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Geometric Control of Fibroblast Growth on Proton Beam-Micromachined Scaffolds

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ABSTRACT

Circular three-dimensional (3D) micropatterns with grooves and ridges of various sizes on the circumference of the structure were micromachined in polymethylmethacrylate, using proton beam micromachining. Fibroblasts were seeded in the center smooth nonpatterned surface of the circle. The circumference grooves could retard the outward spreading of cells after they became confluent in the central smooth surface. The fibroblasts eventually migrated across the grooves and ridges several days later. Wider and deeper grooves were more effective in retarding fibroblast spreading. Our results indicate that groove structures in cellular dimensions can effectively retard fibroblasts invasion. Proton beam micromachining, which has the unique advantage of being the only technique capable of manufacturing direct-write precise 3D microstructure at cellular dimensions, has great potential in generating 3D microscaffolds for studying cell behavior in a 3D microenvironment, which is important for tissue engineering.

INTRODUCTION

TISSUE ENGINEERING is a rapidly developing and interdisciplinary field that applies the principles of cell biology, engineering, and materials science in order to generate artificial tissues or organs for human disease therapy. In natural tissues, the cells are arranged in a three-dimensional (3D) organization that provides the appropriate functional, nutritional, and spatial conditions. The generation of tissues *in vitro* therefore requires biocompatible tissue scaffolds not only to give the cells physical support, but also to mimic the conditions of natural tissues. Three-dimensional amorphous mesh structures made from collagen or polymer fibers are the most common types of tissue scaffolds currently used. However, in general these types of structures do not exhibit the strict geometric environment necessary for efficient tissue growth.[1–7]

Although it is well known that the behavior and function of cells are changed by geometric constraints and substrate surface properties, little work has been carried out on the effects of 3D microsubstrate geometry on cell behavior. This knowledge is important for the success of tissue engineering so that cells can organize in suitable 3D environments and function properly as an organ *in vivo*. The major reason for this lack of information stems from the general unavailability of a precisely patterned 3D microsubstrate. Proton beam micromachining (PBM) is a new technique that can produce 3D high-aspect ratio micromachined surfaces of different shapes and patterns by altering a resist structure, using a focused beam of MeV protons in a fast and direct write process. The depth of the structures can be accurately controlled by varying the proton energy. Three-dimensional microstructures with well-defined geometric shapes have been produced at sizes down to 100 nm, well below cell

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dimensions.[8–11] Although other techniques (e.g., optical lithography and e-beam writing) can produce surface features that are essentially two-dimensional, PBM can produce true 3D structures of high-aspect ratio.[9–11]

Fibroblasts are present in almost all tissue types and organs and they play a central role in the support and repair of tissues and organs. When a tissue is injured, the fibroblasts nearby proliferate and migrate into the wound, and produce a large amount of collagenous matrix, which helps to isolate and repair the damaged tissue.[12] On the other hand, overgrowth and overspreading of fibroblasts can also cause diseases such as liver cirrhosis and nonfunctional scar tissues formed after cardiac infarction.[13,14] Methods to inhibit fibroblast growth and spreading in a 3D tissue microenvironment can be useful in treating diseases associated with fibroblast overproduction and overspreading.

In this article, we present our results on the effectiveness of 3D proton beam micromachined polymethylmethacrylate scaffolds of different geometries to inhibit the spread of fibroblasts in culture.

MATERIALS AND METHODS

Substrate manufacture

Proton beam micromachining (PBM) is a new 3D direct write lithographic process utilizing a focused high-energy (2-MeV) proton beam. Flat sections of polymethylmethacrylate (PMMA—a common polymer resist material), approximately 1 cm square with a thickness of 2 mm, were micromachined by proton beam micromachining. The depth of the structures was controlled by varying the proton energy. After exposure, the samples were developed by procedures described in Sanchez *et al.*[11] The nominal 3D microstructure geometry is shown in Fig. 1, with the smooth nonpatterned surface in the center and three ridges and four grooves of different widths and depths at the circumference. The detailed dimensions of the microstructures are summarized in Table 1.

Cell culture

The micromachined PMMA substrate was sterilized by exposure to UV light for 20 min on each side. Approximately 5×10^2 cells (mouse embryonic fibroblasts, Swiss 3T3, ATCC CCL92) were carefully seeded at the center of the smooth surface of the micromachined circular structure. The substrate was placed in a 35-mm tissue culture dish and incubated in a CO_2 incubator (37°C, 5% CO_2) for about 3 h. After the cells had attached to the surface of the substrate, 3 mL of DMEM (with 10% fetal bovine serum and penicillin–streptomycin at 100 units/mL) was added to cover the PMMA substrate. The cells were allowed to grow for 8–10 days. Cell behavior on the PMMA substrate was monitored with an inverted phase-contrast microscope. All experiments were repeated three times.

RESULTS

Proton beam-micromachined 3D substrate

A schematic diagram and a scanning electron micrograph (SEM) of the 3D microstructure are shown in Fig. 1. Four repeated grooves with vertical walls separated by three ridges surround the central smooth nonpatterned surface area, while the outermost grooves define a constant diameter of 2 mm. The dimensions of the grooves/ridges as well as the size of the central unetched area are summarized in Table 1. The surfaces of the grooves and ridges are both smooth at the light microscope level.

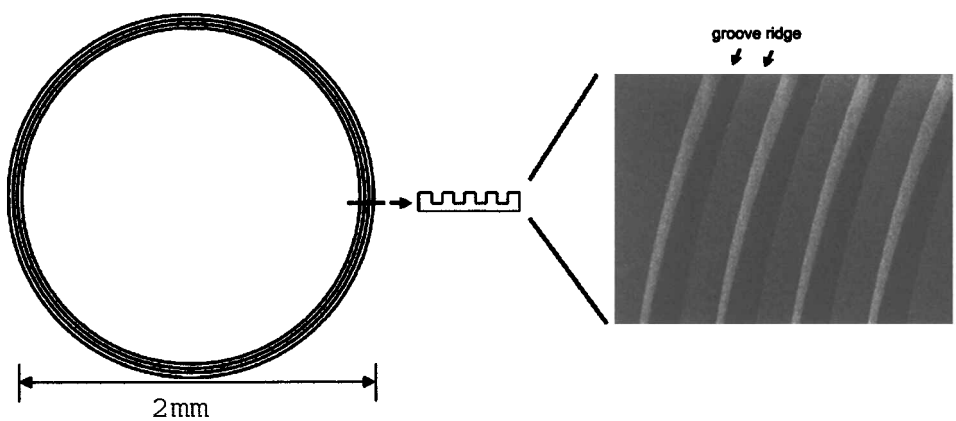

FIG. 1. A diagram of the 3D microstructure. The cross-section of the groove and ridge structure is shown in an amplified view. An SEM photo of the microstructure is also shown.

TABLE 1. PARAMETERS OF PMMA SUBSTRATES

Sample	Width of ridge (μm)	Width of groove (μm)	Depth of groove (μm)	Unetched area (mm^2)
Rw20Gw20Gd5	20	20	5	2.326
Rw20Gw10Gd20	20	10	20	2.545
Rw20Gw20Gd20	20	20	20	2.326
Rw20Gw30Gd20	20	30	20	2.112

Definitions: Rw, ridge width; Gw, groove width; Gd, groove depth.

Fibroblast behavior on 3D PMMA microstructure

Fibroblasts seeded at the center of the structure on day 1 reached confluency and fully occupied the central smooth nonpatterned surface by day 4 (Fig. 2). The 3D microgrooved surfaces then acted as geometric constraints that retarded the further outward spreading of the cells. We observed that grooves with a depth of 20 μm are significantly more effective than shallow grooves of 5 μm in restricting the fibroblasts from spreading outward (Fig. 2). Cells could migrate and cross the 20-μm grooved 3D structure within 2 days when the groove depth was 5 μm, whereas it took about 4 days when the groove depth was 20 μm (Fig. 2). When the depth of the groove was maintained at 20 μm, increasing the width of the groove resulted in more efficient blockage of the fibroblasts, with few cells crossing the 30-μm grooved structure 4 days after cells became confluent in the center (Fig. 3). Changing the ridge size from 20 to 15 μm did not have any obvious impact on the ability of the fibroblasts to migrate and cross the microgroove structures (data not shown). The brown spots in Figs. 2 and 3 represent overcrowded cells that formed multiple cell-layered areas due to the physical restriction of the microgrooves. When this physical restriction is absent, as in the control, this cell overcrowding was not observed.

It is noted that in Fig. 3, where the depth of the grooves were kept constant in all structures, there is a slight decrease in the unetched smooth areas as the width of the grooves increases from 10 to 30 μm (Table 1). Structure Rw20Gw30Gd20, which has the smallest central smooth area for fibroblast growth, retarded fibroblast spreading most efficiently. It the thus clear that it is the microgrooves that effectively retarded fibroblast spreading.

A second 3D structure was designed to test longitudinal movement along ridges: a circular ring structure containing a single 30-μm-wide, 20-μm-deep groove was interrupted with four radial 400-μm-long microridge outlets with widths of 5, 20, 25, and 30 μm, respectively (Fig. 4A). In this structure, cells migrated and spread outward until they reach the circular barrier. Cells were then observed to bypass the circular barrier groove and preferentially spread along the three large ridge channels (Fig. 4C–E), but not along the narrowest ridge of 5 μm (Fig. 4B). This result indicated that fibroblasts have restricted movement along a 5-μm-wide ridge, but can move eas-

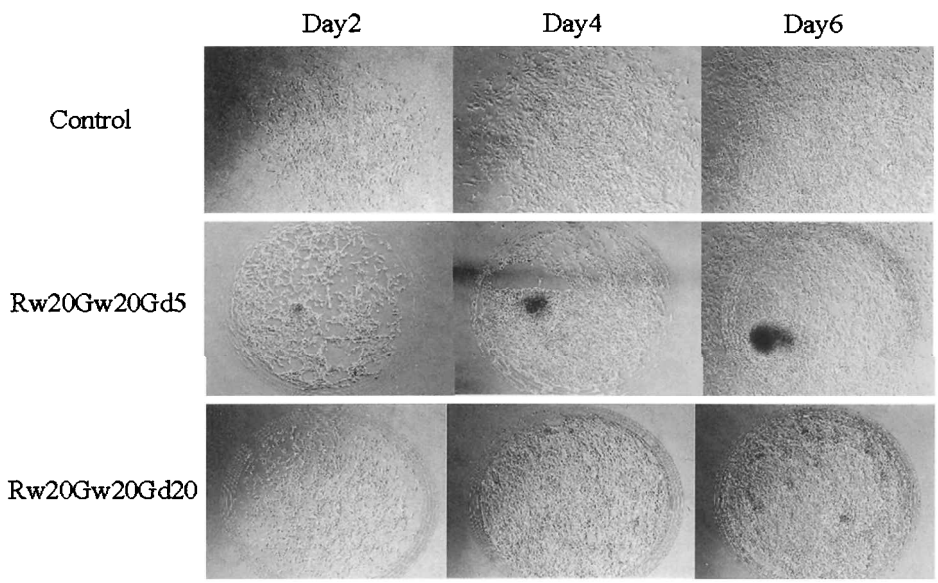

FIG. 2. Deep grooves are more effective in restricting fibroblasts. All dimensions are presented in micrometers. Rw, ridge width; Gw, groove width; Gd, groove depth. Day number indicates days after cell plating onto the central nonpatterned surface.

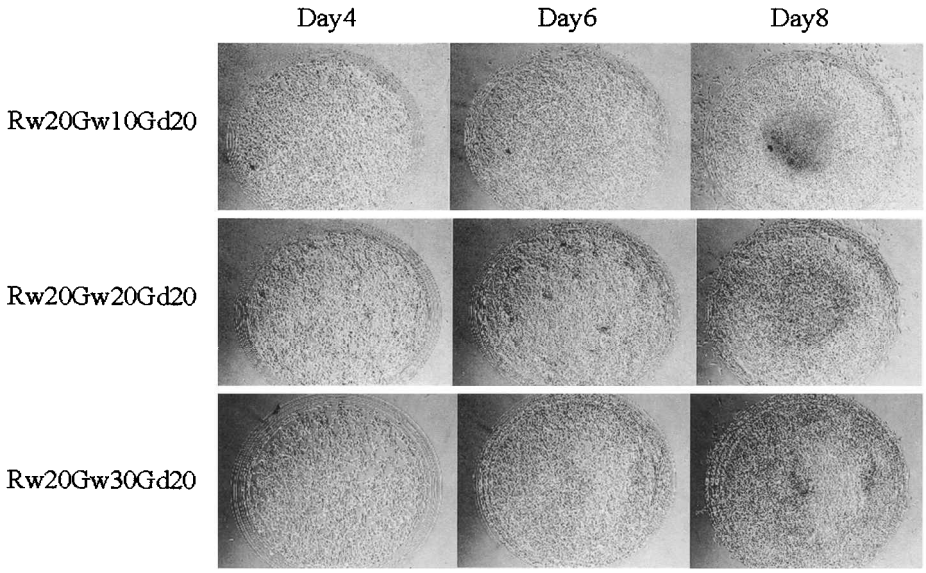

FIG. 3. Wider grooves are more effective in restricting fibroblasts. All dimensions are presented in micrometers. Rw, ridge width; Gw, groove width; Gd, groove depth. Day number indicates days after cell plating onto the central nonpatterned surface.

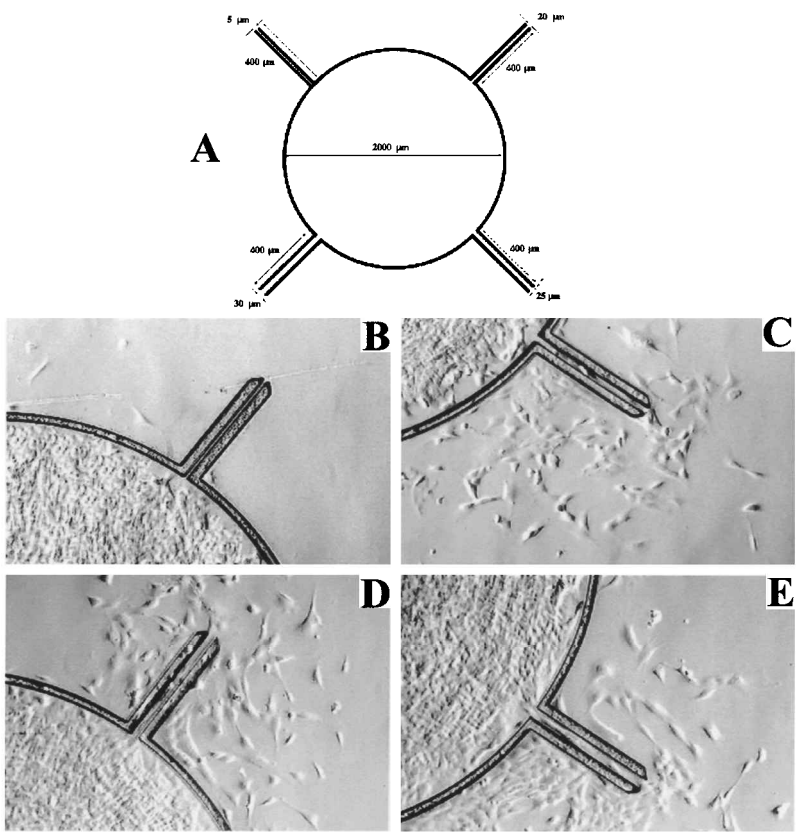

FIG. 4. Fibroblasts selectively migrate and spread through smooth surface channels. A modified 3D microstructure corral containing four smooth channels with widths of 5, 20, 25, and 30 μm at the four corners is shown in **(A)**. **(B–E)** Cell growth and spreading through the four channels on day 7. Channel width: **(B)** 5 μm; **(C)** 20 μm; **(D)** 25 μm; **(E)** 30 μm.

ily along ridges of 20 μm or more. Interestingly, when cells pass along the ridges and move into the region outside the barrier, they then move back toward the main cell cluster, possibly sensing some chemical cues from the main body of cells still trapped within the circular barrier.

DISCUSSION

Although quite a number of studies have been carried out in relation to topographic guidance of cells, especially using parallel groove/ridge micropatterns, as far as we are aware these studies did not particularly focus on the behavior of cells migrating from a smooth nonpatterned 2D surface to precise 3D microgrooved structures. In these previous studies, cells were directly seeded onto the microgrooved surface and their morphology and behavior were then monitored.[15–21] Britland *et al.* showed that cells aligned and elongated along the direction of the grooves and ridges, responding to deeper topographic cues such as 6-μm grooves more strongly compared with shallow grooves of 0.5 μm.[22] Epitenon fibroblasts isolated from the surface of rat flexor tendons moved at higher speed on patterned substrata than on plain surfaces and 5-μm-deep grooved silica substrata facilitated the *in vitro* healing of completely divided rat flexor tendons, compared with a smooth nonpatterned surface.[23] In this work, we have shown that when mouse embryonic fibroblasts were seeded onto a smooth nonpatterned surface, and spread outward toward the 3D microgrooved circular structures, the 3D microstructures can act as geographic constraints that restrict fibroblast growth and migration to the surrounding areas. Grooves that are deeper (up to 20 μm) and wider (up to 30 μm) appear to be more effective in restricting the lateral movement of cells across the groove structures, whereas narrower ridges appear to be more effective in restricting longitudinal movement along the ridges. When both a smooth surface and grooves are present (Fig. 4), fibroblasts preferentially spread along the smooth surface. It remains to be seen whether different fibroblasts respond differently to topographical cues, because it has been observed that epitenon fibroblasts are more sensitive to multiple grooved substrata than same-size baby hamster kidney (BHK) fibroblasts.[15]

Our results indicate that 3D microstructures can potentially deter fibroblasts from spreading and growing into a particular tissue environment. These 3D microstructures may be useful in tissue engineering when multiple cell types are needed to form a proper tissue and fibroblast overgrowth can be detrimental in the proper formation and function of the tissue. Our results also indicate that proton beam micromachining can be used to generate 3D microstructured substrata for studying cell behavior. As a unique direct write micromachining 3D technique, random and complex microstructures can be generated that may reflect more closely the *in vivo* tissue environment, facilitating the study of cell–cell and cell–matrix interactions for tissue engineering.

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