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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 cells 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


Conflict of interest statement: R.L., R.J., and D.G.A. are advisors to Stemgent, and R.L. and R.I. are cofounders of Fate Therapeutics.

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(11), 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 S3.4 and B) confirmed that UV treatment yields chemically distinct surfaces from both virgin polystyrene and conventional tissue culture polystyrene (TCPSS). 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 surfaces (Fig. 1B) and significantly fewer cells grew on TCPSS (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 protocols for high-throughput 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., $C_7H_8O_7$ and $C_8H_8N_2O$ 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). Similarly 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/cm² (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 1:5 dilution ratio used during conventional hESC passaging assays. 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 pluripotency-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. 2C). 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. S5C 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. S6A). Like the polystyrene surfaces (Fig. 1E), an intermediate dose of UV on two other common research materials, polypropylene and hydrogel-coated surfaces, optimally supported colony formation from fully dissociated hESCs (Fig. S6B). 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 morphologies (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. S7A 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 smaller 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. S8B 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 (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. 4 A and B) and a normal karyotype (Fig. S10B). Further, cells after long-term culture robustly expressed all the pluripotency markers tested, including SSEA-4, Oct4, 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 passaging yielded significant increases in the number of undifferentiated cells over mEFs that increased from one cell to 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 single-cell passaging of human pluripotent stem cells. Images of BG01 hESCs (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 8–12 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 mESs, only GFP-positive cells were analyzed for Tra-1-60 and SSEA-4 expression. Therefore, mESs were excluded from the analysis. (E) Normal 46XY 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. 6.** UV-patterned substrate supports single-cell passaging of human pluripotent stem cells. Images of BG01 hESCs (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 8–12 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 mESs, only GFP-positive cells were analyzed for Tra-1-60 and SSEA-4 expression. Therefore, mESs were excluded from the analysis. (E) Normal 46XY 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.

**Table 1.** Design parameters for developing a UV-treated culture system for human pluripotent stem cells. 

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Condition for long-term culture</th>
<th>Points to consider</th>
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<tbody>
<tr>
<td>UV dose</td>
<td>2.5 min/mL</td>
<td>Low dose fail to modify surface enough, high dose creates surface hard that negatively impact colony formation.</td>
</tr>
<tr>
<td>Spot Diameter</td>
<td>200–300 μm</td>
<td>Small spacing increases number of spots per well and therefore maximizes growth area, but increases the likelihood of colonies merging &amp; differentiating.</td>
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**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 could be moved to mESs 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 pHAGE-STEMCCA vector, which is a Cre-excisable polycistronic 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 spot diameter with 200-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 efficiently, 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), wherein ROCK inhibitor was required only briefly for 8–12 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. 6F). 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. S10B) 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 mESF 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 (Figs. 1F and 2A) and creates at least twofold more colonies per cell seeded than conventional cell culture plastics (Fig. 1D). Spatial patterning of the UV treatment can further increase the number of undifferentiated cells per growth area (Fig. 2E). Spatial patterning of the UV treatment could constrain hiPSC colony size in two dimensions; however, hiESCs/hiPSCs could only be consistently patterned into spots when spacing was greater than 100 μm (Fig. S6F). We consider several possibilities to explain these observations. Optimal spatial pattern of the UV treatment may (i) establish favorable gradients of soluble factors using preferential migration toward other hiESCs/hiPSCs (31), (ii) improve crosstalk between soluble factors and mechanical signal transduction (33), and (iii) facilitate the previously noted preference for hiESCs/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 (W). It is possible that the reduced spot diameters enable high densities of colonies and 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. S1OC). It should be possible to produce such plates in many different formats (e.g., multiwell 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

An 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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