anti-mouse Alexa Fluor 546, goat anti-rabbit

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