Surface-engineered substrates for improved human pluripotent stem cell culture under fully defined conditions

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The current gold standard for the culture of human pluripotent stem cell lines requires the use of a feeder layer of cells. Here, we develop a spatially defined culture system based on UV/ozone radiation modification of typical cell culture plastics to define a favorable surface environment for human pluripotent stem cell culture. Chemical and geometrical optimization of the surfaces enables control of early cell aggregation from fully dissociated cells, as predicted from a numerical model of cell migration, and results in significant increases in cell growth of undifferentiated cells. These chemically defined xeno-free substrates generate more than three times the number of cells than feeder-containing substrates per surface area. Further, reprogramming and typical gene-targeting protocols can be readily performed on these engineered surfaces. These substrates provide an attractive cell culture platform for the production of clinically relevant factor-free reprogrammed cells from patient tissue samples and facilitate the definition of standardized scale-up friendly methods for disease modeling and cell therapeutic applications.

Results

UV Treatment to Generate Optimal Surface Chemistry. hESCs and hiPSCs aggregate and undergo apoptosis when passaged as single cells under conventional culture conditions (22, 23). We hypothesized that spatial control of the plating surface chemistry could influence early aggregation and could lead to improved cell propagation. Therefore, we sought to develop methods that would allow the rapid spatially controlled generation of chemically optimized surfaces for hESC/hiPSC propagation. We focused our efforts on polystyrene, the most commonly used plastic in cell culture. Recently, hundreds of polymers were evaluated to define the chemical and material properties of polymers that support the long-term culture of fully dissociated hESCs/hiPSCs (11). In these experiments, a distinct polymer surface chemistry was found to correlate with undifferentiated hESC growth, whereas indentation elastic modulus or roughness properties of biomaterials had less pronounced effects on growth (11). To generate optimized polymer surface chemistry [characterized by time of flight secondary ion mass spectrometry (ToF-SIMS) analysis by certain oxygen-containing ions and hydrocarbon ions]...

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virgin polystyrene was treated with short-wavelength UV (Fig. 1A). Polystyrene surfaces treated with different doses of UV were analyzed using ToF-SIMS (Fig. S1) to reveal mass spectral information from their topmost layers (~10 Å) (24). Comparative analysis of these spectra (Figs. S2 and S3 A and B) confirmed that UV treatment yields chemically distinct surfaces from both virgin polystyrene and conventional tissue culture polystyrene (TCPs). Further, these results were consistent with surface elemental and functional composition resolved by X-ray photoelectron spectroscopy (XPS; Fig. 1 B and C). To test the effect of UV treatment on cell growth, we cultured fully dissociated transgenic Oct4-GFP-positive BG01 hESCs on the surfaces generated from different doses and precoated with fetal bovine serum (FBS) (Fig. 1E). hESCs robustly grew on moderately treated (1.5–3 min) polystyrene surfaces (Fig. 1C), whereas no cells attached to virgin polystyrene surface (Fig. 1B) and significantly fewer cells grew on TCPs (Fig. 1D).

To identify specific functionalities generated by UV that were important for colony formation, a numerical model was developed using a multivariate chemometric technique (25). This technique, using partial least squares (PLS) regression, is particularly useful when analyzing high-dimensional datasets like ToF-SIMS spectra to identify salient features that correlate with another variable. In Fig. 1E, Inset, we correlated surface chemistry contained in the spectra of various polystyrene surfaces to the number of hESC colonies observed on the surfaces. Good agreement between the measured number of colonies and that predicted from the PLS model developed from the ToF-SIMS spectra was seen ($R^2 = 0.87$). In the PLS model, each secondary ToF-SIMS ion from the UV-treated surfaces was listed with its regression coefficient, a quantitative measure of its contribution to hESC colony formation. Further, some secondary ions were identified to associate with certain classes of surface chemical functionality (Fig. 1F; an expanded list is provided in Fig. S3C).

The assignments of surface chemical functionalities were also supported by XPS (Fig. 1 B and C) and are consistent with previous attachment studies involving UV treatment of virgin polystyrene (26). Consistent with the results from the polymer array (11), robust self-renewal of hESCs was supported by ester/carboxylic acid functionalities yielding oxygen-containing ions in the ToF-SIMS spectra (Fig. 1F and Fig. S3C). In particular, polystyrene surfaces treated with UV for 1.5–3 min produced high intensities of several secondary ions in the ToF-SIMS experiments that were identified to support hESC colony formation (e.g., C$_2$H$_4$O$^+$ and C$_2$H$_6$N$^+$ in Fig. S2).

**Increased Number of hESCs/hiPSCs During Short-Term Culture.** At the optimal 2.5-min dose, UV-treated polystyrene (UVPS) provides an attractive platform for stem cell culture. hESCs could be readily passaged directly from standard mEF substrates to UVPS, and vice versa, without any significant cell death, indicating that hESCs do not require an adaptation period to be cultured on UVPS. When coated with human serum, UVPS supported clonal growth in serum-free mTeSR1 media (Stem Cell Technologies) at an efficiency of 30 ± 12%, which is comparable to previous acrylate-based polymer results (11), and cells were highly Oct4-GFP-positive after 7 d of culture (Fig. S4A). Media in these experiments were supplemented with rho-associated kinase (ROCK) inhibitor Y-27632 (Stemgent) for the first 8–12 h of culture to reduce initial apoptosis of completely dissociated hESCs (27). Similar to behavior seen on the best acrylate-based polymers (11), blocking the vitronectin-binding integrin αvβ3 resulted in a significant decrease in day 1 cell adhesion, whereas blocking a matrigel-binding (28) integrin β1 had no effect (Fig. 1G). When surfaces were coated with varying concentrations of a recombinant vitronectin solution in PBS, robust growth occurred above a threshold of 100 ng/cm$^2$ (Fig. S4B). Because clonal cell seeding and extremely high dilution ratios during passaging can lead to the outgrowth of genetically abnormal hESCs (29, 30), we tested the ability of UVPS to support the culture of fully dissociated hESCs at the typical 1:3 dilution ratio used during conventional ESC passaging. At these relevant conditions, UVPS supported about three times more cells per area than traditional feeder-containing mEF substrates (Fig. 1H). These results indicate that the surface chemistry generated by optimal UV treatment, like the surface chemistry of acrylate polymer hits (11), outperforms mEF substrates, the gold standard for hESCs.
Spatial Patterning. After 7 d of culture on UVPS, we observed the appearance of a few scattered large colonies (>1,200 μm in diameter) that were heterogeneous in pluripotency marker expression (Fig. S5A), indicating differentiation at the interior of the colony. To suppress the formation of these large colonies, we spatially controlled UV treatment by inserting a photomask between the polystyrene and radiation source (Fig. 2A). UV treatment generated a chemically heterogeneous surface as assayed by ToF-SIMS surface chemical analysis (Fig. 2B). Features down to ~30 μm could be reliably generated by this masking technique (Fig. 2B). Spatially patterned UV treatment of polystyrene constrained cell migration and adhesion, such that hESCs attached and grew only on the areas of the dish that received UV treatment when precoated with FBS (Fig. 2A). Areas of the dish that were blocked from the UV treatment by the photomask did not support colony growth. Similar results were seen with five other human pluripotent stem cell lines, including three hiPSC lines (Fig. S5B), and when human serum or human vitronectin was used instead of FBS to coat the UV-patterned surfaces (Fig. S5 C and D).

Spatially patterned UV treatment could also generate robust substrates on a hydrogel-coated surface that supported robust colony formation and cell patterning (Fig. S6C). Like the polystyrene surfaces (Fig. 1E), an intermediate dose of UV on two common research materials, polypropylene and hydrogel-coated surfaces, optimally supported colony formation from fully dissociated hESCs (Fig. S6D). Similar surface chemical functionalities supported colony growth on UVPS and polypropylene surfaces (Fig. 1F and Fig. S6D).

In contrast to UVPS without any spatial patterning, on the UV-patterned surfaces with 150- to 450-μm diameter spots, cells showed uniform staining of all pluripotency markers tested (Fig. 2C), and subsequent single-cell flow cytometric analysis indicated a small but significant decrease in the number of differentiating GFP-negative cells on a 300-μm spot pattern (Fig. S6E). Further, we detected poor spatial patterning when spot spacing was less than 200 μm, because cells could bridge the gaps between the UV-treated spots (Fig. S6F). Some of these bridged cells also exhibited low levels of pluripotency markers and exhibited differentiated cell morphology (Fig. S6F). Cells could be patterned in various geometries with sharp edges and curves (Fig. 2D) and could support more cells per well than traditional feeder-containing culture systems (Fig. S7 A and B). This behavior was consistent in two serum-free media formulations (Fig. S7C).

To gain insight into the spatial patterning parameters that control hESC behavior, we generated patterns of varying spot diameter but with the same cumulative UV-treated growth area per well. In this series of patterns, spot diameters varied from 300 to 20,600 μm (additional characteristics are presented in Fig. S10A). As before, we seeded fully dissociated hESCs on these patterns at a density typically used during routine cell culture. As the growth area became progressively partitioned into smaller and larger spots, similar numbers of cells were observed initially 24 h after seeding (Fig. S8A), although more cells were found in large cell aggregates as spot diameters became larger (Fig. S8 B and C). After an additional 7 d of growth, cultures with the smaller spot diameters contained more undifferentiated cells (Fig. 2E) and proliferated faster on average, as indicated by a shorter doubling time (Fig. S8D).

Modeling Cell Movements on Patterned Substrates. To probe why large cell aggregates preferentially form on larger spots, we modeled cell movements on patterned substrates through computer simulations (i.e., in silico) using the following assumptions. Cells were modeled to migrate on the surfaces through a random walk in the absence of neighboring cells, whereas in the presence of neighboring cells, single cells and cell aggregates preferentially migrate toward each other, as observed previously (31). Snapshots of cell migration behavior in silico on small and large spots (300 vs. 1,400 μm in diameter) indicate that the majority of cells form aggregates within 2–3 h postseeding (Fig. 3A and Fig. S9A). This time scale of aggregate formation was consistent with live imaging of cells in vitro on UV pattern (Movie S1). The number of cells in each aggregate after an additional 21 h of migration in silico was distributed differently between the small and large spot sizes (Fig. 3B). The 1,400-μm large spot pattern contained a long tail distribution of many aggregates of five cells or larger, whereas the 300-μm small spot pattern had a narrower distribution with more cells in smaller aggregates. Simulation results indicate that almost all the cells participate in colony formation, because the percentage of cells that are single is negligible (Fig. 3C) when seeded at a typical density during routine cell culture (60,000 cells per well in a 6-well plate). This result was further supported by the experimental data (Fig. S8C), and similar
results were obtained from simulations of reduced cell migration in media with ROCK inhibitor (Fig. S9 B and C). In summary, both experimental and modeling results suggest that spots with a diameter of 300 μm control hESC colony formation by preventing the formation of large cell aggregates soon after seeding, which leads to lower average rates of growth (Fig. S8D).

**Long-Term Culture on Patterned Substrates.** UV-patterned polystyrene was subsequently tested for its ability to support long-term culture. Cells after prolonged culture (>10 passages, 2 mo) on UV-patterned polystyrene using conventional collagenase or mechanical procedures were found to maintain an undifferentiated state at each passage (Fig. 4A and B) and a normal karyotype (Fig. S10B). Further, cells after long-term culture robustly expressed all the pluripotency markers tested, including SSEA-4, Sox2, and Nanog (Fig. 4C). Derivatives of all three embryonic germ layers were seen in teratoma assays (Fig. 4D), demonstrating that hESCs cultured on the UV-patterned polystyrene maintain their full pluripotent potential. Routine cell culture with collagenase passing yielded significant increases in the number of undifferentiated cells over mEFs that increased from passage to passage, indicating its utility for scale-up applications (Fig. 4E). Similar results were seen with two other hiPSC lines after >10 passages on UV-patterned polystyrene, where >80% of cells were positive for Tra-1-60 and >90% of cells were positive for SSEA-4 (Fig. 4F).

**Clonal Growth During Gene Targeting and Reprogramming.** To examine whether these substrates could support clonal outgrowth of gene-targeted cells, hESCs on UV-patterned substrates were subjected to targeting of the AAVS1 locus using zinc-finger nuclease (ZFN)-mediated genome editing (Fig. 5A). Using previously described ZFN pairs and constructs (15), we targeted to the AAVS1 locus a donor plasmid expressing GFP under the control of the constitutively active CAGGS promoter. The targeting construct contained a splice acceptor-2A-puromycin selection cassette, and after using electroporation to introduce the constructs into hESCs, cells were grown in puromycin on UV-patterned polystyrene. After 2 wk of puromycin selection, we isolated clones that remained brightly GFP-positive after more than 2 mo of culture (Fig. 5A).

Given the ability of UV-patterned substrates to support hESC/hiPSC self-renewal with human serum, we also sought to reprogram human fibroblasts on UV-patterned substrates under xeno-free conditions. Fibroblasts established from two patient skin punch biopsies were infected with a Cre recombinase (Cre)-excisable vector carrying the reprogramming factors on UV-patterned polystyrene coated with human serum (Fig. 5B). After 4 wk of culture in serum-free mTeSR1 media, several colonies emerged (Fig. 5B) and hiPSC lines could be established that robustly expressed the pluripotency markers (Fig. 5C). Because residual expression of integrated copies of reprogramming factors can affect the gene expression and biological properties of hiPSCs (3, 32), the factors were excised after expressing Cre recombinase. When grown on UV-patterned polystyrene, >90% of the vector-free cells expressed several pluripotency markers (Fig. 5D). Also, the vector-free reprogrammed cells generated derivatives of all three embryonic germ layers in teratoma assays (Fig. 5E), demonstrating that the cells were fully pluripotent.
after reprogramming and excision of the reprogramming factors on UV-patterned substrates. Given that human serum can be readily replaced with recombinant human vitronectin, the UV-patterned substrate supports reprogramming and gene modification of human pluripotent stem cells. Images of BG01 hESCs (A) and patient-237 hiPSCs (B) cultures on “UV-Pattern” (as described in Fig. 2A) after 7 and 27 passages using single-cell accutase dissociation. The image of hESCs at passage (p) 7 in A contains an overlay of a fluorescent image indicating high expression of Oct4-GFP. Immunostaining of patient-237 hiPSCs at passage 27 indicates expression of the pluripotency marker Nanog (green) in all cell nuclei and high expression of SSEA-4 (red). Surfaces were precoated with 20% (vol/vol) bovine serum, and cells were seeded in ROCK inhibitor for the first 12 h. (C) Flow cytometry of BG01 hESCs with the Oct4-GFP reporter after three consecutive passages on UV-Pattern with accutase. (D) Flow cytometry of cells for pluripotency markers SSEA-4 and Tra-1-60, after >10 consecutive passages on UV-Pattern for five different cell lines. “A” indicates accutase-mediated passaging. For the transgenic Oct4-GFP BG01 cells passaged on mEFs, only GFP-positive cells were analyzed for Tra-1-60 and SSEA-4 expression. Therefore, mEFs were excluded from the analysis. (E) Normal 46.XY karyotype was maintained for patient-237 hiPSCs propagated on UV-Pattern for more than 5 mo (27 passages). (F) Design parameters for developing a UV-treated culture system for human pluripotent stem cells.

**Fig. 5.** UV-patterned substrate supports reprogramming and gene modification of human pluripotent stem cells. (A) Phase-contrast images of BG01 hESCs on UV-patterned substrates (300-μm spot diameter with 400-μm spacing) after electroporation of CAAGS-GFP targeting and ZFN plasmids. Cells were seeded in ROCK inhibitor for the first 8–12 h after electroporation. A successfully targeted clone would be moved to mEFs and was highly GFP-positive after >2 mo of culture. (B) Phase-contrast and immunostained images of “patient-237” fibroblasts on UV-patterned polystyrene infected with a loxP-flanked version of the pFlag-STEMCCA vector, which is a Cre-excisable polyclonal vector encoding the reprogramming factors. The patterned surface was precoated with human serum, and the high serum content of the initial fibroblast media allows fibroblasts to adhere to the untreated areas of the dish. Over 4 wk, cells change morphology and form hiPSC colonies (arrow) on the substrates (10-cm dish, 300-μm spacing) for 4 wk. (C) Pluripotency marker immunostaining of patient-237 hiPSC line on “UV-Pattern” (as described in Fig. 2A). (D) Southern blot analysis for Klf4 on genomic DNA from several patient-237 hiPSC lines after Cre-recombinase expression. Cell lines labeled in red indicate excision of reprogramming vector; these clones show loss of viral KLF4 bands (lower band) on Cre expression, indicating that vector-free hiPSCs can be clonally isolated postexcision. The bar graph on the right indicates % of Max expression. Therefore, mEFs were excluded from the analysis. (E) Nanog / Nuclei SSEA-4 / Nuclei Smooth muscle Neuroepithelium Mesoderm Ectoderm Endoderm Intestinal epithelium Phase CAAGS-GFP

**Fig. 6.** UV-patterned substrate supports single-cell passaging of human pluripotent stem cells. Images of BG01 hESC (A) and patient-237 hiPSC (B) cultures on “UV-Pattern” (as described in Fig. 2A) after 7 and 27 passages using single-cell accutase dissociation. The image of hESCs at passage (p) 7 in A contains an overlay of a fluorescent image indicating high expression of Oct4-GFP. Immunostaining of patient-237 hiPSCs at passage 27 indicates expression of the pluripotency marker Nanog (green) in all cell nuclei and high expression of SSEA-4 (red). Surfaces were precoated with 20% (vol/vol) bovine serum, and cells were seeded in ROCK inhibitor for the first 12 h. (C) Flow cytometry of BG01 hESCs with the Oct4-GFP reporter after three consecutive passages on UV-Pattern with accutase. (D) Flow cytometry of cells for pluripotency markers SSEA-4 and Tra-1-60, after >10 consecutive passages on UV-Pattern for five different cell lines. “A” indicates accutase-mediated passaging. For the transgenic Oct4-GFP BG01 cells passaged on mEFs, only GFP-positive cells were analyzed for Tra-1-60 and SSEA-4 expression. Therefore, mEFs were excluded from the analysis. (E) Normal 46.XY karyotype was maintained for patient-237 hiPSCs propagated on UV-Pattern for more than 5 mo (27 passages). (F) Design parameters for developing a UV-treated culture system for human pluripotent stem cells.

**Single-Cell Enzymatic Passaging.** Lastly, UV-patterned substrates also supported the single-cell enzymatic passaging of hESCs with minimal use of small-molecule supplements. Current hESC/hiPSC passaging protocols use collagenase and/or mechanical dissociation to generate multicellular aggregates that vary in size, whereas dissociation into single cells using proteolytic enzymes (e.g., trypsin, accutase) greatly reduce this variability. Although dissociated hESCs/hiPSCs self-renew inefficiently, with the majority of single cells undergoing apoptosis (22, 23), we reasoned that the rapid establishment of multicellular aggregates on the patterned surfaces within 2–3 h after cell seeding could reduce the single-cell apoptosis and promote colony formation. Using accutase enzymatic dissociation to single cells, cultures could be consistently passaged at a 1:3 dilution every 5–6 d for both hESCs (Fig. 6A) and hiPSCs (Fig. 6B), whereas ROCK inhibitor was required only briefly for 5–12 h after dissociation. Cells robustly maintained expression of pluripotency markers consistently from passage to passage (e.g., Fig. 6C) and after long-term culture for five different cell lines (Fig. 6D). Karyotype analysis confirmed that all five cell lines had the correct number of chromosomes after >10 passages; in particular, patient-237 hiPSCs maintained a normal karyotype for more than 5 mo in culture (Fig. 6E). We note that using extremely harsh passaging conditions (e.g., using long incubations in trypsin, entirely omitting use of ROCK inhibitor postseeding) resulted in cultures with genetic abnormalities (Fig. S1B) as previously described (29, 30). We conclude that single-cell passaging on UV-patterned substrates can remove much of the variability in hESC/hiPSC culture generated by incomplete dissociation during mechanical or collagenase passaging, thus creating a standardized culturing system (e.g., Fig. 6C).
Discussion

Through this work, we were able to generate substrates that replace or outperform mEF substrates, the gold standard in human pluripotent stem cell culture, in several key applications. The UV treatment itself without patterning creates a surface chemistry that supports at least threefold more cells per area (Fig. 1H and Table S7A) and creates a least twofold more colonies per cell seeded than conventional cell culture plastics (Fig. 1D). During routine cell culture, these differences grow exponentially from passage to passage and lead to even larger increases in undifferentiated cell number (Fig. 4E). Spatial patterning of the UV treatment can further increase the number of undifferentiated cells per growth area (Fig. 2E). By optimizing the surface treatment, we identified several parameters that were critical to the success of using UV treatment of polystyrene for hESC/hiPSC culture (Fig. 6F). The dose of UV was determined such that suitable amounts of carboxylic acid/ester and nitrogen-containing moieties were generated on the surface, whereas both under- and overexposure to UV led to undesirable surface chemistry (Fig. 1 E and F). Spatial patterning of UV treatment could constrain hESC/hiPSC colony size in two dimensions; however, hESC/hiPSCs could only be consistently patterned into spots when spacing was greater than 100 μm (Fig. 3F). We consider several possibilities to explain these observations. Optimal spatial pattern of UV treatment may (i) establish favorable gradients of any factors guiding preferential migration toward other hESC/hiPSCs (31), (ii) improve crosstalk between soluble factors and mechanical signal transduction (33), and (iii) facilitate the previously noted preference for hESC/hiPSCs to be in cell-cell contact (22, 23, 34). Constraining cells to spots of small diameters prevented large multicellular aggregates from forming after seeding (Fig. S8 B and C), leading to higher levels of proliferation of undifferentiated cells (Fig. S8D). Small diameters, however, constrain the maximum colony size before cells are passaged (Fig. S7A). This tension spot diameter could support most applications of routine cell culture with hESCs/hiPSCs (Figs. 4–6), and similarly sized colonies were routinely observed on standard feeder substrates before they needed to be passaged (Fig. S10C). It should be possible to produce such plates in many different formats (e.g., multwell plates, dishes) economically, because UV treatment does not require the relatively expensive gas handling and vacuum processing equipment used to manufacture standard tissue culture dishes.

Because use of the engineered substrates did not require any special steps or adaptation from cultures using existing feeder substrates, the surface treatments described here would likely integrate well with many existing protocols of manipulating human pluripotent stem cells. Further, the treated surfaces represent an important advance over the gold standard feeder substrates because they are fully defined synthetic substrates that enhance propagation of undifferentiated cells and support the long-term cell culture, clonal outgrowth of hESCs/hiPSCs, and reprogramming of human somatic cells. These surface-engineered substrates therefore have strong potential to replace feeder-containing substrates in almost any procedure envisioned with human pluripotent cells, enabling broad and rapid scale-up of these cells for both research and clinical applications.

Materials and Methods

A UV unit (Bioforce Nanoscience, Inc., USA) generated high-intensity light to treat surfaces. Before cell seeding, surfaces were coated for 15–30 min with human vitronectin, 20% human serum, or 20% (vol/vol) FBS. Further details are provided in SI Materials and Methods.

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**Supporting Information**

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**SI Materials and Methods**

**UV Radiation Plates.** The UV unit (Bioforce Nanoscience Inc., USA) was used to generate high-intensity UV light, which can excite molecular oxygen to form atomic oxygen and ozone. Compared with the reduced pressure plasma treatment used to manufacture commercial TCPS, UV treatment is a straightforward process that does not require relatively expensive gas handling and vacuum processing equipment. In this study, untreated polystyrene (Corning), polypropylene (Grainger), or Ultra-Low Attachment surfaces containing a neutral hydrophilic hydrogel coating (Corning) were oxidized at a distance of around 4 cm from the UV lamp, and results are reported for exposure times under atmospheric conditions after preheating the UV lamp for 30 min. Chemically heterogeneous surfaces (patterned surfaces) were obtained by a simple masking technique. The custom-made stainless-steel photomask was placed on the surface of the untreated polystyrene dish and treated in the unit as above. Treated surfaces could be stored under ambient room temperature conditions for at least 6 mo.

**Cell Culture.** Before cell seeding, UV-treated surfaces were coated at room temperature for 15 min with either recombinant human vitronectin (0.2–3 μg/mL in DMEM/F12 base medium; R&D Systems), 20% human serum (vol/vol in DMEM/F12; Sigma), or 20% FBS (lot no. AVC63371, cat no. SH30070.03, vol/vol in DMEM/F12; HyClone). For this coating procedure, serum percentage may need to be optimized from 5–40% depending on the batch of serum. Because we saw identical behavior between recombinant vitronectin and serum for any assay on our engineered substrates, we used serum to reduce the cost of many experiments by 10-fold.

hESC (BG01, WIBR1, and WIBR3) and previously established hiPSC lines were maintained on mitomycin C-inactivated mEF feeder layers in standard medium (1). BG01-Oct4-GFP hESCs were made by introducing an Oct4-GFP-puro construct into hESCs (2). hiPSCs (C1 cell line only) were derived through lentiviral infection of Oct4, Sox2, and Klf4 and cultured on mEFs as described previously (3).

For FACS sorting, hESC or hiPSC lines were cultured in 10 μM ROCK inhibitor (Y-27632; Stemgent) for 12–24 h in standard mEF conditions before sorting. Cells were harvested enzymatically with 1 mg/mL collagenase and then with 0.05% trypsin for 5 min at 37 °C. hiPSCs were immunooassayed using SSEA-4. Cells were collected in media with ROCK inhibitor and sorted on a FACS Aria flow cytometer (Becton Dickinson). Cells were subsequently plated on various surfaces in medium supplemented with ROCK inhibitor (4) for the first 8–12 h of culture to reduce initial apoptosis of completely dissociated hESCs. Short-term addition of other small molecules [e.g., blebbestatin (5, 6), thiazovivin (7)] that inhibit apoptosis, enhance motility, or up-regulate E-cadherin could also help single-cell growth. For FBS/human serum/vitronectin-coated UV-treated surfaces, culturing occurred in mTESR1 (Stem Cell Technologies), TeSR2 (Stem Cell Technologies), or AF NutriStem hESC XF (Stemgent) media. Long-term culture on UV-treated surfaces occurred in mTeSR1 media and by passaging at a ratio of 1:3 every 5–7 d using either mechanical passaging and collagenase or accutase (StemPro Accutase; Invitrogen) for single-cell enzymatic passaging. ROCK inhibitor was added during the first 8–12 h for the single-cell enzymatic passaging.

**ToF-SIMS.** A secondary ion mass spectrometer (ION-TOF; IV) was operated using a B+ primary ion source operated at 25 kV and in “bunched mode.” A 1-pA primary ion beam was rastered on an area of 100 × 100 μm. Secondary ions were collected from the same area over a 10-s acquisition time. Ion masses were determined using a high-resolution ToF analyzer allowing accurate mass assignment. The typical mass resolution (at m/z = 41) was just over 6,000. Nitrogen-containing ions in the spectra likely arise from incorporation of air in the UV unit. ToF-SIMS images were acquired using random raster in a high-current bunched mode.

**Numerical Analysis of Surface Chemistry.** Principal component analysis (PCA) and PLS regression were carried out using the Eigenvector PLS_Toolbox 3.5 utilizing the SIMPLS algorithm. For least-squares regression, Excel (Microsoft) was used.

**Image Analysis to Monitor Cell Aggregation.** To quantify cell aggregate size after cell seeding, hESCs were passaged at a seeding density of 12,000 onto six-well plates with varying UV-patterned surfaces. After 24 h, cell aggregates were observed using phase-contrast microscopy and images were recorded at a magnification of 4×. To quantify the size of aggregates, image analysis was performed using ImageJ (National Institutes of Health). The images were converted to binary using an automated thresholding function, and colony areas were computed using particle analysis.

**Flow Cytometry Analysis.** Cells were dissociated using accutase and resuspended in 0.5% paraformaldehyde in PBS. Cells were then analyzed on a FACS Calibur flow cytometer (Becton Dickinson) within 7 d. Histograms were generated in FlowJo (Tree Star, Inc.).

**Stem Cell Migration Modeling and Simulation.** To understand and predict the effects of micropatterned surfaces on the survival and colony formation of stem cells better, a numerical model was implemented.

Individual stem cells were modeled as circles with a 30-μm diameter seeding randomly on a round 2D surface representing a single micropatterned “spot,” with the locations of cells represented as x,y coordinate points. In the absence of neighbors, cells are assumed to move in random motion defined by random unit vectors on the unit circle.

In the presence of neighboring stem cells, single cells appear to migrate preferentially toward others and to form small aggregates (8). This was modeled in the simulation as directed movement toward nearest cells at two-thirds of normal speed under a threshold distance of 110 μm. Cells closer than 30 μm were treated as a colony and allowed to random-walk within 30 μm of each other. This allowed cell aggregates to migrate preferentially toward other single cells and cell aggregates. Cells were assumed to move at 9 μm/h, and simulations were run for 24-h periods at different cell seeding densities and on different-sized micropatterned surfaces.

From the final cell locations on the 2D surface, the number and size of cell colonies were counted using the Statistics Toolbox.
in MATLAB (MathWorks). The pairwise distances between all pairs of cells in the simulation were computed using the PDIST function utilizing the Euclidean distance method:

\[ d_{ab} = \sqrt{(x_a - x_b)\, (x_a - x_b)^T} \]

where \( x_a \) and \( x_b \) represent coordinate \( x,y \) pairs defining the final locations of cells \( a \) and \( b \).

From the pairwise distance calculations, an agglomerative cluster tree relating individual cells by distance was created using the linkage function in MATLAB. Starting from individual cells, clusters were built up of nearest neighbor cells first, and these clusters were linked in levels based on shortest distance clustering, the minimum distance between elements of each cluster:

\[ \min(d_{ab}(a, b) : a \in A, \ b \in B) \]

where \( a \) and \( b \) represent cells \( a \) and \( b \) of clusters \( A \) and \( B \), respectively.

Colonies were defined as consisting of cells located less than 30 \( \mu m \) from at least one other cell in a cluster. Using the MATLAB function cluster, colonies were constructed from the agglomerative hierarchical cluster tree generated by the linkage function above, setting the cutoff criterion distance as 30 \( \mu m \).

**Immunocytochemistry.** As described previously (1), samples were washed with PBS, fixed with 4\% (vol/vol) paraformaldehyde solution for 20 min, permeabilized with 1\% (vol/vol) Triton X-100 in PBS for 45 min if staining for a nuclear protein (Oct4, Sox2, or Nanog), and then stained overnight with following primary antibodies: GFP (with OG5 cultures), SSEA-4, TRA-1-60 (Millipore), TRA-1-81 (Invitrogen), Nanog, Sox2, and Oct4. Alexa-Fluor secondary antibodies were then added. The nuclei were stained with Hoechst.

**Teratoma Assay.** hESC or hiPSC aggregates were collected by enzymatic dissociation, washed with PBS, and fixed in 4\% (vol/vol) paraformaldehyde for 20 min, permeabilized with 1\% (vol/vol) Triton X-100 in PBS, and then washed with PBS, 0.1\% (vol/vol) Tween 20, 0.1\% (vol/vol) Triton X-100, and 0.01\% (vol/vol) saponin. The samples were blocked with 1\% (vol/vol) BSA in PBS and then incubated with primary antibodies: GFP (OG5 cultures), SSEA-4, TRA-1-81 (Invitrogen), Nanog, Sox2, and Oct4. Alexa-Fluor secondary antibodies were then added. The nuclei were stained with Hoechst.

**Karyotypic Analysis.** Chromosomal studies were performed by Cell Line Genetics using standard protocols for high-resolution G-banding.

**Lentiviral Infection and hiPSC Derivation.** Reprogramming used a loxP-flanked version of the pHAGE-STEMCCA vector (9), which is a Cre-excisable polycistronic vector encoding Oct4, Sox2, Klf4, and cMyc reprogramming factors driven by a constitutive EF1\(\alpha \) promoter. Vesicular stomatitis virus G protein (VSVG)-coated lentiviruses were generated in 293 cells as described previously, except human serum replaced FBS and mTeSR1 was used during reprogramming (10). In brief, culture medium was changed 24 h after transfection and virus-containing supernatant was collected 48–72 h after transfection. Viral supernatant was filtered through a 0.45-mm filter. Virus-containing supernatants were pooled for infections and supplemented with an equal volume of fresh culture medium. One million patient-derived human fibroblasts (“237” and “267” samples) were seeded 24 h before transduction in T75 flasks. Four consecutive infections in the presence of 2 \( \mu \)g/mL polybrene were performed over a period of 48 h. Culture medium was changed 12 h after the last infection. Five days after transduction, fibroblasts were passaged with trypsin and replated onto “UV-Pattern.” For the first 4 d, cells were cultured in 20\% human serum in DMEM-F12 media and then switched to mTeSR1 media. The hiPSC colonies were picked manually on the basis of morphology between 2 and 4 wk and manually maintained and passaged.

**Gene Modification and Vector Excision.** For gene targeting or Cre-recombinase-mediated vector excision, hESC/hiPSC lines were cultured in ROCK inhibitor 24 h before electroporation. Cells were harvested with accutase, and 1 \times 10^5 cells resuspended in PBS were transfected with the appropriate plasmids by electroporation (250 V, 500 \( \mu F \), 0.4-cm cuvettes; Gene Pulser Xcell System; Bio-Rad) as described previously (11, 12). For vector excision, 50 \( \mu \)g of pCre-PAC (13) was used. We targeted the promoter of PPP1R12C (the AAVS1 locus) using a gene-trap vector containing a SA-2A-purmycin selection cassette (11). Cells were subsequently plated in medium supplemented with ROCK inhibitor for the first 24 h. Cells were selected with the addition of puromycin (2 \( \mu \)g/mL) 2 d after electroporation for a period of 48 h. Individual colonies were picked 10–14 d after electroporation. Excision of reprogramming transgenes was determined as described previously (12) by Southern blot analysis of EcoR1-digested genomic DNA probed against hKLF4.

**Live-Cell Imaging.** Cells were cultured for the duration of imaging in CO\(_2\)-independent medium (Gibco) supplemented with 10 \( \mu \)M ROCK inhibitor, 10\% (vol/vol) FBS (HyClone), 1 mM glutamine (Invitrogen), 1\% nonessential amino acids (Invitrogen), 0.1 mM \beta-mercaptoethanol (Sigma), and 40 ng/mL FGF2 (R&D Systems). Cells were imaged for a period of 20 h using a DeltaVision (Applied Precision, Inc.) microscope (objective with a magnification of 4\( x \) to 10\( x \)) and an HQ2 (Photometrics) camera. Time lap intervals were 10 min.

**Statistical Testing.** All error bars indicate 95\% confidence intervals. \( P \) values were calculated using Student \( t \) tests for binary comparisons or ANOVA for multiple group comparisons.

6. Chen G, Hou Z, Gulbranson DR, Thomson JA (2010) Actin-myosin contractility is a Cre-exisable polycistronic vector encoding Oct4, Sox2, Klf4, and cMyc reprogramming factors driven by a constitutive EF1\(\alpha \) promoter. Vesicular stomatitis virus G protein (VSVG)-coated lentiviruses were generated in 293 cells as described previously, except human serum replaced FBS and mTeSR1 was used during reprogramming (10). In brief, culture medium was changed 24 h after transfection and virus-containing supernatant was collected 48–72 h after transfection. Viral supernatant was filtered through a 0.45-mm filter. Virus-containing supernatants were pooled for infections and supplemented with an equal volume of fresh culture medium. One million patient-derived human fibroblasts (“237” and “267” samples) were seeded 24 h before transduction in T75 flasks. Four consecutive infections in the presence of 2 \( \mu \)g/mL polybrene were performed over a period of 48 h. Culture medium was changed 12 h after the last infection. Five days after transduction, fibroblasts were passaged with trypsin and replated onto “UV-Pattern.” For the first 4 d, cells were cultured in 20\% human serum in DMEM-F12 media and then switched to mTeSR1 media. The hiPSC colonies were picked manually on the basis of morphology between 2 and 4 wk and manually maintained and passaged.

**Live-Cell Imaging.** Cells were cultured for the duration of imaging in CO\(_2\)-independent medium (Gibco) supplemented with 10 \( \mu \)M ROCK inhibitor, 10\% (vol/vol) FBS (HyClone), 1 mM glutamine (Invitrogen), 1\% nonessential amino acids (Invitrogen), 0.1 mM \beta-mercaptoethanol (Sigma), and 40 ng/mL FGF2 (R&D Systems). Cells were imaged for a period of 20 h using a DeltaVision (Applied Precision, Inc.) microscope (objective with a magnification of 4\( x \) to 10\( x \)) and an HQ2 (Photometrics) camera. Time lap intervals were 10 min.

**Statistical Testing.** All error bars indicate 95\% confidence intervals. \( P \) values were calculated using Student \( t \) tests for binary comparisons or ANOVA for multiple group comparisons.
Fig. S1. Controlled modification of substrate surface chemistry. The complete range (m/z = 0–200) of spectra from ToF-SIMS of UV-treated substrates. Spectra are shown for virgin polystyrene treated for the indicated times and for conventional TCPS substrates. A detailed analysis of these spectra is provided in Figs. S2 and S3.
Virgin Polystyrene

Virgin polystyrene + 2.5 min UV-treatment

Virgin polystyrene + 0.5 min UV-treatment

Virgin polystyrene + 1.5 min UV-treatment

Virgin polystyrene + 3 min UV-treatment

Virgin polystyrene + 5 min UV-treatment

Virgin polystyrene + 10 min UV-treatment

Virgin polystyrene + 30 min UV-treatment

Normalized Ion Intensity

Mass-to-charge ratio (m/z)

Fig. S2. Example of surface chemistry differences generated by UV treatment. The enlarged section (m/z = 30–100) of ToF-SIMS spectra of UV-treated substrates is as shown in Fig. S1. Four specific ions differentially represented on the surfaces are indicated in red, blue, green, and black (ion details are provided in Fig. S3 A–C). C2H5O+ (m/z = 45, green line) and C2H6N+ (m/z = 44, blue line) ions were identified to support hESC colony formation, whereas C2H3O+ (m/z = 43, red line) ions were identified to inhibit hESC colony formation (Fig. S3C). C7H7+ (m/z = 91, black line) ions were identified to associate with unmodified virgin polystyrene.
**Fig. S3.** Surface chemical structures associated with UV treatment. (A) Using principal component analysis (PCA) of the ToF-SIMS spectra, samples with similar surface chemistry cluster together in the PCA graph. Each “PC” value in the figure represents a linear combination of peak intensities from the spectra in Fig. S1. Note that the 1.5–3 min of UV treatment (in the red ellipse) optimally supports colony formation (Fig. 1 C and D). (B) Loadings of PC1 and PC2. Loadings reveal the major ions that cause the different values of samples on the PC axis. Ions with positive loading values contribute to larger PC values, and vice versa. For example, virgin polystyrene, with the highest PC1 values, generates the highest intensity of C7H7+. (C) Using a multivariate PLS model of the ToF-SIMS data, two lists of ions, with the highest or lowest regression coefficients, were identified as supporting (coefficient > 0) or inhibiting (coefficient < 0) hESC colony formation.

**Fig. S4.** Chemical optimized substrates allow for undifferentiated cell growth through integrin engagement with vitronectin. (A) Flow cytometry histogram of Oct4-GFP levels in UVPS-cultured cells from day 7 (red, treated with optimal 2.5-min UV dose; Figs. S1–S3) and on standard mouse embryonic feeder (mEFs)-containing substrates (gray). The peak near 10⁷ relative fluorescence units (RFU) in the gray histogram largely consists of mEFs. UVPS was coated with 20% (vol/vol) FBS in DMEM-F12 base media for 15–30 min at room temperature before cell seeding. Cells were seeded in 10 μM Y-27632 ROCK inhibitor for the first 8–12 h after dissociation. (B) Number of pluripotent cells after 5 d for two different cell lines when coated with different concentrations of recombinant vitronectin in PBS(+). One milliliter of the solution per well of a six-well plate (10 cm²) was used.
Fig. S5. Modulating cell behavior by patterning culture substrates. (A) Large BG01 hESC colony on a UV-treated substrate without any spatial patterning (UVPS). Arrows indicate cells in these large colonies that down-regulate the pluripotency markers Nanog (red) and Oct4 (green). (B) (Upper) Growth of other human pluripotent cell lines on UV-patterned polystyrene substrates ("UV-Pattern"). (Lower) Pluripotency marker staining (colored) after one to three passages is shown for each labeled cell line. Surfaces were coated with 20% (vol/vol) FBS in DMEM-F12 base media for 15–30 min at room temperature before cell seeding. Cells were seeded in 10 μM Y-27632 ROCK inhibitor for the first 8–12 h after dissociation. (C) Immunostaining for the pluripotency marker Nanog on BG01 hESCs on UV-Pattern coated with human vitronectin after 7 d of culture. (D) Phase-contrast images of BG01 hESCs on UV-Pattern coated with 20% (vol/vol) human serum in DMEM-F12 base media after 7 d of culture. The figure is a composite of multiple panels.
Effect on ESC colony formation  | Functionality | Characteristic ions and corresponding regression coefficients for colony formation
--- | --- | ---
Negative Hydrocarbon | C$_2$H-, CH-, C$_3$H$_5$+ C$_4$H$_7$+ | C$_3$H$_7$+
Positive Ester/carboxylic acid | CHO$_2$- |  

Fig. S6. Surface chemical structures associated with UV treatment. (A) UV treatment of hydrogel-coated surfaces and polypropylene substrates supports robust colony formation. A live image of GFP fluorescence of transgenic Oct4-GFP reporter in BG01 hESCs is shown for both surfaces. The image was taken after four passages with collagenase for the hydrogel surface. (B) Colony formation on hydrogel-coated surface (Hydro, black squares) and polypropylene (PP, blue circles) treated with various UV doses. Colony number was assayed on day 5 after cell seeding (n = 3; error bars are 95% confidence intervals). (C) PLS model prediction of the number of colonies plotted against the experimental results for polypropylene. All surfaces were coated with 20% (vol/vol) FBS in DMEM-F12 base media for 15–30 min at room temperature before cell seeding. Cells were seeded in 10 μM Y-27632 ROCK inhibitor for the first 8–12 h after dissociation. (D) Using the PLS model of the ToF-SIMS data, surface ions with high positive or negative regression coefficients were identified as supporting or inhibiting hESC colony formation for polypropylene. (E) Histogram of Oct4-GFP levels of cells on patterns of 300-μm spot diameter vs. no spatial micropatterning as assayed by flow cytometry after 8 d of culture in serum-containing hESC media (15,000 cells seeded per well). The cumulative area UV-treated per well is the same for both patterns. The column graph indicates a lower percentage of differentiating Oct4-negative cells on the 300-μm diameter spots. RFU, relative fluorescent unit. (F) Cells can bridge UV-treated spot areas when spots are spaced ~100 μm apart (arrow indicates cell growth between spots). These cells between spots can differentiate, as indicated by down-regulation of Oct4 (green) and Nanog (red) at the arrows. In the diamond pattern, cells heterogeneously bridge the gaps as indicated by arrows when they are spaced <100 μm apart.

Fig. S7. Patterned substrates allow for undifferentiated cell growth in serum-free media. (A–C) Relative number of hESC colonies on UV-treated substrates with various patterns on day 7 after cell seeding. Error bars indicate 95% confidence intervals (n = 3). UVPS was coated with 20% (vol/vol) FBS in DMEM-F12 base media for 15–30 min at room temperature before cell seeding. Cells were seeded in 10 μM Y-27632 ROCK inhibitor for the first 8–12 h after dissociation. Both serum-free media, Nutristem and mTeSR1, support robust growth on UV-patterned surfaces for two passages.
Fig. S8. Cell behavior on UV-patterned substrates. (A) Number of adhered cells after 24 h of culture in mTeSR1 media on the constant area patterns as described in Fig. 2E (40,000 cells seeded per well). No significant differences were seen across the different patterns. (B) Phase-contrast images of hiPSCs after 24 h of culture in mTeSR1 media on the constant area patterns as described in Fig. 2E (25,000 cells seeded per well). White arrows indicate large aggregates. No large aggregates were seen in the 300-μm diameter spot pattern. (C) Area distribution of cell aggregates seen after 24 h of culture in mTeSR1 media on the constant area patterns as described in Fig. 2E (12,000 cells seeded per well). (Inset) Column graph indicates fraction of cells contained in large (>20,000 μm²) aggregates. As the number of spots increases, larger cell aggregates are observed. (D) Cell population-averaged doubling time for cells cultured on the constant area patterns for 7 d as described in Fig. 2E (15,000 cells seeded per well).

Fig. S9. Simulations of cell behavior on UV-patterned substrates. (A) Representative individual cell trajectories over 24 h predicted from cell migration and aggregation model on a single 600-μm spot at a low seeding density to indicate random walk. Colors indicate different cells. (B) Distribution of cells in each aggregate as predicted from the cell migration model for two different patterns, 300- and 2,300-μm spot diameters, at a low cell density when ROCK inhibitor is added. (C) Distribution of cells in each aggregate as predicted from the cell migration model for two different patterns, 300- and 1,400-μm spot diameters, when ROCK inhibitor is added at routine cell densities. In ROCK inhibitor, cells migrate faster as described by Li et al. (8). The conclusion that 300-μm diameter spots reduce large cell aggregates is consistent for both cases.
Fig. S10. Characteristics of substrates and cells on UV-patterned substrates. (A) Table indicating various dimensions and areas of UV-treated plates used for cell culture. (B) Cell cultures became abnormal within 5 passages on UV-patterned substrates when passaged under harsh conditions (with accutase or trypsin without ROCK inhibitor). In contrast, cell cultures were karyotypically normal for 7–27 passages on UV-patterned substrates when passaged with collagenase or accutase with overnight ROCK inhibitor incubation after dissociation (Fig. 6E). p, passage. (C) Representative phase-contrast images of BG01 hESCs grown on top of a mouse embryonic feeder (MEFs) layer. Arrows indicates large hESC colonies that approach a diameter of 300 μm.

Movie S1. Bright-field video of hESCs after cell seeding on a UV-patterned substrate with a 300-μm diameter spot pattern. Images were taken every 10 min, and a counter on the bottom indicates the elapsed time since cell seeding. CO2-independent media contained 10% (vol/vol) FBS and ROCK inhibitor. The majority of cells attach and aggregate within 3 h. Patterned substrate was coated with 20% (vol/vol) FBS for 15–30 min at room temperature before cell seeding.