phenylindole (DAPI, Wako). Fluorescence micrographs were obtained with a BZ-8100 fluorescence microscope (Keyence, Osaka, Japan) and analyzed with Image J software (NIH).

RESULTS

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

Next we measured the absorbance of a PDMS thin film by means of FT-IR (Fig. 1B). Plasma treatment of PDMS caused a noticeable decrease in the absorbance bands at 800 cm^{-1} derived from CH_3 rocking and ≡Si–C stretching vibrations, indicating a decrease in the number of methyl groups. In contrast, two bands, centered at 1,026 and 1,070 cm^{-1} and attributable to asymmetric ≡Si–O–Si stretching vibrations, were increased by plasma treatment, indicating that the CH3–Si–O groups in PDMS underwent transformation to O–Si–O groups. Taken together, the changes in the FT-IR spectra suggest that a siliceous layer formed at the surface of PDMS film as a result of plasma treatment.

Adsorption of vitronectin on and adhesion of hiPSCs to plasma-treated and untreated PDMS surfaces We next investigated how vitronectin mediated hiPSC adhesion to the PDMS surfaces. It is well known that many types of adhesive cells can be cultured on a plasma-treated hydrophilic polystyrene dish, generally referred to as a tissue culture treated dish, but not on an untreated hydrophobic polystyrene dish. Moreover, formation of a siliceous layer by plasma treatment may facilitate hiPSC adhesion to PDMS surfaces coated with vitronectin, which mediates cell–glass adhesion (20). Thus we hypothesized that vitronectin coating would enable hiPSCs to adhere to plasma-treated PDMS surfaces but not to untreated PDMS surfaces. To test this hypothesis, we coated untreated and plasma-treated PDMS surfaces with vitronectin. Immunostaining with anti-

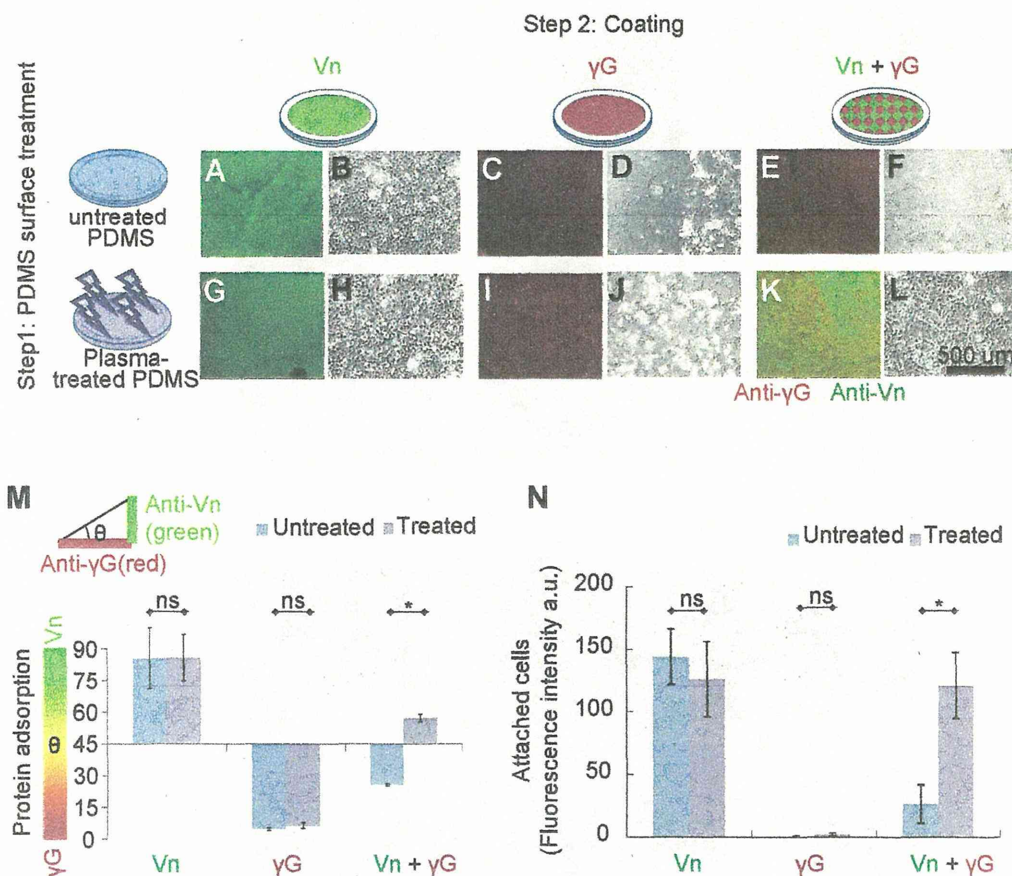

FIG. 2. Vitronectin (Vn) and γ-globulin (γG) adsorption on and hiPSC adhesion to plasma-treated and untreated PDMS surfaces. Untreated (A–F) and plasma-treated (G–L) PDMS surfaces coated with vitronectin alone (A, B, G, H), γ-globulin alone (C, D, I, J), or a mixture of vitronectin and γ-globulin (E, F, K, L). (A, C, E, G, I, K) Double immunostaining of PDMS surfaces with anti-vitronectin (green) and anti-γ-globulin (red) antibodies. (B, D, F, H, J, L) Phase-contrast micrographs of hiPSCs cultured in hESF9a. (M) Adsorption ratio (θ) of vitronectin and γ-globulin on PDMS. $\theta = \tan^{-1}$(anti – vitronectin (green flourescence)/anti-γ-gloubulin (red flourescence)). Fluorescence intensity is given in arbitrary units. Theta is <45° where the red fluorescence (γ-globulin) is greater than the green fluorescence (vitronectin), and θ is >45° where the green florescence (vitronectin) is greater than the red fluorescence (γ-globulin). Data are means ± SE ($n = 3$). *$P < 0.05$, ns: not significant, Holm's multiple comparisons. (N) Attached cells on a 2-mm circle estimated by means of staining with calcein AM. PDMS surfaces were coated with vitronectin alone, both vitronectin and γ-globulin, or γ-globulin alone. Data are means ± SE ($n = 3$). *$P < 0.05$, ns: not significant, Holm's multiple comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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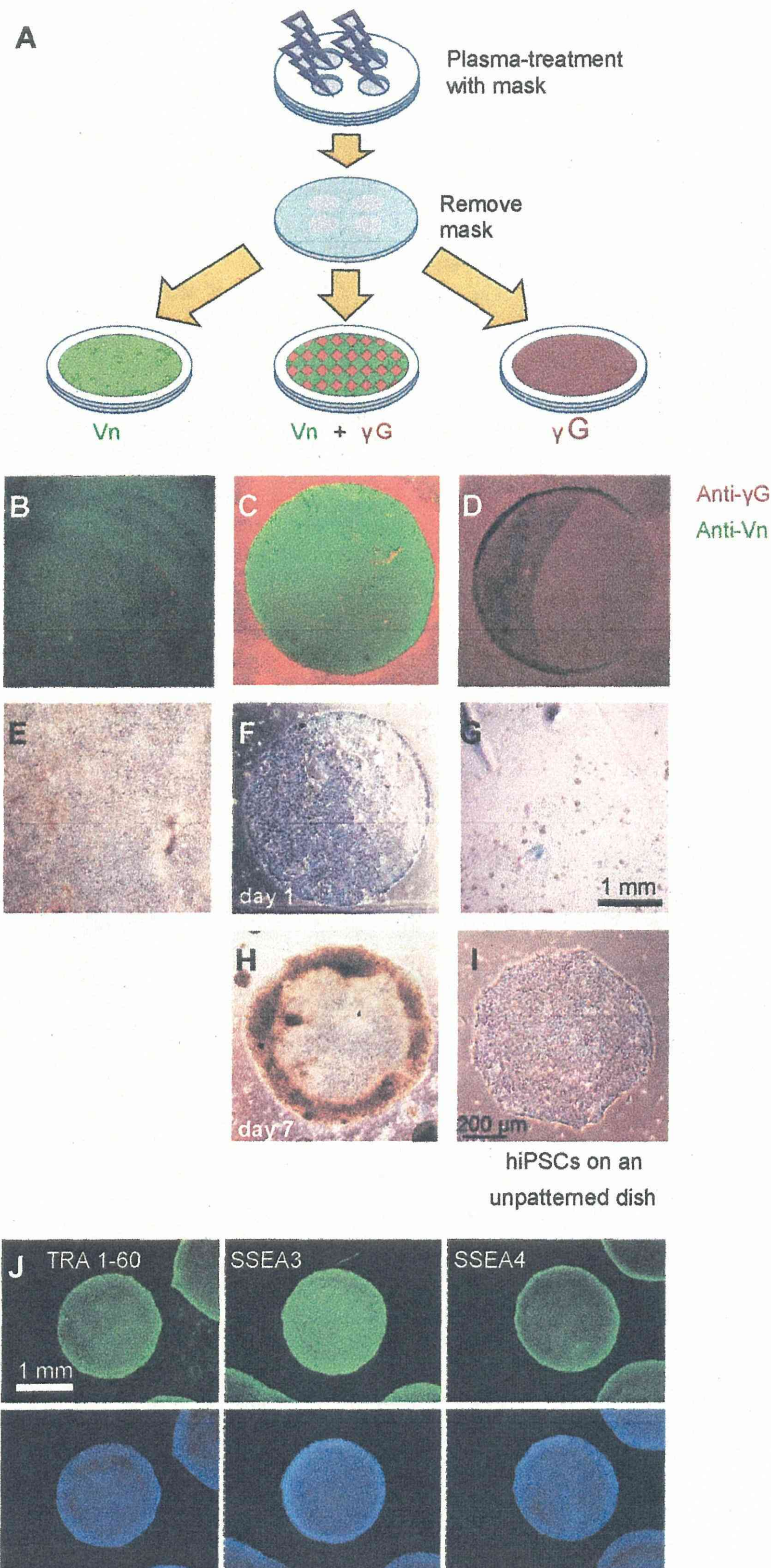

FIG. 3. Patterning of hiPSCs by plasma patterning of PDMS surfaces and subsequent coating with vitronectin (Vn) and γ-globulin (γG). (A) Schematic of preparation of plasma-patterned, protein-coated PDMS. PDMS sheets covered with a perforated mask (2-mm-diameter holes) were treated with plasma and then coated with vitronectin alone (B, E), a mixture of γ-globulin and vitronectin (C, F, H), or γ-globulin alone (D, G). (B–D) Immunostaining of PDMS sheets coated with anti-vitronectin (green) and anti-γ-globulin antibodies (red). (E–H) Phase-contrast micrographs of hiPSCs cultured for 1 day (E–G) or 7 days (H) in a defined culture medium. (B–H) The scales are the same. (I) Phase-contrast micrograph of an hiPSC colony without feeder cells on an unpatterned culture dish. (J) Immunostaining for undifferentiated-cell marker antibodies 4 days after cell plating: anti-TRA 1-60 antibody (green), anti-SSEA3 antibody (green), and anti-SSEA4 antibody (green). Nuclei are stained with DAPI (blue, lower panels). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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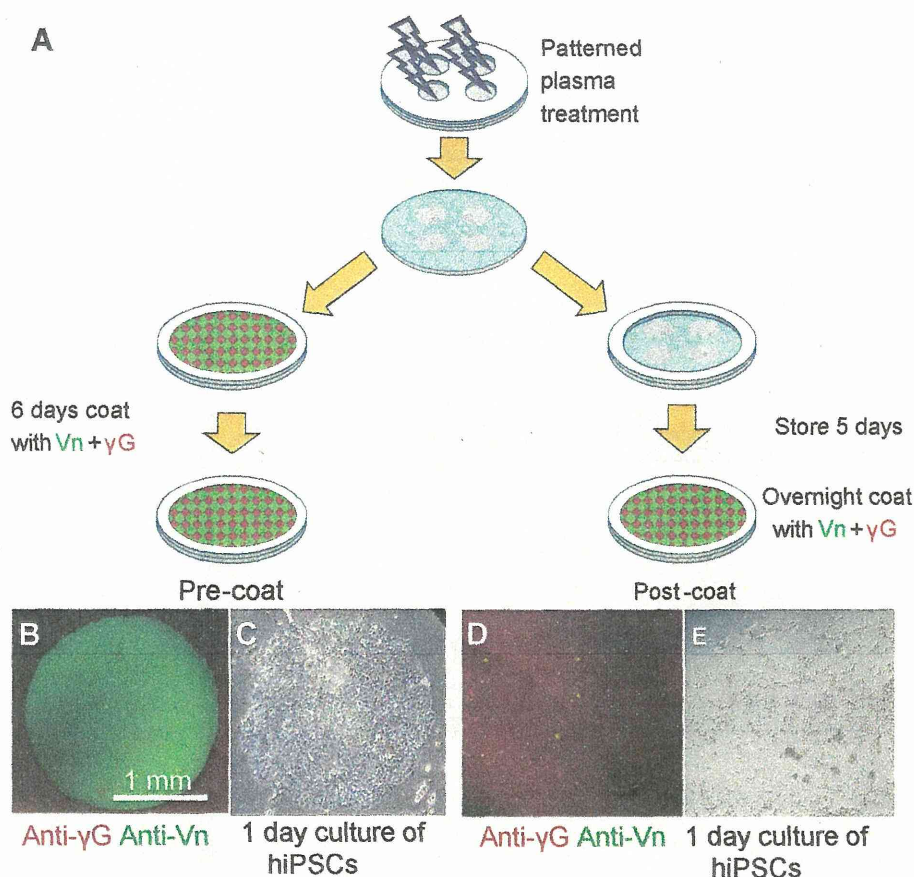

FIG. 4. Storability of plasma-patterned, protein-coated PDMS surfaces. (A) Schematic of pre-coat and post-coat storage experiments. Masked PDMS was treated with plasma and then either pre-coated with a mixture of γ-globulin (γG) and vitronectin (Vn) approximately 30 min after plasma treatment and stored 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vitronectin antibodies showed that vitronectin was adsorbed on both plasma-treated and untreated PDMS surfaces (Figs. 2A, G, M and S2) and that hiPSCs adhered to both surfaces (Figs. 2B, H, N and S2). Thus, these results did not support our initial hypothesis.

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

Patterning of hiPSCs on plasma-patterned PDMS surfaces coated with vitronectin and γ-globulin We speculated that our results might be useful for generating hiPSC patterning on PDMS surfaces, so we carried out the following experiments. We patterned a PDMS surface by applying a PDMS mask perforated with 2-mm-diameter holes and then treating the masked surface with plasma. After removal of the mask, the plasma-patterned surface was coated with vitronectin, γ-globulin, or both (Fig. 3A). Immunostaining showed that when the patterned surface was treated with either vitronectin alone or γ-globulin alone, the protein was adsorbed onto the entire surface (Fig. 3B, D). In contrast, when the patterned surface was treated with a mixture of the two proteins, immunostaining showed bright green staining in the circular (plasma-treated) areas and bright red staining in the surrounding (untreated) areas as shown in Fig. 2E, K, indicating that vitronectin and γ-globulin were preferentially adsorbed onto the plasma-treated and untreated areas, respectively (Fig. 3C). Human iPSCs adhered to the entire surface of PDMS coated with vitronectin alone but did not adhere to the surface coated with γ-globulin alone (Fig. 3E, G). In contrast, treatment of the patterned PDMS with a mixture of vitronectin and γ-globulin allowed hiPSCs to adhere to the plasma-treated circular areas (Fig. 3F), and the hiPSCs remained in those areas for at least 7 days (Fig. 3H). The morphology of patterned hiPSCs appeared to be that of normal hiPSC colonies (Fig. 3I); the

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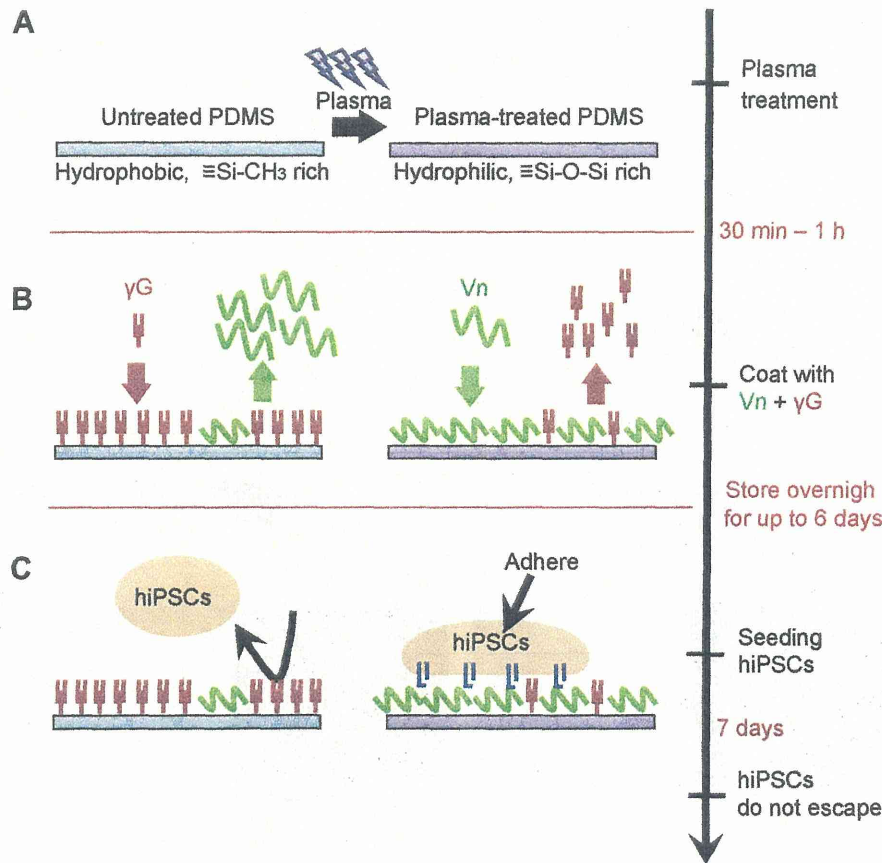

FIG. 5. Schematic of experimental results. (A) Plasma treatment of the PDMS surfaces decreased the number of methyl groups and increased the number of siloxane bonds, resulting in the formation of a siliceous surface (thus increasing hydrophilicity). (B) Coating PDMS with a mixture of vitronectin (Vn) and γ-globulin (γG), 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.

colonies were tightly packed and flat (monolayer) and consisted of cells with large nuclei and scant cytoplasm (1,2,26). Because the cells kept proliferating without escaping from the plasma-treated area, the edges of the colonies became thick (multilayer) and appeared dark under a phase-contrast microscope on day 7 (Fig. 3H). Immunostaining revealed that the patterned hiPSCs expressed undifferentiated-cell markers TRA 1-60, SSEA3, and SSEA4 (Fig. 3J). We obtained the same results by using another hiPSC line, 253G1 (Fig. S3). These results suggest that our method can be used to prepare hiPSCs on plasma-patterned PDMS surfaces.

Storage of plasma-patterned PDMS before plating of cells Next we determined whether the cell patterning would form if the PDMS was stored for several days after plasma patterning. It would be convenient if patterned PDMS sheets could be prepared several days before cells were plated because this would enable batch production of many PDMS plates of the same quality. Although the plasma-hydrophilized surface of the PDMS recovered its hydrophobicity 5 days after plasma treatment (Fig. 1A), the hiPSCs retained the pattern for 7 days (Fig. 3H), which suggests that coating the surface with vitronectin and γ-globulin 30 min after plasma treatment enabled retention of the plasma pattern for 7 days. To test this, we evaluated two preparation methods: a pre-coat method and a post-coat method. In the pre-coat method, PDMS surfaces were plasma-patterned on day 0, the patterned PDMS was coated with vitronectin and γ-globulin 30 min after plasma treatment, and the coated PDMS was then stored in a CO_2 incubator for 6 days (Fig. 4A). In the post-coat method, the PDMS surfaces were plasma-patterned on day 0, stored in a CO_2 incubator for 5 days, and coated with vitronectin and γ-globulin for overnight (Fig. 4A). Under the pre-coat conditions, vitronectin adhered to the circular areas, which were surrounded by 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 are 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 ≡Si–O–Si≡ 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 ≡Si–OH and ≡Si–O–Si≡ groups on the surfaces (28). In our present study, the absorbance band due to ≡Si–O–Si≡ in

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

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

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

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

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

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

In summary, our serum- and feeder-free culture medium allowed us to elucidate the relationships between plasma treatment, protein adsorption, and cell adhesion. We found that γ-globulin prevented hiPSC adhesion to untreated PDMS surfaces by blocking vitronectin adsorption. We used our findings to develop a practical cell-patterning method that has three major advantages over previously reported methods. We believe that the fundamental information about hiPSC adhesion to PDMS revealed by this study will serve as a platform for the development of technology to control the microenvironment of hPSCs.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2014.02.009.

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