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

3 **Running Title:** hiPSC patterning on PDMS by vitronectin and γ -globulin

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

1 **Key words:** PDMS, low-pressure plasma, microenvironment control, microdevice,
2 serum- and feeder-free culture, iPS cells, cell adhesion, competitive adsorption

4 **Introduction**

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

11 The development of new devices for patient-specific drug screening using hPSCs
12 requires control of the microenvironment of the cells, including the spatiotemporal
13 distribution of soluble factors, cell-cell interactions, and cell-substrate interactions; and
14 microfabricated devices are increasingly being developed for this purpose (4, 5).
15 Polydimethylsiloxane (PDMS) is one of the most popular biocompatible materials for
16 such devices because this elastomer is non-toxic, chemically inert, transparent, and gas
17 permeable (6). For the fabrication of microdevices, PDMS surfaces have often been
18 modified by gas-phase processing methods including plasma treatment (in this paper,
19 "plasma" refers to low-pressure plasma, not blood plasma, unless otherwise stated),
20 ultraviolet irradiation, chemical vapor deposition, and sputter coating of metal
21 compounds (7). Plasma treatment is easy to carry out and is used for various purposes,

1 including PDMS–PDMS and PDMS–glass bonding, cleaning PDMS surfaces, and
2 facilitating the coating of surfaces with cell-adhesive extracellular matrix (ECM) proteins
3 (8, 9). Therefore, we frequently use plasma treatment in the fabrication of PDMS
4 microdevices for cell culture (10, 11).

5 One of the most fundamental requirements for PDMS microdevices for hPSC
6 applications is that the cells adhere to the PDMS surface, because hPSCs form flat
7 colonies on culture dishes and cannot maintain their pluripotency without adhesion (1,
8 12). Although there have been many studies of adsorption of cell-adhesive and
9 non-cell-adhesive molecules, including ECM components, on biocompatible surfaces (13,
10 14), the mechanism of adhesion remains to be revealed. The study of cell adhesion is
11 complicated by the fact that the culture environment contains unknown amounts of
12 various undefined molecules, including those in the ECM and in serum (15-17). Thus,
13 investigation of how PDMS surface modifications and cell-adhesive and
14 non-cell-adhesive proteins affect hPSC adhesion under defined culture conditions is
15 urgently needed if medical applications of microfabricated devices for hPSCs are to be
16 developed.

17 Here, we studied the effects of two proteins, vitronectin and γ -globulin, which is one
18 of the most abundant protein in serum, on the adhesion of hiPSCs to plasma-treated and
19 untreated PDMS surfaces under defined culture conditions. We chose vitronectin for
20 three reasons: 1) hPSCs adhere to tissue culture dishes coated with vitronectin (18, 19);
21 2) vitronectin in serum plays a major role in mediating adhesion of cells to the

1 hydrophilic surface of glass, as reflected in the protein's name ("vitro" = "glass," "nectin"
2 = "cell adhesion molecule") (20, 21); and 3) vitronectin may adsorb well on PDMS,
3 because PDMS, like glass, is rich in Si-O bonds (6). In contrast, γ -globulin (an
4 immunoglobulin) has the opposite adhesion properties and thus can be expected to block
5 adsorption of vitronectin on PDMS for three reasons: 1) although adhesion of PSCs to
6 polymers is mediated by integrins, cadherin, and glycans (16, 19, 22, 23), γ -globulin has
7 not been reported to mediate PSC adhesion; 2) γ -globulin has a hydrophobic fragment
8 crystallizable (Fc) region that is involved in adsorption on hydrophobic surfaces (7, 24);
9 and 3) PDMS is rich in hydrophobic methylene groups (6).

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

16

17 **Materials and Methods**

18 ***Culture and subculture of hiPSCs***

19 Two hiPSC cell lines, 201B7 (2) and 253G1 (27), were obtained from RIKEN BRC
20 Cell Bank (Tsukuba, Japan) through the National BioResource Project for the Ministry of
21 Education, Culture, Sports, Science and Technology, Japan. The 201B7 line was used

1 unless otherwise stated. For all experiments, hiPSCs cultured in KSR-based medium on
2 mouse embryonic fibroblast feeder cells were transferred to serum- and feeder-free
3 culture conditions in hESF9a medium (11, 25) on dishes coated with 2 µg/mL fibronectin
4 from bovine blood plasma (F-1141, Sigma-Aldrich, St. Louis, MO, USA) and were
5 passaged at least once before use (11, 26). For subculturing, the cells were detached from
6 the culture dish by using 0.2–0.5 U/mL dispase (17105-041, Life Technologies, Grand
7 Island, NY, USA) in hESF9a medium and replated in hESF9a medium with 5 µM ROCK
8 inhibitor (Y-27632, Wako Pure Chemical Industries, Osaka, Japan), which blocks
9 dissociation-induced apoptosis of hPSCs (12). The hESF9a medium was changed daily.
10 For the adhesion experiments, hiPSCs were dissociated into single cells by incubation
11 and trituration in 0.02% (w/w) ethylenediaminetetraacetic acid (EDTA) in PBS^{-/-} and
12 then plated in hESF9a solution with 5 µM ROCK inhibitor.

13

14 ***Preparation and plasma treatment of PDMS surfaces***

15 PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, Midland, USA)
16 were thoroughly mixed at a 10:1 weight ratio. To make PDMS sheets, we poured the
17 mixture between two polyethylene terephthalate films separated with 0.5-mm rubber
18 spacers and cured it in an oven at 120 °C for 2 h. To make perforated masks, we
19 perforated the sheet with 2-mm-diameter holes by using a hole punch. The resultant
20 0.5-mm-thick PDMS sheets and perforated masks were rinsed with ethanol and sterilized
21 at 160 °C for 2 h

1 PDMS sheets with or without a perforated mask were hydrophilized by treatment with
2 a plasma for 60 s (YHS-R, SAKIGAKE-Semiconductor Co., Kyoto, Japan) after 5 min
3 under vacuum (ultimate vacuum, 2 Pa; TA150XA, Tasco, Osaka, Japan). Between 30
4 min and 1 h later (unless otherwise stated), the perforated mask was removed, if one was
5 used, and the PDMS sheet surface was coated with 5.5 mg/mL rabbit γ -globulin
6 (011-000-002, Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA), 0.6
7 $\mu\text{g}/\text{cm}^2$ human blood plasma-derived vitronectin (2349-VN, R&D Systems), or both, and
8 the coated sheet was incubated overnight at 37 °C.

9

10 *Contact angle measurement and Fourier transform infrared spectroscopy*

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

15 For Fourier transform infrared (FT-IR) spectroscopy, a PDMS thin film was prepared
16 as reported before (28). Briefly, 0.1 g of a mixture of PDMS was dissolved in 30 mL of
17 chloroform, and the solution was spin-coated (3000 rpm, 20 s) onto a oxidized Si(111)
18 substrate. The resulting PDMS film was cured at 65 °C for 12 h. The surface chemical
19 bonding of the PDMS thin film was analyzed by FT-IR spectroscopy (Nicolet, Thermo
20 Fisher Scientific). Spectra were accumulated from 32 scans at a resolution of 1.0 cm^{-1} in
21 transmittance mode in the wavenumber range between 4000 and 400 cm^{-1} .

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2 ***Cell attachment assay and immunostaining***

3 Attached living cells were stained with 1 μ M calcein AM (Dojindo, Kumamoto,
4 Japan), a fluorescent dye that can be transported into living cells, for 20 min at 37 °C. For
5 immunostaining of the surface of PDMS coated with proteins, the PDMS surface was
6 rinsed with PBS containing 0.5 mM CaCl_2 and 0.5 mM MgCl_2 ($\text{PBS}^{+/+}$), fixed in 4%
7 formaldehyde (Sigma-Aldrich) with 0.5 mM MgCl_2 and 0.5 mM CaCl_2 , and reacted with
8 primary antibodies overnight; the primary antibodies were then visualized with
9 secondary antibodies (Table S1). The antibodies were diluted in $\text{PBS}^{+/+}$ containing 10
10 mg/mL bovine serum albumin. For immunocytochemistry, hiPSCs plated on
11 conventional culture dishes were rinsed with $\text{PBS}^{+/+}$, fixed in 4% formaldehyde with 0.5
12 mM MgCl_2 and 0.5 mM CaCl_2 , permeabilized, blocked with $\text{PBS}^{+/+}$ containing 0.2%
13 Triton X-100 and 10 mg/mL bovine serum albumin, and reacted with primary antibodies,
14 which were visualized with secondary antibodies (Table S1). The antibodies were diluted
15 in $\text{PBS}^{+/+}$ containing 0.2% Triton X-100 and 10 mg/mL bovine serum albumin. Nuclei
16 were stained with 0.4 μ M 4',6-diamidino-2-phenylindole (DAPI, Wako). Fluorescence
17 micrographs were obtained with a BZ-8100 fluorescence microscope (Keyence, Osaka,
18 Japan) and analyzed with Image J software (NIH).

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1

2 **Results**

3 *Effects of plasma treatment on PDMS surface structure*

4 To determine how plasma treatment affected PDMS hydrophilicity, we measured the
5 contact angle of a 2- L water droplet and found that 30 min after a 60-s plasma treatment,
6 the contact angle (19°) was significantly lower than the angle before treatment (86° , Fig.
7 1A), indicating that the PDMS surface had been hydrophilized by the plasma treatment.
8 The contact angles after 15- and 60-s treatments were the same, and the angle was stable
9 during the period from 30 min to 2 h after treatment (Fig. S1). The contact angle
10 increased gradually, and by 5 days after treatment, the angle had recovered to
11 approximately 70% ($59^\circ/86^\circ$) of that for the untreated surface (Figs. S1 and 1A),
12 suggesting the hydrophobicity of the PDMS surface had been restored. On the basis of
13 these results, we coated PDMS with vitronectin, γ -globulin, or both during the period
14 between 30 min and 1 h after plasma treatment, unless otherwise stated.

15 Next we measured the absorbance of a PDMS thin film by means of FT-IR (Fig. 1B).
16 Plasma treatment of PDMS caused a noticeable decrease in the absorbance bands at 800
17 cm^{-1} derived from CH_3 rocking and $\equiv\text{Si}-\text{C}$ stretching vibrations, indicating a decrease
18 in the number of methyl groups. In contrast, two bands, centered at 1026 and 1070 cm^{-1}
19 and attributable to asymmetric $\equiv\text{Si}-\text{O}-\text{Si}$ stretching vibrations, were increased by
20 plasma treatment, indicating that the $\text{CH}_3-\text{Si}-\text{O}$ groups in PDMS underwent
21 transformation to $\text{O}-\text{Si}-\text{O}$ groups. Taken together, the changes in the FT-IR spectra

1 suggest that a siliceous layer formed at the surface of PDMS film as a result of plasma
2 treatment.

3 4 *Adsorption of vitronectin on and adhesion of hiPSCs to plasma-treated and untreated* 5 *PDMS surfaces*

6 We next investigated how vitronectin mediated hiPSC adhesion to the PDMS surfaces.
7 It is well known that many types of adhesive cells can be cultured on a plasma-treated
8 hydrophilic polystyrene dish, generally referred to as a tissue culture treated dish, but not
9 on an untreated hydrophobic polystyrene dish. Moreover, formation of a siliceous layer
10 by plasma treatment may facilitate hiPSC adhesion to PDMS surfaces coated with
11 vitronectin, which mediates cell–glass adhesion (20). Thus we hypothesized that
12 vitronectin coating would enable hiPSCs to adhere to plasma-treated PDMS surfaces but
13 not to untreated PDMS surfaces. To test this hypothesis, we coated untreated and
14 plasma-treated PDMS surfaces with vitronectin. Immunostaining with anti-vitronectin
15 antibodies showed that vitronectin was adsorbed on both plasma-treated and untreated
16 PDMS surfaces (Figs. 2A, G, M and S2) and that hiPSCs adhered to both surfaces (Figs.
17 2B, H, N and S2). Thus, these results did not support our initial hypothesis.

18 19 *Effect of γ -globulin on hiPSC adhesion to untreated PDMS surfaces*

20 We suspected that a blocking factor was needed to prevent vitronectin adsorption on
21 the untreated hydrophobic PDMS surface. For this purpose, we chose γ -globulin, which

1 has a hydrophobic Fc region and has not been reported to mediate PSC adhesion (16, 19,
2 22, 23). We hypothesized that γ -globulin would adsorb preferentially to the untreated,
3 hydrophobic PDMS surface and would thus block cell adhesion. To test this hypothesis,
4 we coated untreated and plasma-treated PDMS surfaces with a mixture of vitronectin and
5 γ -globulin. Immunostaining showed that bright red staining (red fluorescence was
6 stronger than green fluorescence) on the untreated PDMS surface and bright green
7 staining (green fluorescence was stronger than red fluorescence) on the plasma-treated
8 PDMS surface, indicating γ -globulin and vitronectin were adsorbed preferentially on the
9 untreated and plasma-treated PDMS surfaces, respectively (Figs. 2E, K, M and S2).
10 Human iPSCs adhered to the plasma-treated vitronectin-rich surface but not to the
11 untreated γ -globulin-rich surface (Figs. 2F, L, N and S2). These results supported our
12 second hypothesis. Interestingly, coating with γ -globulin alone resulted in adsorption of
13 γ -globulin on both untreated and plasma-treated PDMS surfaces, and hiPSCs did not
14 adhere to either surface (Figs. 2C, D, I, J, M, N and S2). These results suggest that
15 γ -globulin blocked adsorption of vitronectin on the untreated PDMS surface and thus
16 prevented hiPSC adhesion.

17

18 ***Patterning of hiPSCs on plasma-patterned PDMS surfaces coated with vitronectin and*** 19 ***γ -globulin***

20 We speculated that our results might be useful for generating hiPSC patterning on
21 PDMS surfaces, so we carried out the following experiments. We patterned a PDMS

1 surface by applying a PDMS mask perforated with 2-mm-diameter holes and then
2 treating the masked surface with plasma. After removal of the mask, the
3 plasma-patterned surface was coated with vitronectin, γ -globulin, or both (Fig. 3A).
4 Immunostaining showed that when the patterned surface was treated with either
5 vitronectin alone or γ -globulin alone, the protein was adsorbed onto the entire surface
6 (Fig. 3B, D). In contrast, when the patterned surface was treated with a mixture of the
7 two proteins, immunostaining showed bright green staining in the circular
8 (plasma-treated) areas and bright red staining in the surrounding (untreated) areas as
9 shown in Fig. 2EK, indicating that vitronectin and γ -globulin were preferentially
10 adsorbed onto the plasma-treated and untreated areas, respectively (Fig. 3C). Human
11 iPSCs adhered to the entire surface of PDMS coated with vitronectin alone but did not
12 adhere to the surface coated with γ -globulin alone (Fig. 3E, G). In contrast, treatment of
13 the patterned PDMS with a mixture of vitronectin and γ -globulin allowed hiPSCs to
14 adhere to the plasma-treated circular areas (Fig. 3F), and the hiPSCs remained in those
15 areas for at least 7 days (Fig. 3H). The morphology of patterned hiPSCs appeared to be
16 that of normal hiPSC colonies (Fig. 3I); the colonies were tightly packed and flat
17 (monolayer) and consisted of cells with large nuclei and scant cytoplasm (1, 2, 26).
18 Because the cells kept proliferating without escaping from the plasma-treated area, the
19 edges of the colonies became thick (multilayer) and appeared dark under a phase-contrast
20 microscope on day 7 (Fig. 3H). Immunostaining revealed that the patterned hiPSCs
21 expressed undifferentiated-cell markers TRA 1-60, SSEA3, and SSEA4 (Fig. 3J). We

1 obtained the same results by using another hiPSC line, 253G1 (Fig. S3). These results
2 suggest that our method can be used to prepare hiPSCs on plasma-patterned PDMS
3 surfaces.

4 5 *Storage of plasma-patterned PDMS before plating of cells*

6 Next we determined whether the cell patterning would form if the PDMS was stored
7 for several days after plasma patterning. It would be convenient if patterned PDMS
8 sheets could be prepared several days before cells were plated because this would enable
9 batch production of many PDMS plates of the same quality. Although the
10 plasma-hydrophilized surface of the PDMS recovered its hydrophobicity 5 days after
11 plasma treatment (Fig. 1A), the hiPSCs retained the pattern for 7 days (Fig. 3H), which
12 suggests that coating the surface with vitronectin and γ -globulin 30 min after plasma
13 treatment enabled retention of the plasma pattern for 7 days. To test this, we evaluated
14 two preparation methods: a pre-coat method and a post-coat method. In the pre-coat
15 method, PDMS surfaces were plasma patterned on day 0, the patterned PDMS was
16 coated with vitronectin and γ -globulin 30 min after plasma treatment, and the coated
17 PDMS was then stored in a CO₂ incubator for 6 days (Fig. 4A). In the post-coat method,
18 the PDMS surfaces were plasma patterned on day 0, stored in a CO₂ incubator for 5 days,
19 and coated with vitronectin and γ -globulin for overnight (Fig. 4A). Under the pre-coat
20 conditions, vitronectin adhered to the circular areas, which were surrounded by
21 γ -globulin, and the hiPSCs adhered to the circular areas (Fig. 4B, C). In contrast, the

entire post-coated PDMS surface adsorbed γ -globulin, and hiPSCs did not adhere to the surface (Fig. 4D, E). These results suggest that vitronectin and γ -globulin coating of the plasma-patterned surface suppressed recovery of hydrophobicity and thus enabled retention of the patterning.

Discussion

There have many reports describing chemical modifications of PDMS surfaces including photo-oxidation and plasma treatment (7-9, 29, 30). In this study, we used plasma treatment, during which a reaction involving a collision between an electron and O_2 generates a singlet oxygen atom, which etches the PDMS surface (29). FT-IR analysis of the PDMS surface before and after plasma treatment indicated that plasma exposure resulted in the formation of $\equiv Si-O-Si\equiv$ groups by oxidation of $\equiv Si-CH_3$ by singlet oxygen radical species. Oxidization of PDMS film surfaces by exposure to vacuum ultraviolet light ($\lambda = 172$ nm) has been reported to reduce the number of $\equiv Si-CH_3$ groups and subsequently increase the numbers of $\equiv Si-OH$ and $\equiv Si-O-Si\equiv$ groups on the surfaces (28). In our present study, the absorbance band due to $\equiv Si-O-Si\equiv$ in the FT-IR spectra significantly increased with the plasma treatment. Taken together, our results and those reported in the reference (28) suggest that the plasma treatment increased the number of hydroxyl groups and siloxane groups to form a siliceous layer on the outermost surfaces of the PDMS film. The end result was generation of hydrophilic groups on the PDMS (Fig. 5A). Five days after plasma treatment, the PDMS surface

1 recovered some its original hydrophobicity, as indicated by the water contact angle,
2 although the FT-IR data did not show recovery of hydrophobicity. Hydrophilized PDMS
3 surfaces have been reported to quickly recover their hydrophobicity owing to migration
4 of unreacted oligomers to the surface (31, 32). However, this migration might occur only
5 on the outermost surface, at a depth that is shallower than that reached by FT-IR
6 measurement. Thus, one possible explanation for our results is that adsorption of the
7 coating protein on the outermost surface anchored the transformed functional groups,
8 preventing their migration and thus suppressing recovery of hydrophobicity (Fig. 5B, C).

9 Because many adhesive cells adhered to the plasma-treated polystyrene dish but did
10 not adhere to an untreated polystyrene dish, we first hypothesized that vitronectin was
11 adsorbed only by the plasma-treated PDMS surface, on which the hiPSCs subsequently
12 adhered. However, we found that vitronectin was adsorbed by both plasma-treated and
13 untreated PDMS surfaces. Coating PDMS with a mixture of vitronectin and γ -globulin
14 generated a concentration difference between the two proteins. The affinity of the
15 untreated hydrophobic PDMS surface for γ -globulin might be larger than that for
16 vitronectin because γ -globulin has a hydrophobic Fc region. Moreover, an increase in the
17 concentration of one protein may have suppressed adsorption of the other by means of
18 competitive adsorption (33-35). As a result, addition of γ -globulin apparently blocked the
19 adsorption of vitronectin and thus blocked subsequent adhesion of hiPSCs to the
20 untreated PDMS surface.

1 Compared to the many previously reported cell-patterning methods (5, 10, 36-39), our
2 method offers three advantages. The first advantage is that the pattern is long-lasting.
3 Although there have been reports of successful patterning of PSCs (37, 39), the cells
4 have been found to escape from the pattern within a few days. Long-lasting patterning is
5 important because hPSC differentiation takes at least a few days (11). The use of
6 serum-supplemented culture medium and the presence of feeder cells can cause cell
7 escape because serum and feeder cells supply ECM, which facilitates cell adhesion (15).
8 In this study, we used our serum- and feeder-free culture medium, which contains no
9 ECM and may have supported long-lasting cell patterning. Moreover, we added
10 γ -globulin to block vitronectin adhesion, and thus the cells could not have escaped even
11 if hPSCs had produced small amounts of ECM by themselves.

12 The second advantage is that the patterned PDMS sheets can be stored for at least 6
13 days before the cells are plated. We previously described micropatterned plasma
14 treatment using a physical mask (10). However, PDMS surfaces treated with plasma
15 generally regain their hydrophobicity within a few days (31, 32). In contrast, the protein
16 coating used in this study might have prevented migration of unreacted oligomers to the
17 outermost surface and enabled retention of the pattern for at least 7 days. Storability
18 enables transportation of patterned PDMS sheets to a remote location and permits quality
19 control because many patterned PDMS sheets of the same quality can be prepared in one
20 batch.

1 The third advantage is that the patterned surfaces are easy to prepare; the PDMS is
2 covered with a sterilized perforated mask, treated with plasma, and coated with a mixture
3 of γ -globulin and vitronectin. In contrast, microcontact printing, which is one of the
4 easiest conventional methods of patterning (36), requires more preparation steps
5 including fabrication of microstamps, surface modification with alkanethiols, uniform
6 inking on microstamps, and pressure-controlled printing. Both γ -globulin and vitronectin
7 are readily available and non-toxic. The protocol is simple enough for use in a standard
8 cell biology laboratory. Thus, our patterning method is practical for routine cell biology
9 experiments.

10 Generally, hPSCs form flat colonies on a dish, as do patterned colonies, but the colony
11 sizes are not well controlled, because pipetting is used to dissociate large colonies into
12 small colonies during passaging (1, 2, 26). Combination of our patterning method with
13 single-cell dissociation (12) may permit equalization of colony size and thus control of
14 self-renewal and differentiation of hPSCs. Moreover, our method may be applicable to
15 other types of ECM components and cells, because hiPSCs also adhere to ECM
16 components such as fibronectin and laminin, which can be coated on glass and on
17 plasma-treated polystyrene culture dishes, and many type of cells adhere to these ECM
18 components (11, 18, 40).

19 In summary, our serum- and feeder-free culture medium allowed us to elucidate the
20 relationships between plasma treatment, protein adsorption, and cell adhesion. We found
21 that γ -globulin prevented hiPSC adhesion to untreated PDMS surfaces by blocking

1 vitronectin adsorption. We used our findings to develop a practical cell-patterning
2 method that has three major advantages over previously reported methods. We believe
3 that the fundamental information about hiPSC adhesion to PDMS revealed by this study
4 will serve as a platform for the development of technology to control the
5 microenvironment of hPSCs.

6

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14

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21

1 **Figure legends**

2 **Figure 1. Effects of plasma treatment of PDMS surfaces**

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

8

9 **Figure 2. Vitronectin (Vn) and γ-globulin (G) adsorption on and hiPSC adhesion to**
10 **plasma-treated and untreated PDMS surfaces**

11 **(A–F)** Untreated and **(G–L)** plasma-treated PDMS surfaces coated with vitronectin alone
12 **(A, B, G, H)**, γ-globulin alone **(C, D, I, J)**, or a mixture of vitronectin and γ-globulin **(E,**
13 **F, K, L)**. **(A, C, E, G, I, K)** Double immunostaining of PDMS surfaces with
14 anti-vitronectin (green) and anti-γ-globulin (red) antibodies. **(B, D, F, H, J, L)**
15 Phase-contrast micrographs of hiPSCs cultured in hESF9a. **(M)** Adsorption ratio (θ) of
16 vitronectin and γ-globulin on PDMS.

$$\theta = \tan^{-1} \left(\frac{\text{anti-vitronectin (green fluorescence)}}{\text{anti-}\gamma\text{-globulin (red fluorescenc)}}$$

17 Fluorescence intensity is given in arbitrary units. Theta is <45° where the red
18 fluorescence (γ-globulin) is greater than the green fluorescence (vitronectin), and is
19 >45° where the green fluorescence (vitronectin) is greater than the red fluorescence

1 (γ -globulin). Data are means $\pm$ SE ($n = 3$). * $P < 0.05$, ns: not significant, Holm's
2 multiple comparisons. (N) Attached cells on a 2-mm circle estimated by means of
3 staining with calcein AM. PDMS surfaces were coated with vitronectin alone, both
4 vitronectin and γ -globulin, or γ -globulin alone. Data are means $\pm$ SE ($n = 3$). * $P < 0.05$,
5 ns: not significant, Holm's multiple comparisons.

6

7 **Figure 3. Patterning of hiPSCs by plasma patterning of PDMS surfaces and**
8 **subsequent coating with vitronectin (Vn) and γ -globulin (γ G)**

9 (A) Schematic of preparation of plasma-patterned, protein-coated PDMS. PDMS sheets
10 covered with a perforated mask (2-mm-diameter holes) were treated with plasma and
11 then coated with vitronectin alone (B, E), a mixture of γ -globulin and vitronectin (C, F,
12 H), or γ -globulin alone (D, G). (B–D) Immunostaining of PDMS sheets coated with
13 anti-vitronectin (green) and anti- γ -globulin antibodies (red). (E–H) Phase-contrast
14 micrographs of hiPSCs cultured for 1 day (E–G) or 7 days (H) in a defined culture
15 medium. (B-H) The scales are the same. (I) Phase-contrast micrograph of an hiPSC
16 colony without feeder cells on an unpatterned culture dish. (J) Immunostaining for
17 undifferentiated-cell marker antibodies 4 days after cell plating: anti-TRA1-60 antibody
18 (green), anti-SSEA3 antibody (green), and anti-SSEA4 antibody (green). Nuclei are
19 stained with DAPI (blue, lower panels).

20

Figure 4. Storability of plasma-patterned, protein-coated PDMS surfaces

(A) Schematic of pre-coat and post-coat storage experiments. Masked PDMS was treated with plasma and then either pre-coated with a mixture of γ -globulin (γ G) and vitronectin (Vn) approximately 30 min after plasma treatment and stored for 6 days (left) or post-coated with the protein mixture 5 days after plasma treatment (right). Immunostaining of pre-coated (B) and post-coated (D) PDMS by anti-vitronectin (green) and anti- γ -globulin antibodies (red). Phase-contrast micrographs of hiPSCs cultured on pre-coated (C) and post-coated (E) PDMS for 1 day in a defined culture medium.

Figure 5. Schematic of experimental results

(A) Plasma treatment of the PDMS surfaces decreased the number of methyl groups and increased the number of siloxane bonds, resulting in the formation of a siliceous surface (thus increasing hydrophilicity). (B) Coating PDMS with a mixture of vitronectin (Vn) and γ -globulin (γ G), but not with either protein individually, resulted in adsorption of γ -globulin on the untreated PDMS surface and adsorption of vitronectin on the plasma-treated PDMS surface. (C) Human iPSCs adhered to the vitronectin-rich plasma-treated surfaces but not to the γ -globulin-rich untreated surfaces; this difference resulted in hiPSCs patterning. Treating the surfaces with vitronectin and γ -globulin prior to storage permitted retention of the protein pattern for at least 6 days of storage. The hiPSCs did not escape from the patterned area for at least 7 days.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5