Microarray with Micro- and Nano-topographies Enables Identification of the Optimal Topography for Directing the Differentiation of Primary Murine Neural Progenitor Cells

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During development and tissue repair, progenitor cells are guided by both biochemical and biophysical cues of their microenvironment, including topographical signals. The topographical cues have been shown to play an important role in controlling the fate of cells. Systematic investigation of topographical structures with different geometries and sizes under the identical experimental conditions on the same chip will enhance the understanding of the role of shape and size in cell–topography interactions. A simple customizable multi-architecture chip (MARC) array is therefore developed to incorporate, on a single chip, distinct topographies of various architectural complexities, including both isotropic and anisotropic features, in nano- to micrometer dimensions, with different aspect ratios and hierarchical structures. Polydimethylsiloxane (PDMS) replicas of MARC are used to investigate the influence of different geometries and sizes in neural differentiation of primary murine neural progenitor cells (mNPCs). Anisotropic gratings (2 μm gratings, 250 nm gratings) and isotropic 1 μm pillars significantly promote differentiation of mNPCs into neurons, as indicated by expression of β-III-tubulin (59%, 58%, and 58%, respectively, compared to 30% on the control). In contrast, glial differentiation is enhanced on isotropic 2 μm holes and 1 μm pillars. These results illustrate that anisotropic topographies enhance neuronal differentiation while isotropic topographies enhance glial differentiation on the same chip under the same conditions. MARC enables simultaneous cost-effective investigation of multiple topographies, allowing efficient optimization of topographical and biochemical cues to modulate cell differentiation.
1. Introduction

Despite the advent of novel methods for neuronal differentiation, such as generation of neurons from fibroblasts and induced pluripotent cells (iPSCs), the yield of functional neurons from such procedures remains low.\textsuperscript{[1–3]} For this reason, it is important to identify factors that enhance neuronal differentiation of precursor cells. The in vivo physical microenvironment of stem cells plays a critical role in differentiation that can be equally important as hormonal signals.\textsuperscript{[4]} Because the nervous system is a complex 3D environment with a wide range of topographical sizes and architectures, it is also possible that such biophysical factors could be an important determinant of differentiation to a neuronal lineage.\textsuperscript{[5]} Thus, topographically patterned biomaterials have been considered a promising solution to generate functional neurons for a variety of applications.\textsuperscript{[6,7]}

Cells respond to topography over spatial dimensions of nano- to micrometer, while the rigidity of the substrate and the presence of different biochemical signals can also modulate cellular response to topography. The availability of nano- and microfabrication technologies to create biomimetic surface structures has allowed topographical regulation of cellular fate and behavior to be increasingly recognized and extensively reviewed.\textsuperscript{[7–10]} Topographies have therefore been incorporated into biomaterials for both in vitro and in vivo applications, for instance, to direct stem cell differentiation or to serve as orthopedic implants, grafts, and nerve conduits.

Various patterned substrates of electrospun fibers, micro- and nanometer patterns, and microbeads have been employed to study the neuronal differentiation of different cell types including embryonic stem cells (ESCs) and human mesenchymal stem cells (hMSCs)\textsuperscript{[11–13]} and neural stem cells/progenitor cells (NSCs/NPCs).\textsuperscript{[14–17]} However, the variation in material properties and surface chemistry among the different experimental materials could also affect cell responses and differentiation. Thus, there is a need for a systematic comparison of different topographies on a single substrate to cause a single population of cells to undergo neuronal differentiation. To date, the majority of studies on cell–topography interactions have focused on a few nano-/micro-topographies, for instance, nano- and/or micrometer gratings, pillars or electrospun fibers.\textsuperscript{[12,16,18]} Investigation of a wide spectrum of individual topographies could be labor-intensive and cost-prohibitive due to the cost of fabrication and cell culture reagents. An array of different surface patterns on a single piece of sample, providing a platform for high-throughput screening under the same conditions, would thus be advantageous. A limited number of micro-topography-arrays, including Biosurface Structure Arrays, which incorporate different combinations of pillars of $>5 \mu m$, have been studied for their effects on osteogenic differentiation of murine ESCs and murine osteoblastic cell line.\textsuperscript{[19,20]} Another group has reported a poly-lactic acid with different 10–28 $\mu m$ topographies of a uniform height to study the proliferation and osteogenic differentiation of hMSCs.\textsuperscript{[21]} Nevertheless, the incorporation of sub-micrometer topographical structures with different heights onto a single chip seems to remain a challenge. This is mainly due to laborious and tedious nature of current patterning technique in fabricating, on a single chip, a range of nano- and micro-topographical structures with different heights.

This study reports the design and fabrication of a poly-carbonate (PC) multi-architecture chip (MARC) that incorporates distinct topographies with various dimensions and architectural complexity onto a single chip. The field of each of the different topographies has an area of approximately 4 mm$^2$, which is large enough to enable a statistically robust comparison of the effects of various geometries on neuronal differentiation of murine NPCs (mNPCs). Polydimethylsiloxane (PDMS) replicas of MARC were used for the neuronal differentiation of primary murine NPCs to elucidate the topographical influence on neuronal and glial differentiation, and to identify the parameters that alter the effect of a given topography in improving neuronal differentiation. We found that the anisotropic topographies enhanced neuronal differentiation, while isotropic topographies promoted differentiation into glial cells. Thus, we conclude that the topography of the physical environment has an important influence on neuronal and glial differentiation.

2. Results

2.1. Description and Characterization of Multi-architecture Chip (MARC)

The MARC is a versatile customizable topography microarray. Vastly different topographies can coexist on a single 2.2 cm $\times$ 2.2 cm chip. MARC was fabricated in two stages, as shown in Figure 1: 1) patterning of topographies via nano-imprinting lithography (NIL); and 2) assembly of topographies onto a single chip. The MARC in this study was a 6 $\times$ 6 array of 18 distinct surface topographies in duplicate and a pristine unpatterned polydimethylsiloxane (PDMS) replicas of MARC were used for the neural differentiation of murine NPCs (mNPCs).

Figure 2A(1)–(18) shows scanning electron microscopy (SEM) images of different topographies on the MARC, while Figure 2A(19) and (20) illustrate the MARC and the layout and arrangement of individual topographies. Two gaps ($\sim$0.5 mm) on the array helped to identify individual surface patterns (arrows in Figure 2A(20)). On the MARC were various geometries of nano- to micrometer resolution: isotropic (pillars, holes, microlenses, and hexagonal sub-micrometer holes, which are also known as moth eye structures) and anisotropic (nano- and micrometer gratings) surface features, and three types of hierarchical structures. The hierarchical structures were composed of additional structures, gratings or dimples, on the surface of the 2 $\mu m$ gratings. All distinct topographies of MARC are listed in the Supporting Information (SI) Table S1 along with their dimensions and abbreviations.

2.2. Characterization of PDMS Replicas of MARC

Table 1 lists MARC topographies on PDMS replicas with their respective dimensions and codes. The codes will be used in the later sections. PDMS replicas of MARC were checked for fidelity of replication with SEM. Figure 2B shows the...
multi-architectural patterns that had been replicated onto PDMS substrates with good fidelity. Among 18 different surface patterns, micro G1, micro G2, micro G3, nano G1, nano G2, 1 μm pillars, 2 μm holes, 1.8 μL concave, 1.8 μL convex, and inverted hierarchical structures (hierarchy 1, hierarchy 2, and hierarchy 3) were replicated with good fidelity, compared to 130 nm pillars, 250 nm pillars, 500 nm pillars, 1 μm pitch microlenses, and sub-micrometer protrusions (Figure S1, SI). Thus our study mainly focused on the 12 well-replicated patterns. Inverted hierarchical structures were essentially micro G1 with additional structures on the 2 μm grooves (250 nm gratings and 250 nm pillars). The unpatterned control area, which was replicated from the pristine PC covering around the array (Figure S2A, SI), was also examined by SEM to confirm the absence of topographical features (Figure S2B, SI). SEM and atomic force microscopy (AFM) confirmed that the topographies on PDMS substrates were not masked by the coating of poly-L-ornithine (PLO) and laminin (Figure S2C–H).

2.3. Characterization of Primary mNPCs

NPC/NSCs are known to be oligopotent with ability to differentiate into three lineages: neurons, astrocytes, and oligodendrocytes. Primary mNPCs were isolated from the hippocampal region of postnatal mouse brain. The undifferentiated mNPCs were characterized by their morphology in phase contrast microscopy and their immuno-reactivity to Nestin, Vimentin, Sox2, and BLBP (Figure 3A(1)–(4)). Primary mNPCs also express Ki67 indicating their proliferative capacity. Further morphological characteristics of the primary mNPCs are detailed in the SI.

Neuronal differentiation of mNPCs involved an induction phase followed by a maturation phase. Figure 3B illustrates the timeline for neuronal differentiation of primary mNPCs and the compositions of optimized differentiation media. The optimal medium for neuronal maturation on PDMS was identified as Dulbecco’s Modified Eagle Medium (DMEM)/F12 supplemented with 0.25 × N2, 1 × B27, and Neurobasal medium, which yielded high neuron-to-astrocyte ratio (Figure S3A,B; SI).

2.4. Effect of MARC Topographical Cues on Neuronal Differentiation

2.4.1. Topographical Influence on Length of Induction Period

Primary mNPCs (P16-P19) were induced to differentiate into neurons on PDMS replicas of MARC under optimized biochemical conditions. Three different induction lengths of neuronal differentiation (0, 3, and 7 days of induction) were investigated to identify the optimal induction length in the topography-assisted neuronal differentiation as illustrated in Figure 3B.

When mNPCs were seeded directly in the maturation medium without the induction phase, few cells survived on PDMS replicas of MARC at the endpoint of 7 days. A small fraction of the remaining attached cells were weakly positive-stained for neuronal or astrocytic markers (data not shown).

Primary mNPCs were also investigated with a 3-day induction period before transition to maturation phase. Neuronal differentiation efficiency on topographies ranged from 39 ± 5.56% to 60 ± 8.34% of TUJ1 (β-tubulin III)-positive cells, which were not statistically different from 49.22 ± 2.41% on the control. GFAP (glial fibrillary acidic protein)-positive fraction was significantly higher on topographies than on the unpatterned control (Figure S4A–C, SI). Analysis of topographical influence on neuronal maturation revealed a significantly higher population of MAP2 (microtubule-associated protein 2) (Figure S4D–F, SI).
protein 2)-positive neurons on micro G1, micro G3, 1 μm pillars and 2 μm holes, compared to nano G2 \( (P < 0.05, \text{Figure 5E, SI}) \). Gene expression data showed that the expression of TUJ1 and MAP2 on micro G1 was nearly twice as high as that on control and other topographies \( (P < 0.05, \text{Figure S4E, SI}) \).

With 7-day induction, higher number of cells was retained on the substrate throughout the differentiation process as evidenced in the immunofluorescence images \( (\text{Figure 4A,B}) \). Gene expression data showed that the expression of TUJ1 and MAP2 on micro G1 was nearly twice as high as that on control and other topographies \( (P < 0.05, \text{Figure S4E, SI}) \).

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Table 1. List of multi-architectural patterns on PDMS replicas of MARC.

<table>
<thead>
<tr>
<th>Description</th>
<th>Topography</th>
<th>Code in text</th>
<th>Corresponding SEM image in Figure 2B</th>
<th>Corresponding SEM of MARC template in Figure 2A</th>
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</thead>
<tbody>
<tr>
<td>Anisotropic</td>
<td>2 μm gratings with 2 μm space and 2 μm depth</td>
<td>Micro G1</td>
<td>1</td>
<td>1</td>
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<tr>
<td></td>
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<td>Micro G2</td>
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<td>2 μm gratings with 1 μm space and 80 nm depth</td>
<td>Micro G3</td>
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<td>3</td>
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<td></td>
<td>2 μm gratings perpendicular to 250 nm gratings</td>
<td>Hierarchy 1</td>
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<td>9</td>
</tr>
<tr>
<td></td>
<td>2 μm gratings with 250 nm dimples</td>
<td>Hierarchy 2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2 μm gratings parallel to 250 nm gratings</td>
<td>Hierarchy 3</td>
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<tr>
<td></td>
<td>250 nm gratings with 250 nm space and 150 nm depth</td>
<td>Nano G1</td>
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<td>4</td>
</tr>
<tr>
<td></td>
<td>250 nm gratings with 250 nm space and 250 nm depth</td>
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<td>13</td>
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<td>Isotropic</td>
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<td>1 μm pillars</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>2 μm pillars 12 μm pitch</td>
<td>2 μm holes</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.8 μm diameter, 2 μm pitch concave microlens</td>
<td>1.8 μL concave</td>
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<tr>
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<td>1.8 μm diameter, 2 μm pitch convex microlens</td>
<td>1.8 μL convex</td>
<td>12</td>
<td>17</td>
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<tr>
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<td>500 nm pillars with 500 nm height and 10 μm pitch</td>
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<tr>
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<td>130 nm pillars with 400 nm pitch</td>
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</tr>
<tr>
<td></td>
<td>250 nm pillars with 250 nm height and 400 nm pitch</td>
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<td>Not replicated</td>
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</tr>
<tr>
<td></td>
<td>1 μm pitch microlens (concave)</td>
<td>-</td>
<td>Not replicated</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1 μm pitch microlens (convex)</td>
<td>-</td>
<td>Not replicated</td>
<td>15</td>
</tr>
<tr>
<td>Sub-micrometer protrusions (inverse moth eyes) 270 nm pitch</td>
<td>-</td>
<td>Not replicated</td>
<td>18</td>
<td></td>
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</table>

microgratings and nanogratings were also detected with the 3-day induction.

Neuronal differentiation efficiency of hierarchy 1 was comparable to micro G1 but higher than those of hierarchy 2 and hierarchy 3. Two microlenses had similar astrocyte populations but 1.8 μL concave had a higher percentage of neurons than 1.8 μL convex, which was not statistically different.

Topographical regulation of cell morphology was also observed, as evidenced in immunofluorescence images (Figure 4A,B). Neurites of differentiated neurons were parallel to the direction of the gratings on micro G1 (Figure 4A(2) and B(2) and on three inverted hierarchical structures (Figure 4A(5),(6) and B(5),(6)), but not on other topographies. Arborization of neurites were observed on isotropic pillars, holes, and microlenses compared to the other surface patterns (Figure S6, SI). Longer 7-day induction led to more complex neuronal morphology in comparison to short induction (Figure S5 versus Figure S6, SI).

The data from immunofluorescence staining were in agreement with gene expression data on selected patterns. TUJ1 expression was significantly higher on micro G1, 1 μm pillars, and nano G2 compared to unpatterned control (P < 0.05) in the 7-day induction experiment (Figure 4E). Thus, we suggest that micro G1 (2 μm gratings with 2 μm space and height), nano G1 (250 nm gratings with 150 nm height), hierarchy 1, and 1 μm pillars topographies are optimal biophysical cues for neuronal differentiation with high neuron-to-astrocyte ratio.

2.4.2. Effect of Topographical Cues on Regional Targeting of Differentiated Cells

The topographical influence to direct differentiation of forebrain mNPCs into midbrain and hindbrain neuronal subtypes was also studied. The expressions of FOXG1, a hindbrain development marker,[24] and Nurr1, a midbrain development marker,[25] were investigated to determine if the differentiated cells on day 12 (3-day induction with 9-day maturation) were regionally targeted. These experiments were repeated twice.

Figure 5A shows the quantitative analysis of FOXG1 expression, based on immunofluorescence staining images in Figure S7A (SI). The fraction of FOXG1-positive cells was the lowest on 1 μm pillars (24.01 ± 2.88%), which was statistically different from the highest fraction on 2 μm holes (47.26 ± 7.5%) (P < 0.05%). The second highest population was observed on nano G2 (41.97 ± 3.73%). The control and the remaining topographies generated similar population of FOXG1-positive cells.

Nurr1 expression on different topographies ranged from 23.26 ± 3.61% on nano G2 to 27.87 ± 9.45% on 1.8 μL concave while the control generated 25.33 ± 8.06% (Figure 5B). No significant difference in Nurr1 expression levels was observed among topographies themselves and control at 95% confidence interval level. The second lowest Nurr1-positive percentage among topographies was detected on 2 μm holes.

Interestingly, these trends in midbrain and hindbrain subtype expression were nearly opposite to the TUJ1 and MAP2 expression observed on the forebrain mNPCs.
The topographical influence on mNPCs into oligodendrocyte differentiation was also investigated with O4 for 7-day induction experiment (Figure S8A,B; S1). O4 is the earliest recognized marker specific for oligodendrocyte lineage and is expressed until the mature stage. Micro G1 generated the lowest population of O4-positive cells (1.91 ± 1.18%). The highest percentage was detected on 2 μm holes (11.2 ± 1.49%), with the second highest on microlenses (9.69 ± 1.91%) and the third highest on 1 μm pillars (7.84 ± 0.87%). Similar fractions of oligodendrocytes were observed on the remaining topographies. Micro G1 has the lowest population (1.19 ± 1.18%), which was statistically different from that on 2 μm holes (P < 0.05).

The topographical regulation of mNPCs differentiation was further investigated by adding serum to the medium to induce astrocytic differentiation (Figure 6A,B). The highest percentage of GFAP-positive astrocytes was observed on 1.8 μm convex (87.95 ± 4.50%), followed by nano G2 (86.36 ± 4.52%), micro G3 (85.56 ± 7.30%), and 1 μm pillars (82.89 ± 9.25%) (Figure 6B). The control and other surface patterns had similar fractions of differentiated astrocytes (approximately 65–70%). A few TUJ1-positive cells were still observed on micro G1 (1.8 ± 1.28%), hierarchy 3 (14.3 ± 11.1%), nano G1 (14.3 ± 9.7%), and 1 μm pillars (12.21 ± 9.03%) under this condition. Thus, we suggest that 2 μm holes, 1 μm pillars, and 1.8 μm microlenses are the best for inducing glial differentiation.

3. Discussion

3.1. Topographical Regulation of mNPC Differentiation

In this study, MARC with distinct topographies was developed and the PDMS replicas were easily fabricated using standard soft lithography. Primary mNPCs were isolated from hippocampus, and the neuronal differentiation was investigated subsequently on the PDMS replicas of MARC. The optimal topographies and biochemical conditions were identified for improving neuronal and glial differentiation. We suggest that, depending on the goal of the research study and the desired application, suitable combination of topographical and biochemical conditions should be applied in order to achieve the most sufficient enhancement in the yield. For example, anisotropic micro G1, in combination with neuronal differentiation medium, can be used to achieve a higher percentage of neuronal cells (high N:A ratio) and thus a more homogeneous differentiated neuronal population. Astrocyte differentiation can be improved with 2 μm holes, which could produce a higher yield of astrocytes. The low N:A ratio on this topography may be useful for development of neuro-pathological models in studying neurological diseases. Topographical cues may also be incorporated in controlling the regional fate of the differentiating cells.

Topographies on MARC were observed to have differential influence in directing the differentiation of primary mNPCs under identical differentiation conditions. Micro G1, hierarchy 1, and nano G1 produced significantly higher fraction of differentiated neurons with relatively fewer astrocytes (high N:A ratio) while 1 μm pillars had high percentages of both population. This suggests that the former three anisotropic topographies can promote differentiation into a more homogeneous neuronal population. In contrast, 2 μm holes, concave and convex microlenses, hierarchy 2 and hierarchy 3 generated low N:A ratio, indicating that a mixture of neurons and astrocytes, or a higher population of astrocytes were yielded on these surface structures, especially with 3-day induction. Thus, the results were indicative of the specific effects that different topographical structures had on the differentiation of mNPCs and high-throughput screening with MARC enables this comparison and analysis. The use
of geometry and dimensions together with appropriate biochemical conditions can therefore provide an inductive microenvironment of differentiation to achieve the desired population of differentiated cells.

This study also examined various biochemical compositions of maturation media and three induction lengths in order to determine optimal conditions for neuronal differentiation of primary mNPCs. The maturation medium composed of Neurobasal medium and B27 was identified to be the best for supporting neuronal differentiation on PDMS (Figure S3A,B; S1).

Our results showed that the induction period could be shortened to 3 days with the use of the topographies, compared to routine 7-day induction. Interestingly, in 3-day induction experiment, a significant increase in mRNA expression of neuronal markers on micro G1 was observed, although the protein expression was not statistically different. This may be because the gene regulation precedes the protein expression. This trend in upregulation of neuronal marker expression on micro G1 was in agreement with the increase TUJ1 and MAP2 protein expression in the 7-day induction experiment. In contrast, a more significant topographical effect on mNPCs’ differentiation was observed with 7-day induction, as evidenced with protein expression and more sophisticated neuronal morphology on topographies. We speculate that
longer exposure to both topographical cues and biochemical cues, including FGF2, led to this more pronounced effect, with better differentiation and survival. Longer induction length might also have contributed to higher cell density and possibly release of more soluble factors since approximately 10–15% of mNPCs remained proliferative in induction medium (data not shown). The cell density had been described to play a role for neuronal differentiation and survival of murine ESCs.

The role of aspect ratios of topographies in controlling the fate and behavior of bovine pulmonary artery smooth muscle cells was reported by Hu and colleagues. In our study, the feature size and aspect ratios of the gratings were observed to influence mNPCs differentiation. Among the three microgratings with different H:W and H:S aspect ratios, micro G1 with the highest aspect ratio (H:S = H:W = 1) favored neuronal differentiation over glial differentiation, compared to micro G2 and micro G3. This trend was observed in both short- and long-induction experiments. However, such trend was not observed with nanogratings. Nano G1 with the H:W and H:S aspect ratio of 0.6 was more inductive to neuronal differentiation than nano G2 with aspect ratio of 1. Thus, we speculate that mNPCs employ different sensing mechanisms for micro- and nanogratings.

The topographical regulation of cell morphology has been documented in the literature. Our study also shows the topographical influence on mNPCs' morphology. Parallel alignment of neurites to the grating direction was observed on micro G1 and three inverted hierarchical structures, which all had a groove depth of 2 μm, while micro G2 with 120 nm depth and micro G3 with 80 nm depth did not show such neurite alignment. These findings were in agreement with the report by Millar et al. that groove depth is important for physical guidance.

The results also indicate that topographical cues could direct the differentiation of mNPCs into a regional phenotype. The forebrain mNPCs could be induced to differentiate into midbrain and hindbrain subtypes with specific topographies, for example, nano G2 and 2 μm holes for hindbrain phenotype, and concave 1.8 μL and nano G1 for midbrain phenotype. These specific topographies have a potential for in vitro biomedical application together with the optimized biochemical conditions to generate the specific subtypes for cell therapies.

Although the topographical regulation of mNPC differentiation has been demonstrated in this study, the mechanism remains unclear. We speculate that the topography can be regulating mNPC differentiation through the mechanotransduction of extracellular biophysical signal to the cell. The topographical signals may have been sensed via integrins of mNPCs and transmitted through focal adhesion signaling and the actin-cytoskeleton to the nucleus, ultimately leading to differential gene expression and differentiation into different lineages. In this study, the cytoskeletal changes were evident in the different morphologies and neurite extensions of mNPCs on various topographies. β1 integrins have also been recognized to be abundant in NSCs and to modulate their proliferation, survival, migration and differentiation in the developing nervous systems. The effects of such mechanotransduction on stem cell differentiation have extensively been reviewed and a number of mechanisms have been proposed and under investigation. However, a detailed investigation of topographical mechanism in neuronal differentiation of mNPCs is still necessary.

In addition to neuronal and astrocytic differentiation, the topographies also influenced the oligodendrocyte differentiation. Isotropic 1 μm pillars and 2 μm holes, which were observed to consistently promote glial differentiation, also enhanced oligodendrocyte differentiation. On the contrary, anisotropic gratings, except micro G2, did not enhance oligodendrocyte differentiation.

Figure 5. Quantification and data analysis of the differentiated cells positive for A) FOXG1 and B) Nurr1. FOXG1 expression on 2 μm holes was nearly twice as high as that on 1 μm pillars (*P < 0.05). No significant difference in Nurr1 expression was observed among the surface topographies and the control at 95% confidence interval level. Error bars indicate standard error of mean.
To further elucidate the hypothesis of appropriate combination of topographical and biochemical cues to enhance differentiation, the mNPCs were exposed to astrocyte differentiation medium containing fetal bovine serum (FBS). A high fraction of astrocytes was detected on all surface topographies after 3 days. The glial-differentiation-promoting structures such as the 2 μm holes showed a very high yield of GFAP-positive cells. Interestingly, the topographies which enhanced neuronal differentiation, namely micro G1, hierarchy 1, nano G1, and 1 μm pillars, could still produce a low level of TUJ1-positive cells even under conditions conducive to glial differentiation. This suggests that topographical cues by themselves can influence mNPC differentiation and lineage specification, even in the different combination of biochemical cues. When working synergistically with biochemical cues in the differentiation medium, laminin-coated topography with appropriate geometry and dimensions can promote neuronal or glial differentiation of mNPCs.

3.2. Novel Characteristics of MARC

MARC as a topography chip is an innovative concept, providing a technology platform for a topography chip array that eliminates the need to analyze one topography at a time. The design of MARC also allows the incorporation of topographies in a range of nano- to micro-scale sizes with different heights. In the conventional methodology of cell–topography interaction studies, where only a few topographies and dimensions could be used per study, a systematic analysis on topographical regulation of cell fate could be costly and labor-intensive to be achieved.

MARC provides a customizable high-throughput solution to the current hurdles. Individual topography field of 2 mm in diameter on MARC was determined from a simple arithmetic of the patterned area and typical cell area. A typical cell, on spreading, will have a maximum area of approximately 0.01 mm² (10 000 μm²). Thus, each 3.42 mm² field allows a sufficient amount of cells (approximately 300) to grow on individual surface topography field for statistical analysis. The chip dimension of 2.2 cm × 2.2 cm can fit within standard cell culture plates or dishes and can be viewed, using standard microscopy specimen holder to allow microscopic screening of the array of topographies. MARC can also significantly reduce the labor and time involved in the conventional platform such as fabrication of individual single-patterned substrates and preparation of culture for separate samples. Consistent results and overall cost saving in reagents, time, and resources can thus be achieved in studying various topographies in the same culture environment. The current study on neuronal differentiation of mNPCs on PDMS replicas of MARC exemplified its
capability to allow systemic analysis and comparison of distinct topographies under identical experimental conditions and biochemical cues. The major distinguishing feature of MARC as a topography chip is overcoming of the fabrication limitations associated with other topography chip-based approaches in incorporating topographies with a wide range of lateral resolution and structure heights.\textsuperscript{[19–21]} Topographies of submicrometer lateral dimension, mixed micro- and nanoscale dimension and/or with different heights will be laborious and challenging to be fabricated on the same chip with photolithography and conventional micro-/nanofabrication. However, utilizing the flexible processing parameters of NIL,\textsuperscript{[35]} different fabrication techniques can be applied to generate various topographies for individual fields on MARC. Thus, MARC platform enables us to incorporate, onto a single chip, a wide range of topographies with different heights, architectural complexity and dimensions (ranging from 150 nm to 2 μm). In the current study, MARC was fabricated to incorporate both anisotropic topographies (micro- and nanogratings with different aspect ratios and heights) and isotropic topographies (micro- and nanopillars and micro-holes). Moreover, hexagonal sub-micrometer holes or moth eyes, microlenses and hierarchical structures composed of 2 μm gratings and other surface topographies, have been incorporated on a topography chip for the first time to the best of our knowledge. The arrangement and combination of different geometries on MARC can also be customized. Additional topographies and dimensions can also be incorporated to MARC according to the requirements of the specific study.

In summary, our data indicate that optimal surface topographies as biophysical cues should be chosen in inducing neuronal differentiation together with suitable biochemical conditions. This approach will provide a more comprehensive biomimetic in vitro microenvironment so that a desirable differentiated population of neurons, astrocytes or oligodendrocytes or a mixture in an ideal ratio can be achieved. Optimal biophysical cues would help address certain drawbacks of the conventional 2D differentiation, for instance, high cost, long duration and low yield, and a poor integration into the host upon transplantation. Selected geometrical architectures and dimensions can be incorporated into the development of tissue engineering devices, for example, nerve conduits. Although neuronal differentiation was the focus of this study, the technology to use a single substrate to control cellular differentiation to different subpopulations is convenient with a potential to be translated to other cell types and lineages. However, a few questions remained to be further addressed in our study. Paracrine signalling from cells on one patterned region to those on nearby regions could possibly affect the analysis and this paracrine effect is yet to be probed. Similarly, it remains questionable whether cell body or neurites could reach and interact with the textures in the grooves of hierarchical structures, and the potential effect of these structures in the groove has not yet been explored. Another intriguing area to study would be the exact spatial relationship between neurites and/or differentiated cells and individual surface geometries for potential applications in contact guidance as well as for investigation of the differentiation mechanisms. A detailed investigation into the mechanism of neuronal differentiation on the surface topography is currently ongoing.

4. Conclusion

In conclusion, this study introduces a simple, customizable, cost-effective topography array, which is capable of incorporating different nano- to micrometer topographies with various aspect ratios and levels of complexity. The MARC enabled thorough topographical screening, yielding a comprehensive analysis and comparison of different geometries and sizes under otherwise identical in vitro experimental conditions. A systematic study of neuronal differentiation of primary mNPCs on different topographies was performed on the PDMS replicas of MARC. Topographical parameters and aspect ratios were shown to influence the lineage commitment of mNPCs and their morphologies. The neuronal differentiation was promoted by anisotropic topographies (2 μm gratings with H:S = H:W = 1 and 250 nm gratings with H:W = H:S = 0.6) and isotropic topography (1 μm pillars) in synergism with biochemical cues of Neurobasal medium and B27 with a 7-day induction and 7-day maturation phases. Isotropic 1 μm pillars and 2 μm holes were shown to be favorable to glial differentiation of primary mNPCs. Overall, we show that MARC enables identification of the optimal combination of topographical and biochemical cues through simultaneous analysis of different topographies to better understand cell–topography interaction. Thus the customizable MARC as a topography chip is a novel and unique platform with a wide range of potential biological applications.

5. Experimental Section

Fabrication of MARC: The schematic diagrams in Figure 1 show the stages employed in the fabrication of the MARC: 1) patterning of topographies via NIL and 2) assembly of topographies onto a single chip. In the first stage, thermal NIL was primarily utilized to fabricate distinct topography separately onto individual free-standing thermoplastic polycarbonate (PC) sheets (0.25 mm thick, Innocx, PC2151). The topographies (line-space gratings, pillars, dimples, lenses and complex 3D hierarchical structures) are listed in Table S1 (SI). The molds with the inverse structures of these topographies were made of silicon, quartz, or nickel and supplied commercially. They were cleaned, treated in oxygen plasma (Reactive Ion Etcher, TRION) at 20 sccm O\textsubscript{2}, 80 W, 250 mTorr for 3 min and further treated with an anti-stiction layer, (1H,1H,2H,2H)-perfluorodecyltrichlorosilane (FDTS) (Sigma) to render the mold surface hydrophobic to facilitate demolding. The thermal imprinting process of the PC sheet was performed on a nanoimprinter system (Obducat, AB), at a controlled imprint temperature above the glass transition temperature ($T_g$ of PC = 150 °C) with an external imprint pressure from the system. Following this, the temperature of the nanoimprinter system was cooled down below the $T_g$. As different topographies were imprinted onto separate PC sheets, the imprinting conditions could be optimized with respect to the specific designs. In the second stage, assembly of these distinct
topographies onto a single chip was carried out in a customized layout such as the example in Figure 2A(20). The details of fabrication conditions for gratings, dimples, and microelos structures are given in the SI, Section 1.

Sections of each imprinted PC sheet were cut into a standard size of 2 mm diameter “field” using an in-house-built puncher system. The arrays of “field”, each containing a unique topography, were assembled onto a single silicon support. The remaining area of the silicon support surrounding the topographical “field” was covered by a pristine PC film without pattern. The bonding was carried out using PDMS (SylgardTM184, Elastomer Kit, Dow Corning) mixed in the ratio of 3:1 (base:curing agent). The assembled substrate was placed in a vacuum oven at 70 °C for 1 h to cure the PDMS, during which the “fields” were bonded firmly onto the solid substrate to form the multi-architecture microarray chip.

Fabrication of PDMS Replicas of MARC: The MARC chip and nano-imprinted PC masters were silanized with FTS and washed with 0.01% Triton X100 (BioRad) in distilled water to lower the surface energy for easy demolding. The elastomer and the curing agent were mixed thoroughly at a ratio of 10:1 and degassed. Subsequently, the mixture was poured on the NIL PC master or MARC chip, degassed, and cured at 65 °C in an oven for a minimum duration of 2 h.

Surface Treatment of PDMS replicas of MARC: The PDMS substrates were cleaned and air-plasma treated (Harrick Expanded Plasma Cleaner). The surrounding unpatterned area outside the array of fields was kept and used as unpatterned control. They were subsequently coated with poly-ornithine (PLO, Sigma Aldrich, 33 μg/mL) in sterile distilled water at 37 °C overnight and consecutively with mouse laminin (Invitrogen, 20 μg/mL) in DMEM/F12 for a minimum of 4 h at 37 °C.

Examination of Replication Fidelity of PDMS Samples by SEM and AFM: 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).

PLO- and laminin-coated samples were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate and 3 mM CaCl₂, and dehydrated in a series of ethanol. Critical point drying was done by finally transferring the samples to 100% hexamethyldisiloxane (HDM, Sigma) through a mixture of 1:1 ethanol and HDMS. Finally, the samples were coated with platinum for imaging with SEM.

PLO- and laminin-coated PDMS samples were checked with tapping AFM (DI NanoScope IV Multimode Atomic Force Microscope) without dehydration.

Culture of Primary mNPCs: Primary mNPCs were isolated from the hippocampal region of a day-5 postnatal mouse brain in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines, using in-house protocol, which was adapted from Natalie D. Bull and Perry F. Bartlett. The details of mNPCs isolation were provided in the SI, Section 2. The mNPCs were expanded in laminin (8 μg/mL)-coated 6-well plates in the medium composed of DMEM/F12 (Biological Industries), 1X N2 supplements (Invitrogen), and penicillin (100 units/mL) and streptomycin (100 μg/mL) (Invitrogen). Fibroblast growth factor-2 (FGF-2, GIBCO, Invitrogen) and epidermal growth factor (EGF, Invitrogen) were replenished to the expansion medium every day at a final concentration of 20 ng/L.

Neutral Differentiation of mNPCs on PDMS Scaffolds: The mNPCs (P16 to P19) were used for differentiation experiments. The mNPCs were detached and dissociated into single cells with Accutase (Stem Cell Technologies) and subsequently seeded on the PDMS substrates at a uniform density of 7500 cells/cm². The differentiation period was divided into two phases: induction phase and maturation phase. Figure 3B lists the compositions of induction media and four different types of maturation media for the study on different differentiation conditions on PDMS substrates. Figure 3B also outlines the experiment design for neuronal differentiation on PDMS replicas of MARC with variation in induction-phase lengths (0, 3, and 7 days) and a fixed maturation phase (7 days). The experiments were repeated three times with unpatterned PDMS control unless otherwise specified.

For astrocyte differentiation experiments, mNPCs were seeded at 6000 cells/cm² on PLO- and laminin-coated PDMS replicas of MARC for 3 days. The astrocyte differentiation medium was composed of DMEM/F12, 1X N2 supplements, FBS (Invitrogen), and penicillin/streptomycin.

Immunofluorescence Staining and Imaging: At the end of the time point, the cells on PDMS scaffolds were fixed in 4% paraformaldehyde and permeabilized with 0.25% Triton X. The primary antibodies with their respective optimized concentrations were: goat anti-nestin (1:500, Santa Cruz); goat anti-vimentin (1:500, Santa Cruz); rabbit anti-Ki67 (1:1000, Millipore); rabbit anti-brain lipid binding protein (BLBP) (1:500, Santa Cruz); goat anti-Sox-2 (1:500, Santa Cruz); rabbit anti-β-tubulin III (Tuj1) antibody (1:600, Sigma Aldrich); mouse anti-microtubule associated protein 2 (MAP2) antibody (1:600, Abcam); mouse anti-glia fibrillary acidic protein (GFAP) antibody (1:600, Millipore); mouse anti-oilogendrocyte marker O4 (7.5 μg/mL, Millipore); rabbit anti-Forkhead box protein G1 (FOXG1) (1:500, Abcam) and mouse anti-nuclear receptor related 1 protein (Nurr1) (1:500, Santacruz). The secondary antibodies were: Alexa Fluor 546 and Alexa Fluor 488 (all at 1:750, Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen). Imaging was performed with Leica epifluorescence microscope equipped with Q imaging camera (Qimaging) and with Q Capture Pro software. From each surface pattern, 7 to 10 random images were captured and images were processed and analyzed with ImageJ (NIH) and Q Capture Pro.

RNA Isolation and Real-Time PCR: RNA was extracted from the differentiated cells using Trizol (Invitrogen) from selected patterned PDMS samples made from NIL master with larger area. RNA was reverse-transcribed to cDNA using Sensiscript RT kit (Qiagen). Real-time PCR was conducted using Taqman probes and 7500 Fast Realtime PCR machine (Applied Biosystems). The Taqman probes were: GAPDH (Mm99999915_g1), Tubb3 (Mm00727586_s1), GαF (Mm01253033_m1), and Mtap2 (Mm00485230_m1). Briefly, 10 ng of cDNA was loaded into each reaction and comparative ∆∆CT data analysis was done with GAPDH as the endogenous control. Data presented are normalized to unpatterned control.

Image Processing and Data Analysis: Images analysis and cell counting were done with ImageJ. Three different experiments were analyzed unless otherwise specified. Analysis on neurite branching was done on TuJ1-positive cells using ImageJ. One-way ANOVA and post-test Tukey analysis were performed at confidence interval levels of 95% and 99%.
Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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