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Spatial coordination of cell orientation directed by nanoribbon sheets

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ABSTRACT

Spatial coordination of cell orientation is of central importance in tissue/organ construction. In this study, we developed microfabricated poly(lactic-co-glycolic acid) (PLGA) nanoribbon sheets with unique structures, using spin-coating and micropatterning techniques, in order to generate a hierarchically assembled cellular structure consisting of murine skeletal myoblasts (C2C12). The nanoribbon sheets were composed of aligned PLGA nanoribbons in the center, and strips on four sides which take a role as bridges to connect and immobilize the aligned nanoribbons. Such unique structures facilitated the alignment of C2C12 cells into bilayer cell sheets, and cellular alignment was directed by the aligned direction of nanoribbons. The nanoribbon sheets also facilitated the construction of multilayer cell sheets with anisotropic (orthogonal) and isotropic (parallel) orientations. The enhanced expression of myogenic genes of C2C12 cells on the bilayer cell sheets demonstrated that the nanoribbons induced C2C12 cell differentiation into mature myoblasts. The micropatterned nanoribbon sheets may be a useful tool for directing cellular organization with defined alignment for regenerative medicine and drug screening applications.

1. Introduction

Recapitulation of hierarchical assembly of cells and extracellular matrix (ECM) is of great significance to engineer functional tissues. The exquisite microscale and nanoscale architectures of tissues induce various cell behaviors, including alterations in cell adhesion, morphology, orientation, and various intracellular signaling pathways [1–3]. For example, cardiac and skeletal muscles possess striated structures consisting of sarcomeres packed into highly organized bundles. Skeletal muscle fibers are organized as parallel bundles, whereas cardiac muscle fibers are arranged at branching angles [4,5]. To engineer an artificial niche or scaffold that simulates the natural topographic landscape of tissues (e.g. muscles), one potential approach is to construct micro-organized cellular structures. Efforts are underway to engineer multilayer cell-material composites using various techniques, including laser-guided directed writing, micro-molding of cell-laden hydrogels, and dielectrophoretic force-cell micropatterning [6–8]. However, these techniques have been limited by complicated process requirements and a lack of suitable biological properties. For example, alginate hydrogels have been widely used to develop microparticles and microfibers; however, anchorage-dependent cells poorly proliferate on these substrates.

Micropatterning, which facilitates the generation of simple or complicated motifs (e.g. grooves, pillars, and wells) on various surfaces with flat or curved features, is a powerful tool for directing cell behaviors, including spatial arrangement and differentiation [9–11]. However, despite the significant efforts, the construction of a three-dimensional (3D) structure with a well-defined cell arrangement using micropatterning remains a challenge [12]. To address this challenge, we aimed to develop a technique that

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combines the superior qualities of micropatterning to build multilayered cellular constructs.

Advances in nanotechnology have led to the development of freestanding, ultrathin polymeric films (referred to as "nanosheets"). Nanosheets, which are composed of biodegradable and biocompatible materials (e.g. polylactic acid and polysaccharides), have unique features, such as strong adhesion to dry or hydrated surfaces, high flexibility (allowing adherence to flat or curved surfaces), and excellent transparency [13–17]. Here, we aimed to utilize these properties to develop engineered matrices for constructing multilayer micropatterned cell sheets. To this end, it was important for the nanosheets to display excellent biodegradability and cell adhesion as well as low cytotoxicity to facilitate cell residence and the regulation of cell orientation [18]. Poly(lactic-co-glycolic acid) (PLGA) is a copolymer approved by the Food and Drug Administration (FDA) for use in a number of therapeutic devices [19,20]. The tunable degradation (regulated by the composition of lactic and glycolic acids) and safety of PLGA (commercial products include Bone-Fix and drug-releasing microspheres) have been confirmed [21].

In this study, we developed microfabricated PLGA nanoribbon sheets with a unique structure using spin-coating and micropatterning techniques to generate a hierarchically assembled cellular structure consisting of aligned murine skeletal myoblasts (C2C12). This material bridges the gap between the well-developed two-dimensional (2D) micropatterning for cell patterning and the 3D construction of multicellular structures. We anticipate that the nanoribbons may be a useful tissue engineering tool for directing cellular organization with defined alignment and microstructure, which could be useful for engineering 3D tissue structures for applications in regenerative medicine and drug screening. For example, freestanding aligned cell sheets could serve as a multilayered cardiac patch, artificial skin, or engineered skeletal muscle.

2. Materials and methods

2.1. Materials

PLGA (70/30; MW: 97,000) was purchased from Polysciences Inc. (PA, US). Dichloromethane and PVA (MW: 13,000–23,000) were purchased from Kanto Chemical Inc. (Tokyo, Japan). Alexa Fluor 594-conjugated phalloidin and 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) were pursued from Sigma–Aldrich (US). A mouse monoclonal anti-fast skeletal myosin antibody and an Alexa Fluor 488-conjugated goat anti-mouse antibody (ab-7784) were purchased from Abcam (US) and Invitrogen (US), respectively. All cell culture-related reagents were obtained from Invitrogen. Petri dishes (60 mm Ø) were purchased from Orange Scientific (Belgium).

2.2. Fabrication and characterization of freestanding PLGA nanoribbon sheets

PLGA nanoribbon sheets were developed by a combination of micropatterning and spin-coating techniques. First, PLGA nanoribbon sheets were generated by spin-coating 5 mg/mL PLGA-dichloromethane solution (4000 rpm, 40 s) on a micro-grooved polydimethylsiloxane (PDMS) replica (2 × 2 cm^2), and baked on a hot plate (80 °C, 90 s). Then, a thin PVA layer (10 wt%, 300 μL) was cast onto the PDMS replica, which was dried in a desiccator overnight in order to remove the residual water in the PVA layer. After being dried, the PLGA/PVA bilayer was carefully detached from the PDMS replica using tweezers. Next, freestanding PLGA nanoribbon sheets were obtained by soaking the PLGA/PVA bilayer film in a water-filled Petri dish in order to dissolve the PVA layer. Then, the rinsing water was completely removed and the dish was dried in a desiccator overnight, allowing for the physical adhesion of the freestanding PLGA nanoribbon sheet onto the dish surface. Subsequently, the dish was filled with water and left for 2 days for the complete removal of residual PVA. Prior to the cell seeding, the nanoribbon sheet-coated dish was sterilized under the UV lamp for 1 h. In case of disordered PLGA nanoribbons, the flat edge part of the PLGA/PVA bilayer film was cut by scissors and gently released in water. Additional rising steps were the same as the process described above.

2.3. Morphology and surface properties of the nanosheets

The thickness of PLGA nanoribbon sheets was analyzed by a surface profiler (Dektak150, Veeco Instruments Inc., Plainview, NY). The morphology of the PLGA nanoribbon sheets was observed using a field emission scanning electron microscope (FE-SEM; JIB-4600F).

2.4. Fabrication of bilayer cell sheets

PLGA nanoribbon sheets (1 cm × 1 cm) were physically adhered on a Petri dish, and thirty thousand C2C12 murine skeletal myoblasts (American Type Culture Collection (ATCC), US) were seeded. After 7 days of culture, the myoblasts exhibited approximately 80% confluence. The first cell layer was then coated with a 50 μg/mL fibronectin solution to promote anchoring the cells of the second layer to those in the first layer. After 30 min of incubation, the first cell layer was covered with another PLGA nanoribbon sheets, which was then seeded with sixty thousand C2C12 myoblasts. The bilayer cell sheets were placed in an incubator for 5 h to facilitate cell adhesion. Additionally, 500 μL of culture medium was added to compensate for the consumption of medium. Next, 10 mL of cell culture media (DMEM, 10% FBS, and 1% penicillin/streptomycin) was added and the cells were cultured for another 5 days.

2.5. Cell viability and morphology

The Viability/Cytotoxicity Kit (Invitrogen) was used to determine cell viability. The calcein AM and ethidium homodimer-1 in the kit stain live and dead cells green and red, respectively. Thirty thousand cells were seeded on a single-layer (1 cm × 1 cm) PLGA nanoribbon sheet. After 3 days of culture, the cells on the single-layer nanoribbon sheets were washed with PBS and incubated in 2 mL of PBS for 15 min at 37 °C. The cells were washed with PBS 3 times, and imaged using a fluorescence microscope (Zeiss, Germany).

2.6. Quantification of myoblast alignment

After 3 days of culture, the cells on the single-layer nanoribbon sheets were washed with PBS, and then fixed and permeabilized using 4% (w/v) formaldehyde for 15 min followed by 0.1% (v/v) Triton X-100 for 5 min. The cells were stained with phalloidin-conjugated Alexa-Fluor 594 (Invitrogen, USA) and DAPI (Sigma–Aldrich, USA) to visualize F-actin and cell nuclei, respectively. The F-actin (in red color) and cell nuclei (in blue color) were then imaged using fluorescence microscopy (Zeiss, Germany). Cellular alignment was quantitatively evaluated based on the alignment angle, which was defined as the angle between the long axis of a nucleus considered as elliptical and the groove direction (200 nuclei were analyzed using ImageJ software package). Alignment angles were then categorized in 10° increments. Cells with alignment angles less than 10° were considered aligned.

2.7. Cell staining using CellTracker

To visualize the cell organization on the bilayer cell sheets, we used CellTracker to distinguish the cells in two layers. C2C12 myoblasts were cultured on the first layer of the PLGA nanoribbon sheet, and then stained using CellTracker™ Green dye (Invitrogen) after 7 days of culture. Briefly, CellTracker™ Green dye was dissolved in anhydrous dimethylsulfoxide (DMSO) to a concentration of 10 mM and then diluted to 5 μM in serum-free medium. The prepared working solution was added to the cell layer for 30 min at 37 °C. The working solution of dye was then replaced with fresh medium, and the cell layer was incubated for an additional 30 min at 37 °C. The cell layer was then washed with PBS and cultured in culture medium overnight. Subsequently, the second cell layer was constructed on the surface of the first layer with fibronectin treatment. The cells in the second layer were stained with CellTracker™ Orange dye before seeding on the microgrooved nanosheets. Briefly, when C2C12 cells cultured in a flask (170 cm^2) reached confluence, 12 mL of a working solution of CellTracker™ Orange dye (5 μM in serum-free medium) was added to the flask and incubated for 30 min at 37 °C. The dye was then replaced with fresh medium, followed by incubation for another 30 min at 37 °C. The cells cultured in the flask were then washed with PBS and cultured in culture medium overnight. The cells stained with CellTracker™ Orange dye were suspended and then seeded onto the second set of nanoribbon sheets. At 8 days of culture, a fluorescence microscope (Zeiss, Germany) was used to observe the cell orientation of the generated bilayer cell sheet.

2.8. Myotube formation

To induce myotube formation in the bilayer cell sheet, the cell culture medium was replaced after 8 days of culture with a differentiation medium composed of DMEM supplemented with 2% horse serum (HS), and 1% penicillin/streptomycin.

2.9. Immunocytochemistry

After 12 days of culture, the cell sheets with a number of generated myotubes were fixed in a 4% paraformaldehyde solution for 15 min at room temperature, then washed twice with PBS and placed into a 0.1% Triton X-100 solution for 5 min. Non-specific binding sites were blocked using 10% goat serum (Invitrogen) for 1 h at room temperature. The cells were incubated with primary antibody (mouse monoclonal anti-fast skeletal myosin antibody, 1:1000 dilution) overnight at 4 °C. The cell sheets were washed 3 times with PBS and then incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse antibody, 1:1000 dilution) for 1 h at room temperature. Alexa Fluor 594-conjugated phalloidin (for F-actin staining) and DAPI (for nucleus staining) were then added to the cell sheets and incubated for 30 min at 37 °C.