Impact of vitronectin concentration and surface properties on the stable propagation of human embryonic stem cells[a)]

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The standard method for culturing human embryonic stem cells (hESC) uses supporting feeder layers of cells or an undefined substrate, Matrigel™, which is a basement membrane extracted from murine sarcoma. For stem cell therapeutic applications, a superior alternative would be a defined, artificial surface that is based on immobilized human plasma vitronectin (VN), which is an adhesion-mediating protein. Therefore, VN adsorbed to diverse polymer surfaces was explored for the continuous propagation of hESC. Cells propagated on VN-coated tissue culture polystyrene (TCPS) are karyotypically normal after >10 passages of continuous culture, and are able to differentiate into embryoid bodies containing all three germ layers. Expansion rates and pluripotent marker expression verified that a minimal VN surface density threshold is required on TCPS. Further exploration of adsorbed VN was conducted on polymer substrates with different properties, ranging from hydrophilic to hydrophobic and including cationic and anionic polyelectrolyte coatings. Despite differing surface properties, these substrates adsorbed VN above the required surface density threshold and were capable of supporting hESC expansion for >10 passages. Correlating wettability of the VN-coated surfaces with the response of cultured hESC, higher cell expansion rates and OCT-4 expression levels were found for VN-coated TCPS, which exhibits a water contact angle close to 65°. Importantly, this simple, defined surface matches the performance of the benchmark Matrigel, which is a hydrogel with highly complex composition. [DOI: 10.1116/1.3525804]

I. INTRODUCTION

Human embryonic stem cells (hESC) are isolated from the inner cell mass of blastocysts and thus retain the ability to differentiate into the three primary germ layers (endoderm, ectoderm, and mesoderm). They offer the potential for applications in tissue repair and drug discovery.[1] Obtaining significant numbers of hESC is a prerequisite for their use in both research and industrial scale applications. This drives efforts to explore cell culture environments that are capable of hESC propagation, which is defined as the expansion of hESC over multiple passages while preserving their karyotypic stability and pluripotency.[2] The current standard method for hESC culture employs a Matrigel™ (MG) cell culture substrate,[3] which is a commercial product distributed

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by Becton Dickinson. It consists of a gelatinous membrane extracted from a mouse sarcoma, rich in laminin-111, and contains collagen, entactin, heparan sulfate proteoglycans, and several growth factors.[3] Several methods also employ a cell culture medium that is conditioned by exposure to inactivated mouse or human feeder cells.[1,4–6] This conditioning, when supplemented by basic fibroblast growth factor, maintains pluripotency of hESC. However, these poorly defined cell culture environments may be a source of xenogenic risk and are not suitable for generating clinical-grade cells for medical applications. Recent efforts have focused on developing a defined cell culture environment that is capable of the stable and prolonged expansion of hESC. Hakala *et al.* recently showed the performance of MG to be superior to other cell culture substrates when used to expand hESC in defined cell culture media.[7] As a natural product, MG suffers from batch-to-batch variability and introduces a complex protein mixture. It may also expose cultured hESC to non-human sialic acid, an immunogenic molecule that can limit its industrial scale-up for clinical applications.

With the aim of defining surface properties that are suitable for hESC culture, a study by Derda *et al.* screened an array of surface-immobilized short peptide sequences.[8] Specific surface properties showed promise and the study identified a surface density threshold. However, the surface density threshold was not quantified and hESC pluripotency was inferred from expression of the OCT-4 genetic marker, which is necessary but not sufficient criterion for establishing hESC pluipotency. A later study demonstrated the stable propagation of hESC in a defined culture medium on a synthetic surface consisting of selected peptide sequences covalently bound to an acrylate polymer matrix.[9] Karyotypic stability of the expanded hESC was verified and their ability to differentiate into the three germ layers was established *in vitro*. Kolhar *et al.* showed that a cyclic arginine-glycine-aspartic acid (RGD) peptide sequence conjugated to amine moieties presented on tissue culture plastic is capable of supporting the long-term propagation of hESC and the peptide surface density was estimated at 10–30 fmol/cm^2 from fluorescence measurements.[10] A full synthetic polymer surface with tunable wettability and rigidity has been shown to support hESC expansion in conditioned cell culture medium.[11] However, this surface could not support the long-term propagation of multiple hESC lines in defined, serum-free medium. Indeed a recent comprehensive study reports that wettability may be optimally tuned to enhance hESC proliferation but integrin engagement with surface-presented vitronectin is required to promote hESC colony formation and ensure a viable platform.[12]

To develop a cell culture platform based on defined extracellular matrix (ECM) proteins, Ludwig *et al.* used human collagen IV, laminin, fibronectin, and vitronectin to expand hESC in a defined cell culture medium.[13] Although the propagated hESC expressed pluripotency markers and were able to differentiate *in vivo*, prolonged expansion resulted in karyotypic instability, which may result from adaptation to the new platform. Similar studies have found that single-component matrices provide viable substrates for the stable propagation of hESC: laminin, fibronectin, laminin-511, and vitronectin.[14–17]

With the emergence of a defined substrate as a realistic possibility, Braam *et al.* identified that surface-presented vitronectin (VN) enables the adhesion of hESC and activates a key integrin, which is believed to play a role in supporting the long-term propagation of hESC.[16] This study was extended to the propagation of human induced pluripotent stem cells cultured on VN by Rowland *et al.*[18] They identified the same integrin as mediating cell attachment but added that its subsequent inhibition enhanced cell proliferation.[18] VN is an ECM protein that promotes cell adhesion and spreading.[19] A recent study by Yap *et al.* demonstrated the long-term propagation of multiple hESC lines on VN immobilized by adsorption on tissue culture polystyrene (TCPS).[20] A novel aspect of this work was the quantification of the adsorbed VN surface density. Correlation of this data with the response from cultured hESC established that a threshold VN surface density of 250 ng/cm^2 is required to successfully expand these cells while preserving their pluripotency.[20] Moreover, VN surface density on TCPS equal to or greater than this threshold provides a cell culture surface that matches the performance of MG in both defined and conditioned cell culture media and provides the opportunity to adapt this platform to the culture of hESC at scale.

Having established that VN adsorbed to TCPS is a viable substrate capable of supporting the long-term propagation of multiple hESC lines,[20] the present study examines VN presented by polymer substrates with a variety of surface properties, namely: bare, coated with cationic and anionic polyelectrolyte, and rendered hydrophilic from plasma treatment. Heparin, which presents affinity-binding sites for VN,[19] was also introduced as an anionic polyelectrolyte. Following an initial screening of these surfaces, with and without VN coating, the long-term propagation of a single hESC line is tested on four promising candidates, whose performance is correlated with quantification of the adsorbed VN surface density. The relationship between the surface wettability and the propagation of hESC was also assessed. The scale-up potential of these surfaces was probed by measuring the hESC proliferation rate and estimating the population doubling time using optical detection followed by a final cell count.

II. EXPERIMENT

A. Preparing the cell culture substrates

1. Coating tissue culture polystyrene with varying vitronectin surface density

TCPS surfaces were coated with human plasma-purified VN at different surface densities, as described by Yap *et al.*[20] Briefly, VN solutions of varying concentration were prepared by diluting 1 mg/ml stock VN solution (Millipore CC080) with sterile 1× phosphate buffered saline (PBS), diluted from 10× PBS (Sigma P5493) using pure water (PURELAB® Option Q, Elga) to 1.25, 2.5, 5, and 10 μg/ml (VN1.25, VN2.5, VN5, and VN10, respectively). These so-

lutions were used to coat 60 mm center-well TCPS organ culture dishes, OCD, (Becton Dickinson Biosciences) by incubating the OCD with 300 μl of the solution at 4 °C for 15 h. The VN-coated OCDs were rinsed briefly with PBS before using them as substrates for cell culture.

2. Preparation of VN-coated polymer substrates with different surface properties

Biaxially oriented polyethylene terephthalate (PET) and polystyrene (PS) films, 100 and 125 μm thick, respectively, were sourced from Goodfellow (Cambridge, UK). Circular disks, 16 mm in diameter, were cut from the films and were cleaned by soaking in isopropanol. These were rinsed in pure water and blow-dried with nitrogen before use. An *in situ* polymerized, grafted poly(acrylic acid) (PAA) brush was developed on PET using the method described by Racine *et al.*,[21] with a UV activation time of 15 min and a UV polymerization time of 5 min. The surface density of the carboxylic acid groups on PET-PAA was quantified by Toluidine Blue O (Sigma-Aldrich T3260) staining and titration, as described by Racine *et al.*[21]

Bare PET films and PET-PAA films were briefly rinsed with water and dried under nitrogen airflow before sterilizing them for 15 min within 70% ethanol, followed by twice rinsing in sterile PBS, in the sterile environment of a biological safety cabinet (NuAire Nu-425-400E). The PET-PAA surfaces were activated by a 15 min exposure to a *p*H 10 buffer solution, consisting of 0.0214*M* NaOH (Goodrich Chemical Enterprise, Singapore) and 0.05*M* sodium hydrogen carbonate (Merck), which ensured charging of the PAA brush. This was followed by two rapid rinses in PBS before the surfaces were coated with poly-L-lysine (PLL). The PET and PET-PAA disks were coated with PLL in an OCD, which was filled with 400 μl of 50 μg/ml PLL (Sigma-Aldrich P6282) in PBS solution. The samples were incubated for 20 min followed by three rinses in PBS. The PLL surface density on PET was measured by Ponceau S staining, using the method described by Yap *et al.*[20] Similarly, surfaces were coated with heparin in an OCD by incubating with 400 μl of a 100 μg/ml heparin (Sigma H3149) solution in PBS for 30 min, followed by three rinses with PBS. Autoclaving was used to sterilize PBS and the *p*H 10 buffer solution. PLL and heparin solutions were sterilized by filtration through sterile NALGENE® syringe filter membranes with a pore size of 0.2 μm (Nalge Nunc International Corporation) using a 10 ml sterile Injekt® syringe (B. Braun Melsungen AG).

Oxygen-plasma-treated PET and PS films (PET-plasma and PS-plasma, respectively) were activated in a plasma chamber (Triple P, Duratek, Inc.) using a 100 W, 2.45 GHz radio frequency discharge applied to an oxygen gas pressure of 3.5×10^{-1} Torr for 1 min. These films were subsequently sterilized by a 15 min exposure to the UV light in the biological safety cabinet.

Coating of PET-plasma, PS-plasma, bare PET, PET+PLL, PET+PLL+heparin, PET-PAA, PET-PAA+PLL, and PET-PAA+PLL+heparin disks with VN was achieved by placing them in sterile (as-received) OCDs, where they were weighed down by cylindrical glass rings (borosilicate glass, outer diameter of 17 mm, wall thickness of 1.5 mm, and height of 10 mm). The surfaces were coated from a 400 μl volume of 5 μg/ml vitronectin in PBS by incubating them for 15 h at 4 °C, followed by a brief rinse with PBS immediately prior to cell seeding.

3. Control cell culture substrates coated with Matrigel™

Control cell culture plates consisted of OCDs coated with Matrigel™ (Becton Dickinson and Company, Franklin Lakes, NJ). Cold KnockOut (KO) medium, consisting of 85% KO-Dulbecco's modified eagle medium (Invitrogen 10829-018), 15% KO serum replacement (Invitrogen 10828-028), 1 mM *L*-glutamine (Invitrogen 25030-032), 1% nonessential amino acids (Invitrogen 11140-050), 10 ng/ml FGF-2 (Invitrogen PHG0021), 0.1 mM 2-mercaptoethanol (Invitrogen 21985-023), 25 u/ml penicillin, and 25 μg/ml streptomycin (Invitrogen 15140-122), was used to dilute MG in a 1:30 ratio. Each OCD was coated with 1 ml of this coating solution for 15 h at 4 °C, followed by a brief rinse in KO medium before cell passaging.

B. Biological responses of hESC on vitronectin surfaces

1. Culture of hESC on TCPS coated with different VN surface densities

HES-3 (46, XX) cells (ES Cell International) were cultured in conditioned medium (CM), prepared as described by Yap *et al.*[20] The received cells were first cultured at 37 °C/5% CO_2 on TCPS dishes coated with MG. They were then adapted to TCPS dishes coated with VN5 for over ten passages. Cells from VN5 were then tested on TCPS dishes coated with VN1.25, VN2.5, VN5, and VN10. In these experiments, the cell culture medium was changed daily and the cells were passaged weekly, as described by Yap *et al.*[20]

The passaging process for HES-3 cells grown on MG coated TCPS involved the removal of differentiated cells by pipetting and treatment with collagenase IV (200 u/ml) for 3 min at 37 °C. They were dissociated by scraping and repeated pipetting into small clumps and seeded into freshly coated OCD at a 1:5 split ratio. The passaging process for HES-3 cells cultured on all VN-coated surfaces followed the one used for MG. The hESC passage is abbreviated as P*n*, where *n* denotes the passage number (e.g., passage 2, P2).

2. Growth tracking of hESC on different matrices

In the first set of experiments, 1×10^5 HES-3 cells/cm^2 were seeded into TCPS coated with VN1.25, VN2.5, VN5, and VN10, as well as a MG control. These experiments were performed over 6 weeks and growth curves over 7 days were measured at P2, P6, and P7. Samples were run in triplicate at each passage and for each condition.

Freshly-seeded cells were cultured in an incubator (Galaxy R, RS Biotech Laboratory Equipment Ltd.) for 48 h, after which the media in the dish was refreshed and the cells were imaged with the stem cell imaging system (SCIS), de-

veloped by Oh *et al.*[22] The cell culture medium was subsequently refreshed daily and the cells were also imaged daily from day 2 to day 7. SCIS image analysis measured the surface area covered by cells. A threshold value was set on the system such that it detected cell colonies observed on the OCD, without the opaqueness of the different cell culture substrates impinging on the calculation of the total area covered by the cells. From the zone of linear cell area expansion, the expansion rate was calculated as the time-rate increase in area. Cells harvested on day 7 were resuspended in 1 ml and counted with a NucleoCounter® NC-100™ (ChemoMetec A/S, Denmark). Cell coverage of the surface and cell expansion rates on the OCD or polymer disks were normalized to the surface area available for cell attachment within the image or to the inner diameter of the glass ring. These surface areas in the OCD and on the disk are approximately 2.4 and 2 cm^2, respectively.

The second set of experiments used the same procedure as described above with HES-3 cells that were previously cultured for over ten passages on TCPS coated with VN5. HES-3 cells were mechanically passaged weekly and seeded onto PET, PET+PLL, PET-PAA, PET-plasma, or PS-plasma disks at a split ratio of 1:5. TCPS+VN5 and TCPS+MG were included as the positive controls in this experiment. At P13, 2.5×10^4 HES-3 cells/cm^2 were seeded onto these freshly coated surfaces, and their growth rates, maximum cell numbers, and pluripotent marker expression were monitored in triplicate.

3. Flow cytometry analysis

Flow cytometry, also known as fluorescence activated cell sorting (FACS), was performed by targeting the cell surface marker mAb 84[23] and transcription factor OCT-4, with an isotype control. After dissociating into a single cell suspension with TrypLE™ Express (Invitrogen), cells are fixed and permeabilized with reagent *A* and *B* (Caltag Laboratories, Invitrogen). During the cell permeabilization step, mAb 84 (1:20),[23] a cytotoxic antibody to hESC and OCT-4 (1:20, Santa Cruz) mouse monoclonal antibodies were added to reagent *B*. Cells were then washed with 1% bovine serum albumin in PBS, followed by incubation with fluorescein isothiocyanate conjugated goat antimouse antibody (1:500, DAKO) in the dark. They were then washed and resuspended with 1% bovine serum albumin in PBS and analyzed on a FACSCalibur (Becton Dickinson) as described by Yap *et al.* Briefly, data were collected from 10 000 cells, and the percentage of cells positive for the marker was gated at the intersection of the control and marker histograms.

4. Spontaneous differentiation and quantitative real time polymerase chain reaction analysis

Embryoid bodies were generated with HES-3 cells from P6, which were cultured on VN5 OCD for 1 week, after which they were scraped off and cultured in an ultralow attachment six-well plate (Corning, Inc.) in a differentiation medium, prepared as described in Chin *et al.*[24] Following their growth in suspension for 1 week, the cell aggregates were dissociated by repeated pipetting into small clumps and replated onto an OCD coated with 0.1% gelatin, then cultured for 2 weeks, with differentiation medium replaced every 2 days. On day 21 of differentiation, total ribonucleic acid (RNA) was harvested from these cells following the protocol of the RNeasy mini kit (Qiagen GmbH, Germany).

For each well of the MicroAmp® optical 96-well reaction plate (Applied Biosystems BV 4306737), 26 ng of total RNA was converted to complementary DNA using Superscript II (Invitrogen 18064-022). Quantitative real time polymerase chain reaction (qRT-PCR) was performed with a SYBR® green PCR master mix (Applied Biosystems BV) kit, used in an ABI PRISM® 7500 (Applied Biosystems BV), whose software analyzed the optical absorption data. The primers used for qRT-PCR are listed in the supplementary Table S1.[25]

5. Karyotype analysis

HES-3 cells expanded on TCPS coated with VN1.25, VN2.5, VN5, and VN10 were harvested following P10 and grown on an OCD coated with VN5 for 4 days. Karyotype analysis of these cells was carried out on 20 *G*-banded metaphases by the Cytogenetics Laboratories of the KK Women's and Children's Hospital.

6. Screening assay for HES-3 cells cultured on polymer substrates with and without VN5 coating

The short-term adhesion assay was designed to screen surfaces for HES-3 cell adhesion and subsequent expansion. If no significant numbers of cell colonies attached within 1 day, the surface was designated as nonviable. This designation is also applied when cultured cells did not attain sufficient numbers for passaging after 7 days. Continuous passaging of the cells relied on the number of colonies attached to the surface generating sufficient cells for the next passage. HES-3 cells were prepared as described above and seeded onto PET, PET+PLL, PET+PLL+Heparin, PET-PAA, PET-PAA+PLL, PET-PAA+PLL+Heparin, PET-plasma, and PS-plasma disks with and without VN5 coating. Surviving colonies from different surfaces were passaged for up to 8 weeks.

C. Surface characterization

1. Determination of VN surface density on surfaces by Ponceau S staining

Protein surface density was determined by Ponceau S staining.[20] For this experiment, 400 μl VN solutions of 0, 2.5, 5, 10, and 20 μg/ml were used to coat PET, PET-PAA, PET-plasma, and PS-plasma surfaces for 15 h at 4 °C, as described above for cell culture substrates. The top surface of the coated polymer films was exposed to Ponceau S staining solution (Sigma-Aldrich) for 15 h at room temperature. After three rinses in water and blow-drying, each sample was placed into a new OCD and 400 μl of 0.1*M* NaOH was added to each sample. The samples were then shaken gently for 20 min, allowing desorption of Ponceau S stain from the sample surface. Samples were run in duplicates, yielding four 200 μl aliquots per VN concentration. Each aliquot was

placed in a flat-bottom 96-well plate and neutralized with 15 μl of 50% acetic acid (J. T. Baker). Colorimetric absorption was measured at 515 nm on an Infinite® 200 multimode microplate reader (Tecan, Switzerland). This was compared to a standard curve, which was generated using Ponceau S concentrations ranging from 0 to 10 μg/ml in 5% (w/v) acetic acid. The Ponceau S surface density was converted into a VN surface density by establishing a relationship between the adsorbed Ponceau S stain and the quantification of VN adsorbed to TCPS, using data reported by Yap *et al.*[20] Assuming similar accessibility to Ponceau S stain of VN adsorbed to PET, PET-PAA, PET-plasma, and PS-plasma surfaces, the VN surface density was thus inferred from the Ponceau S stain surface density. The same Ponceau S staining and its subsequent quantification were used to estimate the PLL surface density on the PET+PLL substrate.

2. Contact angle measurements

The water contact angles (θ_w) of TCPS, PET, PET+PLL, PET-PAA, PET-plasma, and PS-plasma surfaces with and without different VN coating concentrations were measured at ambient temperature on a manual contact angle goniometer (model 100, Ramé-Hart, Inc.). Five 1 μl sessile pure water drops were measured on each sample. The average contact angle and its standard deviation were determined from contact angles measured on duplicates of each polymer surface.

D. Statistical analysis

Data values are reported as mean and standard deviation. Graphs were plotted and data were transformed with Microsoft Office Excel 2007 software. Student *t*-tests were performed between experimental and control conditions. *P* values < 0.05 were considered to represent statistically significant differences.

III. RESULTS AND DISCUSSION

A. Growth of HES-3 cells on TCPS coated with different concentrations of VN

Figure 1 shows representative images from triplicate cultures on different VN surface densities at P6. Growth of HES-3 cells on TCPS coated with VN1.25 and VN2.5 appears patchier than TCPS coated with VN5, VN10, and the MG control. Bare patches, devoid of hESC, appear on the VN1.25 and the VN2.5 samples at day 2 and growth over 7 days appears to be more clustered. In comparison, cells spread more evenly in the other three conditions. After 7 days' culture, cells have not fully occupied the surface of VN1.25. The SCIS quantifies this growth trend by plotting the total area of the OCD occupied by HES-3 colonies, showing the most rapid hESC expansion rates on VN5, VN10, and MG, while VN2.5 and VN1.25 substrates show progressively lower expansion rates [Fig. 1(b)]. Cell culture images at days 2 and 3 [Fig. 1(a)] indicate poorer cell attachment and lower numbers of spreading HES-3 colonies on VN1.25 and VN2.5. The area expansion rates in Fig. 2(a)

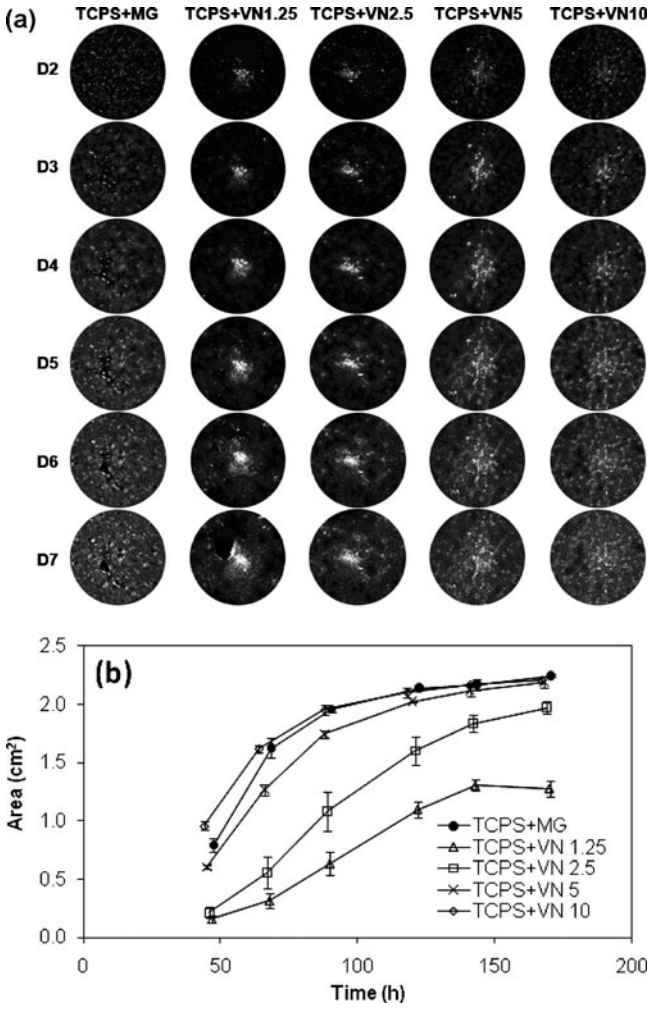

FIG. 1. (a) Images of HES-3 colonies cultured on TCPS coated with MG, VN1.25, VN2.5, VN5, and VN10, respectively, from day 2 to day 7 of P6. The diameter of each image circle is 14.5 mm. (b) Area data representing the surface covered by HES-3 cells from day 2 to day 7 on the same TCPS surfaces. These are calculated by SICS from triplicate runs of images similar to those shown in (a).

were derived from the linear growth portion of this data and similar data at P2 and P7. Although growth rates of HES-3 at P2 appear similar at all VN concentrations, the images taken from P6, shown in Fig. 1, indicate that hESC adapted to lower VN concentrations lose viability, resulting in a sharp decrease in growth rates of HES-3 cells cultured on TCPS coated with VN1.25 at P7 [Fig. 2(a)]. Indeed, despite the early signs of viable hESC culture on VN1.25, cells passaged on this surface all but lose their ability to adhere and expand by P7, highlighting the importance of testing the robustness of new culture platforms beyond five passages. Figure 2(b) shows the final cell numbers, measured with a cell counter, which differ slightly from the estimates generated by the SCIS area measurement. Moreover, the folded layer seen on day 7 [Fig. 1(a)], which is a consequence of the poor adherence of cells cultured on VN1.25, leads to larger discrepancies between the calculated areas on the TCPS surface and the final cell numbers, as observed for P7 in Figs. 2(a) and 2(b). Despite these differences, final cell numbers follow the

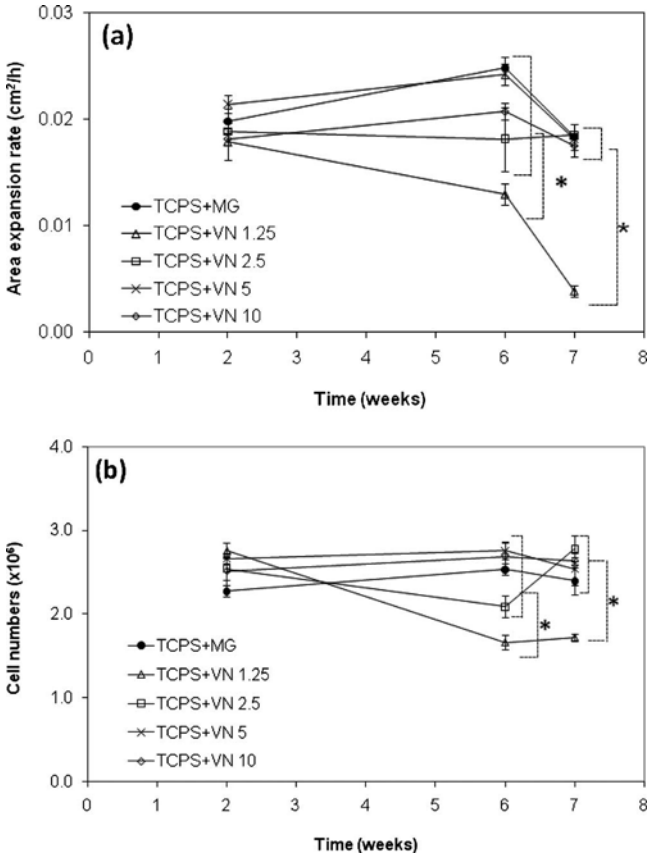

FIG. 2. (a) Area expansion rate during linear expansion phase and (b) cell numbers at day 7 for HES-3 cells at P2, P6, and P7 on TCPS surfaces coated with MG, VN1.25, VN2.5, VN5, and VN10, respectively. At P6 and P7, TCPS coated with VN1.25 shows a significant decrease in growth rate as compared to the other surfaces. $*=P<0.05$.

trends established from area expansion rates: both parameters are significantly lower on VN1.25 at P6 and P7 than on higher VN surface densities and the MG benchmark. Moreover, the VN1.25 OCD surface covered by hESC at day 7 of P6 is approximately half that of the other TCPS surfaces and the MG control. These results confirm that TCPS coated with VN1.25 is insufficient for optimal hESC expansion and that VN5 provides the required surface density to support hESC culture without compromise. The latter propagates hESC with an efficiency equal to VN10 and the MG positive control.

B. Pluripotency of HES-3 cells on TCPS coated with different concentration VN solutions

Flow cytometry analysis of OCT-4 and mAb 84 pluripotency markers at P10 are shown in Fig. 3. HES-3 cells cultured on TCPS coated with VN2.5, VN5, VN10, and the MG control surface express an equally high percentage of these pluripotent markers. Distinctly lower expression levels are exhibited by HES-3 cultured on TCPS coated with VN1.25, for which the downregulation of mAb 84 implies a probable loss of pluripotency. Moreover, visual examination of HES-3 cells cultured on VN1.25 at P10 reveals a more fibroblastic morphology and loss of the hESC phenotype (data not shown).

HES-3 cells cultured on TCPS coated with VN1.25, VN2.5, VN5, and VN10 were tested at P6 for their *in vitro* differentiation capability. As shown in Fig. 4(a), differentiated cells show downregulation of the NANOG and OCT-4 pluripotency markers and upregulation of GATA6, Hand1, and PAX6, which are representative of the endoderm, mesoderm, and ectoderm lineages, respectively. This verifies the ability of cultured HES-3 cells at P6 to differentiate into the three primary germ layers. Moreover, chromosome analysis of HES-3 at P10 indicates that the cells retain their normal

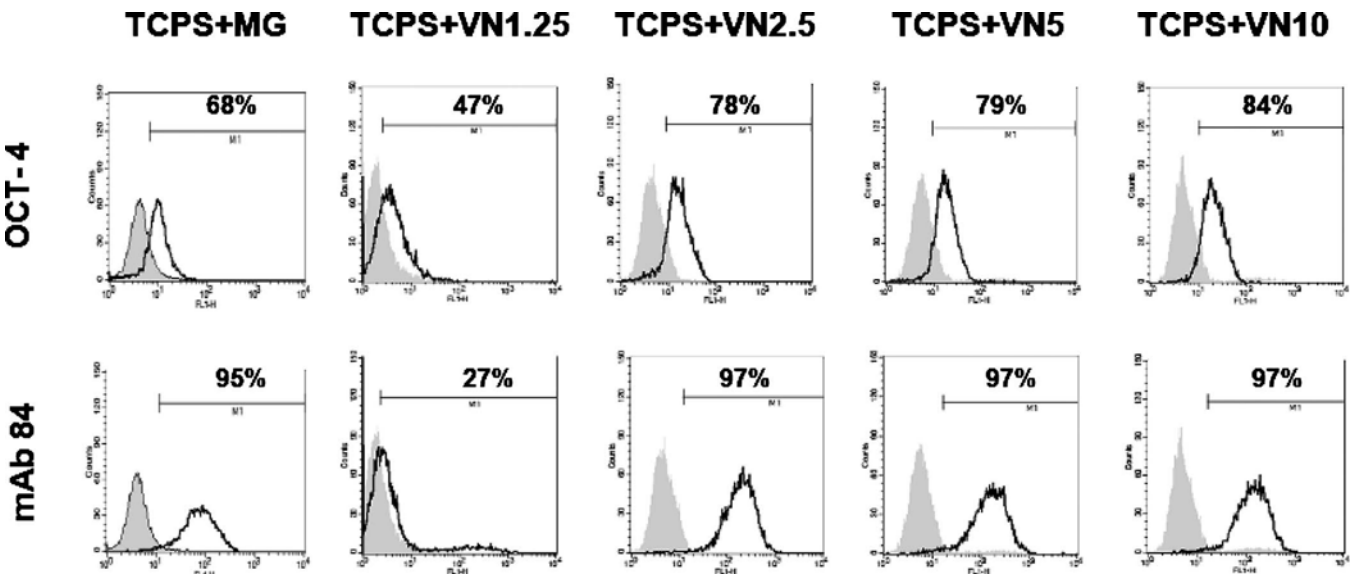

FIG. 3. FACS analysis of OCT-4 and mAb 84 pluripotent markers for P10 HES-3 cells cultured on surfaces of TCPS+MG, TCPS+VN1.25, TCPS+VN2.5, TCPS+VN5, and TCPS+VN10. Cells grown on MG were used as a positive control.

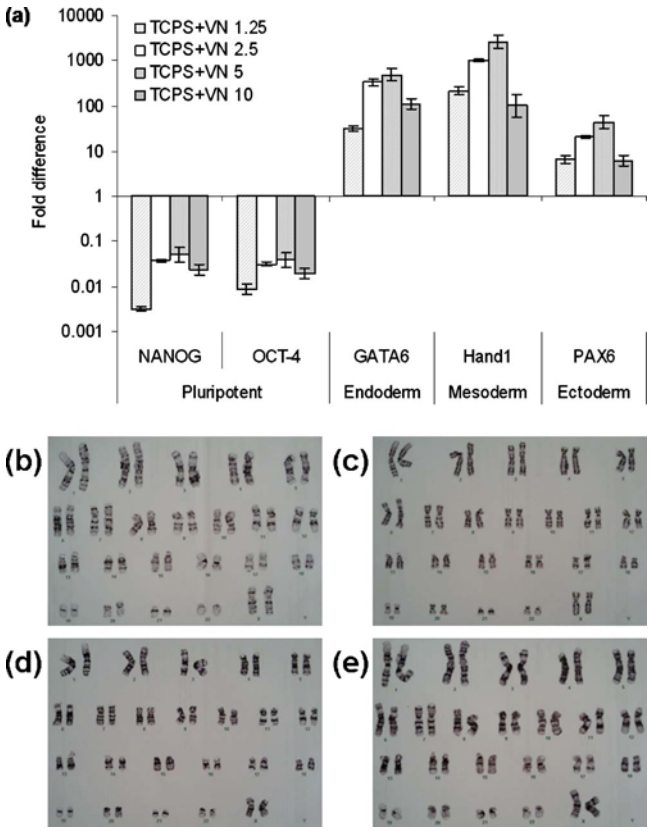

FIG. 4. (Color online) (a) Expression of genetic markers following the *in vitro* 21 days' spontaneous differentiation of P6 HES-3 cells cultured on TCPS coated with VN1.25, VN2.5, VN5, and VN10. Karyotype maps of P10 HES-3 cells cultured on TCPS coated with (b) VN1.25, (c) VN2.5, (d) VN5, and (e) VN10.

diploid karyotype, as depicted in Figs. 4(b)–4(e). Thus, cells cultured on TCPS coated with VN, irrespective of the surface density, retain their ability to differentiate (at least up to P6 on VN1.25) and maintain stable karyotype.

C. Growth of HES-3 cells on different polymer substrates coated with VN5

Preliminary cell adhesion and proliferation were used to screen the polymer substrate surface properties, bare and coated with VN5. As reported in Table I, surfaces without VN coating do not promote HES-3 attachment and cannot support the formation of viable HES-3 cell colonies, thus indicating that exogenous VN is required to promote the adhesion and support the expansion of hESC. Moreover, for surfaces with polyelectrolyte multilayer coatings comprising ≥ 4 layers (PET-PAA+PLL+heparin+VN5), the substrate cannot support HES-3 expansion, despite it being coated from VN5 solution. With a smaller number of multilayers, the surface is capable of supporting HES-3 propagation to at least P13 (TCPS+VN5, PET+VN5, PET+PLL+VN5, PET-PAA+VN5, PET-plasma+VN5, PS-plasma+VN5, PET+PLL+heparin+VN5, and PET-PAA+PLL+VN5).

SICS was used to track HES-3 proliferation on model polymer surfaces coated with VN5. Figure 5(a) shows images of P13 cells from day 2 to day 7, indicating that all the VN-coated surfaces promote cell adhesion and support their expansion. The increase in area covered by expanding cells, shown in Fig. 5(b), depicts a similar trend across all the surfaces tested, including the TCPS+VN5 and MG positive controls. The area expansion rates for the two positive control surfaces are moderately but distinguishably higher than those for other surfaces [Fig. 5(c)]. The PET+PLL+VN5 surface differs by having an initial high area expansion rate, followed by a lower area expansion rate after day 4. Figure 5(c) reports the final cell numbers, which are measured independently of the SICS data. Again, hESCs cultured on MG and VN5 show the highest cell densities compared to the other polymer surfaces at P10 ($P<0.05$). Looking more closely at the day 7 images [Fig. 5(a)], PET+VN5 and PET+PLL+VN5 appear to grow a thinner layer of HES-3 cells. This observation arises from the absence of brighter white areas, which are seen on other substrates. It is reflected in the final cell numbers, which indicate that these two surfaces yield the lowest cell numbers at day 7.

TABLE I. Screening results for short-term adhesion assay of HES-3 cells on different surfaces.

Surfaces	Attachment at P0	
	Without VN	With VN
TCPS+MG	√	···
TCPS	×	√
PS-plasma	×	√
PET-plasma	×	√
PET	×	√
PET+PLL	×	√
PET+PLL+Heparin	×	√
PET-PAA	×	√
PET-PAA+PLL	×	√
PET-PAA+PLL+Heparin	×	×

D. Undifferentiated state of HES-3 cells on different plastics coated with VN5

Flow cytometry measurements of mAb 84 and OCT-4 expression levels serve to indicate whether P13 HES-3 cells are differentiating. Figure 6 shows that greater than 50% and 90% of the cells are positive for OCT-4 and mAb 84, respectively, across the different plastic surfaces. Lower OCT-4 levels of expression occur for HES-3 cells cultured on VN-coated PET and PS-plasma. However, this decrease is not reflected by the levels of mAb 84 expression for these two surfaces. In general, hESC appear to express pluripotent markers across all polymer surfaces over several passages, implying that these substrates may be suitable for long-term culture.

E. Surface characterization

1. Quantification of surface-adsorbed VN

Ponceau S stain adsorbed to VN-coated polymer surfaces was quantified following its desorption. Assuming a linear

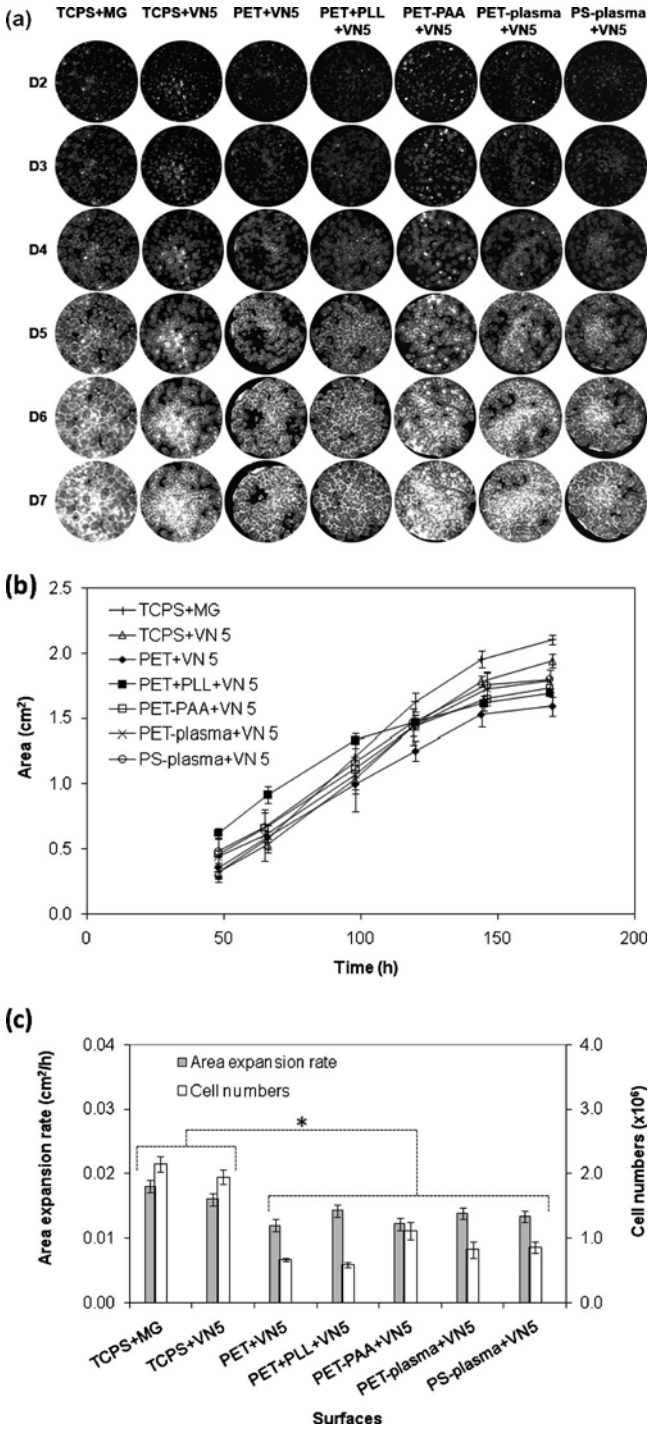

FIG. 5. (a) Representative images of HES-3 from day 2 to day 7 of P13 on the following surfaces: TCPS+MG, TCPS+VN5, PET+VN5, PET+PLL +VN5, PET−PAA+VN5, PET-plasma+VN5, and PS-plasma+VN5, respectively. Diameter of the image circles is 14.5 mm. (b) Area covered by HES-3 cells on these surfaces from day 2 to day 7 at P13. (c) Area expansion rate of HES-3 cells at P13 and total cell numbers on day 7 at P13. The data on the area expansion rate and HES-3 cell numbers are calculated by SICS from triplicate runs of images similar to those shown in (a). $*=P<0.05$, which applies only to the area expansion rate data.

increase in adsorbed VN surface density for solution concentrations below 10 μg/ml, the constant relating VN surface density to Ponceau S was determined to be $S_{VN}=22\,752^{*}S_P$, where S_P is the Ponceau S surface density in nmol/cm^2 and S_{VN} is the VN surface density in ng/cm^2. Figure 7 shows the adsorbed VN surface density, calculated from Ponceau S staining data using this relationship.

From Fig. 7, both PET and PET-PAA surfaces adsorb higher VN surface densities than PET-plasma and PS-plasma. Quantification of VN adsorbed to PET+PLL was not feasible due to Ponceau S staining of both PLL and VN. For these polymer substrates, the surface density adsorbed from VN5 is $\geq$250 ng/cm^2, which is the threshold established by Yap *et al.*[20] for culturing hESC on TCPS. This threshold is corroborated by HES-3 cell expansion rates on TCPS coated from $\geq$VN5 (Fig. 1). Thus, these polymer substrates immobilize a VN surface density that is expected to be sufficient for supporting the expansion of HES-3 cells.

2. Effects of surface wettability on the propagation and pluripotency of HES-3 cells

Substrate wettability can influence the bioresponse of cultured cells.[12,26–28] Figure 8(a) shows the sessile water drop wettability of polymer surfaces coated with increasing VN surface density. Adsorbed VN reduces the wettability of hydrophobic PET and increases the wettability of hydrophilic PS-plasma. As the VN surface density reaches saturation, the wettability of these two substrates converges to 47°–48°. For TCPS and PET-plasma, adsorbed VN gives rise to a modest rise in contact angle, from 58° to 66° and from 49° to 58°, respectively. Similarly, for PET-PAA and PET+PLL, the water contact angle increases from 52° to 67° and from 60° to 68°, respectively. With saturating VN adsorption, we may consider that the polymer substrate is fully coated with adsorbed protein molecules. Atomic force microscopy measurements reported by Yap *et al.*[20] discern aggregates of adsorbed VN molecules but are unable to resolve individual molecules, which are presumed to coat the intervening regions of the polymer substrate.

TCPS consists of plasma-treated PS, which has been stored and shipped, probably over several weeks or months. Given that bare PS exhibits a water contact angle approaching 90° and that freshly plasma-treated PS is fully wettable, the 58° measured contact angle of as-received TCPS reflects the evolution of this surface since its fabrication. The wettability increase following plasma treatment is due to the free-radicals and polar groups that were generated by polymer chain scission. During storage, these groups partially resorb into the bulk polymer, giving rise to a progressive decrease in wettability.[29] Even after their long-term storage, TCPS surfaces present polar groups within a surface layer of 3–5 nm.[29] Freshly plasma-treated PET and PS expose randomly charged hydrophilic polar moieties, such as C—O, C=O, O—C=O, and O—(C=O)—O components.[29,30] In contrast, PET+PLL exposes a cationic polymer with —NH— and —NH$_2$ moieties, and PET-PAA presents an anionic grafted polymer brush, with a carboxyl acid group (—COOH) surface density of 11.5 ± 1.4 nmol/cm^2. PET+PLL exhibits a Ponceau S density of 0.23 ± 0.4 nmol/cm^2, which is comparable to that of a saturated VN coating on

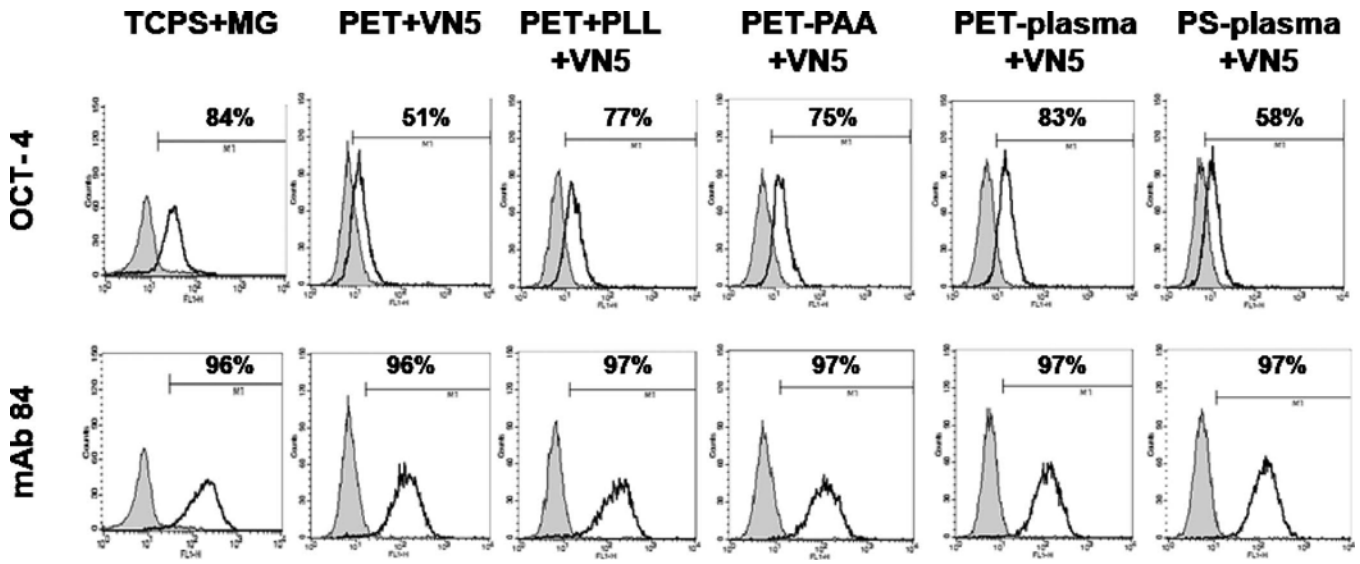

FIG. 6. FACS analysis of OCT-4 and mAb 84 surface marker for HES-3 cells at P13 cultured on the following surfaces: TCPS+MG, PET+VN5, PET +PLL+VN5, PET-PAA+VN5, PET-plasma+VN5, and PS-plasma+VN5, with MG used as a positive control.

TCPS.[20] Thus, polymer substrates without an overriding net surface charge are hydrophobic bare PET, hydrophilic PET-plasma and PS-plasma, and TCPS and PET-plasma, both of which have intermediate wettability. VN adsorbed by hydrophobic interactions is expected to expose hydrophilic groups. Conversely, VN coated on a hydrophilic surface may expose hydrophobic segments of the protein, thus decreasing the surface wettability.[31] While this may explain the convergence in wettability when VN adsorbs to bare PET and to PS-plasma substrates, the mere presence of a saturated VN coating may account for the observed wettability change. Similarly, VN adsorption to PET+PLL and PET-PAA is dominated by charge interactions and the adsorbed VN molecules contribute to the final surface wettability.

Given the surface charge presented by PET+PLL and PET-PAA, the HES-3 response to these VN-coated surfaces may also be influenced by the protein's configuration. VN

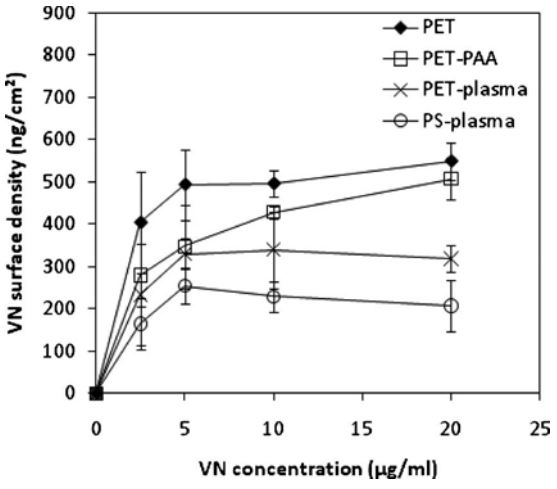

FIG. 7. Adsorbed VN surface density vs the concentration of the depositing solution for PET, PET-PAA, PET-plasma, and PS-plasma surfaces.

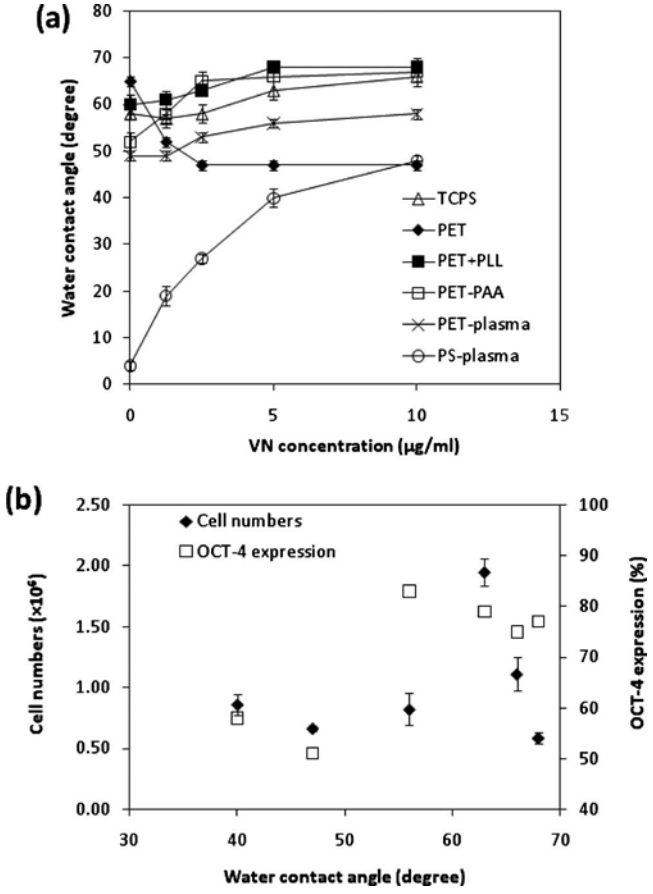

FIG. 8. (a) Water contact angles of VN-coated surfaces as a function of the depositing VN solution concentration. These surfaces include the following: TCPS, PET, PET+PLL, PET-PAA, PET-plasma, and PS-plasma. (b) Total cell numbers and expression of the OCT-4 pluripotency marker for HES-3 cells at P13. These are plotted vs the water contact angle of the following surfaces: TCPS+VN5, PET+VN5, PET+PLL+VN5, PET-PAA+VN5, PET-plasma+VN5, and PS-plasma+VN5.

contains a similar number of negatively (66) and positively (56) charged residues (ExPASy, human VN: P04004), which allow it to adsorb to both cationic and anionic surfaces, with similar efficiency but *via* different domains. The bare PET and PS-plasma surfaces represent the hydrophobic and hydrophilic extremes, respectively, of underlying substrate properties and are not expected to carry a strong net charge. When coated with VN5, these surfaces adsorb the highest and lowest levels of VN, which differ by almost a factor of 2 (Fig. 7). From Schvartz *et al.*,[19] the integrin-binding domain lies at the opposite end of the VN molecule, with respect to the heparin-binding domain. The latter is presumed to be cationic, adsorbing more favorably to PET-PAA. The collagen-binding domain, adjacent to the integrin-binding segment, may be expected to bind more favorably to PET+PLL. Thus, it is surprising that VN adsorbed to different surfaces is capable of promoting the adhesion and supporting the expansion of hESC. This is likely due to the protein's flexibility and the presence of several redundant RGD-binding sequences,[32] which facilitates their accessibility.

Long-term culture on these polymer substrates gives rise to different HES-3 proliferation rates and levels of pluripotency. Given that these surfaces are coated with a VN surface density ≥ 250 ng/cm^2, above the threshold for the HES-3 bioresponse, this leads to an examination of how their wettability may influence the expansion of HES-3 cells. Data from Figs. 5(c) and 6 are plotted versus surface wettability [Fig. 8(a)] to yield Fig. 8(b). VN-coated PS-plasma and PET exhibit the highest wettability and give rise to the lowest levels of OCT-4 expression for cultured HES-3 cells [Fig. 8(b)]. These surfaces, with underlying substrates that represent the uncharged extremes of the wettability spectrum, also generate lower final cell numbers. The highest efficiency for HES-3 culture, as measured by final cell number and OCT-4 expression level, is obtained on VN-coated TCPS with a contact angle of 63°. Lim *et al.* found that the adhesion efficiency of human fetal osteoblastic cells correlates strongly with substrate wettability, obtaining high rates of cell attachment on relatively hydrophilic surfaces and low rates on hydrophobic surfaces, with $\theta_w = 65°$ as the defined boundary.[12,28] For the cationic and anionic surfaces: PET+PLL+VN5 and PET-PAA+VN5, OCT-4 expression remains high but total cell numbers fall with respect to VN-coated TCPS. These data indicate that, the VN-coated polymer substrate properties, including surface wettability, influence the rate of HES-3 cell proliferation and their pluripotency.

The data reported in the present study and by Yap *et al.*[20] provide a clear indication that the TCPS substrate coated from VN5 provides an performance equivalent to MG, which is the current industry standard and benchmark.[7] Given that Matrigel is a water-swellable coating and contains a number of ECM proteins and polysaccharides,[33] it is interesting to note that VN-coated TCPS, which is a comparatively rigid substrate coated with only one ECM protein, is equally capable of supporting the expansion of multiple hESC lines. This efficiency has been measured by cell pluripotency, karyotype stability, and cell proliferation rates.

IV. CONCLUSIONS

The perspective of hESC manufacturing in respect of clinical-grade requirements for therapeutic applications drives the replacement of MG with a defined substrate that supports the stable propagation of hESC. VN-coated polymer surfaces were used for the long-term expansion of HES-3 cells in CM. TCPS coated with solution concentrations $\geq$VN5 provides a viable substrate, capable of expanding these cells for over ten passages. Cells maintain karyotypic stability and are able to spontaneously differentiate *in vitro*, where embryoid bodies show evidence of the three primary germ lineages. HES-3 cells cultured on TCPS coated with VN2.5 show a slight decrease in their cell expansion rate, as measured by SICS at P6. When cultured on VN1.25, the cells initially appear viable, but show a marked decrease in their adherence to the cell culture substrate at P6, terminating in a sharp reduction in the number of viable cell colonies at P7. This adaptation of the cells is also reflected in a decrease in their expression of the OCT-4 and mAb 84 pluripotency markers, which are expressed normally in HES-3 cultured on higher VN surface densities.

Other forms of VN surface presentation were pursued using diverse polymer surfaces, ranging from hydrophobic to hydrophilic, with the inclusion of anionic and cationic polyelectrolyte coatings. An initial screening confirmed that all these polymer substrates require a VN coating to promote the adhesion and support the expansion of HES-3 cell colonies. The only exception was a four multilayer coating (PET-PAA+PLL+heparin+VN5), which did not support the growth of HES-3 colonies. Coatings of $\leq$3 multilayers were able to support HES-3 expansion over at least 13 passages, indicating that VN adsorbed to these surfaces with different properties is capable of the long-term expansion of hESC. The performance of simple model surfaces (TCPS, PET, PET+PLL, PET-PAA, PET-plasma, and PS-plasma) coated with VN5 was correlated with their wettability to sessile water drops. From the expression of OCT-4 and mAb 84 markers at P13, VN-coated PET and PS-plasma exhibit a higher wettability and generate a potential loss in HES-3 pluripotency. Cationic and anionic PET+PLL and PET-PAA, respectively, give rise to slower expansion rates at P13. The highest expansion rates and pluripotency marker expression levels are observed with VN-coated TCPS, which exhibits a contact angle of 65°, a result that compares favourably with data generated by Mei *et al.*[12]

TCPS+VN5 and the benchmark MG matrix show equal performance, both for the proliferation rate and the expression of pluripotency markers in HES-3 cells. These surfaces are capable of the long-term expansion of multiple hESC lines,[20] while preserving karyotypic stability and pluripotency. This similarity in performance between VN-coated TCPS and Matrigel is remarkable, given that the former is an adsorbed ECM protein, while the latter is a hydrogel coating of complex composition.

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