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Fabrication of transferable micropatterned-co-cultured cell sheets with microcontact printing

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ABSTRACT

The purpose of the present study is to develop a novel method for the fabrication of transferable micropatterned cell sheets for tissue engineering. To achieve this development, microcontact printing of fibronectin on commercially available temperature-responsive dishes was employed. Primary rat hepatocytes were seeded on the dish surfaces printed with fibronectin. Under serum-free conditions, hepatocytes were attached onto fibronectin domains selectively. Then, a second cell type of endothelial cells was seeded in the presence of serum. Double fluorescent staining revealed that endothelial cells successfully adhered to the intervals of hepatocyte domains. Finally, all the cells were harvested as a single contiguous micropatterned cell sheet upon temperature-reduction. With a cell sheet manipulator having a gelatin layer for the support of harvested cell sheets, harvested micropatterned cell sheets were transferred to new dish surfaces. This technique would be useful for the fabrication of thick tissue constructs having a complex microarchitecture.

1. Introduction

More than a decade ago, we proposed a new kind of tissue engineering, named cell sheet engineering. Since then, we successfully performed animal experiments and clinical applications in different fields [1–5]. The key technology of cell sheet engineering is the fabrication of transplantable cell sheets by utilizing temperature-responsive dishes. Since a temperature-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm), is covalently immobilized on the dish surfaces, cell adhesion and detachment can be noninvasively controlled by temperature change across 32 °C. At 37 °C, the surface of the dishes is relatively hydrophobic, and therefore suitable for cell culture. When the temperature is reduced to the lower critical solution temperature of PIPAAm, 32 °C, however, the surface of the dish becomes very hydrophilic, and hence confluent sheets of cultured cells can be spontaneously released from the dish surface [6].

Recently, we have published several works on producing micropatterned temperature-responsive culture surfaces for the fabrication of micropatterned-co-culture cell sheets. By micropatterning cells, capillary and neural networks can be introduced in the cell sheets. Furthermore, more complex tissue architectures such as renal glomerulus as well as liver lobules can be reproduced by the same techniques. In the previous studies, we utilized various methods to achieve a co-culture of different cell types [7–10]. For example, we reported successful co-culture of two types of cell sheets in a stratified manner. Endothelial cell sheets were harvested from temperature-responsive dishes, and transferred onto confluent monolayer hepatocytes. These heterogeneously layered cell sheets maintained the differentiated functions and viability of hepatocytes during prolonged culture [11]. In our previous works, the patterning was achieved using electron beam polymerization method and porous metal masks [7–10]. With this method, simple micropatterns such as islands-in-sea could be successfully fabricated, but complex patterns could not be achieved. Moreover, during the polymerization of the monomer solution, generated radical species diffused from the exposed area toward vertical or lateral directions, broadening the polymerized features. We found that the minimal feature size of patterned dual temperature-responsive surfaces was limited to 100 μm [7,8]. In order to circumvent these shortcomings, the utilization of microfabrication techniques such as photolithography and softlithography is promising for surface micropatterning. For rapid prototyping of surface micropatterning and microfluidic channel without expensive photomasks and light sources for photolithography, we developed a maskless micropattern exposure system [12,13]. This system consisted of a commercially available liquid crystal device projector that had been modified for the fabrication of

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micropatterned surfaces. Using this system, recovery of micropatterned endothelial cells was successfully achieved on microfabricated temperature-responsive culture surfaces [14]. These micropatterned endothelial cells were sandwiched between two homogeneous fibroblast sheets, and co-cultured [14]. This technique can be useful for the fabrication of prevascularized tissue constructs.

Although these methods provided sufficient reproducibility and resolution, the procedures were more time-consuming or complex than desired. In order to overcome these constraints, in the present study, microcontact printing (μCP) was employed to fabricate micropatterned surfaces onto temperature-responsive culture dishes. Commercially available temperature-responsive culture dishes, on the surface of which PIPAAm is uniformly immobilized, were used as a substrate for micropatterning. Elastomeric polydimethylsiloxane (PDMS) stamps were prepared with the maskless technique described elsewhere [12]. In the present study, micropatterned-co-cultured cell sheets were fabricated on temperature-responsive surfaces, and later on were harvested and transferred to new dishes.

2. Materials and methods

2-Methoxy-1-methylethyl acetate was purchased by Wako Pure Chemical Industries (Tokyo, Japan). PDMS prepolymer and catalysts (Sylgard 184) were purchased from Dow Corning (Midland, MI USA). Tissue culture polystyrene dishes (35-3002) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA). Trypsin ethylenediamine tetraacetic acid disodium salt solution, antibiotics (streptomycin and penicillin), and Hanks' balanced salt solution were obtained from Gibco BRL Life Technologies (Grand Island, NY, USA). Epidermal growth factor (EGF) was available at PeproTech Inc. (Rocky Hill, NJ, USA).

2.1. Fabrication of PDMS stamps

Silicon wafers (p-type, 50 mm in diameter and 280 μm in thickness, Mitsubishi Materials, Tokyo, Japan) were treated by oxygen plasma (irradiation intensity: 400 W; oxygen pressure: 0.2 mmHg) for 180 s in a plasma dry cleaner (PX-1000; SAMCO International, Kyoto, Japan), then covered with a negative thick photoresist for visible light (XP SU-8-3050G-1, Nippon Kayaku, Tokyo, Japan) by spinning at 7000 rpm for 50 s, pre-baked for 30 min at 100 °C, then exposed to visible white light for 10 s, using a previously reported maskless micropattern exposure system (MMP-100S, Sanyo Electric, Osaka, Japan) [12,13]. After postexposure baking at 80 °C for 30 min followed by heating-up to 110 °C for 30 min, the photoresist-coated silicon wafers were immersed in 2-methoxy-1-methylethyl acetate as a developer for 5 min to strip non-exposed area of photoresist on the silicon wafer. Micropatterned surfaces were air dried, and examined under a scanning electron microscope (SEM) (VE-9800, Keyence, Osaka, Japan). The mixture of PDMS prepolymer and catalysts was poured onto the master mold and cured for 30 min at 80 °C, for 1 h at 60 °C or for 24 h at 40 °C. The cured PDMS was cut and peeled from the master mold and then trimmed to provide the stamp.

2.2. Microcontact printing

PDMS stamps were treated by oxygen plasma (irradiation intensity: 400 W; oxygen pressure: 0.2 mmHg) for 30 s in a plasma dry cleaner (PX-1000; SAMCO International, Kyoto, Japan), and sterilized under UV light for 3 min. Then, the micropatterned surfaces (2×2 cm^2) were coated with fibronectin solution (100 μg/mL in PBS, Biomedical Technologies, Stoughton, MA), and dried at room temperature for 10 min. After removing the remaining fibronectin solution, the coated stamps were contacted with temperature-responsive culture dishes (UpCell®; CellSeed, Tokyo, Japan) under a weight (2×3 cm, 120 g) for 3 min. Then dish surfaces were rinsed with PBS and stored at 37 °C in Hank's solution until use. The surfaces were subjected to cell culture or surface characterization by atomic force microscopy

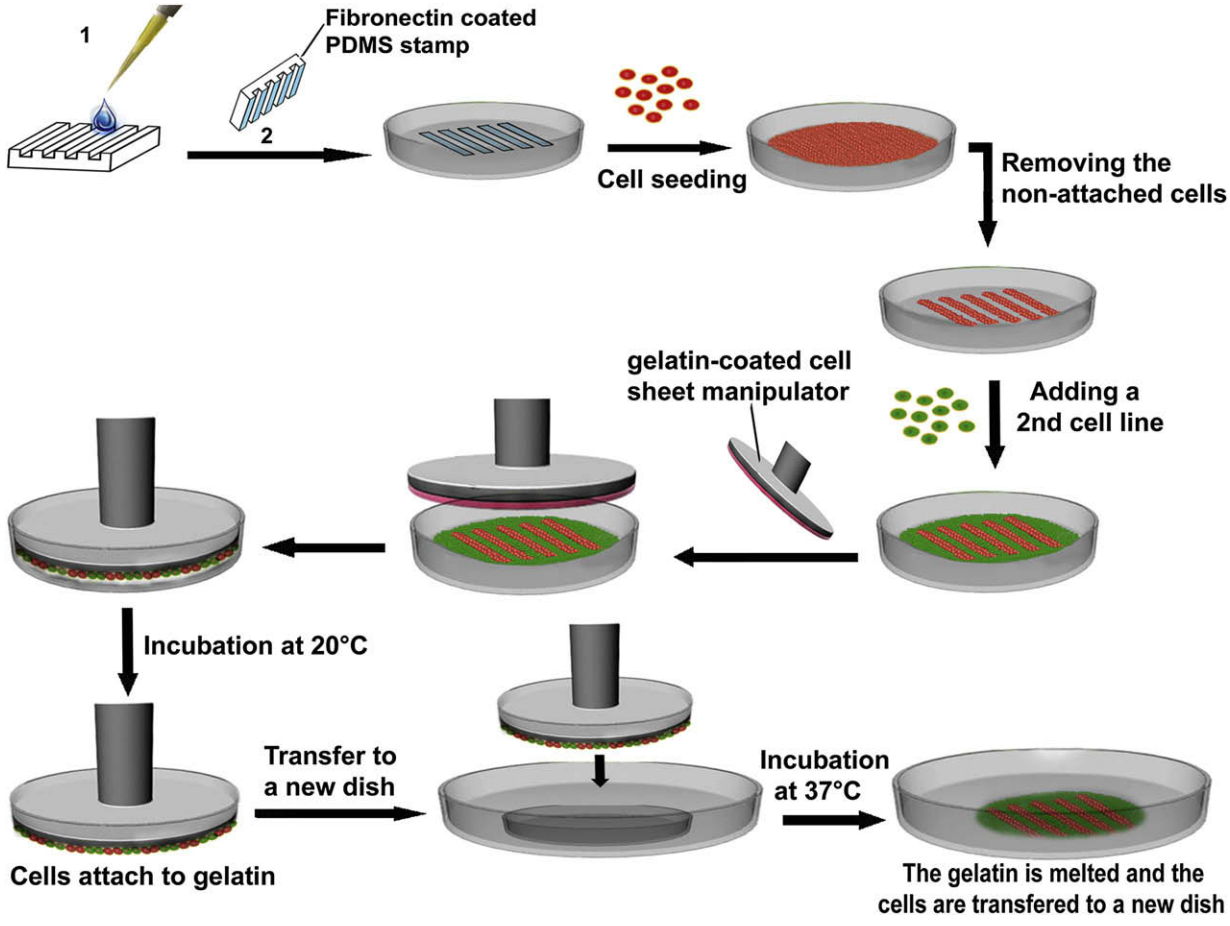

Fig. 1. Procedures for fabrication and transfer of micropatterned-co-cultured cell sheets.

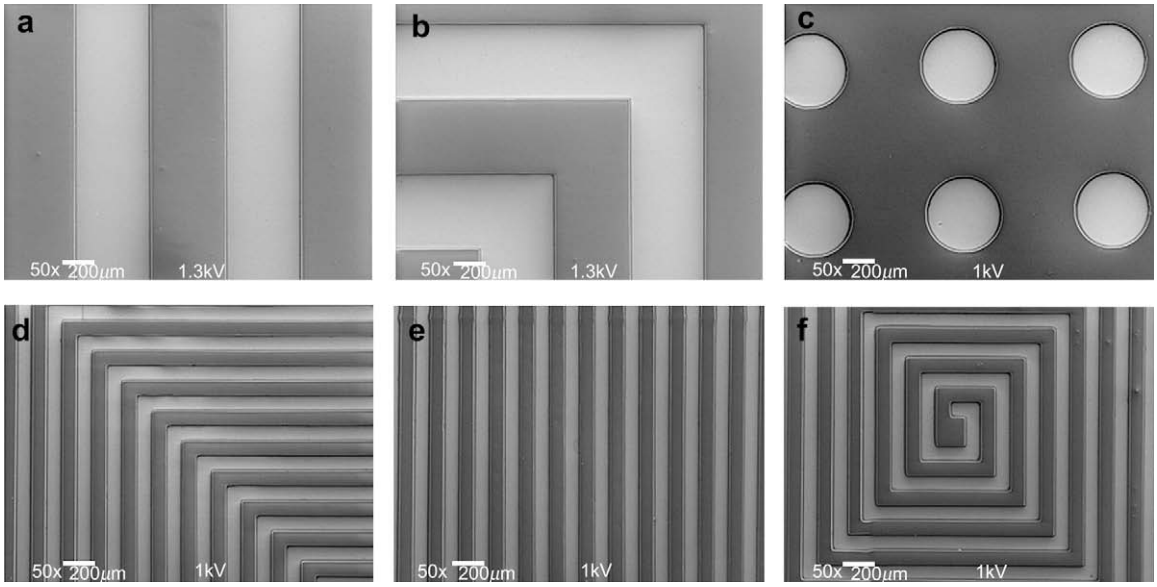

Fig. 2. SEM images of micropatterned photoresist on silicon wafers. (a) Stripes of 500-μm wide and 500-μm intervals. (b) L-shaped lines (a part of maze-like figures) of 500-μm wide and 500-μm intervals. (c) Circles of 500 μm in diameter and 500-μm intervals. (d) L-shaped lines (a part of maze-like figures) of 100-μm wide and 100-μm intervals. (e) Stripes of 100-μm wide and 100-μm intervals. (f) Maze-like figure of 100-μm wide and 100-μm intervals. Bars represent 200 μm.

(AFM) and immunofluorescence. Before surface characterization, the surfaces were fixed with 4% paraformaldehyde in PBS for 15 min at 37 °C, and rinsed with PBS. AFM was performed with NanoScope V (Veeco Instruments, Plainview, NY, USA) under a tapping®-mode (spring constant of 0.03 N/m) with silicon nitride tips (Veeco Probes; Veeco Instruments) at room temperature. For immunofluorescence, dish surfaces were blocked (Protein Block, Serum-Free; DakoCytomation, Glostrup, Denmark), rinsed with PBS, and reacted with rabbit polyclonal anti-bovine fibronectin antibody (1:200 diluted, Biogenesis, Kingston, NH, USA) at room temperature for 1 h, and rinsed with PBS. After incubation with the secondary antibody, goat anti-rabbit IgG antibody conjugated with AlexaFluor® 488 (1:200 diluted, Invitrogen, Carlsbad, CA, USA) at room temperature in darkness for 1 h and then rinsed with PBS. The surfaces were observed under a fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan). Obtained images were processed with software (AxioVision 4.6; Carl Zeiss, Jena, Germany).

2.3. Cell culture

Rat primary hepatocytes were isolated from 9 to 12-week old F344/NSlc male rats by the modified Seglen method [15] as previously described [16]. Isolated hepatocytes possessed 90–95% viability under trypan blue dye exclusion test. Bovine aortic endothelial cells were provided from the Japan Health Science Foundation (JCRB0099), and were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Moregate BioTech, Queensland, Australia), 100 U/mL penicillin, and 100 μg/mL (Sigma). For co-culture, first, primary hepatocytes were seeded onto temperature-responsive culture dishes having micropatterns with fibronectin at a density of 1.5×10^5 cells/cm^2 in a serum-free medium composed of DMEM, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL EGF, 10 mM nicotinamide, and 0.2 mM L-ascorbic acid 2-phosphate, and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for

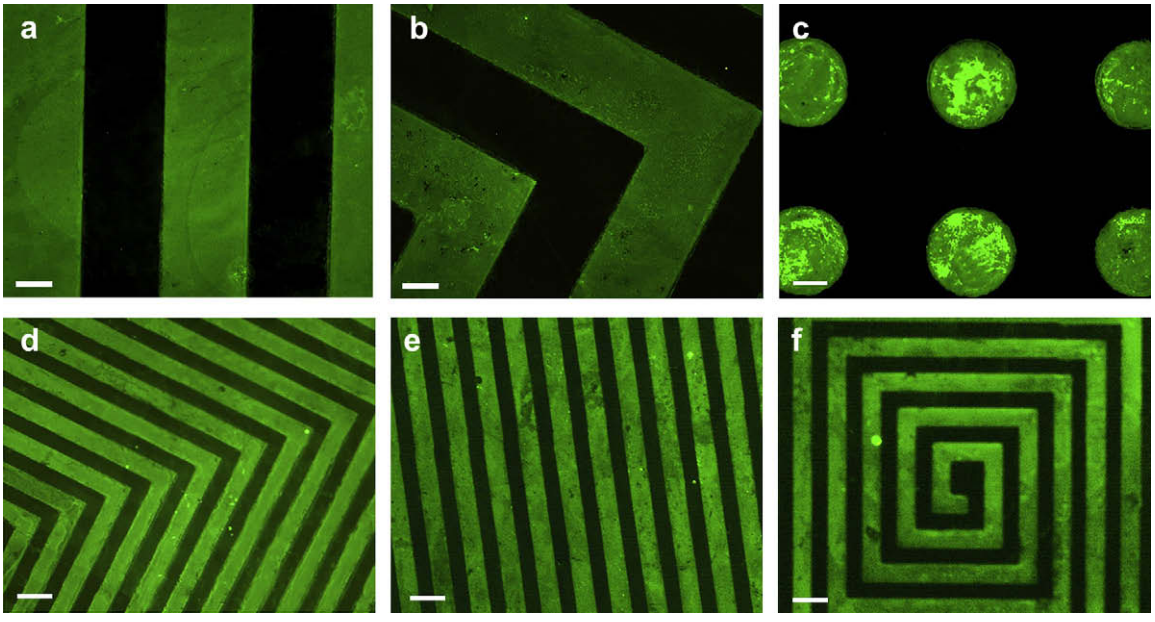

Fig. 3. Immunofluorescent micrographs of temperature-responsive surfaces microcontact-printed with fibronectin. Each panel corresponds its counterpart in Fig. 2. Bars represent 200 μm.

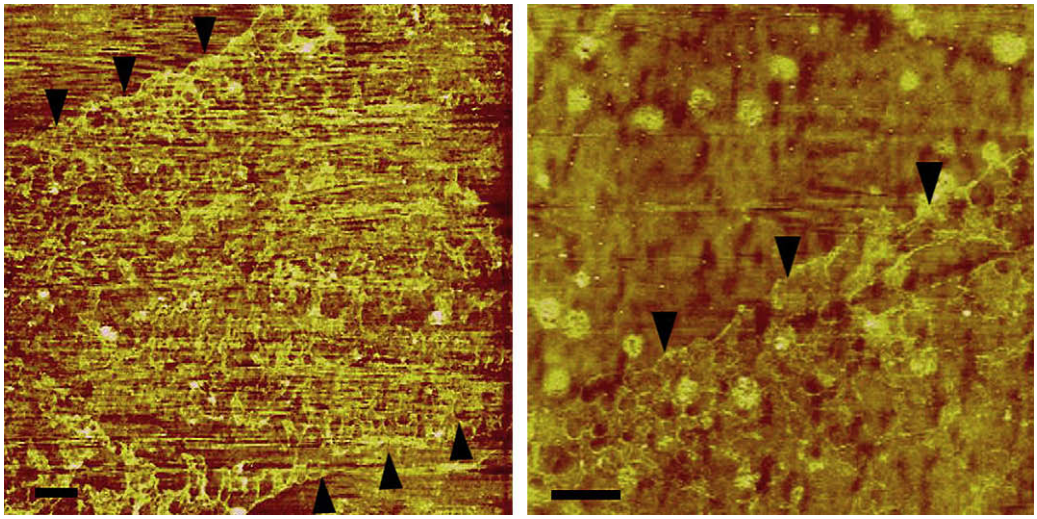

Fig. 4. AFM images of temperature-responsive dish surfaces printed with 100-μm width fibronectin stripes. Arrows show the edges of stripes. Bars represent 10 μm.

2 h. After unattached cells were removed by a gentle exchange of culture medium, endothelial cells were seeded at 6.0×10^5 cells/cm^2 and cultured at 37 °C for 4 h. Then, the culture medium was again exchanged to DMEM, supplemented with 10% fetal bovine serum. Random co-culture of hepatocytes and endothelial cells was also performed on temperature-responsive culture dishes uniformly coated with fibronectin for comparison with the patterned co-culture. Cells were monitored under a phase contrast microscope (TE300; NIKON, Tokyo, Japan).

2.4. Cell sheet manipulation

A cell sheet manipulator having a gelatin gel layer (1.5 mm in thickness) described previously [14,17] was used (Fig. 1). For cell sheet harvest and transfer, the manipulator was placed over cells on temperature-responsive culture dishes in the presence of the culture medium, and incubated for 1 h in a 5% CO_2 incubator set at 20 °C. Then, the manipulator was gently lifted to harvest all the cultured cells as a single contiguous cell sheet, and moved onto a new fibronectin-coated culture dish or a poly-L-lysine-coated glass bottom dish (Matsunami, Osaka, Japan) on which fibronectin was co-coated. After incubation at 20 °C for 30 min, an excessive amount of culture medium at 37 °C was added to melt the gelatin and separate the cell sheet from the manipulator. After 20 min incubation at 37 °C, the culture medium was changed to remove melted gelatin. This step was repeated two more times to completely remove gelatin from the culture.

2.5. Immunofluorescence

Cells were washed with PBS, which was warmed at 37 °C to avoid hydration of the UpCell® dishes, and fixed with 4% paraformaldehyde in PBS for 20 min. After permeabilization with 0.5% Triton X-100 in PBS for 4 min, the cells were blocked

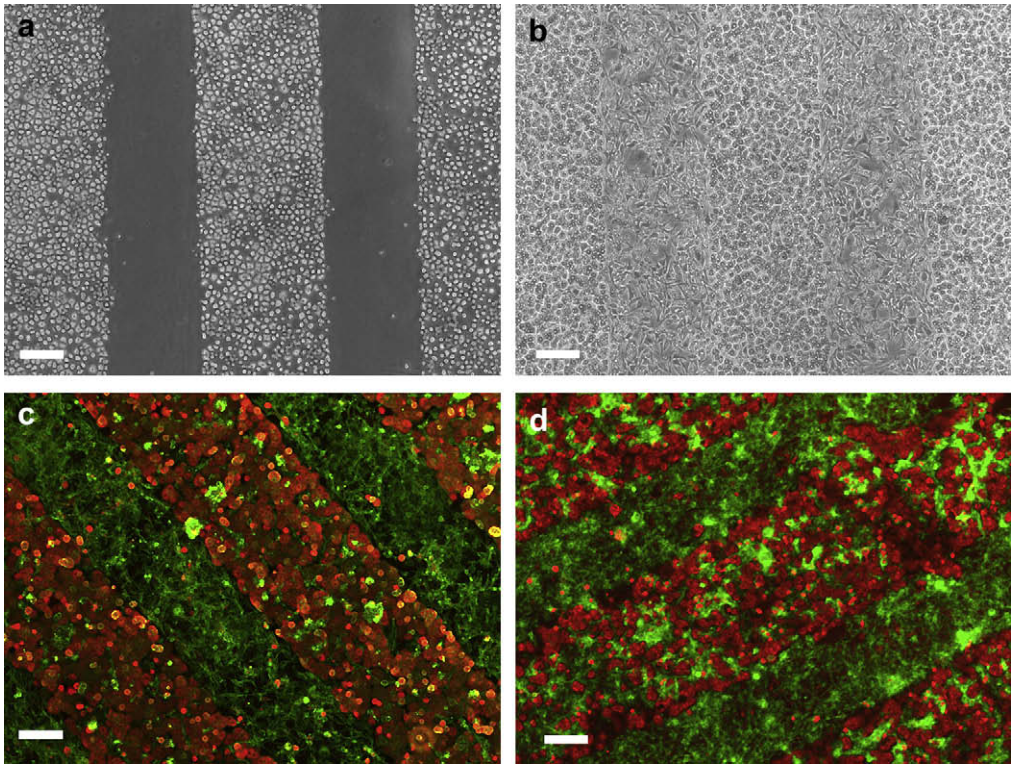

Fig. 5. Microscopic views of micropatterned-co-culture of hepatocytes and endothelial cells. (a) Phase contrast micrographs of hepatocytes seeded on fibronectin stripes in a serum-free condition. (b) Phase contrast micrographs of co-cultured hepatocytes and endothelial cells. (c) Fluorescent micrographs of co-cultured hepatocytes and endothelial cells before cell sheet harvest. Hepatocytes were fluorescently labeled with AlexaFluor® 594 (red) before seeding. Endothelial cells were stained with AlexaFluor® 488-conjugated isolectin GS-IB4 (green). (d) Fluorescent micrographs of co-cultured hepatocytes and endothelial cells after cell sheet transfer. Bars represent 200 μm.

Table 1
Pattern size fidelity: values were measured according to the SEM observations (a), according to fluorescent laser scanning micrograph of a PIPAAM surface which was microcontact-printed with an aqueous fibronectin solution, and subsequently tagged with fluorescent dye (b) and according to the phase contrast laser scanning micrographs of patterned HCs (c).

	100-μm wide patterned domains		500-μm wide patterned domains	
	Patterned area	Interval	Patterned area	Interval
a	112.36 μm ± 4.82	90.97 μm ± 6.59	471.80 μm ± 3.50	441.05 μm ± 10.55
b	104.33 μm ± 3.42	102.00 μm ± 3.65	504.25 μm ± 7.46	497.00 μm ± 10.8
c	109.00 μm ± 4.95	96.80 μm ± 4.76	557.4 μm ± 48.72	464.2 μm ± 43.93

with a Protein Block, Serum-Free solution (Dako) for 15 min and reacted with a 1:100 dilution of sheep anti-rat albumin antibody (1 mg/mL, Bethyl laboratories, Montgomery, TX, USA) at 4 °C overnight. This was followed by an incubation with a 1:500 dilution of AlexaFluor® 594 labeled donkey anti-sheep IgG(H + L) (2 mg/mL) at 25 °C for 1 h. To visualize endothelial cells, cells were double stained with a 1:100 dilution of AlexaFluor® 488-conjugated isolectin GS-IB4 (*Griffonia simplicifolia* lectin; Invitrogen, Carlsbad, CA, USA) at 25 °C for 2 h. The stained cells were observed under a fluorescence microscope (TE2000-U, NIKON) and processed with AxioVision 4.6 software.

3. Results and discussion

Micropatterned photoresist surfaces (masters) were observed under an SEM (Fig. 2). Micropatterns were well fabricated regardless of the sizes and shapes in the ranges of 20–500 μm (Fig. 2 and Supplementary data). Then, a PDMS prepolymer was poured onto the master and cured. The shape and size of PDMS stamps fitted well the negative pattern on the mold. PDMS stamps were treated with oxygen plasma to make the surface temporally hydrophilic [18], coated with fibronectin, and subjected to μCP onto temperature-responsive dishes. No significant difference was observed in the quality of the printing of fibronectin regardless of the curing temperature at 40 °C, 60 °C, or 80 °C (data not shown), but a temperature scale of 30 min at 80 °C was chosen for the rest of the experiments.

Cross-sectional images of the PDMS stamps were obtained with SEM (data not shown). The average depth of the patterns was 13.2 ± 0.2 μm. Although the surfaces of PDMS stamps were slightly rough compared to the photoresist surfaces, fibronectin was well printed on temperature-responsive dishes surfaces as shown in Fig. 3, and in a previous study [17]. The contact-printed surface was fully covered with fibronectin, and the boundary between the patterned area and the non-patterned area was clearly distinguished. Fibrous materials were observed only on fibronectin-printed areas, and the boundary was also clearly seen under AFM (Fig. 4).

After successful transfer of fibronectin with μCP onto temperature-responsive dishes, hepatocytes were seeded in a serum-free medium. Within 3 h of culture at 37 °C, hepatocytes attached only onto fibronectin-printed domains (Fig. 5a). Seeded hepatocytes were well defined in the fibronectin domains (Table 1). The micropatterns of hepatocytes did not fade even after 48 h of culture in a serum-containing medium. Then, endothelial cells were plated on the dishes. Almost all the endothelial cells attached onto the intervals between the patterned hepatocytes (Fig. 5b). Thus, micropatterned-co-culture of hepatocytes and endothelial cells was successfully achieved. Double fluorescent staining with anti-albumin antibody specific for hepatocytes, and isolectin GS-IB4 specific for endothelial cells [19] also revealed cell micropatterning was well obtained (Fig. 5c). The patterned hepatocytes were homogenously stained in red – specifically to albumin, and the patterned endothelial cells were homogenously stained in green – specifically to isolectin GS-IB4. In both cell types, non-specific staining occurred to some extent. The signal obtained due to non-specific staining was attributed to background noise. Indeed, with endothelial cells, which do not express albumin on their surface,

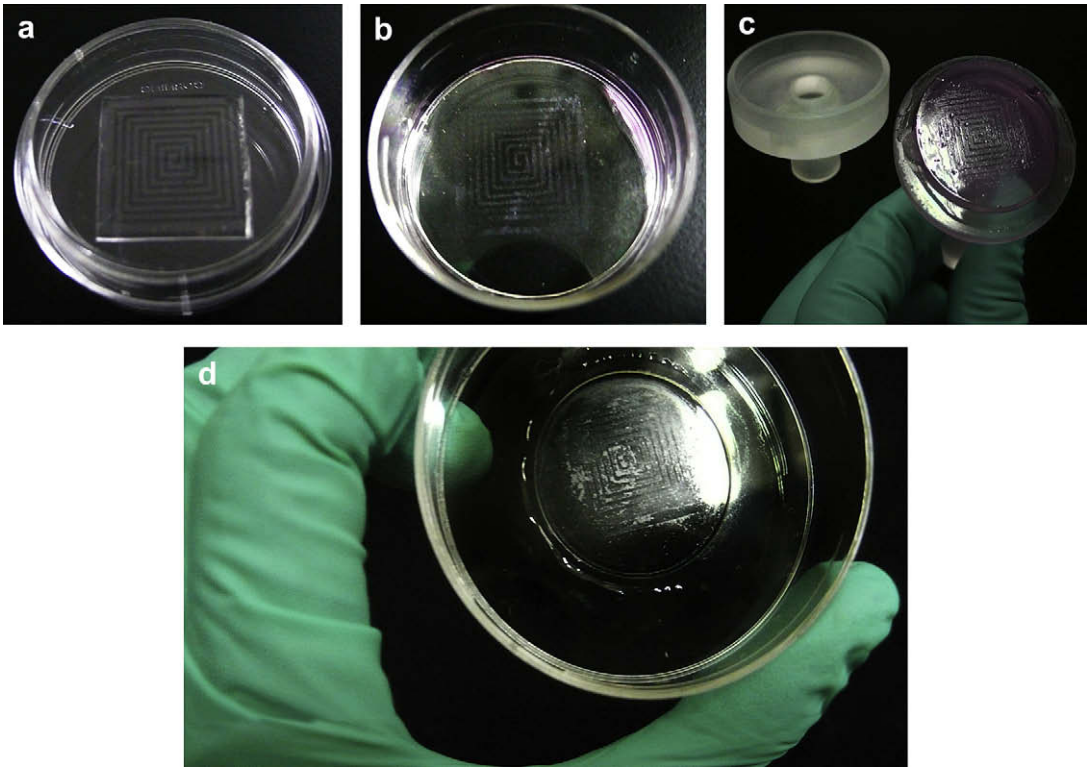

Fig. 6. Macroscopic views. (a) PDMS stamps. (b) Micropatterned hepatocytes cells. (c) Harvest of micropatterned cell sheets with gelatin-coated stamps. (d) Cell sheets transferred onto larger dishes.

the background showed a very low homogeneous red signal. With hepatocyte cells, which express carbohydrates on their surface, but are stained with isolectin GS-IB4 which binds specifically to carbohydrates expressed on the surface of endothelial cells [19], the background showed some green spots on the surface of the cells (Fig. 5c). Our controls showed that the first cell type (hepatocytes) attached well to the fibronectin-patterned areas and that the second cell type (endothelial cells) attached onto the fibronectin-free areas only (data not shown).

Finally, micropatterned-co-cultures of hepatocytes and endothelial cells were subjected to cell sheet harvest and transfer. By using a cell sheet manipulator (Figs. 1 and 6), micropatterned cell sheets were reproducibly harvested and transferred to new dish surfaces. Double fluorescent staining of the transferred micropatterned cell sheets revealed that the micropatterned orientation of two cell types was kept intact during cell sheet harvest and transfer (Fig. 5d). Though well defined stripe patterns were observed in the transferred patterned co-cultured cell sheet, the amount of green precipitates at the level of hepatocytes and the amount of red spots on endothelial cells increased. One cell type might have contaminated the other patterned cell type while moving the plates. Our current work, focusing on the automation of the procedure [20], aims at improving the current results and reduces the level of contamination.

In this study, harvest and transfer of micropatterned cell sheets were successfully achieved with μCP and commercially available temperature-responsive culture dishes. Since a temperature-responsive polymer, PIPAAm is covalently grafted on the dish surfaces, all the cells are detached from the surfaces upon temperature-reduction below 32 °C without the need of proteolytic enzymes like trypsin, while cells attach, spread and proliferate at 37 °C [6]. Here, by the utilization of μCP on temperature-responsive culture dishes on which temperature-responsive polymer is uniformly grafted, various designs of micropattern were fabricated in a rapid prototyping manner. Compared with our previous work to obtain micropatterned cell sheets, expensive masks for electron beam radiation, electron beam sources, and laborious grafting of two kinds of temperature-responsive polymer on the surfaces were eliminated in the present method.

For cell sheet harvest and transfer, a cell sheet manipulator was employed. With this manipulator, we elsewhere showed that five cell layers were repeatedly stratified, and thick cell sheet constructs were fabricated [14]. We reported elsewhere [21] the successful harvest of non-patterned hepatocytes and endothelial cell sheets from temperature-responsive dishes where the viability of transferred cells and the adequate organization of the functional cell–cell junctions were demonstrated. Therefore, the proposed approach is promising for fabricating thick cell sheet constructs having tissue-like microarchitecture inside. Such constructs might be useful for basic research of cell biology to understand cell–cell interactions.

4. Conclusions

By using μCP, micropatterning of fibronectin was achieved on commercially available temperature-responsive culture dishes. After two-step cell seeding, hepatocytes and endothelial cells were co-cultured in a micropatterned manner. All the cells were harvested as a single micropatterned cell sheet, and transferred onto new dishes. This technique would be useful for the fabrication of thick tissue constructs having a complex microarchitecture.

Acknowledgments

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.06.033.

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