入手も早い。iPS細胞作成という素晴らしい日本における発明が世界に先駆けて産業応用されるために、横並びで国内企業が互いに牽制しあうようなムードは撤廃し、各企業のオリジナリティーをいかしてアカデミアと連携して幹細胞実用化に向けて進むことを願ってやまない。

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生命と医療の倫理学　第2版

現代社会の倫理を考える〈第2巻〉

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要旨　初版刊行から10年が経ち、医療倫理を巡る情勢の激変に伴い全面改訂。とりわけ「終末期医療の決定プロセスに関するガイドライン」等、終末期医療ガイドラインの整備や、ヒトゲノム・遺伝子解析研究に関する三省指針改正、臓器の移植に関する法律の改正等、最新の状況を反映した内容に改めた。また未曽有の大震災の経験から、危機管理に関する内容を加え、医療現場スタッフの便も図った。
目次　第1章 患者の権利、医師の裁量、臨床倫理の原則、チーム医療、医療安全；第2章 告知、インフォームドコンセント；第3章 医療情報開示、個人情報保護；第4章 臨床試験、GCP、利益相反；第5章 遺伝子診断、遺伝子治療、遺伝カウンセリング、分子標的治療；第6章 クローン技術、幹細胞研究、再生医療、生殖補助医療、エンハンスメント；第7章 脳死、臓器移植；第8章 緩和ケア、QOL；第9章 終末期医療、安楽死、尊厳死、自殺幇助、生命維持治療の不開始・中止；第10章 平時と大災害の医療
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命を守る材料－人工血管から再生医療の最先端へ　東京理科大学坊っちゃん科学シリーズ〈3〉

編者／東京理科大学出版センター
著者／菊池明彦、曽我公平、牧野公子、柴建次、大塚英典
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要旨　人工血管、人工心臓、人工関節、金ナノ粒子による診断と治療、ドラッグデリバリーシステム、そして、最先端の再生医療を解説。
目次　第1章 血液に触れて使われる材料（人工血管;人工心臓 ほか）；第2章 骨などの硬い組織に用いられる材料（人工関節-金属・無機・高分子からなる複合材料；人工骨材料-セラミックスの多孔性材料）；第3章 診断に用いられる材料（ナノ粒子による蛍光バイオイメージング；金ナノ粒子による診断と治療 ほか）；第4章 ドラッグデリバリーシステム；第5章 再生医工学（組織工学の手法を用いた再生医療；生分解性材料を用いる再生医療 ほか）
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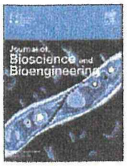

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Control of adhesion of human induced pluripotent stem cells to plasma-patterned polydimethylsiloxane coated with vitronectin and γ-globulin

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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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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-

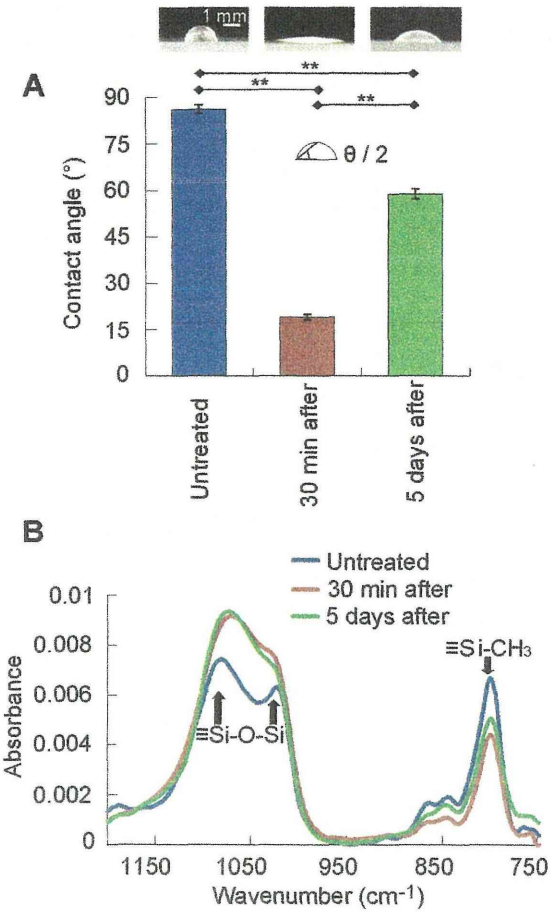

FIG. 1. Effects of plasma treatment of PDMS surfaces. (A) Contact angle (θ) of water. Data are means ± SE ($n = 5$). ** $P < 1 \times 10^{-10}$, Tukey's multiple comparison. Insets are photographs of 2 μL water droplets. (B) FT-IR absorbance spectra of PDMS before plasma treatment (blue) and 30 min (red)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

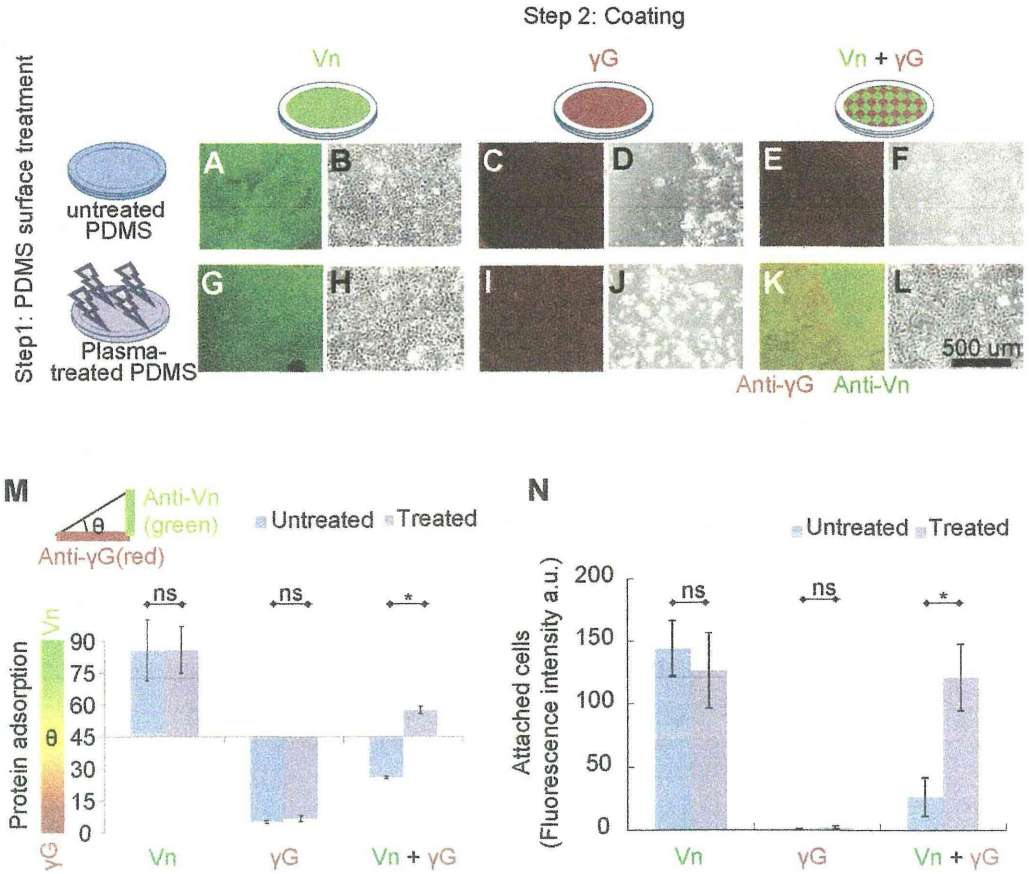

FIG. 2. Vitronectin (Vn) and γ-globulin (γG) adsorption on and hiPSC adhesion to plasma-treated and untreated PDMS surfaces. Untreated (A—F) and plasma-treated (G—L) PDMS surfaces coated with vitronectin alone (A, B, G, H), γ-globulin alone (C, D, I, J), or a mixture of vitronectin and γ-globulin (E, 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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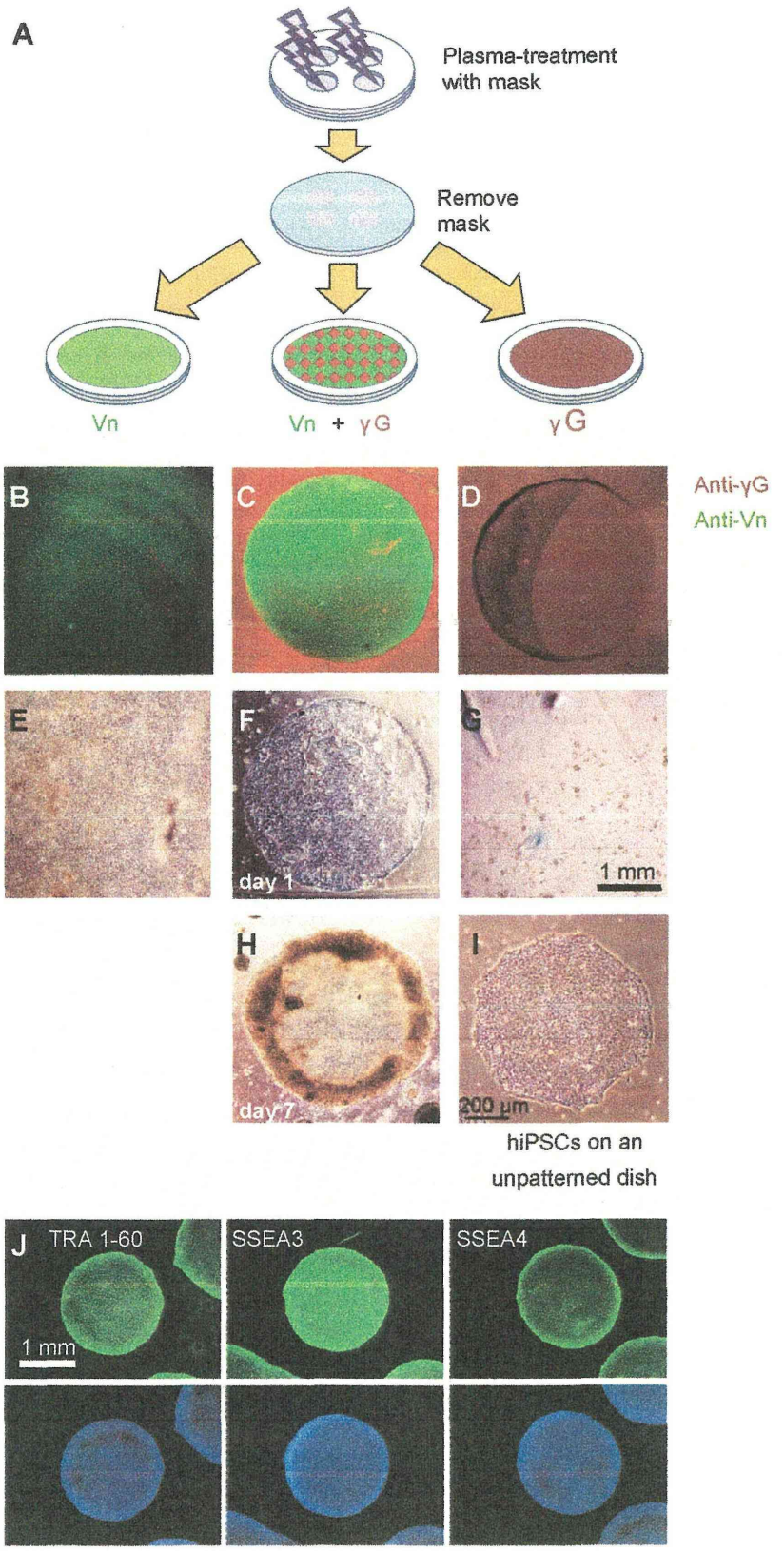

FIG. 3. Patterning of hiPSCs by plasma patterning of PDMS surfaces and subsequent coating with vitronectin (Vn) and γ-globulin (γG). (A) Schematic of preparation of plasma-patterned, protein-coated PDMS. PDMS sheets covered with a perforated mask (2-mm-diameter holes) were treated with plasma and then 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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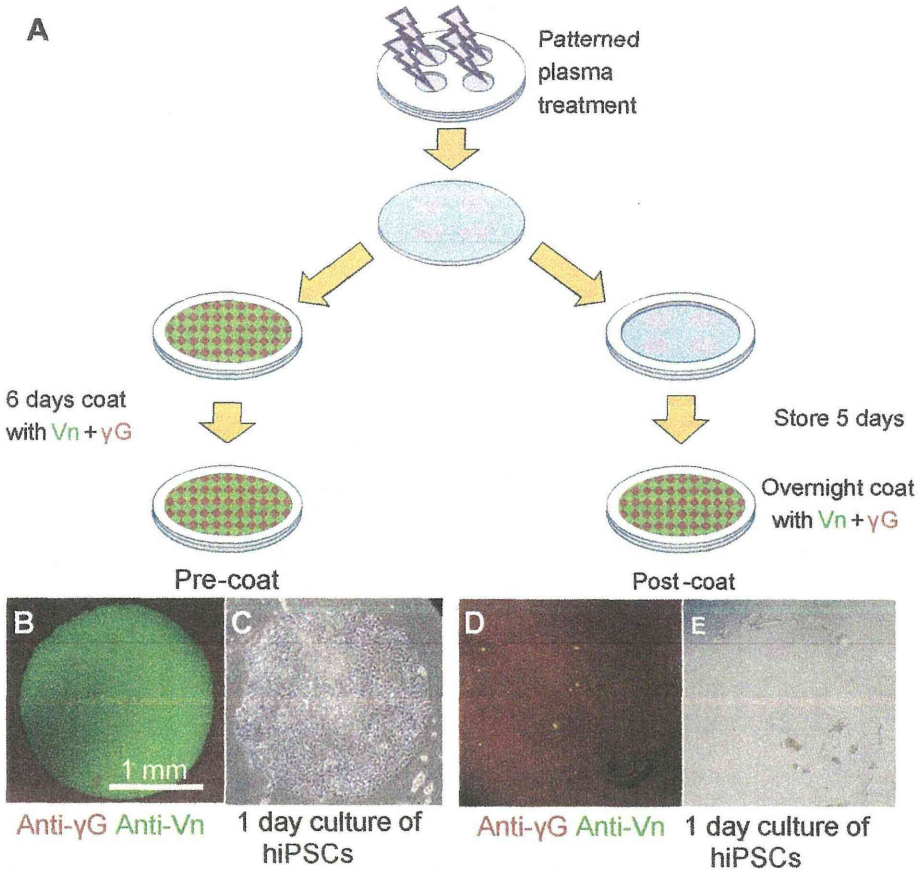

FIG. 4. Storability of plasma-patterned, protein-coated PDMS surfaces. (A) Schematic of pre-coat and post-coat storage experiments. Masked PDMS was treated with plasma and then either pre-coated with a mixture of γ-globulin (γG) and vitronectin (Vn) approximately 30 min after plasma treatment and stored 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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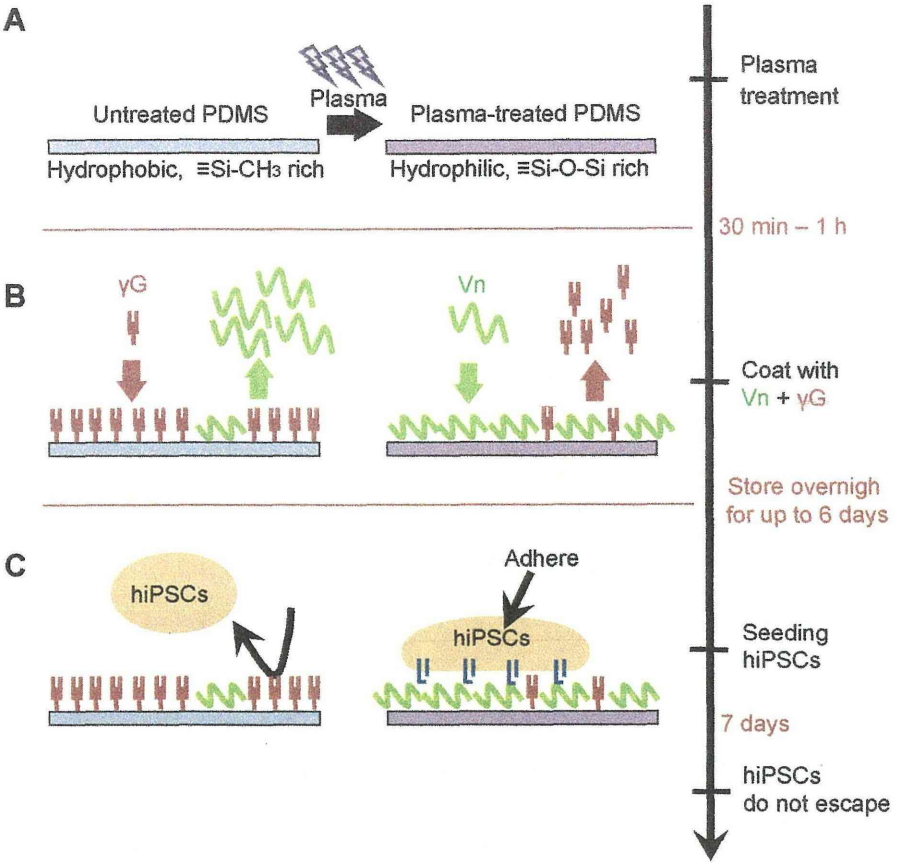

FIG. 5. Schematic of experimental results. (A) Plasma treatment of the PDMS surfaces decreased the number of methyl groups and increased the number of siloxane bonds, resulting in the formation of a siliceous surface (thus increasing hydrophilicity). (B) Coating PDMS with a mixture of vitronectin (Vn) 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 ≡Si–CH_3 by singlet oxygen radical species. Oxidization of PDMS film surfaces by exposure to vacuum ultraviolet light (λ = 172 nm) has been reported to reduce the number of ≡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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