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Effective Spatial Separation of PC12 and NIH3T3 Cells by the Microgrooved Surface of Biocompatible Polymer Substrates

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Abstract

Most organs and tissues are composed of more than one type of cell that is spatially separated and located in different regions. This study used a microgrooved poly(lactic-*co*-glycolic acid) (PLGA) substrate to guide two types of cocultured cells to two spatially separated regions. Specifically, PC12 pheochromocytoma cells are guided to the inside of microgrooves, whereas NIH3T3 fibroblasts are guided to the ridge area in between neighboring parallel microgrooves. In addition, the microgrooved structures can significantly promote the proliferation and neural differentiation of PC12 cells as well as the osteogenic differentiation of NIH3T3 cells. Therefore, the microgrooved PLGA surface with separated PC12 and NIH3T3 cells can serve as a potential model system for studying nerve reconstruction in bone-repairing scaffolds.

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

Potential applications of cell separation. Characterization of a microgrooved PLGA substrate. Proliferation behaviors of PC12 and NIH3T3 cells after being cultured on microgrooved PLGA substrates. Comparison of the G/R ratios under mono- and coculture conditions. LSCM images of PC12 and NIH3T3 cells monocultured for 2 h. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b01018.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

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INTRODUCTION

Most organs and tissues are composed of more than one cell type. Various types of cells and the relevant extracellular matrix (ECM) secreted by them usually occupy different spatial locations in order to perform their specific yet inherently interconnected functions. Thus, tissue engineering requires the development of a strategy for controlling the spatial organization of different cell types through regional enrichment or spatial separation. How to design and build tissue engineering scaffolds convenient for various cells to grow and distribute at appropriate places is now becoming a worldwide research hot spot.^{1–3} For example, Jiang et al. fabricated vessel-like tubes with endothelial, muscle, and fibroblast cells at different sites on the tube wall via first patterning those cells on a 2D surface and then rolling the surface into a 3D tubular structure.⁴

Although cell type varies in different parts of the body, nerve cells are one of the few existing in almost all organs and tissues. Nerves serve as the main communication bridge between the brain and the rest of the body. Tissue damage caused by accident or disease is often accompanied by local nerve damage. Therefore, nerve reconstruction is of significant importance in the tissue repair process. The failure in introducing nerve cells into scaffolds and stimulating them to form a network may eventually lead to the failure of tissue repair. Unfortunately, there are few studies in the literature dealing with this issue, especially under the coculture condition.

To control the spatial distribution of different cells in a scaffold, one promising solution is to use the unique architecture of the scaffolds to guide the different cells under the coculture condition to different areas of the scaffolds. Here, we show the effective spatial guidance of PC12 pheochromocytoma cells and NIH3T3 embryo-derived fibroblasts by using a microgrooved poly(lactic-*co*-glycolic acid) (PLGA) substrate that is widely used as a biomedical engineering scaffold (Figure 1). NIH3T3 fibroblasts are large, highly motile,⁵ and can be reprogrammed to differentiate into osteoblast-like cells under the induction of bone morphometric protein 2 (BMP2).⁶ On the other hand, PC12 is an important model cell for the study of neuronal growth and differentiation due to its well-recognized neuron-like behavior.⁷ Thus, we aim to coculture these two types of cells on the microgrooved PLGA substrate and then use the substrate to spatially separate them into two isolated populations and guide them into different areas of the scaffold. Specifically, the NIH3T3 cell population, capable of differentiation into osteoblasts to promote bone formation, and the PC12 population, capable of differentiation into neurons and responsible for forming nerves, are

guided to the ridge and microgroove region, respectively (Figure 1). We believe that the resultant scaffold, made of well-isolated cells on the microgrooved PLGA substrate, can be potentially used to produce bone tissues embedded with nerves after rolling and layer-by-layer assembly because the neurons in a microgroove can be developed into nerve fibers in a matrix of osteoblast-derived bone tissue (Figure S1, ESI). Thus, success in the separation and guidance of PC12 cells into microgrooves from a coculture of NIH3T3 cells and PC12 cells can possibly lead to a model system for studying nerve reconstruction in bone-repairing scaffolds.

2. MATERIALS AND METHODS

2.1. Preparation of Microgrooved PLGA Substrate

Soft lithography was employed to prepare a poly(dimethylsiloxane) (PDMS) template. In brief, a silicon wafer was first spin-coated with a negative photoresist (NR21-20000P, Futurrex, USA). After baking at 80 °C for 10 min and 150 °C for 5 min, the resist was exposed to UV light through a photomask (3 in. × 3 in.) and developed in RD6 developer solution. A mixture of PDMS base (Sylgard 184, Dow Corning, USA) and curing agent (10:1 w/w) was then poured onto the silicon wafer and cured at 60 °C to make a PDMS master. A poly(lactic-*co*-glycolic acid) (PLGA, 50:50, $M_w = 50\,000$ g/mol) replica was finally obtained by a melt-casting method (150 °C for 4 h) using a PDMS master as a template. Figure 1A–C shows the schematic illustration of the fabrication procedure. The morphology of the grooved PLGA substrates was characterized using scanning electron microscopy (SEM, Quanta 200 SEM, FEI, Netherland).

2.2. Cell Culture

Mouse embryo-derived fibroblast cells (NIH3T3) and rat pheochromocytoma cells (PC12, Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI medium 1640 (PAA, Germany) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Gibco, USA). PLGA samples were placed in 24-well plates and sanitized in 75% (v/v) ethanol for 2 h, followed by rinsing with sterilized phosphate-buffered saline (PBS) three times. After pretreatment with culture medium for 12 h, cells were seeded at a density of 1.5×10^4 cells/cm². Cells were allowed to grow for 12, 24, 48, or 72 h before inspection. For coculture experiments, equal numbers of NIH3T3 and PC12 cells were thoroughly mixed, seeded, and then imaged in the same manner as for monoculture experiments.

2.3. Immunofluorescence Staining

Laser scanning confocal microscopy (LSCM, Leica, Germany) was used to investigate the effects of culture time and microgroove width on NIH3T3 and PC12 cells. In brief, NIH3T3 and PC12 cells cultured on microgrooved PLGA substrates with various groove width (25, 50, 100 μm) for different times (12, 24, 48, 72 h) were washed with PBS and then fixed using 4% formaldehyde for 15 min at 37 °C. Thereafter, cells were immersed in 0.1% Triton X-100 for 10 min and DAPI (Invitrogen, USA) for 5 min (37 °C). In mono- and coculture, NIH3T3 cells were preloaded with live cell dye Cell Tracker Green CMFDA (Invitrogen,

USA), and PC12 cells were preloaded with live cell dye Cell Tracker CM-Dil (Invitrogen, USA) following the manufacturer's protocols.

2.4. Cell Proliferation and Differentiation

Cell proliferation was assessed by widely used Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan). Specifically, NIH3T3 and PC12 cells were seeded onto the PLGA substrates at densities of 5×10^3 and 1.5×10^4 cells/cm², respectively. At the prescribed time points (1, 3, 5, 7 days), the samples were transferred to new 24-well plates. Then the CCK-8 working solution was added to each sample and incubated at 37 °C for 1 h. Subsequently, the supernatant medium was extracted and the absorbance at 450 nm was measured with a Thermo 3001 microplate reader (Thermo Scientific, USA) ($n = 6$).

NIH3T3 cells were cultured on the microgrooved substrates for 9 days in the presence of 300 ng/mL recombinant human BMP2 (PeproTech, USA). PC12 cells were cultured on the other microgrooved substrates for 9 days in the presence of 50 ng/mL NGF (R&D Systems, Wiesbaden, Germany). Cells were then washed with PBS and homogenized in TRIzol reagent (Invitrogen, USA). RNA of the homogenized samples was then extracted following the manufacturer's protocol. Total RNA concentrations were quantified using NanoDrop2000 (Thermo Scientific, USA). Subsequently, first-strand cDNA was synthesized using oligo(dT)-adaptor primer and AMV reverse transcriptase (TaKaRa, Tokyo, Japan). A real-time polymerase chain reaction (RT-PCR) was achieved using the SYBR green system (Genecopoeia, USA). Amplifications for cDNA samples were carried out at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The following primer sequences were used: β -Tubulin III gene: forward, 5'-CCTTCATCGGCAACAGCACG-3'; reverse, 3'-GCCTCGGTGAACTCCATCTC-5'; GAP-43 gene: forward, 5'-ATGCTGTGCTGTATGAGAA GAACC-3'; reverse, 3'-GAAATTCTTTGCCGAAAGGTGCAACGG-5'; Osteopontin (OPN) gene: forward, 5'-TGCAAACACCGTTGTAACCAAAAGC-3'; reverse, 3'-TGCAGTGGCCGTTT GCATTTCT-5'; Col1A1 gene: forward, 5'-ATGCCGCGACCTCAAGATG-3'; 3'-TGAGGCACAGACGGCTGAGTA-5'; GAPDH gene: forward, 5'-TGTGTCCGTCGTGGATCTGA-3'; reverse, 3'-TTGCTGTTGAAGTCGCAGGAG-5'. The relative quantification of the target gene was normalized to GAPDH and calculated using the 2^{-Ct} method. Melting curve profiles were produced at the end of each PCR so as to confirm the specific transcriptions of amplification.

Western blot was used to analyze the special marker proteins, Tubulin III for the neural differentiation of PC12 cells and OPN for the osteogenic differentiation of NIH3T3 cells. Briefly, cells were cultured, washed with PBS, and homogenized in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, added to 100 µg/mL phenylmethanesulfonyl fluoride prior to use) to extract the total protein. After 15 min on ice and then centrifugation at 13 000 rpm for 5 min, the resulting suspension was mixed with 2× SDS sample buffer (100 mM Tris-HCl PH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerin) and boiled for 5 min. Samples were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked by 5% dried nonfat milk for 45 min at room temperature, incubated with anti-Tubulin III, anti-OPN

(Santa Cruz, CA, USA), and anti-GAPDH antibodies in a 1:500 dilution overnight at 4 °C, washed, and further incubated with HRP-conjugated secondary antibodies (Abclonal, USA) in a 1:5000 dilution for 1 h at room temperature. Immunoreactive bands were detected using Western blue (Promega, Madison, WI, USA). GAPDH was used as an internal control. Quantitative densitometric analysis of the image was carried out using ImageJ software, with GAPDH as a loading control.

2.5. Image and Statistical Analysis

All images were analyzed with ImageJ software. Cell nuclei were manually counted in order to quantify the number of cells proliferating in the grooves or on the ridges. A one-way ANOVA followed by a Tukey test for means comparison was performed to assess the level of significance by employing the SPSS 19.0 statistics software. Results are expressed as the mean $\pm$ standard error, and $p < 0.05$ was designated as statistically significant.

3. RESULTS

3.1. Fabrication and Characterization of Microgrooved PLGA Substrates

In this study, the spatial separation and guidance of different cell types were investigated on a PLGA substrate because PLGA is biodegradable and well accepted as a bone-repairing scaffold material. Melt casting instead of solvent casting was used to fabricate grooved microstructures on the PLGA substrates so that the contamination of residual solvent could be avoided because the solvent can hardly be removed completely from PLGA. Repeated tests proved that melt casting was an accurate and facile method of producing a large number of microgrooved PLGA substrates via PDMS templates, which were fabricated using standard soft lithography procedures. Figure 2 shows the SEM images of microgrooved PLGA substrates. The groove depth was set as 50 μm , and the groove width varied among 25, 50, and 100 μm , respectively. As can be seen, the as-prepared samples exhibit a clean surface without impurity particles, and the microgroove features including shape and size are in good agreement with the design, indicative of the precise pattern transfer between the PDMS template and the PLGA replica. In addition, the energy-dispersive X-ray spectra (EDS) and high-resolution SEM images were also collected to compare the surface properties of microgrooved substrates such as roughness and chemical composition with the results shown in Figure S2 (ESI). Evidently, there are no significant differences between the grooves and ridges in terms of surface roughness and chemical composition. The substrates were termed G100R200 (groove width 100 μm , ridge width 200 μm), G50R200 (groove width 50 μm , ridge width 200 μm), and G25R200 (groove width 25 μm , ridge width 200 μm). The groove depth was fixed at 50 μm unless otherwise stated.

3.2. Regional Enrichment of PC12 and NIH3T3 Cells under the Monoculture Condition

The respective responses of PC12 and NIH3T3 cells to microgrooves under the monoculture condition were first investigated. In our study, the cells were confined either in a groove (including its wall and bottom) or on a ridge. Figure 3 illustrates the images of PC12 and NIH3T3 cells after being cultured for 48 h separately. As can be observed, both cell types exhibit remarkable regional enrichment phenomena. Specifically, PC12 cells show an apparent preference for the grooves (Figure 3a–c), and NIH3T3 cells display a clear cellular

enrichment on the ridge surface (Figure 3d–f), indicating their distinct responses to microgroove topography. To characterize this observation in a quantitative manner, the ratios of cell density (namely, cell numbers per unit area) in the grooves to that on the ridge (G/R ratio) were calculated for all PLGA substrates with different geometries. Three independent samples for each groove width and three randomly chosen regions on each sample were imaged with LSCM. As a result, a total of nine images were used for the statistical analysis of cell locations for each groove width and each cell type. The results reveal that G/R ratios for PC12 are ca. 4.96 ± 1.45 on G25R200 and then increase to ca. 11.73 ± 2.41 on G50R200 and eventually reach 20.84 ± 1.74 on G100R200, demonstrating an enhanced regional enrichment and spatial guidance to the microgroove with the increase in groove width. In contrast, the G/R ratios for NIH3T3 are 0.38 ± 0.06 on G25R200, 0.29 ± 0.04 on G50R200, and 0.36 ± 0.09 on G100R200.

3.3. Spatial Separation of PC12 and NIH3T3 Cells under the Coculture Condition

The large discrepancy in G/R ratios between monocultured PC12 and NIH3T3 cells implies possible spatial separation and guidance induced by microgrooves under the coculture condition. To confirm this assumption, the mixed suspension of NIH3T3 and PC12 cells was exposed to microgrooved PLGA substrates and cultured for a certain period of time before inspection. For the sake of observation, PC12 cells were marked with red fluorescent cell tracker CM-Dil and NIH3T3 cells were prestained with green fluorescent cell tracker CMFDA prior to coculture and imaging. All cell nuclei were labeled with DAPI. It is proven in Figure 4a–c that the two types of cells retain their preference for growing either in the grooves or on the ridges even in coculture mode, indicating effective spatial separation by microgrooves. In comparison, NIH3T3 and PC12 cells cocultured on smooth PLGA surfaces are found to distribute irregularly and randomly (Figure 4d). Figure 4e,f shows the G/R ratios for cocultured PC12 and NIH3T3 cells as a function of time. It can be seen that PC12 cells show an obvious preference for staying in the microgrooves as early as 2 h after being seeded on these substrates, leading to a G/R ratio of close to 5.0. The highest G/R ratios are reached at 48 h, indicating that the vast majority of PC12 cells are concentrated in the grooves especially on G50R200 and G100R200 ($G/R_c \gg 1$). By 72 h, G/R ratios decrease a little compared to those at 48 h but are maintained at ca. 10 for G50R200/G100R200 and at 3.5 for G25R200. In contrast, NIH3T3 cells do not show an obvious preference for the ridge at the beginning of 2 h, leading to a $G/R \approx 1.7$ (Figure 4f). However, the G/R value decreases dramatically to 0.7 at 12 h and tends to decrease further over time, showing a strong preference for the ridge. A significant difference ($p < 0.05$) is found only at 48 h between G25R200 and the other two substrates. With the extension of culture time, no significant difference can be seen among them.

Moreover, it should be pointed out that the spatial separation between PC12 and NIH3T3 cells is dependent not only on the groove width but also on the ridge width and groove depth. Figure 5 lists the LSCM images of PC12 and NIH3T3 cells cocultured for 48 h on PLGA substrates with various ridge widths and groove depths. It can be observed from Figure 5a–c that the narrower the ridge, the higher the G/R ratios for both PC12 and NIH3T3 cells. The increase in the G/R ratio indicates an improved preference of PC12 cells for the grooves but a declined preference of NIH3T3 cells for the ridge (Figure 5g), resulting

in a poor spatial separation of these two cells. Although the groove depth also exhibits a significant influence on the spatial separation of PC12 and NIH3T3 cells, the G/R ratios are quite different (Figure 5d–f). Namely, the G/R ratio of PC12 cells increases dramatically when the groove depth rises from 25 to 50 μm but remains almost unchanged when the groove depth rises from 50 to 100 μm , indicating the existence of a threshold value of groove depth, beyond which the preference of PC12 cells for the grooves is saturated.

Comparatively, the groove depth hardly shows any obvious effect on the preference of NIH3T3 cells for the ridge, and the change in G/R ratios is ignorable (Figure 5h).

3.4. Cell Proliferation and Differentiation Behaviors of PC12 and NIH3T3 Cells Monocultured on Microgrooved PLGA Substrates

Cell proliferation behaviors of PC12 and NIH3T3 cells after being monocultured on microgrooved PLGA substrates were assessed via CCK-8 assay (Figure S3). Interestingly, although PC12 and NIH3T3 cells show almost reverse responses to the groove and ridge, their proliferation behaviors are quite similar, i.e., both cells show significantly accelerated growth on G100R200 during the whole culture time when compared to G50R200, G25R200, and smooth control groups. In addition, it should be noted that cells cultured on G50R200 also exhibit significantly different cell growth from those on G25R200 and smooth substrates for a short period of time, for example, on the third day. However, with the prolonged culture time (on the fifth and seventh days), no significant difference can be observed.

In addition to cell proliferation, the neural differentiation of PC12 cells and the osteogenic differentiation of NIH3T3 cells were also investigated in detail. G100R200 was chosen as the substrate because of the optimal regional enrichment effects for both NIH3T3 and PC12 under the monoculture condition. The real-time PCR results in Figure 6a indicate that β -Tubulin III and GAP-43 as marker genes for neural differentiation are significantly up-regulated after PC12 cells are cultured for 9 days on G100R200 in a culture medium containing neural growth factor (NGF), in comparison to the smooth control ($p < 0.05$). Meanwhile, OPN and COL1A1 as marker genes for osteogenic differentiation are also up-regulated after NIH3T3 cells are cultured for 9 days on G100R200 in a culture medium containing BMP-2 (Figure 6b). To further verify the results of real-time PCR, the protein expressions of β -Tubulin III and OPN were measured by Western blot. The primary outcomes confirm that the expression levels for both proteins are up-regulated on the microgrooved substrates in contrast to smooth controls (Figure 7a,b). Therefore, it can be reasonably concluded that microgrooved structures can significantly promote PC12 and NIH3T3 cells to differentiate into neuron-like cells and osteoblast-like cells, respectively, in the presence of the corresponding growth factors.

4. DISCUSSION

Physical and biochemical cues embedded in scaffold materials are well accepted as effective means to regulate cellular behaviors such as adhesion, proliferation, migration, and differentiation. In this work, we show that topological microstructures on the biodegradable

PLGA substrates can not only affect the proliferation and differentiation of PC12 and NIH3T3 cells but also guide them into different areas of the scaffolds.

Herein soft lithography and melt-casting are combined to fabricate microgrooved PLGA substrates. After PC12 and NIH3T3 cells are cultured on these substrates using either monoculture or coculture mode, PC12 cells show a clear preference for growing in the grooves whereas NIH3T3 cells exhibit an obvious preference for migrating and proliferating on the ridge. To facilitate the quantitative analysis, the G/R ratio is defined to compare the cell density in the groove with that on the ridge, which confirms the preference of PC12 and NIH3T3 cells for the microgroove (G/R ratio >1) and the ridge (G/R ratio <1), respectively, in both mono- and coculture modes. It should be noted that the G/R ratios achieved under the coculture condition are not significantly different from those under the monoculture condition (Figure S4, ESI), suggesting the relatively independent responses of these two cell types to microtopography. Moreover, a comparison of G/R ratios on different substrates further reveals that the spatial enrichment of PC12 cells can be improved when the groove width increases from 25 to 100 μm , when the groove depth increases from 25 to 50 μm , or when the ridge width decreases from 200 to 50 μm . However, the close relationship between regional enrichment and groove geometry (including groove width and depth) is not found in the case of NIH3T3 cells. Although the preference of NIH3T3 cells for the ridge is enhanced by the ridge width, the change in the G/R ratios for NIH3T3 cells is much less than that for PC12 cells. In our study, the best spatial separation of PC12 and NIH3T3 cells occurs at G100R200 and G50R200 (groove depth 50 μm). If the groove width or depth is decreased to below 25 μm , then PC12 cells would not show an obvious preference for the grooves; namely, a large portion of PC12 cells can be found on the ridge. Comparatively, if the groove width increases to above 200 μm or if the ridge width decreases to below 100 μm , then NIH3T3 cells would also lose their preference for the ridge and mix with PC12 cells in the grooves (Figure 5). In such cases, effective spatial separation between PC12 and NIH3T3 cells cannot be realized.

Although we did not form protein micropatterns on the substrates as reported in the literature prior to cell culture in this study, the analysis of the elemental composition and surface roughness of the PLGA substrate showed that there was no obvious difference between grooves and ridges. Moreover, because the size of the microgrooves was wide enough (wider than 20 μm), we believe that it was not likely that microgrooves could preferentially exclude or attract some kind of protein deposition from the culture medium compared to the ridge surface. As a result, the spatial separation and guidance of PC12 and NIH3T3 cells can be induced only by the microgroove structure, especially considering the fact that such phenomena were not observed on the smooth PLGA surface (Figure 4d). Figure 4e shows that PC12 cells aggregate in the groove as early as 2 h after being seeded, resulting in a G/R ratio of about 5.0. Consequently, the density of PC12 cells is increased in the groove. A possible reason is that PC12 cells form small clusters when being seeded and are likely to roll off into the groove before being attached to the substrate surface (Figure S5a, ESI). Although further experiments should be performed to fully confirm this event, the higher cell density in the groove can accelerate the local proliferation rate. With the extension of culture time, more PC12 cells are found in the groove than on the ridge, finally leading to an obvious regional enrichment. However, if the groove is too narrow for PC12

cells to move in, they might be forced to give up the preference for the groove and occupy the ridge surface (Figure 4c). Because PC12 cells are prone to differentiation into neuron-like cells, it is reasonable to assume that their initiative behaviors including preferential migration and proliferation are function-driven so that the cell orientations and cell-cell interactions can be reinforced, as required by the properties of neurons. In contrast to the sustained favor of PC12 cells for the microgroove, the preference of NIH3T3 cells for the ridge is variable, depending on several factors such as culture time and the relative width ratio between the groove and ridge. Two hours after cell seeding, NIH3T3 cells even show a slight preference for the groove-like PC12 cells and the resultant G/R ratio is larger than 1.0. Thereafter, NIH3T3 cells seem to like the wider surface, no matter whether it is located inside the groove or on the ridge. When the ridge width is much larger than the groove width, NIH3T3 cells show an obvious preference for the ridge, as proven by the data obtained on G25R200, G50R200, and G100R200. However, when the groove width is much larger than the ridge width, NIH3T3 cells show an obvious preference for the groove, as proven by G200R100 and G200R50. It is possible that the narrow groove or ridge would hinder NIH3T3 cell spreading and migration. Their preference for a wider surface indicates that tight junctions among NIH3T3 cells may be undesirable. Overall, the effective spatial separation between PC12 and NIH3T3 cells should meet two requirements: a high ridge/groove width ratio and a wide enough groove ($>50\text{ }\mu\text{m}$). Nevertheless, it is worthy to note that although the preferential growth was observed only for PC12 and NIH3T3 cells in the current study, similar phenomena are also possible for other types of cells.

In addition to the induced regional enrichment and spatial separation of PC12 and NIH3T3 cells, microgrooves also promote cell proliferation and differentiation, especially in the case of G100R200, which are in good agreement with the literature. The ease with which PC12 and NIH3T3 cells differentiate into neuron-like and osteoclast-like cells, respectively, when cultured on the microgrooved PLGA surface, together with the effective spatial separation and guidance, makes them a potential model system for studying nerve reconstruction in bone-repairing scaffolds.

5. CONCLUSIONS

Microgrooved PLGA samples with various dimensions are successfully fabricated via soft lithography and melt-casting methods. PC12 and NIH3T3 cell responses to these microgrooves are investigated in detail. It is found that effective spatial separation and guidance of PC12 and NIH3T3 cells can be realized by either monoculturing or coculturing on the microgrooved PLGA substrates; PC12 cells show an apparent preference for the grooves whereas NIH3T3 cells exhibit a clear partiality for migration and proliferation on the ridge. Except for the physical confinement caused by microgrooves, cell properties seem to play another important role in guiding the spatial organization of these two cell types. In addition, microgrooves also significantly promote cell proliferation, neural differentiation of PC12 cells, and osteogenic differentiation of NIH3T3 cells in cooperation with the corresponding growth factors. The remarkable spatial separation of PC12 and NIH3T3 cells as well as the promotional effect for cell differentiation when cultured on the microgrooved PLGA surfaces makes them a potential model system for investigating nerve reconstruction in bone-repairing scaffolds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic illustration of the spatial separation of PC12 (red color) and NIH3T3 (purple color) cells on the microgrooved PLGA substrate. (A) PDMS was poured onto the silicon wafer to make a PDMS template. (B) PLGA was melted at 150 °C for 4 h on the PDMS template surface. (C) Microgrooved PLGA was peeled off of the PDMS template. (D) Equally mixed cells were seeded and cocultured on the microgrooved PLGA substrate. (E) The spatial separation of PC12 and NIH3T3 cells was achieved on a microgrooved PLGA substrate.

Figure 2. Top-view (a–c) and side-view (d–f) SEM micrographs of microgrooves with different widths on the PLGA substrate: (a, d) 100 μm ; (b, e) 50 μm ; and (c, f) 25 μm . The ridge width was 200 μm , and the groove depth was 50 μm .

Figure 3.

Regional enrichment of PC12 and NIH3T3 cells under the monoculture condition. PC12 cells (blue) were cultured on (a) G100R200, (b) G50R200, and (c) G25R200 for 48 h and subsequently stained for cell nuclei. Meanwhile, NIH3T3 cells (green) were cultured on (d) G100R200, (e) G50R200, and (f) G25R200 for 48 h, which were preloaded with live cell dye Cell Tracker Green CMFDA. The depth of the microgrooves was 50 μm for all samples, and their locations were indicated in the images using blue triangles.

Figure 4.

Spatial separation of PC12 and NIH3T3 cells under the coculture condition as a function of groove width. PC12 cells (red) and NIH3T3 cells (green) were cocultured on (a) G100R200, (b) G50R200, (c) G25R200, and (d) flat PLGA for 48 h and then imaged by LSCM. The depth of the microgrooves was 50 μm for all samples. Prior to culturing and imaging, PC12 cells were preloaded with red fluorescent cell tracker CM-Dil and NIH3T3 cells were prestained with green fluorescent cell tracker CMFDA. Triangles indicate the locations of microgrooves. The ratios of cell density in the grooves to that on the ridge (G/R ratios) were calculated for all PLGA substrates with different geometries as a function of time. (e) G/R ratio for PC12 cells. (f) G/R ratio for NIH3T3 cells. By 48 h the (*) significant difference ($p < 0.05$) indicates the comparison of G100R200 and G50R200 to G25R200.

Figure 5.

Effects of ridge widths (a, b, c, g) and groove depths (d, e, f, h) on the spatial separation of cocultured PC12 (red) and NIH3T3 (green) cells. Blue triangles indicate the locations of grooves. The depth of grooves in (a) G200R200, (b) G200R100, and (c) G200R50 was 50 μm . The widths of groove and ridge in d–f were 100 and 200 μm , but the depth of the groove varied in the range of (d) 100, (e) 50, and (f) 25 μm . The G/R ratios were summarized as functions of ridge width (g) and groove depth (h). Culture time: 48 h.

Figure 6. Characterization of neural differentiation of PC12 cells and osteogenic differentiation of NIH3T3 cells by measuring the gene expression level of markers. Both types of cells were monocultured on the G100R200 substrate with the addition of corresponding growth factors to the culture medium. (a) β -Tubulin III and GAP-43 mRNA levels measured for PC12 cells by RT-PCR; (b) OPN and COL1A1 mRNA levels measured for NIH3T3 cells by RT-PCR. * significant difference ($p < 0.05$).

Figure 7.

(a) Western blot photograph of β -Tubulin III and quantitative densitometric analysis of the Western blot image using ImageJ software. (b) Western blot photograph of OPN and quantitative densitometric analysis of the Western blot image using ImageJ software. The data are presented as the means $\pm$ standard deviation. * significant difference ($p < 0.05$).