Journal of Bioscience and Bioengineering
VOL. 118 No. 3, 315–322, 2014

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Control of adhesion of human induced pluripotent stem cells to plasma-patterned polydimethylsiloxane coated with vitronectin and γ-globulin

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Received 19 December 2013; accepted 11 February 2014
Available online 18 March 2014

Human induced pluripotent stem cells (hiPSCs) are a promising source of cells for medical applications. Recently, the development of polydimethylsiloxane (PDMS) microdevices to control the microenvironment of hiPSCs has been extensively studied. PDMS surfaces are often treated with low-pressure air plasma to facilitate protein adsorption and cell adhesion. However, undefined molecules present in the serum and extracellular matrix used to culture cells complicate the study of cell adhesion. Here, we studied the effects of vitronectin and γ-globulin on hiPSC adhesion to plasma-treated and untreated PDMS surfaces under defined culture conditions. We chose these proteins because they have opposite properties: vitronectin mediates hiPSC attachment to hydrophilic siliceous surfaces, whereas γ-globulin is adsorbed by hydrophobic surfaces and does not mediate cell adhesion. Immunostaining showed that, when applied separately, vitronectin and γ-globulin were adsorbed by both plasma-treated and untreated PDMS surfaces. In contrast, when PDMS surfaces were exposed to a mixture of the two proteins, vitronectin was preferentially adsorbed onto plasma-treated surfaces, whereas γ-globulin was adsorbed onto untreated surfaces. Human iPSCs adhered to the vitronectin-rich plasma-treated surfaces but not to the γ-globulin-rich untreated surfaces. On the basis of these results, we used perforated masks to prepare plasma-patterned PDMS substrates, which were then used to pattern hiPSCs. The patterned hiPSCs expressed undifferentiated-cell markers and did not escape from the patterned area for at least 7 days. The patterned PDMS could be stored for up to 6 days before hiPSCs were plated. We believe that our results will be useful for the development of hiPSC microdevices.

[**Key words:** Polydimethylsiloxane; Low-pressure air plasma; Microenvironment control; Microdevice; Serum-free culture; Feeder-free culture; iPS cells; Cell adhesion; Competitive adsorption]

Human pluripotent stem cells (hPSCs), including both human embryonic stem cells and human induced pluripotent stem cells (hiPSCs), exhibit infinite self-renewal capacity and pluripotency (1–3). Because hiPSCs and embryonic stem cells generated by somatic cell nuclear transfer contain the donor's genetic information, medical applications of autologous stem cells offer the hope of rejection-free transplantation of tissues and patient-specific drug screening (2,3).

The development of new cell culture devices for patient-specific drug screening using hPSCs requires control of the microenvironment of the cells, including the spatiotemporal distribution of soluble factors, cell–cell interactions, and cell–substrate interactions; and microfabricated devices are increasingly being developed for this purpose (4,5). Polydimethylsiloxane (PDMS) is one of the most popular biocompatible materials for such devices because this elastomer is non-toxic, chemically inert, transparent, and gas permeable (6). For the fabrication of microdevices, PDMS surfaces have often been modified by gas-phase processing methods including plasma treatment (in this paper, plasma refers to low-pressure air plasma, not blood plasma, unless otherwise stated), ultraviolet irradiation, chemical vapor deposition, and sputter coating of metal compounds (7). Plasma treatment is easy to carry out and is used for various purposes, including PDMS–PDMS and PDMS–glass bonding, cleaning PDMS surfaces, and facilitating the coating of surfaces with cell-adhesive extracellular matrix (ECM) proteins (8,9). Therefore, we frequently use plasma treatment in the fabrication of PDMS microdevices for cell culture (10,11).

One of the most fundamental requirements for PDMS microdevices for hPSC applications is that the cells adhere to the PDMS surface, because hPSCs form flat colonies on culture dishes and cannot maintain their pluripotency without adhesion (1,12). Although there have been many studies of adsorption of

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http://dx.doi.org/10.1016/j.jbiosc.2014.02.009

cell-adhesive and non-cell-adhesive molecules, including ECM components, on biocompatible surfaces (13,14), the mechanism of adhesion remains to be revealed. The study of cell adhesion is complicated by the fact that the culture environment contains unknown amounts of various undefined molecules, including those in the ECM and in serum (15–17). Thus, investigation of how PDMS surface modifications and cell-adhesive and non-cell-adhesive proteins affect hPSC adhesion under defined culture conditions is urgently needed if medical applications of microfabricated devices for hPSCs are to be developed.

Here, we studied the effects of two proteins, vitronectin and γ-globulin, which is one of the most abundant protein in serum, on the adhesion of hiPSCs to plasma-treated and untreated PDMS surfaces under defined culture conditions. We chose vitronectin for three reasons: (i) hPSCs adhere to tissue culture dishes coated with vitronectin (18,19); (ii) vitronectin in serum plays a major role in mediating adhesion of cells to the hydrophilic surface of glass, as reflected in the protein's name ("vitro" = "glass", "nectin" = "cell adhesion molecule") (20,21); and (iii) vitronectin may adsorp well on PDMS, because PDMS, like glass, is rich in Si–O bonds (6). In contrast, γ-globulin (an immunoglobulin) has the opposite adhesion properties and thus can be expected to block adsorption of vitronectin on PDMS for three reasons: (i) although adhesion of PSCs to polymers is mediated by integrins, cadherin, and glycans (16,19,22,23), γ-globulin has not been reported to mediate PSC adhesion; (ii) γ-globulin has a hydrophobic fragment crystallizable (Fc) region that is involved in adsorption on hydrophobic surfaces (7,24); and (iii) PDMS is rich in hydrophobic methylene groups (6).

We investigated the relationships between plasma treatment of PDMS surfaces, vitronectin and γ-globulin adsorption, and hiPSC adhesion under defined culture conditions using hESF9a, a serum- and feeder-free culture medium (25,26); this medium allowed us to study these relationships without masking by undefined factors derived from serum and feeder cells. We used the results of our initial investigations to pattern a PDMS surface with hiPSCs.

MATERIALS AND METHODS

Culture and subculture of hiPSCs Two hiPSC cell lines, 201B7 (2) and 253G1 (27), were obtained from RIKEN BRC Cell Bank (Tsukuba, Japan) through the National BioResource Project for the Ministry of Education, Culture, Sports, Science and Technology, Japan. The 201B7 line was used unless otherwise stated. For all experiments, hiPSCs cultured in KSR-based medium on mouse embryonic fibroblast feeder cells were transferred to serum- and feeder-free culture conditions in hESF9a medium (11,25) on dishes coated with 2 μg/mL fibronectin from bovine blood plasma (F-1141, Sigma–Aldrich, St. Louis, MO, USA) and were passaged at least once before use (11,26). For subculturing, the cells were detached from the culture dish by using 0.2–0.5 U/mL dispase (17105-041, Life Technologies, Grand Island, NY, USA) in hESF9a medium and replated in hESF9a medium with 5 μM ROCK inhibitor (Y-27632, Wako Pure Chemical Industries, Osaka, Japan), which blocks dissociation-induced apoptosis of hPSCs (12). The hESF9a medium was changed daily. For the adhesion experiments, hiPSCs were dissociated into single cells by incubation and trituration in 0.02% (w/w) ethylenediaminetetraacetic acid (EDTA) in $PBS^{-/-}$ and then plated in hESF9a solution with 5 μM ROCK inhibitor.

Preparation and plasma treatment of PDMS surfaces PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, Midland, MI, USA) were thoroughly mixed at a 10:1 weight ratio. To make PDMS sheets, we poured the mixture between two polyethylene terephthalate films separated with 0.5 mm rubber spacers and cured it in an oven at 120°C for 2 h. To make perforated masks, we perforated the sheet with 2-mm-diameter holes by using a hole punch. The resultant 0.5-mm-thick PDMS sheets and perforated masks were rinsed with ethanol and sterilized at 160°C for 2 h.

PDMS sheets with or without a perforated mask were hydrophilized by treatment with a low-pressure air plasma for 60 s (YHS-R, Sakigake-Semiconductor Co., Kyoto, Japan) after 5 min under vacuum (ultimate vacuum, 2 Pa; TA150XA, Tasco, Osaka, Japan). Between 30 min and 1 h later (unless otherwise stated), the perforated mask was removed, if one was used, and the PDMS sheet surface was coated with 5.5 mg/mL rabbit γ-globulin (011-000-002, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), 0.6 $\mu g/cm^2$ human blood plasma–derived vitronectin (2349-VN, R&D Systems), or both, and the coated sheet was incubated overnight at 37°C.

Contact angle measurement and Fourier transform infrared spectroscopy The water contact angle of the PDMS surfaces was analyzed in air by the sessile drop method using a droplet of distilled water (2 μL). Droplets were photographed with a digital camera (CX3, Ricoh, Tokyo, Japan), and the angles were estimated by half-angle contact methods using Image J software (NIH, Bethesda, MD, USA).

For Fourier transform infrared (FT-IR) spectroscopy, a PDMS thin film was prepared as reported before (28). Briefly, 0.1 g of a mixture of PDMS was dissolved in 30 mL of chloroform, and the solution was spin-coated (3000 rpm, 20 s) onto an oxidized Si(111) substrate. The resulting PDMS film was cured at 65°C for 12 h. The surface chemical bonding of the PDMS thin film was analyzed by FT-IR spectroscopy (Nicolet, Thermo Fisher Scientific). Spectra were accumulated from 32 scans at a resolution of 1.0 cm^{-1} in transmittance mode in the wavenumber range between 4,000 and 400 cm^{-1}.

Cell attachment assay and immunostaining Attached living cells were stained with 1 μM calcein AM (Dojindo, Kumamoto, Japan), a fluorescent dye that can be transported into living cells, for 20 min at 37°C. For immunostaining of the surface of PDMS coated with proteins, the PDMS surface was rinsed with PBS containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$ ($PBS^{+/+}$), fixed in 4% formaldehyde (Sigma–Aldrich) with 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, and reacted with primary antibodies overnight; the primary antibodies were then visualized with secondary antibodies (Table S1). The antibodies were diluted in $PBS^{+/+}$ containing 10 mg/mL bovine serum albumin. For immunocytochemistry, hiPSCs plated on conventional culture dishes were rinsed with $PBS^{+/+}$, fixed in 4% formaldehyde with 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, permeabilized, blocked with $PBS^{+/+}$ containing 0.2% Triton X-100 and 10 mg/mL bovine serum albumin, and reacted with primary antibodies, which were visualized with secondary antibodies (Table S1). The antibodies were diluted in $PBS^{+/+}$ containing 0.2% Triton X-100 and 10 mg/mL bovine serum albumin. Nuclei were stained with 0.4 μM 4′,6-diamidino-2-

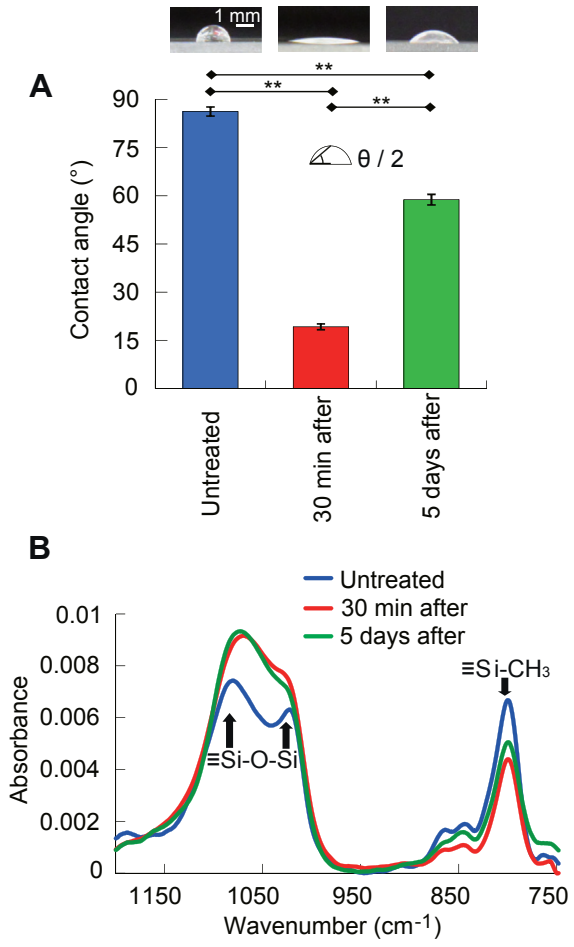

FIG. 1. Effects of plasma treatment of PDMS surfaces. (A) Contact angle (θ) of water. Data are means ± SE ($n = 5$). ** $P < 1 \times 10^{-10}$, Tukey's multiple comparison. Insets are photographs of 2 μL water droplets. (B) FT-IR absorbance spectra of PDMS before plasma treatment (blue) and 30 min (red) or 5 days (green) after 60-s plasma treatment. The arrows represent functional groups whose absorbances were changed by plasma treatment.