ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Maintenance of Pluripotency in Human Embryonic Stem Cells Cultured on a Synthetic Substrate in Conditioned Medium

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ABSTRACT: Realizing the potential clinical and industrial applications of human embryonic stem cells (hESCs) is limited by the need for costly, labile, or undefined growth substrates. Here we demonstrate that trypsin passaging of the hESC lines, HUES7 and NOTT1, on oxygen plasma etched tissue culture polystyrene (PE-TCPS) in conditioned medium is compatible with pluripotency. This synthetic culture surface is stable at room temperature for at least a year and is readily prepared by placing polystyrene substrates in a radio frequency oxygen plasma generator for 5 min. Modification of the polystyrene surface chemistry by plasma etching was confirmed by X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS), which identified elemental and molecular changes as a result of the treatment. Pluripotency of hESCs cultured on PE-TCPS was gauged by consistent proliferation during serial passage, expression of stem cell markers (OCT4, TRA1-60, and SSEA-4), stable karyotype and multi-germlayer differentiation in vitro, including to pharmacologically responsive cardiomyocytes. Generation of cost-effective, easy-to-handle synthetic, defined, stable surfaces for hESC culture will expedite stem cell use in biomedical applications.

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KEYWORDS: human embryonic stem cells (hESCs); radio frequency plasma etching; synthetic culture surface; pluripotency; oxygen; nitrogen; XPS; ToF-SIMS

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Introduction

The ability to maintain human embryonic stem cell (hESC) pluripotency during long-term culture and yet induce differentiation into multiple lineages potentially offers novel cell sources for toxicological screening, in vitro modeling of genetic disorders or therapeutic cell replacement (Cohen et al., 2006; Pouton and Haynes, 2005). However, before these endpoints can be fully realized at industrial scale, it will be necessary to identify growth matrices that possess a range of characteristics. These include ease of handling, chemical and physical stability, cost-effectiveness, reproducibility of manufacture, well-defined composition and compatibility with good manufacturing practice (GMP).

Traditionally, hESCs were cultured on mouse embryonic fibroblast (MEF) feeder layers in serum containing media (Thomson et al., 1998). However, in light of concerns over mouse–human transfer of viruses (Amit et al., 2004) and immunogenic epitopes, such as *N*-glycolylneuraminic acid (Neu5Gc) non-human sialic acid (Martin et al., 2005), the utility of feeder layers from a variety of human tissues has been investigated (Richards et al., 2003). While human foreskin fibroblasts have been used to isolate GMP grade hESC lines (Crook et al., 2007), isolation/preparation of the feeders is a cumbersome task and batch-to-batch variability remains a concern (Mallon et al., 2006).

As an alternative to feeder cell/hESC co-cultures, the ability of various isolated or recombinant extracellular matrix (ECM) components to support hESC attachment and proliferation has been evaluated. Isolated from Engelbreth–Holm–Swarm mouse sarcoma, Matrigel is a solubilized basement membrane preparation rich in laminin, collagen IV, heparan sulfate proteoglycans, and enactin (Greenlee et al., 2004) that has been used to support

pluripotency in many hESC lines (Braam et al., 2008a; Xu et al., 2001). Similarly, either "natural" or recombinant sources of collagen IV, fibronectin, laminin, enactin, and vitronectin have been used successfully (Amit et al., 2004; Braam et al., 2008b; Ludwig et al., 2006a). Nevertheless, fibronectin from natural sources has been shown to exhibit mixtures of isoforms and highly fragmented proteins (Wondimu et al., 2006), raising questions regarding the reproducibility of the surface properties of substrates prepared from these materials. Moreover, cost is often prohibitive for all ECM preparations, which usually also have a limited shelf life, require cold storage, and can lose their biological properties upon dehydration.

For the reasons above, development of a completely synthetic substrate is desirable for the culture of hESCs. This has been achieved for the majority of primary and transformed cells, which attach and grow on standard tissue culture polystyrene (TCPS), but this is not so for hESCs. Interestingly, although production methods of TCPS vary slightly between manufacturers, cell adhesion to materials is often rationalized in terms of an increase to the hydrophilic nature of the surface (Amstein and Hartman, 1975; Curtis et al., 1983). Nevertheless, the specific requirements in terms of surface chemistry needed to support hESC growth and proliferation remain elusive. Therefore, we set out to evaluate whether altering the surface composition of TCPS, particularly in relation to oxygen and nitrogen content, would enable hESC attachment and proliferation. Since radio frequency plasma etching (PE) is widely employed to increase the oxygen content at the surface of polymeric substrates (Stoffels et al., 1995), we used this approach to modify the surface chemistry of TCPS, raising the abundance of oxygen species by 1.6-fold on PE-TCPS. Following the culture of hESC lines, HUES7 and NOTT1, on PE-TCPS pluripotency was retained, as gauged by (i) consistency of proliferation during serial passage, (ii) marker expression, (iii) karyotype stability, and (iv) differentiation to representatives of the three germ layers, including the production of pharmacologically responsive cardiomyocytes. In this work, we discuss the differences in the ability of the two substrates (TCPS and PE-TCPS) to support hESC culture in terms of their surface properties.

Materials and Methods

Materials and General Culture

All tissue culture reagents were purchased from Invitrogen (Paisley, UK, http://www.invitrogen.com/) and chemicals from Sigma (Poole, UK, http://www.sigmaaldrich.com/), unless otherwise stated. MEF and hESC cultures were maintained at 37°C, 5% CO_2 in a humidified atmosphere. Medium was changed daily for hESC culture and every 3–4 days during differentiation.

Culture Surface Preparation

Spin-cast thin polystyrene (PS) films and standard tissue culture treated polystyrene (TCPS) surfaces were prepared for use by coating with Matrigel or by radio frequency PE. Matrigel coating was done by adding 0.8 mL/cm^2 of a 1:100 dilution of Growth Factor Reduced Matrigel (BD Biosciences, Oxford, UK; www.bdbiosciences.com/) in cold DMEM and allowing polymerization to occur at room temperature for 45 min. Prior to use, medium was aspirated from flasks and the culture surface washed with PBS. For PE (Fig. 1), PS films and TCPS 25-cm^2 flasks (Sarstedt AG & Co, Nümbrecht, Germany, www.sarstedt.com; and Nunc, Roskilde, Denmark, www.nuncbrand.com) were placed in a home-built vacuum chamber and pumped to a base pressure of <20 mT. Oxygen gas was then allowed to flow through the chamber at a pressure of 300–350 mT. A radio frequency source with an input power of 20 W was then used to sustain a plasma in the oxygen gas. Samples were exposed to this plasma for 5 min. After PE was complete the chamber was vented to nitrogen. All plasma-treated substrates were sealed with parafilm after etching and could be stored at room temperature for at least 1 year before use.

Characterization of Plasma Etched Substrates

Plasma etched substrates were characterized by (i) atomic force microscopy (AFM; Asylum Research UK Ltd., Oxford, UK, www.AsylumResearch.co.uk) to inform on topography;

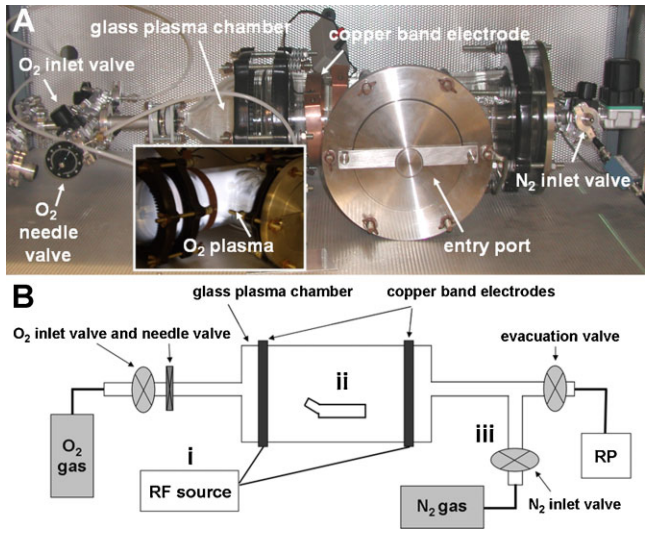

Figure 1. Radio frequency plasma etching. Photograph (**A**) and schematic (**B**) of the radio frequency (RF) plasma etching system. A 20.0 ± 0.4 W radio frequency source (i) was used to excite oxygen gas and create a plasma. Samples were exposed to this plasma for 5 min (ii). After plasma etching was complete, nitrogen gas (iii) was introduced into the chamber (to ensure substrate sterility) until ambient pressure was reached. RP, rotary pump. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

(ii) water contact angle (WCA) measurements to inform on surface wettability; and (iii) X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectroscopy (ToF-SIMS) to examine changes in substrate chemistry.

AFM images were obtained using a MFP-3D atomic force microscope (Asylum Research UK Ltd., www.AsylumResearch.co.uk) operating in tapping mode and using NSC16/AlBS cantilevers (MikroMasch, Tallinn, Estonia, www.spmtips.com). IGOR Pro software was used to process image data and to evaluate the root mean square (rms) surface roughness from $40\,\mu m \times 40\,\mu m$ scans that were collected from both TCPS and PE-TCPS samples.

WCA measurements were performed on both TCPS and PE-TCPS substrates using a CAM 200 Optical Contact Angle Meter (KSV Instruments Ltd, Helsinki, Finland, www.ksvltd.com). Upon contact of the ultra pure water droplet with the surface, 20 images were collected at 1 s intervals and the average contact angle of the droplet was calculated (see Results Section). WCAs of less than 10° cannot be measured accurately but indicate that the substrate is very hydrophilic.

XPS scans were performed at different locations on each of the test substrates and were used to determine the elemental composition at the surface. XPS spectra were recorded using a Kratos Axis Ultra spectrometer (Kratos Analytical Ltd, Manchester, UK, www.kratos.com) employing a monochromated Al K_α X-ray source ($h\nu = 1{,}486.6$ eV), hybrid (magnetic/electrostatic) optics, hemispherical analyzer and a multi-channel plate and delay line detector (DLD) with a collection angle of 30° and a take off angle of 90°. During each XPS scan, the X-ray gun power was set to 150 W and all spectra were recorded using an aperture slot of $300\,\mu m \times 700\,\mu m$ with a pass energy of 80 eV for wide angle survey scans and 20 eV for high-resolution core level scans. All XPS spectra were recorded using the Kratos VISION II software; data files were translated to VAMAS format and processed using the CASA XPS^{TM} software package (Version 2.3.2 and later). During experiments charge compensation was used (Kratos AXIS Nova charge neutralization system: a coaxial low energy electron source within the field of the magnetic lens) and samples were earthed via the stage using a standard BNC connector. All of the measured binding energies are referenced to the C 1 s core level at 285.0 eV.

Secondary ion mass spectrometric analysis for a number of areas on each sample was carried out using a SIMS IV time-of-flight instrument (ION-TOF GmbH, Münster, Germany, www.iontof.com) equipped with a bismuth liquid metal ion gun and a single-stage reflectron analyzer. Typical operating conditions utilized a primary ion energy of 25 kV and a pulsed target current of approximately 1.0 pA. Low energy electrons (20 eV) were used to compensate surface charging caused by the positive primary ion beam on insulating surfaces. Both positive and negative secondary ion species were analyzed using Bi^{3+} clustered ions. A raster area of $200\,\mu m^2$ was analyzed at a resolution of 256×256 pixels. The total primary ion beam dose for each area of analysis was kept below 1×10^{12} ions cm^{-2} throughout, ensuring static conditions. All data analyses were carried out using software IonSpec and IonImage (version 4.1). The ToF-SIMS spectra obtained from a given sample were normalized using the total counts obtained from that sample. Peaks for ion masses that varied between different substrates were identified and quantified.

Surface Analysis in Conditioned Medium

A quartz crystal microbalance (QCM, Q-Sense D300) was used to measure the total mass of material adsorbed on the PS and PEPS surfaces from conditioned medium (CM). Water was flowed over the polystyrene spin-coated QCM quartz sensor crystal before CM was injected. The crystal responded with a decrease in the resonant frequency within one-hundredth of a second indicating mass gain. This frequency decrease was converted to mass increase using the established Sauerbrey method: $M_{qcm} = -C^*(\Delta f/i)$, where Δf is the change in resonant frequency, i is the overtone number, and C is a mass sensitivity constant dependent on the intrinsic properties of the quartz crystal (here $C = 17.7$ ng cm^2 Hz) (Sauerbrey, 1959). All calculations presented here correspond to the third harmonic overtone at 15 MHz. The CM solution was left in contact with each surface for 15 min, after which it was rinsed with water four times; the amount of material adsorbed on each surface was determined by subtracting the weight measured before CM injection from the weight after rinsing.

A Fourier transform infra red spectrometer (FTIR, FTS 40 Pro) with an attenuated total reflection (ATR) accessory was used to determine if the mass increases observed on PEPS and PS surfaces measured using QCM are due to the presence of adsorbed protein. PS and PEPS-coated ZnSe ATR crystals were loaded into the ATR cell. Water was then injected into the cell and a background spectrum was collected immediately. The water was then exchanged for CM and spectra (collated from 100 scans) were collected every hour for 4 h. These were then ratioed to the water background scan to monitor changes in the surface excess of material adsorbed at the interfaces. All spectra were collected with a resolution of $4\,cm^{-1}$ and were baseline flattened before being corrected for small amounts of water vapor signal. All data analyses were carried out using DIGILAB Resolutions Pro version 4.0.0.030 software.

Human Embryonic Stem Cell Lines and Culture

The hESC lines, HUES7 (Cowan et al., 2004) between passages (p) 24 and 37 and NOTT1 (Burridge et al., 2007) between p 26 and 43, were cultured in MEF-CM on Matrigel or PE-TCPS. Passaging was by incubation with 0.05% trypsin for 1 min at 37°C, coupled with tapping the flasks to liberate single cells or preferably small clumps of cells. Population doublings (PDs) at each passage were calculated using the formula [$\log_{10}$(total cell counts/cells seeded)/

$\log_{10}(2)$], where the cell number seeded was 6×10^4 cells/cm^2 for HUES7 and 8×10^4 cells/cm^2 for NOTT1. Proliferation rates were calculated by hours in test culture/cumulative PDs (Denning et al., 2006). Preparation of CM was as described previously (Burridge et al., 2007; Denning et al., 2006). Briefly, MEFs (strain CD1, 13.5dpc) were mitotically inactivated with mitomycin C (10 μg/mL, 2.5 h) and seeded at 6×10^4 cells/cm^2. The next day, inactivated MEFs in T75 flasks were washed with PBS and incubated with 25 mL hESC medium for 24 h, at which time CM was harvested and made ready for use by supplementing with an additional 4 ng/mL basic fibroblast growth factor (bFGF). hESC medium comprised DMEM-F12 supplemented with 15% KnockOut Serum Replacement, 100 μM β-mercaptoethanol (β-ME), 1% non-essential amino acids (NEAA), 2 mM GlutaMAX, and 4 ng/mL bFGF. Each flask of inactivated MEFs was used to condition the medium for seven consecutive days (Burridge et al., 2007).

Immunostaining

For assessment of pluripotency, samples were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X 100. Non-specific binding was blocked with 8% bovine serum albumin (BSA) in phosphate-buffered saline for 30 min at room temperature. Samples were incubated with diluted mouse primary antibodies directed toward OCT4 (1:200; Santa Cruz Biotech, Heidelberg, Germany, http://www.scbt.com/), SSEA-1, SSEA-4, or TRA1-60 (all 1:50; Chemicon, Billerica, MA, http://www.millipore.com/) for 1 h at room temperature. Cy3-conjugated goat anti-mouse IgG + IgM secondary antibody (1:250; Jackson Immuno Research, Inc., West Grove, PA, http://jacksonimmuno.com/) was applied for 45 min at room temperature. Differentiated cells were incubated with mouse monoclonal anti-α-actinin (1:800; Sigma), α-feto protein (clone C3, 1:1000; Sigma), or β-III tubulin (MMS-435P, 1:1,000; Cambridge Bioscience, Cambridge, UK, www.bioscience.co.uk/) overnight at 4°C. Incubation with Cy3 goat anti-mouse IgG + IgM secondary antibody (1:250) was for 1 h at room temperature. Samples were mounted in Vectorshield Hardset containing DAPI (Vector Labs, Peterborough, UK, http://www.vectorlabs.com/) and imaged using a Nikon ECLIPSE-90i fluorescence microscope (Nikon Instruments, Inc., Melville, NY, www.nikoninstruments.com).

For FACS, live hESCs were analyzed by incubating a single cell suspension with diluted mouse primary antibodies directed toward SSEA-4 (1:50, Chemicon) for HUES7 and TRA1-60 for NOTT1 for 20 min at room temperature. Incubation with Alexa488-conjugated secondary antibody (1:200, Invitrogen) was for 20 min at room temperature. FACS was performed on 50,000 cells per sample (Beckman Coulter Cell Lab Quanta SC flow cytometer, Becton Dickinson, Franklin Lakes, NJ, www.bd.com) and data were analyzed using WinMDI software (http://facs.scripps.edu/software.html).

Karyotype Assessment

Exponentially growing cultures of at least 1×10^5 cells were treated with 100 ng/mL colcemid (Karyomax) for 45 min and harvested with 0.05% trypsin–EDTA. Pelleted cells (200*g* for 4 min) were resuspended in 0.6% sodium citrate and incubated at 37°C for 20 min. Cells were then centrifuged (300*g* for 4 min) and fixed by resuspension in 16.7% glacial acetic acid in methanol before washing with two further changes of fixative. Chromosome spreads were prepared by dropping cells onto glass slides, which were air-dried and heated to 70°C overnight. Chromosomes were G banded by staining with Leishman's reagent. For each culture, 30 metaphase spreads were examined; full analysis involving band by band comparison between chromosome homologs was performed on three spreads and presence of gross abnormalities visually examined in 27 spreads, in accordance with ISCN Human Cytogenetic Nomenclature International Guidelines (Mitelman, 1995).

Differentiation

Embryoid body (EB) formation was performed by forced aggregation of defined numbers of hESCs, as described previously (Burridge et al., 2007). Briefly, on day 0 of differentiation, EB formation was initiated by seeding V-96 plates with 3,000 or 10,000 cells/well in CM and centrifuging at 950*g* (~2,800 rpm) for 5 min at room temperature. EBs were maintained in V-96 plates for 4 days in CM. EBs were then transferred to an untreated 90 mm dish in D-FBS (DMEM supplemented with 20% fetal bovine serum, 100 μM β-ME, 1% NEAA, and 2 mM GlutaMAX) where they were maintained in suspension for 6 days to allow further differentiation before transfer to untreated U-96 well plates (one EB/well) in D-FBS.

For qualitative RT-PCR, reverse transcription was carried out using Superscript II (Invitrogen) with 400 ng RNA from day 10 EBs. A total reaction volume of 20 μL was used comprising 2 μL cDNA, Applied Biosystems (Foster City, CA) TaqMan Universal PCR Master Mix, No AmpErase UNG and Assay on Demand primers/probe sets for *Brachyury T* (Hs00610080_m1), *SOX1* (Hs00534426_s1), and *SOX17* (Hs00751752_s1), with *HPRT1* (Hs99999909_m1) as an internal reference. Cycle conditions were 95°C for 5 min, one cycle followed by 95°C for 10 s/60°C for 1 min, 50 cycles. Two independent PCR reactions, each in triplicate, were run and relative quantification performed using Applied Biosystems 7500 Fast Real-Time PCR System & Software. Immunostaining or electrophysiological analysis was carried out onwards from day 18 of differentiation.

Field Potential Analysis by Microelectrode Array

For electrophysiological extracellular field potential measurements, whole beating EBs were plated directly onto Matrigel-coated microelectrode arrays (MEAs; Scientifica,

Uckfield, UK, http://www.scientifica.uk.com/). Each MEA culture dish contains 60 titanium nitride electrodes, with one acting as a reference electrode. Electrodes are 30 μm in diameter and spaced at 200 μm intervals. Contact tracks are made of indium tin oxide (ITO), which is light transmissible thereby allowing visualization of the beating areas. All recordings were made at 37°C in D-FBS using 10 kHz sampling rate and captured using the Multi Channel Systems data acquisition system (Reutlingen, Germany, http://www.multichannelsystems.com/). Measurements were taken at rest and in response to 10^{-6} M isoprenaline (Tocris, Bristol, UK, www.tocris.com).

Results

hESC Culture on a Synthetic Polymer Substrate

We recently described a generic protocol that successfully maintains at least 14 hESC lines on Matrigel in MEF-CM by trypsin passaging (Anderson et al., 2007; Braam et al., 2008a; Burridge et al., 2007; Denning et al., 2006). Therefore, this system was used as a positive control for HUES7 and NOTT1 attachment and proliferation. Unmodified standard TCPS substrates did not support hESC attachment, whereas cells readily attached to Matrigel-coated TCPS and to PE-TCPS (Fig. 2A and B). Notably, even after storage for at least 1 year on the shelf at room temperature, PE-TCPS could be used as a substrate for maintenance of pluripotency in hESCs. Comparison of Matrigel and PE-TCPS cultures showed that both HUES7 and NOTT1 displayed a high nuclear to cytoplasmic ratio and the prominent nucleoli that typify hESC morphology (Fig. 2A and B). Analysis of proliferation rates during serial passages indicated that the average PD interval for HUES7 on Matrigel was 42.8 ± 0.2 h and on PE-TCPS was 52.2 ± 0.1 h (Fig. 2C). NOTT1 on Matrigel was 42.8 ± 0.6 h and on PE-TCPS was 49.7 ± 0.2 h (Fig. 2D). This indicated that surface modification of TCPS by radio frequency PE could render these substrates suitable for hESC culture.

hESC Maintenance of Pluripotency on a Synthetic Culture Substrate

We next evaluated the pluripotency of the hESC cultures on the basis of their marker expression, karyotype, and in vitro differentiation potential (Figs. 3–5). After culture for between 10 and 14 consecutive passages on PE-TCPS, immunostaining showed reactivity to the pluripotency-associated

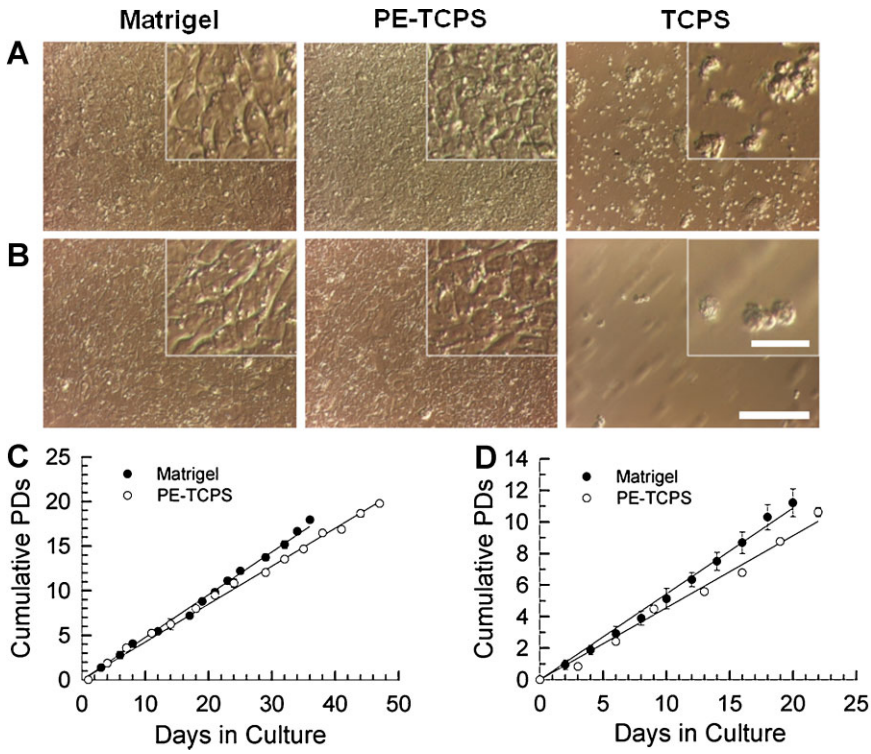

Figure 2. Culture of hESCs on different substrates. HUES7 (**A**) or NOTT1 (**B**) cells cultured on Matrigel and on PE-TCPS show similar morphology, with high nuclear to cytoplasmic ratio and prominent nucleoli, which is distinct from the lack of attachment on TCPS. Images were taken with a Nikon Hoffman objective lens, which uses polarized light and provides a 3-D view of the cell. Bar in main image = 200 μm; in insets = 50 μm. Growth curves are shown for HUES7 (**C**) and NOTT1 (**D**) on Matrigel (solid symbols) or PE-TCPS (open symbols). PDs, population doublings. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

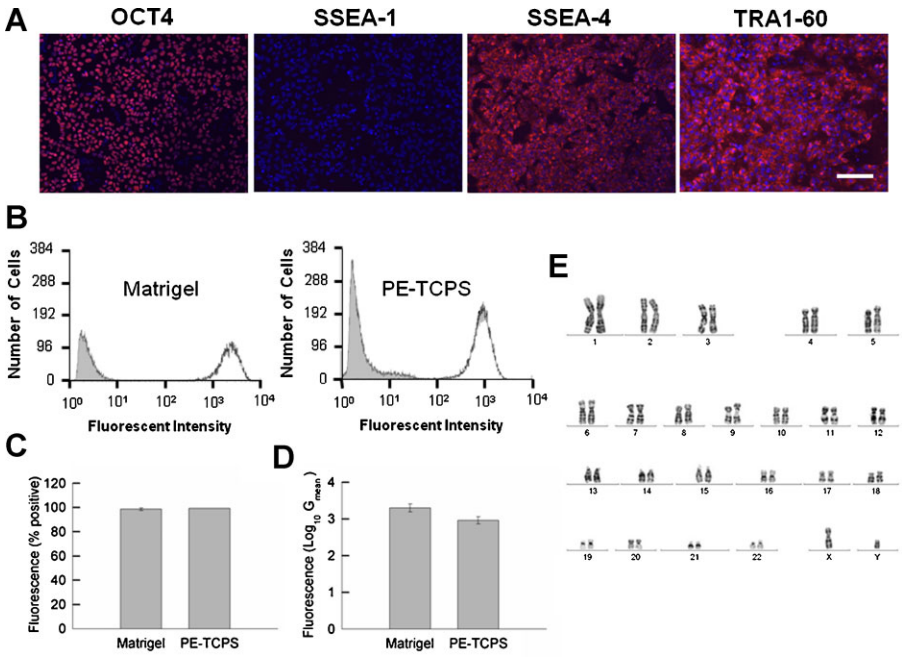

Figure 3. Characterization of HUES7 pluripotency. **A**: Immunostaining after the culture period on PE-TCPS indicated expression of pluripotency markers (red) but absence of SSEA-1. DAPI (blue) shows nuclei. Bar = 100 μm. **B**: Representative FACS plots are shown for Matrigel and PE-TCPS cultures after SSEA-4 labeling (gray, no primary antibody; white, SSEA-4). Quantification of FACS data by (**C**) percent positive cells or (**D**) G_{mean} fluorescence. **E**: Assessment of >60 G-banded chromosome preparations of HUES7 cells between p31 and p38 showed that karyotypic stability was retained in PE-TCPS cultures.

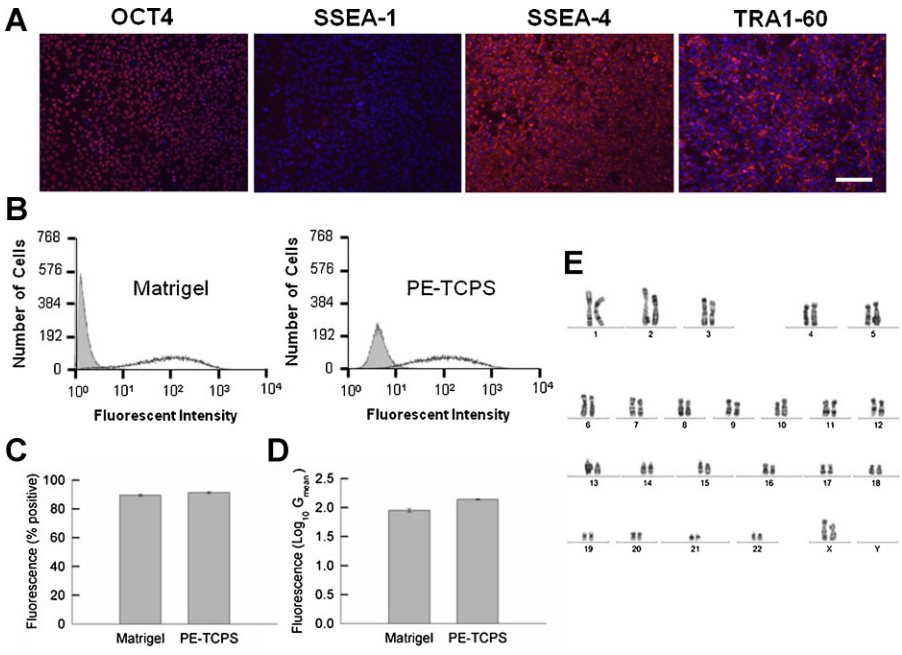

Figure 4. Characterization of NOTT1 pluripotency. **A**: Immunostaining after the culture period on PE-TCPS indicated expression of pluripotency markers (red) but absence of SSEA-1. DAPI (blue) shows nuclei. Bar = 100 μm. **B**: Representative FACS plots are shown for Matrigel and PE-TCPS cultures after TRA1-60 labeling (gray, no primary antibody; white, TRA1-60). Quantification of FACS data by (**C**) percent positive cells or (**D**) G_{mean} fluorescence. **E**: Assessment of 60 G-banded chromosome preparations of NOTT1 cells between p40 and p42 showed that karyotypic stability was retained in PE-TCPS cultures.

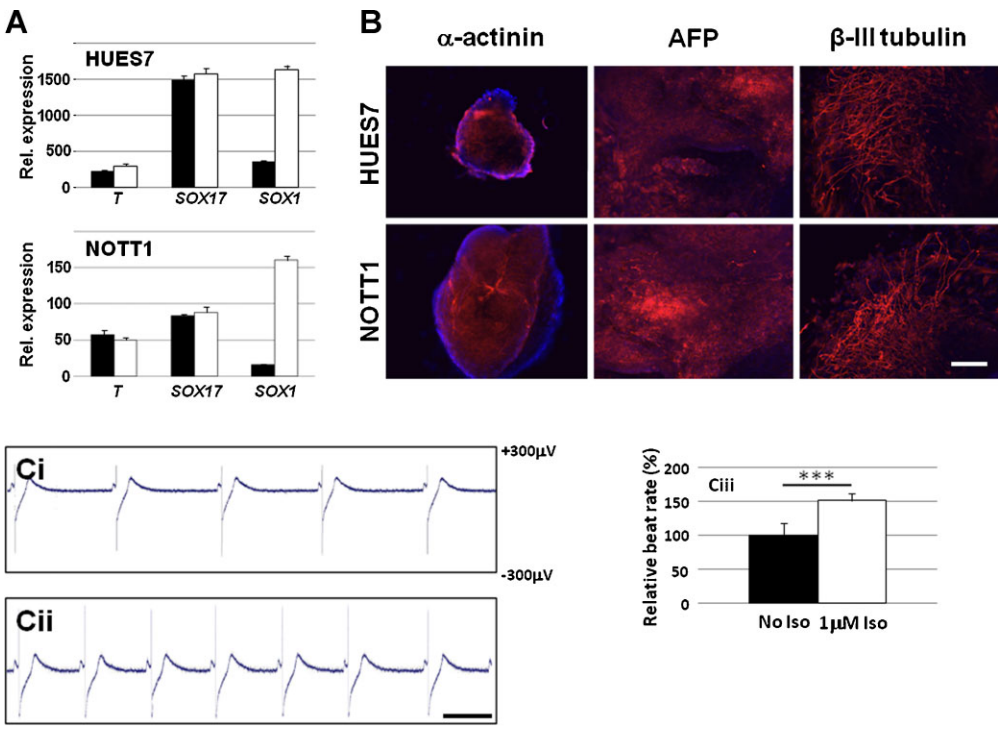

Figure 5. Differentiation potential of hESCs cultured on PE-TCPS. **A**: HUES7 or NOTT1 cells cultured on PE-TCPS or Matrigel were induced to differentiate by formation of embryoid bodies by forced aggregation. Quantitative RT-PCR was carried out on d10 of differentiation for *Brachyury T* (mesoderm), *SOX17* (endoderm), and *SOX1* (ectoderm). **B**: Immunostaining after day 18 of differentiation indicated expression of genes associated with mesodermal (α-actinin), endodermal (AFP), and ectodermal (β-III tubulin) lineages. Red is antibody staining, while blue is DAPI. Bar = 100 μm. **Ci**: Beating clusters derived by differentiation of NOTT1 PE-TCPS cultures displayed cardiac field potentials when analyzed with microelectrode arrays and (**Cii** and **iii**) responded to the β-adrenoceptor agonist, isoprenaline. = 2 s.

transcription factor, OCT4, keratan sulfate-related antigen TRA-1-60, and glycolipid stage-specific embryonic antigen SSEA-4 (Figs. 3A and 4A), but not SSEA-1, which is a marker associated with differentiation (Draper et al., 2002). FACS quantification showed similar G_{mean} levels of SSEA-4 expression for HUES7 cells cultured on Matrigel and PE-TCPS, with $99.3 \pm 0.1\%$ and $98.5 \pm 1.2\%$ cells staining positive, respectively (Figs. 3B–D; $P = 0.49$; *T*-test). Comparable results were observed for NOTT1 with TRA1-60 staining, with Matrigel and PE-TCPS showing $89.9 \pm 0.8\%$ and $91.6 \pm 0.2\%$ positive cells, respectively (Fig. 4B–D; $P > 0.05$; *T*-test). Correct karyotype was confirmed by analysis of 30 metaphase spreads per flask (60 spreads per line; Figs. 3E and 4E).

Differentiation of HUES7 and NOTT1 cells cultured on PE-TCPS was assessed by forcing aggregation of defined numbers of hESCs into EBs, a technique we developed recently (Anderson et al., 2007; Burridge et al., 2007). Quantitative RT-PCR analysis of markers that represent early germ layer formation indicated that expression of *Brachyury T* (mesoderm) and *SOX17* (endoderm) were highly similar in EBs derived from hESCs cultured on Matrigel or PE-TCPS (Fig. 5A). *SOX1* (ectoderm) expression was significantly higher when differentiation was initiated using cells cultured on PE-TCPS (Fig. 5A; $P < 0.001$; *T*-test). Immunodetection of late stage differentiation markers showed expression of α-actinin (mesoderm), AFP (endoderm), and β-III tubulin (ectoderm) (Fig. 5B). Due to our interests in heart development, we evaluated whether hESCs cultured on PE-TCPS could subsequently differentiate into functional cardiomyocytes. Beating outgrowths were mechanically isolated from the main body of EBs and seeded to MEAs, where extracellular field potentials were recorded. Traces typical of cardiomyocytes were observed at rest (Fig. 5Ci) and beat rate increased significantly to $151 \pm 10\%$ (Fig. 5Cii and Ciii; $P < 0.001$; *T*-test) relative to untreated cells in the presence of 1 μM isoprenaline, a β-adrenoceptor agonist known to have a positive chronotropic effect in the human heart. This pharmacological response also indicated that the clusters of beating cells contained hESC cardiomyocytes, since contraction of skeletal or smooth muscle cells would be unaffected or inhibited by isoprenaline (Harding et al., 2007).

PE Substrate Characterization

Because the culture of hESCs on PE-TCPS was successful in enabling attachment and maintaining pluripotency, we

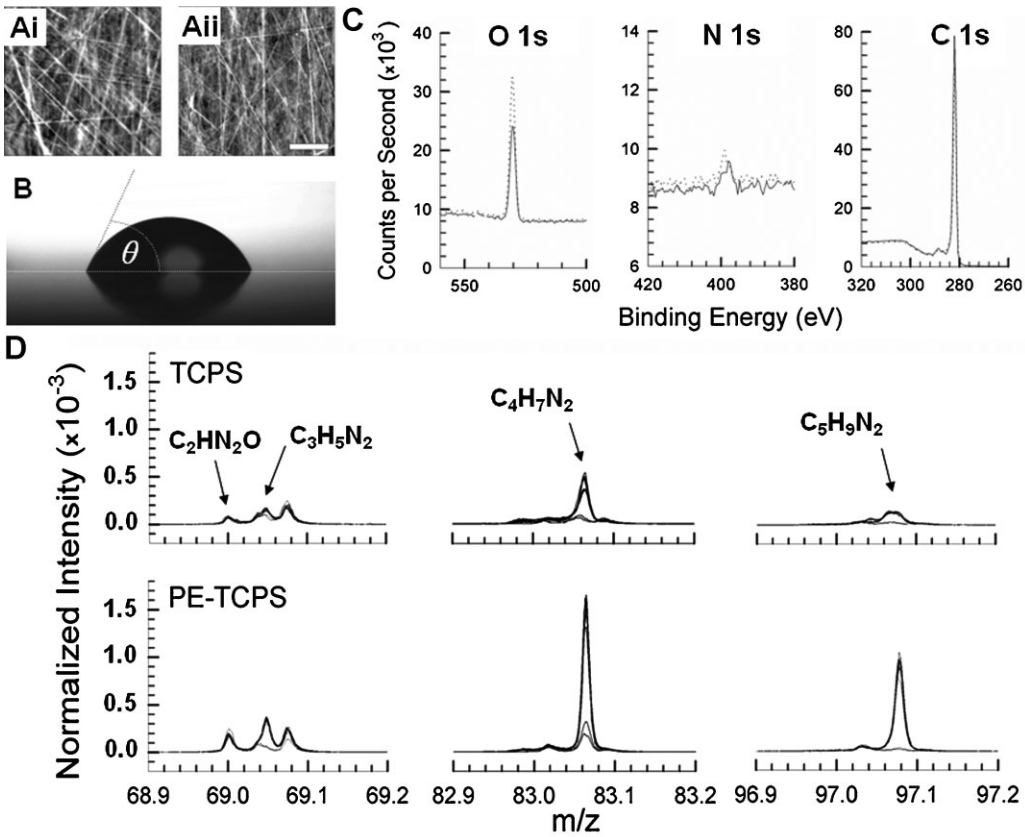

Figure 6. Surface analysis. Representative atomic force microscopy (AFM) images of TCPS (**Ai**) and PE-TCPS (**Aii**) substrates; AFM images were used to evaluate surface topography. Bar = 10 μm. Water contact angle (WCA) measurements indicated that TCPS (**B**) substrates were more hydrophobic (65.5 ± 0.6°) than PE-TCPS surfaces (<10°). Representative X-ray photoelectron spectroscopy (XPS) spectra (**C**) of TPCS (solid line) and PE-TCPS (dotted line) for quantified O1s, N1s, and C1s peaks. PE-TCPS surfaces showed a 1.6-fold increase in oxygen content. Time-of-flight secondary ion mass spectroscopy (ToF-SIMS) analysis identified 14 ions significantly increased on PE-TCPS surfaces; $C_3H_5N_2$, C_2HN_2O, $C_4H_7N_2$, and $C_5H_9N_2$ are examples from this group of ions (**D**).

characterized the surface of the plasma etched material (Fig. 6). Initial analysis of surface topography by AFM (Fig. 6A) did not reveal any differences in roughness between TCPS ($r_a = 5.96 \pm 0.31$ nm) and PE-TCPS ($r_a = 6.42 \pm 0.37$ nm) (Fig. 6A; $P = 0.38$; T-test), indicating that the enhanced hESC response on these substrates relative to untreated TCPS was not caused by differences in the physical structure. The WCA measurements for PE-TCPS revealed contact angles of less than 10° compared with a value of 65.6 ± 0.6° for the TCPS before oxygen plasma treatment (Fig. 6B), indicating that the oxygen etched surfaces were highly hydrophilic. The elemental surface composition determined by XPS (Fig. 6C) demonstrated that elemental oxygen had increased by 63% from 7.84 ± 0.28% on the TCPS samples to 12.81 ± 0.22% on PE-TCPS samples ($P < 0.001$; T-test). The concentration of nitrogen also increased by 11% from 0.77 ± 0.06% on TCPS samples to 0.86 ± 0.03% on PE-TCPS samples. ToF-SIMS analysis (Fig. 6D) showed that relative to TCPS, the levels of 14 ions were significantly increased on PE-TCPS (all $P < 0.001$; Table I). These included both small hydrocarbon fragments ($C_2H_3^+, C_2H_5^+, C_3H_5^+, C_3H_7^+$) and oxygenated hydrocarbons (CHO), but principally they were nitrogen-containing fragments ($C_2H_3N_2^+, C_2HN_2O^+, C_3H_5N_2^+, C_3H_7N_2^+, C_3H_9N_2^+, C_4H_7N_2^+, C_5H_9N_2^+, C_3H_9NO_3^+, C_3H_9NO_4$).

To determine whether the PE altered adsorption of components of CM to the surface, we used quartz crystal microbalance analysis (QCM) and FTIR spectroscopy with an ATR accessory (FTIR ATR). QCM detects changes in overall mass absorbed to the surface while FTIR ATR can be used to monitor adsorption of components from the culture medium, generally regarded to be dominated by proteins. Incubation of polystyrene substrates with CM resulted in adsorption that rapidly plateaued and after washing the bound mass was 619 ng/cm^2 (Supplementary Fig. 3A). In contrast, plasma etched substrates showed rapid initial followed by gradual continued adsorption during the test period, with a mass of 1,086 ng/cm^2 being recorded after washing, which represents an increase of 1.75-fold relative to non-etched substrates (Supplementary Fig. 3A). FTIR ATR analysis of etched and non-etched surfaces incubated with CM showed increases in adsorption for amide peaks I and II,

Table I. ToF-SIMS analysis of TCPS and PE-TCPS shows that plasma treatment changes the composition of specific molecular species at the surface.

m/z	Ion assignment	TCPS (normalized intensity)	PE-TCPS (normalized intensity)
27.0257	C_2H_3	15.14 ± 0.43	23.57 ± 1.11
29.0046	CHO	2.12 ± 0.05	5.31 ± 0.18
29.0416	C_2H_5	12.67 ± 0.53	17.16 ± 1.17
41.0397	C_3H_5	23.61 ± 0.76	32.65 ± 2.01
43.0558	C_3H_7	5.92 ± 0.75	13.00 ± 1.21
55.0217	$C_2H_3N_2$	9.97 ± 0.44	12.09 ± 0.24
69.0007	C_2HN_2O	1.39 ± 0.03	3.02 ± 0.12
69.0463	$C_3H_5N_2$	3.82 ± 0.16	5.99 ± 0.57
71.0563	$C_3H_7N_2$	3.03 ± 0.48	6.85 ± 0.84
73.0756	$C_3H_9N_2$	1.41 ± 0.15	4.56 ± 0.63
83.061	$C_4H_7N_2$	8.50 ± 0.96	22.65 ± 2.56
97.0771	$C_5H_9N_2$	3.48 ± 0.38	13.48 ± 1.81
107.0549	$C_3H_9NO_3$	3.10 ± 0.11	4.54 ± 0.25
123.0505	$C_3H_9NO_4$	0.76 ± 0.05	1.37 ± 0.11

Relative to TCPS, 14 ions were found at significantly higher levels on PE-TCPS surfaces (all $P < 0.001$). *m/z*, mass-to-charge ratio. Spectral data were normalized using total counts from each respective spectrum.

which identify protein secondary structure (Supplementary Fig. 3B), consistent with protein adsorption accounting for the irreversible mass again observed in the QCM experiment.

Discussion

This report has demonstrated that chemical modification of TCPS by radio frequency PE can produce a surface compatible with hESC culture and pluripotency that is cost-effective, stable at room temperature for at least 1 year, reproducible, and easy to handle. Notably, the PE process described here has also been used to successfully change the surface chemistry of TCPS from several manufactures (Nunc, Sarstedt, BD Biosciences), as well as spin-cast polystyrene films, to make them suitable for hESC culture (data not shown). This indicates that the plasma treatment method used has generic applicability in hESC biology and should represent a considerable improvement over the use of feeder cells and isolated or recombinant biological matrices, particularly when producing hESCs at scale for industrial application (Thomas et al., 2009).

ToF-SIMS analysis of PE-TCPS indicated that 14 molecular species were observed at significantly higher concentrations than on TCPS and comprised oxygen- or nitrogen-containing hydrocarbons (Table I). This was reflected by an overall increase in the percentage content of these elements in the substrate, as determined by XPS. WCA measurements observed ($<10^\circ$) for PE-TCPS recorded an increase in the hydrophilic nature of the surface relative to untreated TCPS substrates. This is to be expected based upon the data obtained from the XPS and ToF-SIMS spectra. The increase of the surface nitrogen concentration measured by XPS after plasma treatment is most likely a result of residual nitrogen gas inclusion in the plasma, although post-treatment reaction of the surface with nitrogen in the vent gas or the ambient atmosphere cannot be ruled out entirely.

Identifying the characteristics of the PE-TCPS substrate presents exciting opportunities for custom modification of surfaces to further enhance the characteristics of polystyrene for hESC culture. Notably, we have also found that Primaria (BD Biosciences) and CellBIND (Corning Inc., Corning, NY) culture surfaces, where the "standard" TCPS substrate chemistry has been modified by the manufacturer, can support hESCs to some extent. However, proliferation was typically slower when compared to PE-TCPS and the level of spontaneous differentiation was often higher, as evidenced by expression of the differentiation marker, SSEA1 (Supplementary Fig. 1). The differences in cell behavior between the commercial surfaces and PE-TCPS may relate to their distinct elemental composition and to the distribution of molecular species (Supplementary Fig. 2). Nevertheless, PE-TCPS, Primeria, and CellBIND are all highly hydrophilic, suggesting alterations to surface chemistry that increase hydrophilicity may be the primary consideration to further improve hESC attachment and proliferation.

PE produced a surface that was compatible with enhanced adsorption of CM components, including proteins, as judged by QCM and FTIR ATR analysis (Dixon, 2008; Welle, 2004). QCM analysis showed that adsorption to hydrophobic non-etched polystyrene surfaces was rapid and produced a stable plateau, whereas on hydrophilic etched surfaces adsorption continued throughout the analysis period and a stable plateau was not reached. Such kinetics have been observed previously (Vroman, 1962), which may shed light on the ability of etched surfaces to support hESC attachment. For example, adsorption and saturation of albumin to hydrophobic surfaces occur more readily than to hydrophilic surfaces (Messina et al., 2009). However, hydrophilic substrates show greater protein exchange at the surface, where loosely bound molecules such as albumin are continuously desorbed and replaced by larger, higher molecular weight proteins such as fibronectin, which can enhance cell attachment (Renner et al., 2004).

The notion that plasma etched surfaces enhanced protein adsorption was corroborated by FTIR ATR analysis. Increases were observed in amide I peak at 1,643 cm^{-1}, comprising mainly of C=O stretching vibrations, and the amide II peak at 1,537 cm^{-1}, representing N–H bending and C–N stretching vibrations, are characteristics of protein secondary structure (Stuart, 1996). Increased protein binding and cell attachment is consistent with the physicochemical changes induced by PE. Indeed, hydrophilicity, predominance of hydroxyl groups, and increased interfacial energy of the surface have been proposed to enhance adsorption of proteins that promote cell attachment (Curtis et al., 1983; Ramsay et al., 1997). Nevertheless, a major challenge for the future will be to determine which of the >100 proteins identified in CM (Lim and Bodnar, 2002) is crucial for hESC attachment and proliferation.

Optimizing the substrate further will help tackle the fundamental challenges still remain in culturing hESCs. In the current report, passaging of hESCs on PE-TCPS was successful with trypsin (Fig. 2) or accutase (data not shown), enzymes that dissociate cultures to single or small clumps of cells. However, cultures passaged with collagenase, which produces large clumps, failed to spread successfully and high levels of differentiation were observed. In addition, we relied on medium conditioned from MEFs to maintain pluripotency in the hESCs cultured on the defined surfaces. CM from human fibroblasts has also been used to culture hESCs on plastic (Bigdeli et al., 2008), although in this previous study no surface modification or characterization was performed and so a route to rationally improving the properties of the culture substrate may be more challenging. While CM is still considered the ''gold standard'' culture system to which new developments are compared (Desbordes et al., 2008), this medium is poorly defined and will impede the development of clinical grade products because of the potential for viral transmission (Amit et al., 2004; Cobo et al., 2008) or transfer of xeno-epitopes, such as Neu5Gc, to hESCs (Martin et al., 2005).

Recently, various defined media have been formulated by evaluating the pluripotency networks expressed in hESCs (Avery et al., 2006; Liu et al., 2006; Lu et al., 2006; Ludwig et al., 2006a; Vallier et al., 2005). These media may provide an important starting point for culture improvement, although their reliance on numerous growth factors highlights the need for high-throughput strategies to find cheaper molecules to maintain pluripotency (Desbordes et al., 2008). Our preliminary data with mTeSR defined medium (Ludwig et al., 2006b) and StemPro indicate that culture is successful with trypsin passaging on Matrigel (C. Denning, M.M. Mahlstedt, R. McGilvray, unpublished data) but is not for cultures on PE-TCPS. Interestingly, pre-coating PE-TCPS surfaces with CM did enhance attachment in StemPro (Supplementary Table I) and this observation supports the notion that PE enhances protein adsorption from the medium and hence subsequent cell attachment. However, even with pre-coating with CM, attachment was limited in StemPro and the hESCs could not be maintained through serial passage. Therefore, it would seem likely that CM not only provides proteins that absorb to the surface but also induces a gene/protein expression profile that permits cell–matrix interaction. Indeed, changes to passaging method, culture substrate and medium have all been shown to contribute to gene expression changes in hESCs, including integrins (Braam et al., 2008b; Dvorak et al., 2005; Skottman et al., 2006). Culture of hESCs on PE-TCPS also influenced gene expression during differentiation. Elevated levels of *SOX1* were observed in EBs formed from hESCs cultured on PE-TCPS as compared to those on Matrigel (Fig. 5A).

The observations above highlight physicochemical modification of polystyrene as a route to improving the culture surface for maintenance of hESC pluripotency. The challenge now is to identify the changes necessary such that defined surfaces, media formulations, and passaging regimes can be combined successfully to maintain pluripotency in a broad range of hESCs and newly developed induced pluripotency stem cells (iPSCs). This may entail use of polymer microarrays (Anderson et al., 2005; Pernagallo et al., 2008) or physical manipulation of polymer substrates to present micro- and nanoscale features (e.g., by polymer demixing, Dalby et al., 2004; lithography, Denis et al., 2002; micropatterning, Moroni and Lee, 2009; and spinodal wrinkling, Sharp et al., 2006). Realizing these goals should expedite the use of hESCs and iPSCs in biomedicine.

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