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Morphological investigations of cells that adhered to the irregular patterned polydimethylsiloxane (PDMS) surface without reagents

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ABSTRACT

Polydimethylsiloxane (PDMS) surface consisting irregular pattern was investigated to develop cell-based biochip using PDMS. PDMS surface was modified with nano- and micro-combined patterns using surface deformation technology. Hydrophobicity of nano-patterned PDMS surface was sustained. Nevertheless it has irregular patterns consisting of micro- and nano-patterns. According to atomic force microscopy (AFM), scanning electron microscopy (SEM) and confocal microscopy results by immunostaining method, human mammary epithelial cells (HMEC) adhered well on irregularly patterned surface without any reagents such as gelatin and collagen, compared to commercial culture dish. It implies PDMS material can be utilized as template for cell-based biochip without any reagents.

1. Introduction

For several decades, micro-fabricated chip format has been widely applied to biosensing technologies of biomolecules such as DNA, protein and specific chemicals because its integration capability enables high throughput measurement with extremely low quantities of reagents [1]. Currently, biochips consisting of epithelial cells itself have been developed to detect their physiologic responses against the change of environment such as pH, various chemicals and so on [2–4]. In development of cell-based biochip, *in vivo*-like environments in micro-chip system are required to maintain specific properties of epithelial cells, which are represented in inherent *in vivo* biomatrix. To establish *in vivo*-like environments in micro-chip consisting of epithelial cells as sensing materials, investigations of cell adhesion and proliferation on artificial surface in chip are preferentially required. Therefore, various materials, surfaces and structures have been investigated to create *in vivo*-like environments to artificial chip systems [5,6].

Hydrogel, poly D-L-lactic-coglycolde (PLGA), polystyrene and polydimethylsiloxane (PDMS) have been applied to fabricate cell-based micro-chip because their physical and chemical properties are effective on cell to surface interaction [7]. In particular, PDMS is one of promising materials to fabricate micro-cell chip because it has modifiable surface as well as its moldable properties [8].

Nevertheless, most materials cannot be directly applied in cell-based micro-chip because of their nonsuiting surface properties [9]. Therefore, various technologies have been introduced to modify surface for cell adhesion and proliferation. The surface functionality as well as its wettability and rigidity which can affect cell–surface interaction can be controlled using adhesion proteins such as gelatin and collagen. Lately, peptide has been also utilized to specify and reinforce cell adhesion on surface because peptide has only one cell recognition motif whereas most extracellular matrix proteins usually possess various motifs [9–11].

However, biological surface modification methods using adhesion proteins have disadvantages in clinical uses. Protein might degrade when used over a long time or when used in harsh conditions, which can induce surface property to be altered. Therefore, chemical treatments of surface using 3-aminopropyltrimethoxysilane (APTES) and diethylenetriamine (DETA) have been utilized for modifying the surface [12]. However, chemically modified method also requires additional processes to modify the surface. Some researchers investigated that epithelial cell adhesion on surface *in vitro* can be controlled by nano-scaled structured surface in different ways [13]. Nano-sized pattern technologies using conventional and soft lithographic technologies have been introduced to surface modification to stabilize cell

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adhesion on artificial surfaces and to mimic *in vivo* environment [14]. The size effects of well-defined nano- or micro-patterns on cell adhesion were investigated with various materials [15]. However, specific reagents have been still utilized to modify surface properties and the pure effect of pattern type on cell adhesion has not been investigated without adhesion reagent.

Direct treatments of PDMS surface were investigated by plasma or solvent [16]. Air or oxygen plasma treatment induces PDMS surface become negatively charged, resulting in hydrophilic surface. Such oxygen plasma-treated PDMS was directly applied for cell adhesion and their proliferation rate was compared with that on commercial cell culture dish coated by gelatin [17]. Adhesion and proliferation of cells on plasma-treated PDMS are similar to those on commercial product. However, adhered cell could be removed when medium is flowing in micro-chip. Additionally, hydrophilic property of surface in micro-chip induces bubble formation.

Therefore, the development of surface is required for cell-based micro-chips to satisfy both of two demands, proper hydrophobic property (contact angle: 70–110°) for in micro-fluidics and adhesive properties for cell proliferation without chemical and biological reagent.

We represent in this study suitable PDMS surface for the adhesion of epithelial cell, which consists of irregular patterns using simple surface deformation methods without any reagents. To investigate the possibility cell cultivation of irregularly patterned PDMS surface, PDMS surfaces with various chemical and biological reagents are compared to commercial culture dish with respect to adhered shapes of cells using bright-field optical microscopy. Nano-patterned PDMS surfaces are prepared using simple soft lithography and surface deformation method. The topologies of nano-patterned surfaces are investigated by atomic force microscopy (AFM). The cell morphologies adhered to PDMS surface modified here are also studied using AFM, SEM and confocal microscopy in order to validate the suitability of the surface for cell adhesion.

2. Methods

2.1. Cell cultivation

Human mammary epithelial cells (HMEC) was purchased from Lonza, Ltd. (Wakersville, MD, USA). It was cultivated using RPMI-1640 (PAA, Austria) containing 10% FBS (GIBCO, NY, USA), 1% penicillin-streptomycin (GIBCO, NY, UAS) and 1% of GutaMAX (GIBCO, NY, USA). Commercial culture dish used as positive sample was purchased from Falcon (NJ, USA). HMEC cells were cultivated under the normal condition of 37 °C and 5% of CO_2 with $5 \times 10^5/3.8\,cm^2$ of initial seeding amount.

2.2. Surface preparation

Basic PDMS surface was prepared using 1:10 ratio of PDMS elastomer:PDMS hardener (Sylgard 184, Dow corning, MI, USA) at 80 °C for 1 h. Its thickness and surface area are 8 mm and 3.8 cm^2, respectively.

The 0.2% of gelatin or collagen (sigma, MO, USA) were incubated on basic PDMS surface at 37 °C for 30 min to prepare gelatin or collagen coated PDMS surface prior to washing the surface with DPBS (PAA, Austria).

To prepare chemically treated PDMS surface, 3-aminopropyl-trimethoxysilane (APTS) or diethylenetriamine (DETA) (sigma, MO, USA) were diluted with 95% ethanol to 10%. These were spread on raw PDMS surface and incubated at 25 °C for 10 min. Chemically treated surface was washed by 95% ethanol prior to dry at 80 °C.

The plasma-treated surface of PDMS prepared by direct treatment was obtained using oxygen plasma (Femto Science Cograde, 60 W, Korea) for 120 s.

PDMS surface consisting of irregular nano-patterns was prepared with simple methods already published [18]. Briefly, basic PDMS prepared above was stretched and placed on glass substrate. This stretched basic PDMS was treated by oxygen plasma (Femto Science, Cograde, 240 W, Korea) for 15 min. The PDMS substrate was detached from the glass substrate.

PDMS surface consisting of regular nano-pattern was fabricated by soft lithographic technology using nanoporous aluminum template formed by electrochemical anodization [19]. Nanoporous aluminum surface was coated by octadecyl-triethoxylsilane (C18-silane) to prevent PDMS stick on its surface when nano-patterned PDMS surface was created by soft lithography.

2.3. Fluorescence microscopic investigation

Cells cultivated on various PDMS surfaces were investigated using fluorescence microscope (Nikon, Eclipse TE2000-U, Japan). The viability of cells was measured using Live–dead Cell Staining Kit (Bio Vision, CA, USA) which provides fluorescence of 518 nm for live cells and 615 nm for dead cells. All microscopic experiments were performed with 1 day cultivated cells on PDMS.

2.4. Immunostaining investigation by confocal microscopy

Cells cultivated on various PDMS surface for 1 day were investigated using immunostaining method. Cells on surface were fixed using 4% paraformaldehyde (sigma, MO, USA) in PBS (1X). Fixed cells were permeabilized in 0.2% Triton X-100 (sigma, MO, USA) in PBS and then blocked with 1% BSA (sigma, MO, USA). First antibody (for β-actin, Santa Cruz, CA, USA) diluted with 1:100 ratio was added to the cells and then incubated for 2 h at 37 °C. Cells were rinsed three times with PBS. Second antibody Alexa 647 (5 μg/ml, invitrogen, molecular probes, CA, USA) was added to cells and incubated for 1 h. DAPI (4′,6′-diamidino-2-phenylindole) (0.1 μg/ml, sigma, MO, USA) was utilized for nuclear staining.

As a result, the image containing nucleus stained to blue by DAPI and actin representing to red by Alexa 647 is obtained by confocal microscopy (Nikon, EZ-C1, Japan).

2.5. Atomic force microscope (AFM)

Topologies of nano-patterned PDMS surfaces were investigated by two AFMs (contact mode, PSIA, USA; veeco, USA). All cells were fixed by 4% paraformaldehyde (sigma, MO, USA). Morphologies of cells fixed on various PDMS were measured by AFM (semi-contact mode, NT-MDT NTEGRA SPECTRA, Russia). Scan size is $30 \times 30\,\mu m^2$ and scan rate is 0.8 Hz.

2.6. Scanning electron microscope (SEM)

Images of cells that adhered to irregularly patterned surface with negative sample and positive sample was obtained by SEM (Hitachi S-520, Japan) at accelerating voltage of 15 kV after the fixation of cells by 4% paraformaldehyde (Sigma, MO, USA).

3. Experimental results and discussion

We focused on cell adhesion rather than cell proliferation because most epithelial cells require adhesion step prior to cell

proliferation. To investigate the suitable surface for cell adhesion, we measured mainly morphologies of the cells adhered to various surfaces using confocal microscopy, SEM and AFM. Since doubling time of HMEC is known as over 1 day [20], 1-day-cultured cells were utilized to study cell to surface interaction before cell aggregation by increasing cell density.

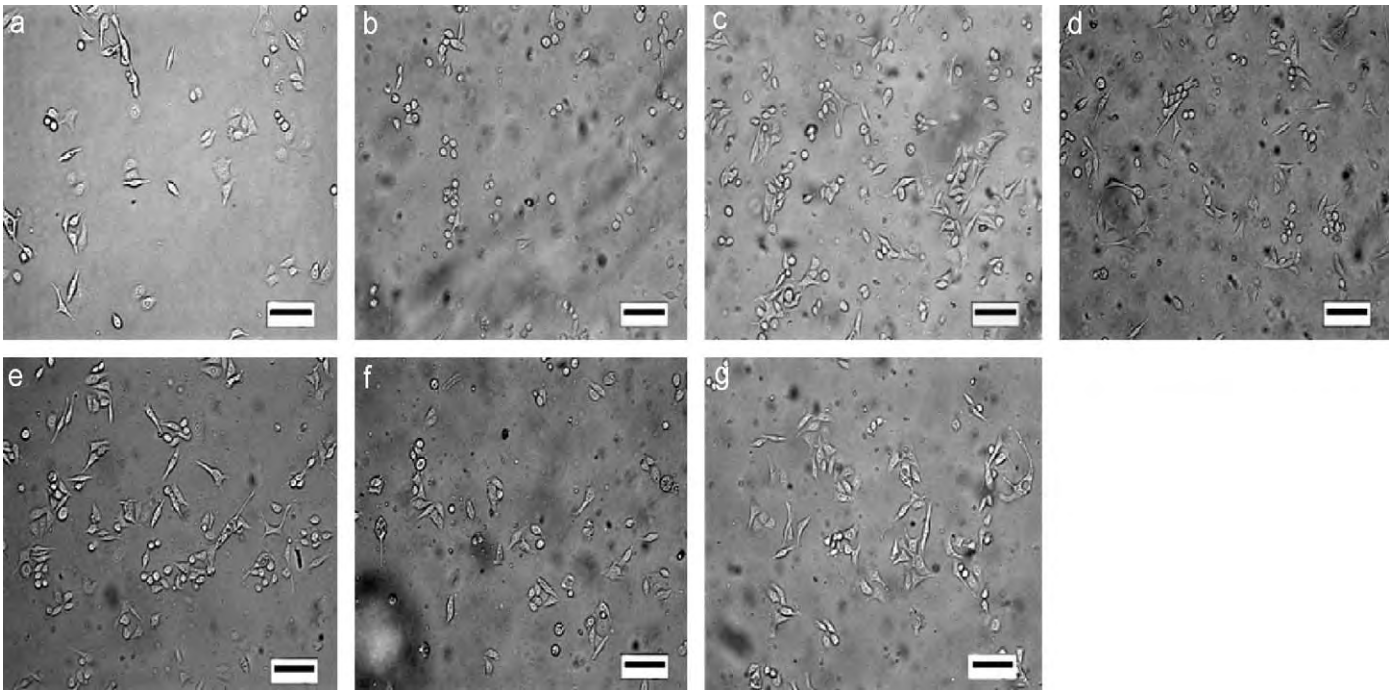

Fig. 1. Bright-field microscopic images of cells that adhered to various surfaces treated by chemical and biological reagents (a) commercial culture dish, (b) basic PDMS, (c) plasma-treated PDMS (20 W, 120 s), (d) APTS coated PDMS, (e) DETA coated PDMS, (f) collagen coated PDMS and (g) gelatin coated PDMS. (Initial cell seeding amount: 5×10^4 cells/3.8 cm^2.) (Scale bars indicate 100 μm.)

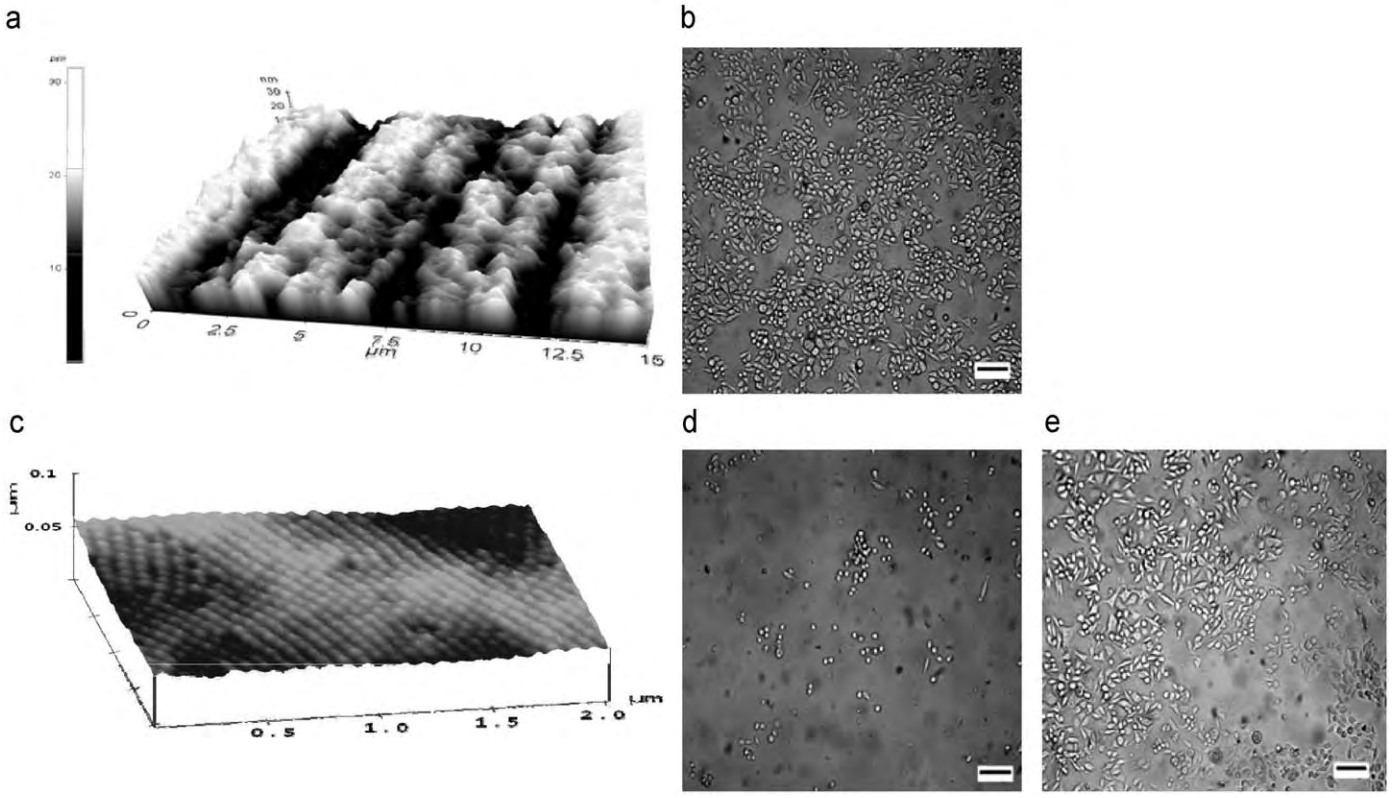

Fig. 2. Comparison of irregular pattern and regular pattern on PDMS: (a) AFM image of irregular patterns on PDMS, (b) bright-field microscopic image of cell adhered to irregular patterns on PDMS, (c) AFM image of regular patterns on PDMS, (d) bright-field microscopic image of cells that adhered to regular patterns on PDMS (not treated) and (e) bright-field microscopic image of cell that adhered to regular patterns on PDMS (Plasma treated, 20 W, 120 s). (Scale bars indicate 100 μm).

3.1. Other simple surface treatment technologies

The typical surface treatment methods for enhancing HMEC cell adhesion, chemical treatments using APTS and DETA and biological treatments using gelatin and collagen were first investigated with commercial culture dish as positive sample and basic PDMS surface as negative sample. As shown in Fig. 1, HMEC cells were not adhered to basic PDMS surface, compared to others. In case of oxygen plasma-treated PDMS surface (c), cells seems to be well adhere, similar to cells on commercial culture dish (a) and on chemically treated surface, with APTS (d) and DETA (e). Morphologies of most cells show that cells were well spread out on surface treated by APTS, DETA and adhesion proteins. However, cells that adhered to chemically treated surface and plasma-treated surface were well detached and removed while washing surface to count them (data are not shown). Therefore, chemical treatment and oxygen plasma treatment methods are not suitable to modify PDMS surface for cell-based micro-chip consisting of micro-fluidics.

3.2. Nano-patterned surface

To develop simple and robust surface modification method without adhesion proteins for cell adhesion, the nano-patterned surface was fabricated with PDMS itself. Fig. 2 presents two different nano-patterned surfaces and their suitability of cells. As shown in Fig. 2(a), irregular pattern consisted of 1–2 μm bigger patterns and less than 1 μm irregular smaller structures (roughness: $R_q = 4.618$ nm). Bigger pattern lanes were formed in the direction perpendicular to stretching of PDMS whereas smaller patterns were irregular. This PDMS surface was hydrophobic with contact angle of over 100° (ca. 105.7°), which was similar to basic PDMS, not treated by oxygen plasma. Fig. 2(b)

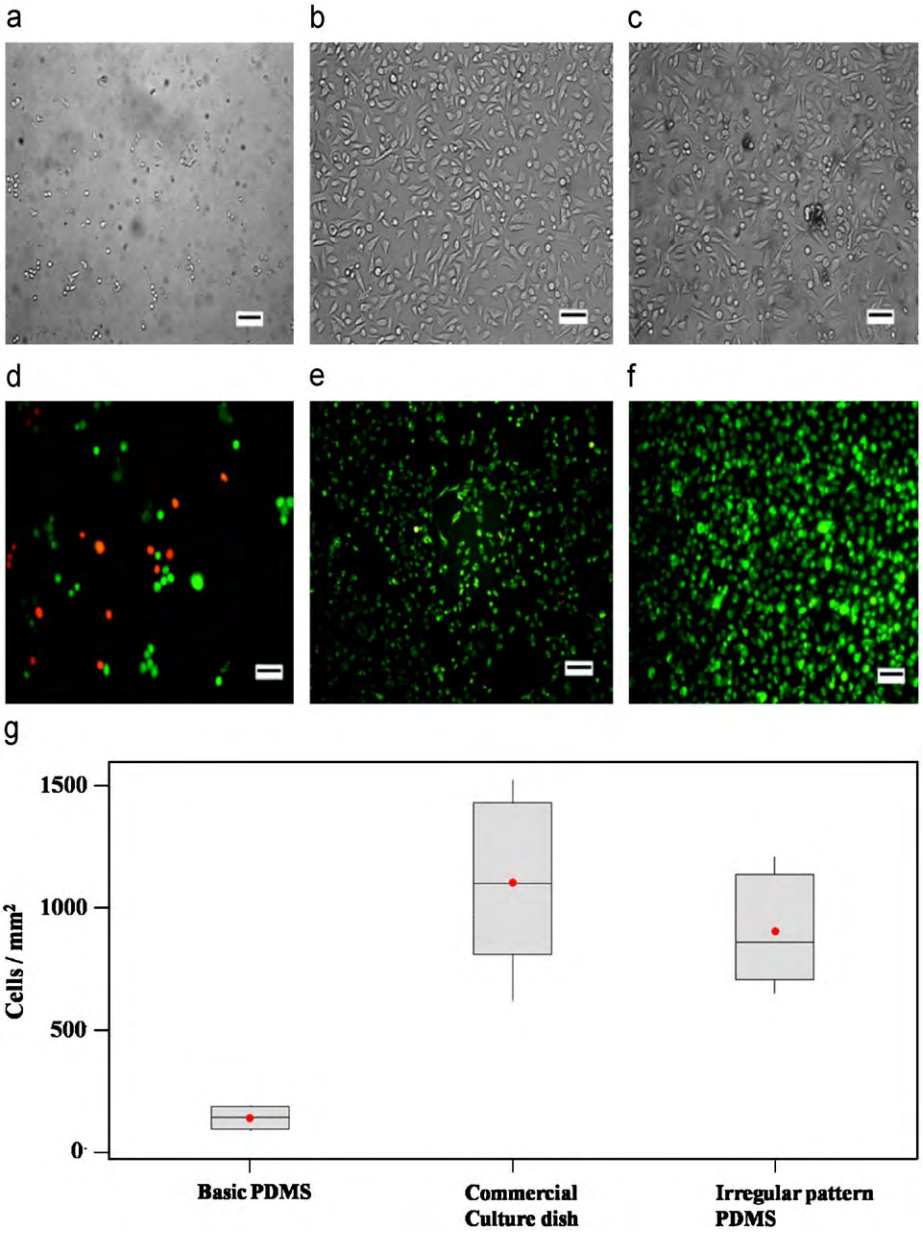

Fig. 3. Bright-field microscopic images of HMEC that adhered to (a) basic PDMS, (b) commercial culture dish, (c) irregularly patterned PDMS surface and fluorescence microscopic images (stained by live and dead cell staining kit) of HMEC that adhered to (d) basic PDMS, (e) commercial culture dish, (f) irregularly patterned PDMS surface (scale bars indicate 100 μm), (g) the box plot of the amount of cell adhered to basic PDMS, commercial culture dish and irregularly patterned PDMS surface (decuplicated).

shows 1-day-cultured HMEC cells on irregular pattern of PDMS. Cells seem to adhere well on surface without any reagents. These adhered cells were maintained after washing step. Fig. 2(c) is the AFM image of regular pattern (roughness: $R_q = 1.867$ nm) on PDMS surface consisting of about 80 nm circular patterns with 3–4 nm in height, fabricated by soft lithography (contact angle, ca. 92°). This small pattern was designed to sustain their inherent hydrophobic property because nano-pattern induces hydrophobic surface to super hydrophobic [21]. HMEC cells adhered to regular pattern is shown in Fig. 2(d) and (e), and this represent HMEC cells that adhered to regular patterns, 2(d) corresponds to the case not oxygen plasma treated and 2(e) to the oxygen plasma treated (120 s, 60 W). In the case of not treated regular pattern by oxygen plasma, HMEC cells did not adhere well on the surface, nevertheless nano-pattern was present on the surface. However, HMEC cells adhered well to the surface treated by oxygen plasma as shown in Fig. 2(e) because regular pattern surface became hydrophilic by oxygen plasma treatment. These results imply irregularly patterned PDMS surface is suitable for cell adhesion in micro-chip because it has capability to attach cell to their surface sustaining hydrophobic property, which makes micro-flow profile stable in micro-channel. Therefore irregular pattern is considered as one of the good candidates for epithelial cell adhesion without reagents in micro-chip.

3.3. Cell adhesion on irregular pattern of PDMS

Fig. 3 presents the irregularly patterned PDMS surface compared with commercial cultural dish as positive sample and basic PDMS as negative sample. As shown in Fig. 3(a–c), cells adhered well on irregularly patterned surface, compared to basic PDMS similar to those on commercial cultural dish. Viabilities of cells that adhered to the surface were measured their with live and dead cell kits. As shown in Fig. 3(d–f), cells on basic PDMS started to die due to poor adhesion whereas cells on irregularly patterned surface and commercial culture dish adhered well and survived. This result was validated with cell counting results as shown in Fig. 3(g). There was no statistical difference between commercial culture dish and irregular pattern surface ($p > 0.05$).

3.4. Morphologies of cells that adhered to irregular pattern PDMS

Fig. 4 shows AFM and SEM images of cells that adhered to surfaces and also their profiles which can predict the quality of cell adhesion with their morphologies. The morphology of single cell on basic PDMS surface as negative sample is shown in Fig. 4(a). The height of cell is 2.5–3.0 μm and its length is about 20 μm. This morphology implies that cell did not adhere to the surface. However, cell image is not clear as shown in Fig. 4. It is likely that cell position is altered while AFM scanning is in progress because the cell on basic PDMS did not adhered firmly. It may provide wrong information of cell morphology. However, SEM image show that cell is not spread out on surface. The morphology of cell on commercial culture dish indicates that cell adheres well and spreads on the surface as shown in Fig. 4(b). The length of cell increased to 30 μm whereas height of cell is not changed. That implies cells adhered well to the surface. This estimate can be validated with SEM result. The morphology of cell that adhered to irregular pattern PDMS surface is shown in Fig. 4(c). The length of cell is about 30 μm with same height. This implies the PDMS surface consisting irregular patterns can provide similar environments provided by commercial culture dish coated with gelatin or collagen. As shown in Fig. 4(c), cells tend to be directionally spread out. It is likely that cells spread along the big lanes pattern on surface. We could not measure both images, cell morphology (micro-scale) and surface topologies (nano-scale) in single image.

Fig. 5 shows the confocal images of cells that adhered to basic PDMS using immunostaining method to investigate their morphologies in detail. Comparing with Fig. 4, cytoplasm of

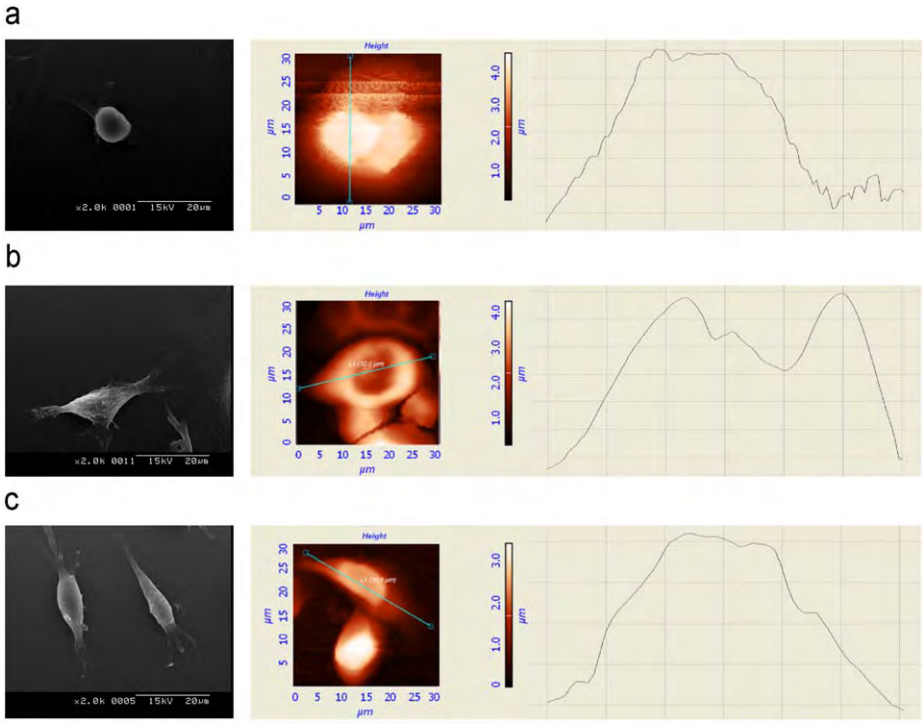

Fig. 4. AFM morphologies of HMEC cells that adhered to (a) basic PDMS, (b) commercial culture dish, (c) irregularly patterned PDMS surface. (*X* and *Y* grids indicate 5 and 0.5 μm, respectively).

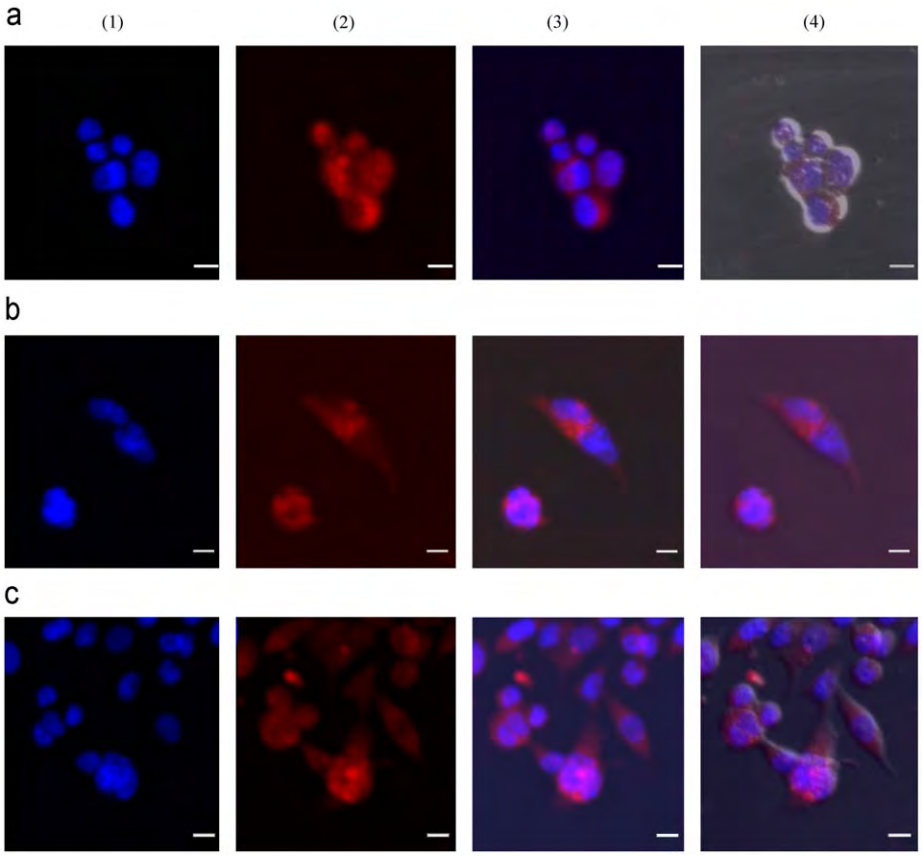

Fig. 5. Confocal images of (a) basic PDMS, (b) commercial culture dish, (c) irregularly patterned PDMS surface (with (1) DAPI, (2) β-actin, (3) merged and (4) merged+DIC. (Scale bars indicate 10 μm.)

cells that adhered to basic PDMS were not generated whereas cytoplasm of cells on commercial culture dish and irregular nano-pattern spread out well. This result represented HMEC cells can adhere to surface using cytoplasm's spread and their morphologies can be simple indicator to decide the cell adaptive surface. Irregular pattern consisting of micro- and nano-combined pattern can be a good surface for cell adhesion by the morphology of cytoplasm and nucleus division even though cells were incubated just for 1 day as shown in Fig. 5(c).

4. Conclusion

To utilize PDMS directly as the surface for HMEC adhesion and proliferation, PDMS surface was modified by fabrication of nano- and micro-combined patterns using surface deformation method. Hydrophobicity of PDMS surface did not vary from basic PDMS surface nevertheless the pattern was irregular in micro- and nano-size. It might be due to the combination of plasma effect (it makes PDMS surface hydrophilic) and nano-pattern effect (it induces PDMS more hydrophobic). According to AFM, SEM and confocal image results using immunostaining method, it can be validated that HMEC adhered to irregularly patterned surface without any reagent such as gelatin and collagen. It represents PDMS material can be utilized as template for cell-based biochip without any reagent. This result can provide key solution to develop micro-fluidic cell-based biochip which requires robust cell immobilization as well as stable micro-flow. Commonly, moderate hydrophobic surface (70–90°) is needed for micro-volume of solution to flow in the micro-chamber when its width is over 1 mm to prevent surface flow formation. This result can be also utilized directly to cell patterning in micro-PDMS chip without surface treatment by chemical and biological reagents.

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