Supplementary Data

A spatially and chemically defined platform for the uniform growth of human pluripotent stem cells

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S1. Supplementary Methods

S1.1. Polymerase Chain Reaction

RNA (1 µg) was extracted using the RNeasy Mini Kit (Qiagen) and first strand synthesis was generated using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). PCR was performed using the AccuPrime Taq DNA polymerase System (Invitrogen). Quantitative RT-PCR was performed using FastStart SYBR Green Master mix (Roche). Differences in expression are calculated using the $2^{-\Delta\Delta C_T}$ method where all samples are first normalized to the standard housekeeper gene GAPDH and then compared to the control sample to generate relative fold differences. Details of primer sequences used in these studies are provided in Table S1 of the supplementary data.

S1.2. Fluorescence Activated Cell Sorting

HPSCs were dissociated into single cells and resuspended in FACS buffer (1% BSA in PBS) for 10 minutes at 4°C. TRA-1-81 (1:100 from Santa Cruz Biotechnology) or SSEA4 (1:100 from Developmental Studies Hybridoma Bank) was added for 20 minutes at 4°C. For intracellular staining, cells were fixed with 2% paraformaldehyde for 10 minutes at 37˚C and then permeabilized using 90%

methanol for 30 minutes at 4˚C. Cells were stained with the following antibodies (Cell Signaling Technology) for 1 hour at room temperature: Oct4 (1:600), Nanog (1:200) or Sox2 (1:200). Stained cells were washed with PBS and secondary antibodies (1:250) were added for 20 minutes at 4°C. FACS acquisition was performed using a LSR II FACS sorter and analyzed using the instrument's FACS DIVA software (BD Biosciences).

S1.3. Teratoma Formation and Analysis

HPSCs were dissociated using 1 mg/ml collagenase Type IV (Invitrogen) for 5 minutes and centrifuged at 1,000 RPM. 1 X 10^6 cells were resuspended in 50 µl of PBS and injected into the testis of adult SCID beige mice (Charles River) as described previously.[1] After 8 weeks, teratomas were isolated and fixed in 4 % paraformaldehyde for 24 hours. Teratomas were sectioned and stained with haematoxylin and eosin.

S1.4. Alkaline Phosphatase (AP) Staining

HPSCs were fixed using 4% paraformaldehyde for 30 minutes at room temperature and rinsed with PBS. A solution consisting of 1 mg/ml Fast Red TR Salt and 40 µl/ml of Naphthol AS-MX phosphate (Sigma) was added to cells for 0.5 - 1 hour.

S1.5. Image Analysis of AP Staining Uniformity

The uniformity of alkaline phosphatase (AP) expression was compared in µCP patterned H9 hESC colonies in CM and standard MEF-supported H9 colonies by image analysis of cells stained with Fast Red AP chromogenic substrate (Sigma). Stained colonies were prepared as described in the Experimental section. Images were obtained using a 5x objective on a Zeiss Observer Z1 inverted microscope equipped with an AxioCAM HRc color CCD. All images were taken with a single white balance setting and analyzed using ImageJ software. Line profiles for the red, blue and green channels were obtained across images of the colonies for both substrate types. At all points in the line profile, the smallest value between all three channels, representing the "white" level, was subtracted from value of the red channel. This produced a positive signal representing the presence of red color relative to a white background and allowing for the presence of other absorptive colors. AP uniformity was assessed by calculating the standard deviation and subsequently the coefficient of variance (COV) of the modified red signal within the boundaries of the colonies. To eliminate edge effects, the colony boundaries were defined by the full-width, half-max (FWHM) of each colony.

S1.6. Fast Red-AP UV-Visible Absorbance

Purified alkaline phosphatase (2,000-4,000 DEA units/mL, Sigma) was diluted to 0.2 units/mL in a 0.01% naphthol AS-MX phosphate aqueous solution. A solution of 1 mg/ml Fast Red TR salt was prepared in aqueous 0.01% naphthol AS-MX phosphate. The two solutions were combined in equal parts and subsequently kept at room temperature for 30 min. The resulting red product was centrifuged and rinsed with PBS and then with deionized water. After drying overnight at 60ºC the

product was dissolved in ethanol. The absorbance of the ethanol solution as a function of wavelength was analyzed with a Varian Cary 100 Bio UV-visible spectrophotometer using pure ethanol in the reference cell.

S1.7. Karyotype Analysis

HPSCs were sent to Cell Line Genetics (Madison) for karyotype analysis according to company protocols. Briefly, cells were passaged onto Matrigel in T-25 flasks in CM. Live cultures were sent for analysis of at least 20 metaphase spreads per sample.

S1.8. Embryoid Body Formation

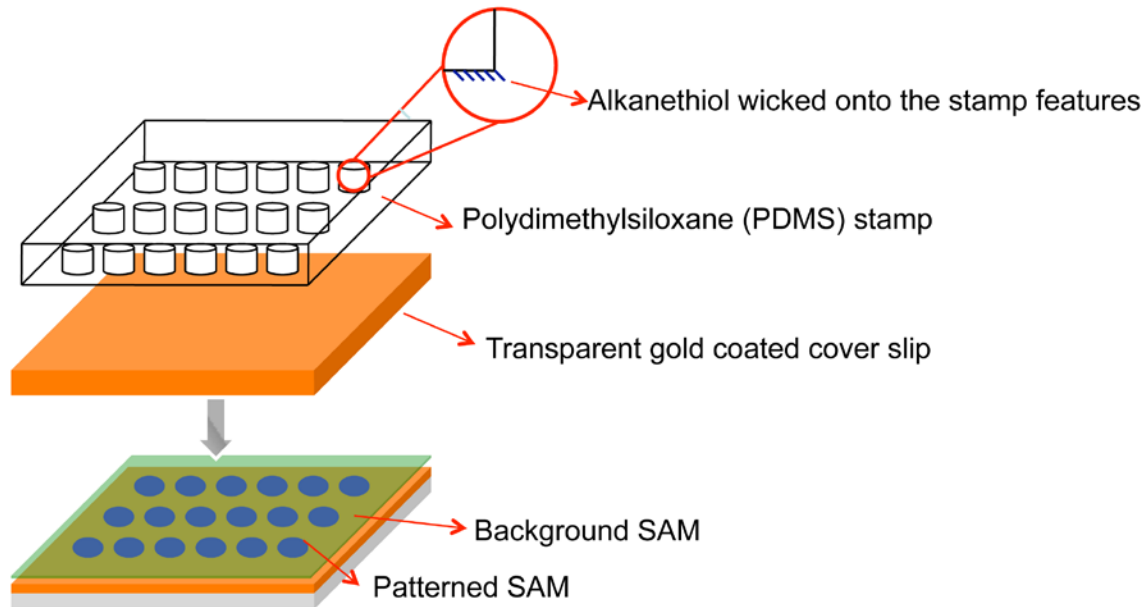
Patterned hPSC colonies were displaced from the surface on the third or fourth day after seeding by treating each well with a 1 mg/ml collagenase solution for 30 minutes. After a gentle PBS rinse, the intact colonies subsequently became detached from the surface and were re-plated into low-attachment 24-well plates in EB medium consisting of Dulbecco's Modified Eagle Medium-F12 (Invitrogen) supplemented with 15% defined fetal bovine serum (Hyclone), 5% Knockout Serum Replacement (Invitrogen), 1 mM L-Glutamine (Invitrogen), 2 mM 2-mercaptoethanol and 0.1 mM Non-essential Amino Acids (Invitrogen). Colonies that did not immediately detach were treated with collagenase for an additional 15 minutes and collected.

S2. Supplementary Discussion

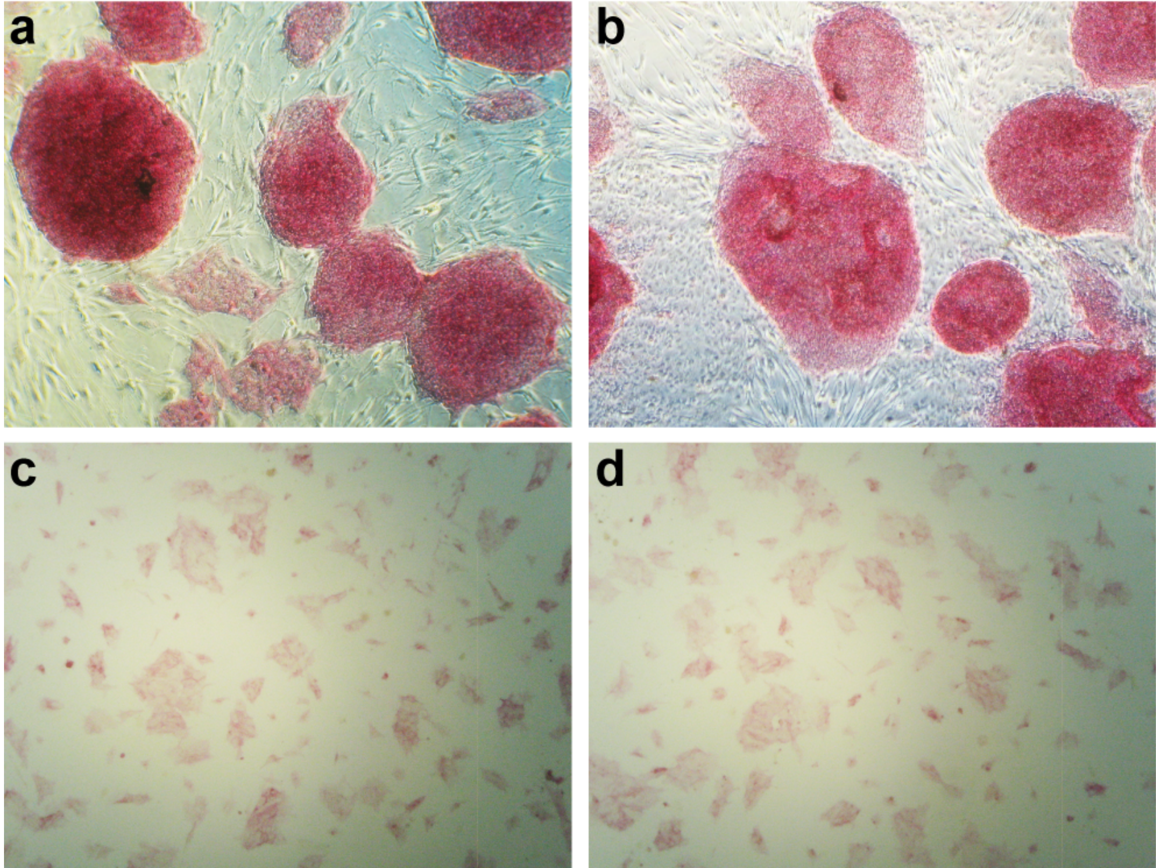
S2.1. Image Analysis of Alkaline Phosphatase stained hPSC colonies

µCP-patterned and MEF-supported colonies stained for alkaline phosphatase (AP) expression were compared using an image analysis protocol directed toward quantifying variabilities within individual colonies. The monitoring of AP expression by Fast Red staining is a widely recognized analytical technique to assess hPSC pluripotency.[2] In this study, AP expression was correlated to the intensity of the red signal measured from bright field images obtained with a standard optical microscope. Briefly, red intensity was associated with the concentration of the Fast-Red-AP precipitate that forms and collects within cells during a typical AP staining experiment. The basis for this approximation is illustrated in Supplementary Fig. S5, which shows the UV/Vis spectrum of the Fast Red-AP precipitated product. The strong absorption peak observed at 590 nm compliments the spectral sensitivity of the Zeiss CCD used to capture images for this analysis such that the light absorbed will attenuate the green and blue channels almost exclusively. Intensity of the red signal in images obtained with this CCD can be used to estimate the concentration of the Fast-Red-AP precipitate at levels that do not saturate absorption in the green or blue channels. An analysis of AP stained hPSC colonies with this method showed a two-fold improvement in the coefficient of variance compared to MEF supported colonies, indicating improved uniformity in the µCP patterned hPSC population (Fig. 4). The coefficient of variance of the staining within patterned colonies was found to be 0.1968, while the result for MEF supported colonies was 0.3969. A total of 5714 pixels from 7 images of patterned colonies and 6456 pixels from 5 images of MEF supported colonies were evaluated.

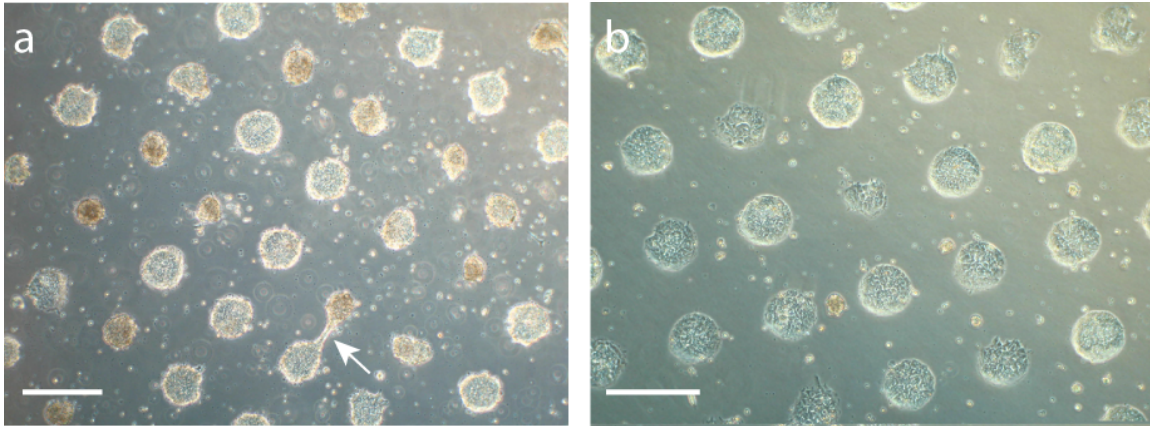
References
[1] A.E. Conway, A. Lindgren, Z. Galic, A.D. Pyle, H. Wu, J.A. Zack, M. Pelligrini, M.A. Teitell, A.T. Clark, Stem Cells, 27 (2009) 18-28.
[2] O. Adewumi, B. Aflatoonian, L. Ahrlund-Richter, M. Amit, P.W. Andrews, G. Beighton, P.A. Bello, N. Benvenisty, L.S. Berry, S. Bevan, Nat Biotech, 25 (2007) 803-816.



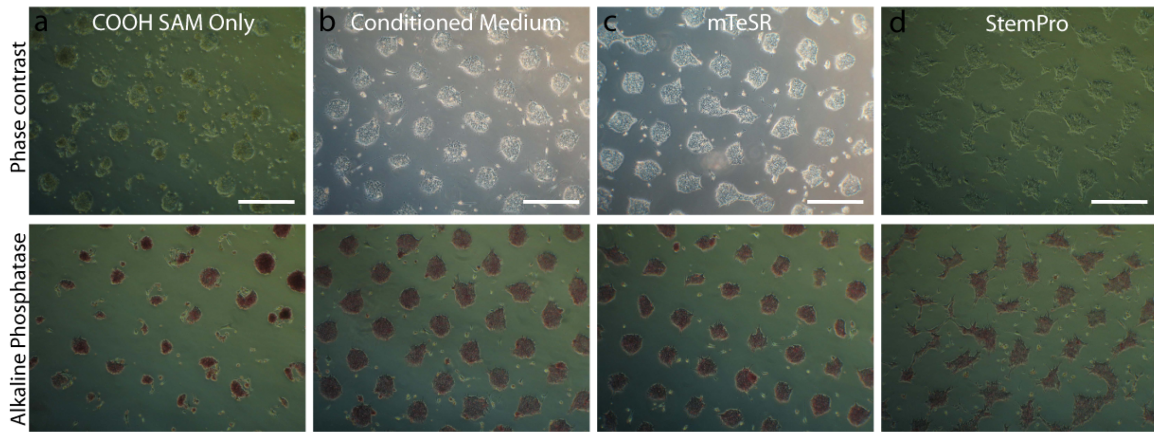
Scheme 1. An illustration of the μCP process used to pattern SAMs for supporting the growth and long-term maintenance of uniform hPSC colonies.



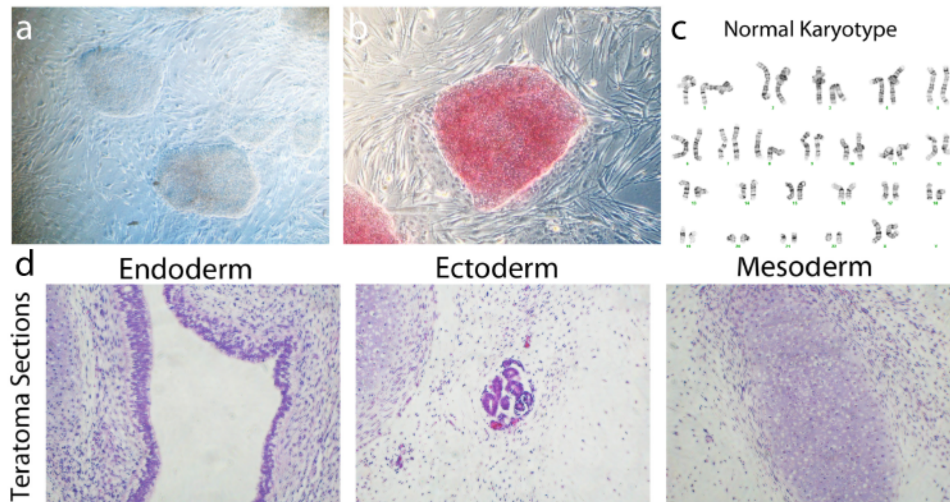
Supplementary Fig. S1. AP staining is heterogeneous for hESC colonies cultured on MEFs and Matrigel: a-b) Bright-field images of AP stained H9 hESC colonies cultured on MEFs under standard conditions. Conventional MEF culture of hESCs typically yields heterogeneously stained colonies. Images were obtained at 10X magnification. c-d) Similarly, H9 hESC colonies cultured on Matrigel in standard conditions also stain heterogeneously. Images for Matrigel cultured colonies were collected at 5X magnification.



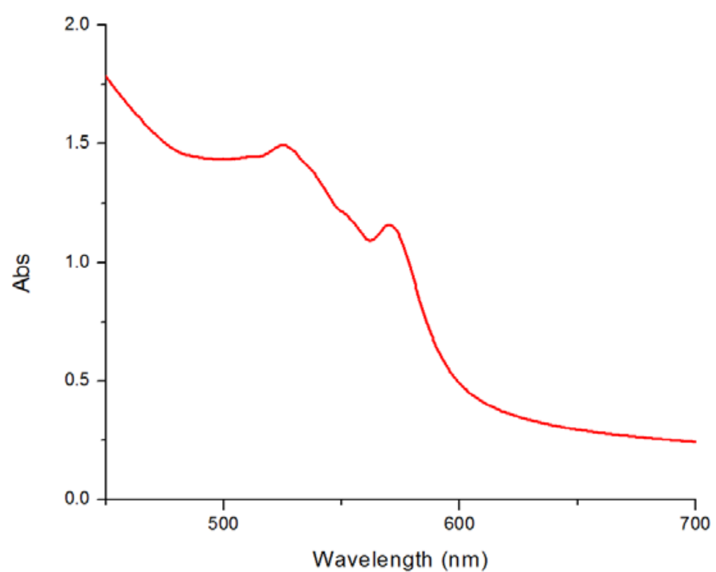
Supplementary Fig. S2. Background functionality affects pattern fidelity: Brightfield microscopy images comparing H9 hESCs cultured on μCP SAMs with either $-CH_3$ (a) or –PEG (b) SAMs in the unpatterned background area. At times colonies on substrates with the $-CH_3$ SAM background can out grow their spatially defined area and merge with an adjacent colony (white arrow). Scale bars = 400 μm.



Supplementary Fig. S3. Laminin supports patterned colony growth: a) Cell adhesion and subsequent colony formation on pristine SAMs is insufficient for continuous passaging. b-d) μCP SAMs in which the patterned areas are exposed to the ECM protein laminin are able to support the growth of H9 hESC colonies in MEF conditioned medium (b) and the commercial defined medium preparations mTeSR (c) and StemPro (d). In these experiments a –COOH presenting SAM was used in the patterned areas while a $-CH_3$ SAM comprised the background of the μCP surface. The hESC colonies from each condition stain positive for AP, indicating that the stem cells remain pluripotent on the chemically defined SAMs (Lower panels).



Supplementary Fig. S4. HPSCs are normal at P0: (a) Bright-field image of H9 hESCs cultured on MEFs prior to use on patterned SAM surfaces showing normal phenotype. (b) P0 colonies stain positive for AP, indicating that the hESCs are pluripotent. (c) P0 hESCs have a normal karyotype with no evidence of trisomies or other anomalies. (d) H&E-stained sections of teratomas formed from P0 cells confirm differentiation toward endodermal, ectodermal and mesodermal lineages.



Supplementary Fig. S5. Ultraviolet-visible absorption spectrum of the product obtained in a typical Fast Red-AP reaction. The plot shows a strong absorption band beginning near 590 nm, which closely corresponds to the shift from red to green sensitivity for the CCD in the image analysis of AP stained hPSC colonies.

Table S1. Primers used for RT-PCR and qRT-PCR studies.

Gene	*Forward*	*Reverse*
Oct4	*ACATCAAAGCTCTGCAGAAAGAACT*	*CTGAATACCTTCCCAAATAGAACCC*
Nanog	*CAGCTGTGTGTACTCAATGATAGATTT*	*ACACCATTGCTATTCTTCGGCCAGTTG*
Sox2	*CTTTTGTTCGATCCCAACTTTC*	*ATACATGGATTCTCGGCAGACT*
UTF1	*TCGCTGAACACCGCCCTGCT*	*CTCCACGTGCTGGTTCAAGGT*
DNMT3B	*AAGGTGCGTCGTGCAGGCAG*	*TCTTGAGGCGCTTGGGTGCG*
DPPA2	*AGGCTTCATAGGCATGCTTAC*	*TGAAGCCTTGCTCTCTTGGTC*
GATA4	*CCTCCAAGGAGTAAGACCCC*	*TGTGAGGAGGGGAGATTCAG*
GAPDH	*TCGACAGTCAGCCGCATCTTCTTT*	*ACCAAATCCGTTGACTCCGACCTT*