A simple and robust fabrication of microwell array by PDMS on a glass substrate for cell-to-cell adhesion

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ABSTRACT

We propose a simple approach to fabricate a microwell array by using Polydimethylsiloxane (PDMS) to study cell-to-cell interaction of Human Bone Marrow Mesenchymal Stem Cells (BM-MSCs). The microwell array of PDMS is replicated from an SU-8 master mold, and then is transferred to a glass substrate by ripping the array that is bonded to the glass substrate by an oxygen plasma treatment. 4 different types of PDMS array (e.g., rectangle, bowtie, wide-rhombus, and rhombus) are formed to vary cell-to-cell contact length. The pattern transfer success rate and applied force are investigated. This method of generating the microwell array will enable a simple and robust construction of PDMS array for various cell-to-cell contact studies.

Keywords: PDMS microwell array, cell-to-cell adhesion, pattern transfer

1 INTRODUCTION

Developing efficient fabrication techniques is important since it enables widespread use of cell studies for many different applications [1-3]. Micro-sized patterns and structures using the PDMS have been used in manipulating and culturing cells, and thus studying cell behavior in a microenvironment since the PDMS is an optically transparent, non-toxic, and biocompatible [4].

In an early stage of cell-to-cell interaction studies, Petri dishes are used to form cell-to-cell contact [5]. However, the observation of cells grown is limited due to a low density of a pair of cells. Moreover, in the conventional cell culture condition, it is hard to manipulate the cell formation.

In order to realize a controlled cell growing environment, a surface patterning method with cell adhesive molecules using PDMS stamping method is typically used [6]. To regulate the cell-to-cell contact with various shape, density, and contact of cells, micropatterns can be printed onto a cell culture substrate using the PDMS stamp inked by adhesive molecules [7-9]. The technology can offer cell culture environment with well-controlled sizes, shapes, and positions on a substrate, thus providing a useful tool for cell studies. Despite its huge potential, the contact printing method should be carefully controlled to avoid nonuniform patterns by a deformation when handling the stamp. As an alternative, an agarose pattern on a glass substrate is fabricated using the PDMS stamp method [10]. The agarose is perfused under the PDMS stamp attached on the glass. Thus, the region sealed against glass remian free of agarose. After curing of agarose, the PDMS stamp is removed, leaving defined geometry of agarose patterns. While the microwells used previously facilitated the cell-to-cell formation by PDMS stamping method, it is difficult to create thick patterns that allow the cells to be trapped into the microwells. In addition, air bubbles can be easily trapped on the PDMS patterns during the filling process of agarose.

We describe a simple microwell array fabrication method to isolate the effects of cell-to-cell contact by transferring PDMS patterns on a glass substrate. To overcome the technical challenges, a simple and robust fabrication method is demonstrated to study the role of intercellular adhesion forces in myogenic differentiation of stem cells and molecular pathways governing this process. Specifically, we use microwell array to control the extent of cell-to-cell contact independent of cell spreading and density. However, cell spreading as controlled by integrins and focal adhesion can also have effects on smooth muscle differentiation. By preserving the cell-matrix contact area by controlling the size of each pattern, but changing cell-to-cell contact length by changing shape of patterns, Adherens Junctions (AJs) between the cells can be varied, as studied before in different cell types [11-12]. Extent of cell-to-cell adhesion can then be examined by immunostaining various cadherin molecules which control BM-MSCs differentiation towards smooth muscle lineage. In particular, we study levels of Cadherin-11 (Cad-11) and its effect on smooth muscle genes α-SMA (Alpha- Smooth Muscle Actin), CNN-1 (Calponin) and MYH11 (Myosin-Heavy Chain). We expect that this tool can be further extended to 3 or 4 neighboring cells in micropatterns, thereby establishing Cadherin-11 as a master regulator of BM-MSCs to smooth muscle differentiation.

2 DESIGN/EXPERIMENT

Fig. 1 shows the configuration of 20 μm thick PDMS pattern fabrication on the slide glass for cell-to-cell adhesion studies in the microwell array. By a conventional soft-lithography, 4 different types of PDMS pattern (e.g., rectangle, bowtie, wide-rhombus, and rhombus) were replicated from a master mold. The density of patterns and contact surface area between the patterns and the glass were listed in Table 1.

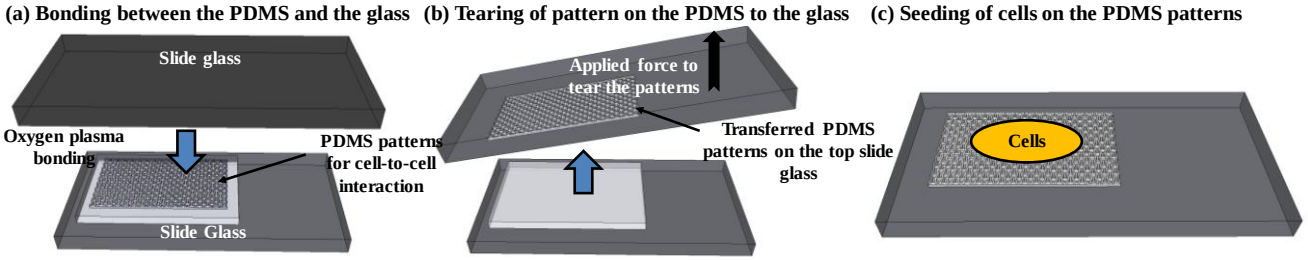

Fig. 1 Working principle for the PDMS microwell array on the slide glass. (a) The replicated PDMS patterns are bonded to the bottom glass to hold the pattern to be torn by the top glass. (b) By applying the mechanical force to the top glass, the patterns can be easily transferred to the top glass since the bonding strength between the bottom glass and the PDMS slab is higher, compared to the bonding strength between the top glass and PDMS patterns due to the large contact area. (c) The cell seeding is performed on the PDMS patterns.

The cell loading density is optimized to obtain the maximum number of properly occupied patterns. The patterns with the pair of cell in each pattern are considered properly occupied. Low density of cells resulted in few of the patterns occupied with cells, with the number of cells per pattern increasing with seeding density. The optimal cell loading density was 0.4 million cells. The two slide glasses were used to sandwich the patterns by oxygen plasma bonding method. The top glass was bonded to the side of the patterns while the bottom glass was bonded to back side of the patterns. To create the microwell patterns, the glass bonded to the patterns was lifted up by applied mechanical force that enables destructive processes such as crack formation or propagation so that the designed patterns were to be transferred on the glass. To applied the force to lift up the glass, we used home-made equipment.

Table 1 PDMS patterns for cell-to cell adhesion

Shape	Rectangle	Bowtie	Wide Rhombus	Rhombus
Density of pattern (#/mm^2)	937.11	797.20	641.46	946.84
Contact area of PDMS (%)	59.91	50.18	41.43	40.82

For the cell studies, Human Bone Marrow Mesenchymal Stem Cells (BM-MSCs) between passage 4 and 8 were trypsinized from normal culture conditions (Dulbecco's Modified Eagle Medium containing 10% Fetal Bovine Serum.) and plated onto PDMS patterns on glass which were placed in 6-well plates. For experiments on PDMS patterns, cells are trypsinized and counted using a standard hemacytometer and the cells were used in each pattern, and cells settled by gravity into the patterns, and were allowed to attach for 48 hours. Following this, Cells trapped in PDMS micropatterns were fixed using 4% Paraformaldehyde, blocked with 5% goat serum and exposed to primary antibody β-catenin in 1:100 dilution. After incubation overnight at 4 degree, corresponding secondary antibodies were used in 1:200 dilution for 1 hour at room temperature. Samples were counterstained with DAPI (Hoechst 33342; 10mg/ml; 1:200 dilution; 5 min at room temperature) for nuclei. Images were obtained using a Zeiss Axio-observer and analyzed by Image J software.

3 METHOD AND MATERIALS

Film photomask for creating a SU-8 master mold were designed for using AutoCAD commercial software (Autodesk, USA) and were printed on transparencies (CAD/Art Service Inc., Bandon, OR, USA). The size of each PDMS was 5 mm × 10 mm and the array of each pattern has 10 μm distance between patterns.

The PDMS patterns were fabricated by using conventional soft-lithography techniques. In order to make a master mold, negative photoresist (SU-8 2015, Micro-Chem Corp, Newton, MA, USA) was used, and then spin coated with target thickness (e.g., 20 μm) at 2000 rpm on the cleaned wafer using a spin processer (WS-650Mz NPP from Laurell Technologies, North Wales, PA, USA). Before the coating process, the silicon wafer was submerged into BHF (buffered hydrofluoric acid) at room temperature for 5 min to remove a thin oxide layer that can make a weak adhesion between the SU-8 and the surface of the wafer. Afterward, it was cleaned with acetone, followed by methanol. It was then rinsed by DI water and blown dry with filtered nitrogen gas. After spin coating process, a soft bake process was conducted at 95 ℃ for 4 min. After UV exposure through the film photomask and subsequent post exposure baking process was conducted at 95 ℃ for 5 min, the wafer was developed in SU-8 developer (Microchem, USA) and washed with isopropyl alcohol to completely remove the developer. Using a gentle stream of nitrogen gas, the wafer with SU-8 patterns was dried. The measured thickness of SU-8 pattern was 20 ± 2 μm.

The PDMS patterns were fabricated from PDMS prepolymer and curing agent (Sylgard 184, Dow Corning Co., Midland, MI). The two materials were thoroughly mixed at a ratio of 10 : 1 (wt/wt). The mixed PDMS was degassed in a vacuum chamber to remove air-bubbles for 20 min. The SU-8 master mold was silanized using hexamethyldisilazane (Sigma Aldrich, Saint Louis, MO, USA) in a vacuum chamber for 3 hours to easily peel off the PDMS from the SU-8 master mold. To mold the PDMS against the master mold, it was carefully poured onto the

SU-8 master mold and cured at 65℃ for 30 min. The PDMS replica was peeled off and bonded irreversibly by exposing O_2 plasma. The flat surface of PDMS was bonded to the glass, and then the surface of PDMS with the patterns was sandwiched with another glass. Finally, the glass bonded to the PDMS patterns was released from the patterns.

4 RESULT/DISCUSSION

To create microwell patterns on the glass substrate, PDMS patterns are fabricated that have 4 different shapes that are able to trap a pair of cells. The PDMS patterns were successfully transferred to the glass by causing a crack

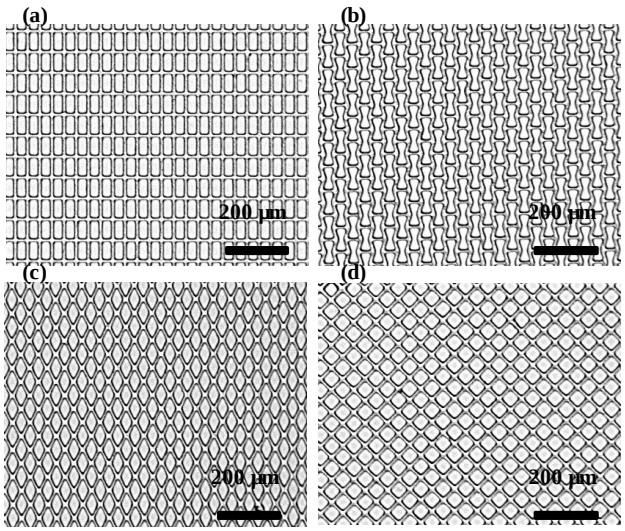

Fig. 2 Photographs of PDMS patterns on the glass substrate. The patterns have 20 μm thick and 10 μm gap among the patterns (a) Rectangle. (b) Bowtie. (c) Wide Rhombus. (d) Rhombus shape.

since the bonding force between the flat surface of PDMS and the bottom glass is larger than that of between the surface of pattern of PDMS and the top glass. After the patterns are bonded, it is simply torn from the bottom glass using the home-made equipment to release the top glass. Only patterns tear at the locations where the patterns are bonded to the bottom glass. It is best to tear the PDMS patterns, applying torque force at the position 50 mm away from the edge of the glass in order to locally initiate the crack. As shown in Fig. 2, the patterns were successfully fabricated on the glass by causing the crack. The patterns

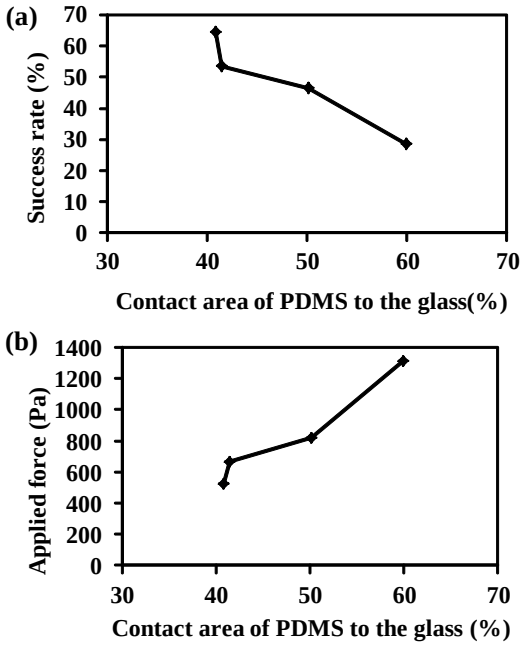

Fig. 3 (a) Success rate of patterns transferred to the glass according to the contact area of PDMS patterns. (b) Applied force to the top glass to create the crack on the patterns.

have 20 μm thick and 10 μm gap among the patterns. The thickness was enough to trap the cells into the wells.

In the Fig. 3(a), the percentages show the success rate of patterns transferred to the glass for each condition given in Table 1. It can be seen that the patterns with smaller contact area are more easily created since the crack resistance is low so that the crack starts more uniformly along the edge of patterns at mechanically instable point. But, with increasing contact area, the success rate was gradually decreased due to nonuniform crack. We investigated the maximum force applied at the top slide glass to tear the pattern by homemade equipment. The mechanical force was applied at the position of glass 50 mm away from the edge of the PDMS patterns. The measured moment of force was

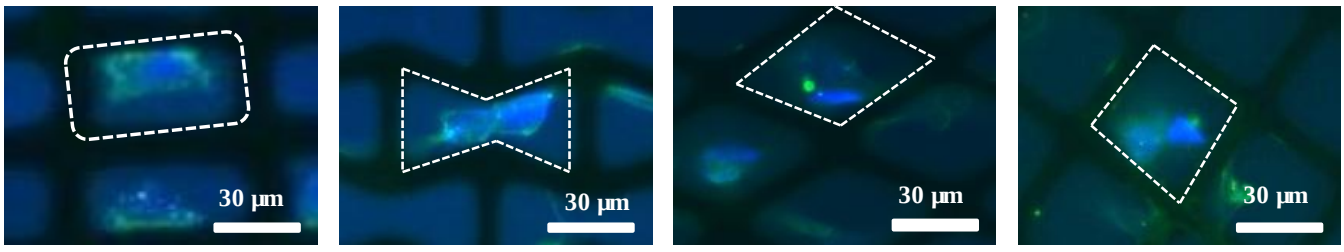

Fig. 4 Fluorescent images showing cell-to-cell adhesion in each pattern. The trapped cells in the patterns were allowed to attach for 48 hours. The samples were counterstained with DAPI for nuclei.

ranged from 0.5 to 1.31 kPa (Fig. 3(b)). As a demonstration of the effectiveness of this method, the cell-to-cell adhesion was performed on the each pattern. Cells are loaded into microwells so that one pair of cells is cultured within each microwell, resulting in contacting a single neighboring cell as shown in Fig. 4. The cell-to-cell adhesion can be examined by immunostaining various cadherin molecules which control BM-MSCs differentiation towards smooth muscle lineage.

5 CONCLUSION

We have demonstrated the simple and low-cost fabrication method of microwell array for cell-to-cell adhesion-mediated differentiation to smooth muscle cells. 4 different types of patterns can be created on the glass substrate and the pattern transfer rate and applied force was investigated as the function of contact area of PDMS to the glass. We believe that the isolated cell pairing into the microwells and the separate analyses of the cell patterns that were cultured allows more efficient analysis from a small quantity of cells, which is more important when using rare samples.

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