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Engineering cell–material interfaces for long-term expansion of human pluripotent stem cells

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ABSTRACT

Cost-effective and scalable synthetic matrices that support long-term expansion of human pluripotent stem cells (hPSCs) have many applications, ranging from drug screening platforms to regenerative medicine. Here, we report the development of a hydrogel-based matrix containing synthetic heparin-mimicking moieties that supports the long-term expansion of hPSCs (≥20 passages) in a chemically defined medium. HPSCs expanded on this synthetic matrix maintained their characteristic morphology, colony forming ability, karyotypic stability, and differentiation potential. We also used the synthetic matrix as a platform to investigate the effects of various physicochemical properties of the extracellular environment on the adhesion, growth, and self-renewal of hPSCs. The observed cellular responses can be explained in terms of matrix interface-mediated binding of extracellular matrix proteins, growth factors, and other cell-secreted factors, which create an instructive microenvironment to support self-renewal of hPSCs. These synthetic matrices, which comprise of "off-the-shelf" components and are easy to synthesize, provide an ideal tool to elucidate the molecular mechanisms that control stem cell fate.

1. Introduction

Since the isolation of human embryonic stem cells (hESCs), there has been a tremendous interest in developing defined, scalable *in vitro* culture conditions that can support their growth. These efforts have led to the development of multiple defined growth media, but these still require either feeder layers such as mouse embryonic fibroblasts (MEFs) or biologically derived matrices such as Matrigel for maintenance of pluripotency and self-renewal of hPSCs [1–6]. Development of chemically defined matrices is a challenging task because the myriad of physicochemical signals that MEFs and Matrigel provide. Within these limitations, recent advances in the field of biomaterials have led to identification of substrates—both naturally derived and synthetic—for the self-renewal of hPSCs [7–16]. High-throughput screening technologies have contributed significantly toward the development of these chemically defined, synthetic materials [10,17].

Accumulating evidence suggests that heparin molecules play a key role in maintaining self-renewal of hPSCs [4,12,18]. Studies by Levenstein et al. showed the role of MEF-secreted heparan sulfate proteoglycans on self-renewal of hESCs [18]. To harness the beneficial effects of heparin moieties on the self-renewal of hPSCs, Klim et al. have developed synthetic matrices that display heparin-binding peptides to support long-term self-renewal of hPSCs [12]. The role of heparin moieties in self-renewal of hPSCs is not surprising given that heparin molecules can bind to soluble bFGF molecules and modulate their bioactivity [19–21]; bFGF is a crucial biomolecule required for maintenance of self-renewal of hPSCs *in vitro*. Additionally, heparin molecules have been shown to protect bFGF from denaturation and proteolytic degradation, thereby increasing its longevity and function [21,22].

Recently we have shown that synthetic heparin mimics such as poly(sodium 4-styrenesulfonate) (PSS) can bind to soluble bFGF and regulate FGF signaling akin to heparin molecules [19]. Based on these findings along with the known role of bFGF molecules on *in vitro* self-renewal of hPSCs, we developed synthetic hydrogels containing PSS moieties to support long-term culture of hPSCs while maintaining their pluripotency. Employing hydrogel-based synthetic matrices, we further elucidated the role of physicochemical cues of the matrix on self-renewal of hPSCs. Such easy to

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synthesize and cost-effective synthetic matrices would not only accelerate the translational potential of hPSCs, but also provide a platform to decipher the interplay between various physico-chemical cues on self-renewal of hPSCs. Additionally, these matrices would help to identify the myriad of molecular and signaling pathways that dictate stem cell fate and commitment.

2. Materials and methods

2.1. Materials

N-acryloyl amino acid (AA) monomers, such as N-acryloyl 2-glycine (A2AGA), N-acryloyl 4-aminobutyric acid (A4ABA), N-acryloyl 6-aminocaproic acid (A6ACA), and N-acryloyl 8-aminocaprylic acid (A8ACA), were synthesized from glycine (Fisher Scientific, Inc.), 4-aminobutyric acid, 6-aminocaproic acid, and 8-aminocaprylic acid (Acros Organics Inc.), respectively, as described elsewhere [23]. Sodium 4-vinylbenzenesulfonate (SS), 3-sulfopropyl acrylate potassium salt (SPA), and [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)ammonium hydroxide (MEDSAH) were purchased from Aldrich. Acrylamide (Am) was purchased from Invitrogen and N,N′-methylenebisacrylamide (BisAm), ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Sigma. The monomers used in this study are summarized in Supplementary Table S1.

2.2. Hydrogel synthesis

The hydrogels containing varying functional groups and hydrophilicity were synthesized through copolymerization of acrylamide with monomers containing either carboxylate or sulfonate groups. The PSS-based hydrogels (PAm_6-co-PSS_2, PAm_6-co-PSS_1, PAm_6-co-$PSS_{0.5}$) were synthesized by copolymerizing acrylamide (Am, 7.5 mmol) with sodium 4-vinylbenzenesulfonate (SS, 2.5 mmol) at 6:2, 6:1, and 6:0.5 mole ratios. The monomers were dissolved in deionized (DI) water, and polymerized in Bio-Rad 1 mm spacer glass plates at room temperature using 0.26, 0.19, and 0.10 mmol of BisAm as a crosslinker and 1.3% w/v of APS/TEMED (redox initiator/accelerator). Hydrogels containing SPA and MEDSAH moieties (PAm_6-co-$PSPA_2$, PAm_6-co-$PMEDSAH_2$) were synthesized by copolymerizing Am (7.5 mmol) with SPA (2.5 mmol) or MEDSAH (2.5 mmol) at a mole ratio of 6:2. The precursors were dissolved in DI water and polymerized using 0.26 mmol of BisAm and 1.3% w/v of APS/TEMED. Lastly, hydrogels with carboxyl groups were synthesized by copolymerizing Am (7.5 mmol) with AA monomers (2.5 mmol) at a mole ratio of 6:2 as described elsewhere [23]. Briefly, the monomers were dissolved in 1 M NaOH and polymerized using 0.26 mmol of BisAm and 1.3% w/v of APS/TEMED. The compositions and nomenclature of the hydrogels are summarized in Supplementary Table S2. The hydrogels were sterilized with 70% ethanol and washed with fresh phosphate buffered saline (PBS) solution for 72 h. The rinsed hydrogels were incubated in culture media (high glucose DMEM with 2 mM L-glutamine and 50 units/ml penicillin/streptomycin) containing 10% fetal bovine serum (premium select) overnight before plating the cells.

2.3. Surface roughness

Surface roughness of hydrogels was evaluated using a Multimode AFM equipped with a Nanoscope IIIA controller from Veeco Instruments (Santa Barbara, CA) run by Nanoscope software v5.30 as previously reported [23]. AFM images were acquired in contact mode at forces of ~4 nN with an "E" scanner (maximum scan area 12×12 mm^2) using Si_3N_4 cantilevers (Veeco) with 0.06 N/m nominal spring constants. Hydrogels were prepared as described above. Upon synthesis, hydrogels were washed in PBS for 36 h to leach out unreacted reactants and to reach equilibrium swelling. For a given scan area, the reported roughness value is the average root mean square (RMS) roughness obtained from two different spots of triplicate samples. Using the nanoscope software, data analysis was carried out where a flattening order 3 was applied to all images to correct for tilt and bow before roughness analysis.

2.4. Elastic modulus

Equilibrium swollen hydrogels in PBS were used for compression measurements [24]. The measurements were performed using Bose ElectroForce 3200 Test Instrument (*Bose*, Minnesota, USA). Samples were compressed by two parallel plates at a maximum loading of 225 N with a crosshead speed of 0.1 mm/min. The elastic moduli were calculated from the linear region of the stress–strain curve (0–5% strain). All measurements were carried out as quadruplicates for each set of parameters.

2.5. Water contact angle

The water contact angle of the hydrogels was determined by a sessile drop method at room temperature using a contact angle meter (CAM100, KSV Instruments Ltd.) [23]. A 5 μl droplet of water was placed on the surface of hydrogels. All samples were prepared as triplicates and results were shown as a mean value with standard deviation.

2.6. HUES9-Oct4-GFP

The lentiviral construct that was used to generate the Oct4-GFP reporter line was kindly provided by Dr. Alexey Terskikh. The reporter line was generated as described earlier [14]. In short, the HUES9 cells were infected overnight with lenti Oct4-GFP and single clones were isolated and screened for stable GFP expression levels.

2.7. Culture of hPSCs

HUES9, HUES9-Oct4-GFP, HUES6, and hiPSC were expanded in defined medium (StemPro; DMEM/F-12 supplemented with StemPro supplement, 2% bovine serum albumin (BSA), 55 μM 2-mercaptoethanol, and 1% Gluta-MAX) or in MEF-conditioned medium. The MEF-conditioned medium was collected after culturing MEF for 24 h using growth medium (Knockout DMEM supplemented with 10% Knockout Serum Replacement, 10% human plasmonate (Talecris Biotherapeutics), 1% non-essential amino acids, 1% penicillin/streptomycin, 1% Gluta-MAX, and 55 μM 2-mercaptoethanol) as described elsewhere [14]. The hPSCs were cultured on mitotically inactivated MEF at an initial seeding density of 10^4 cells/cm^2 in MEF-conditioned medium prior to their culture on Matrigel or synthetic matrices. The hPSCs were manually passaged as small clumps of 30–40 μm size after 6 days of culture onto different matrices (Matrigel and synthetic matrices) by using a splitting ratio of 1:4. All the sequential passages were carried out similarly by passaging the cells manually. The hPSCs on PAm_6-co-PSS_2 hydrogels were passaged after 10–12 days depending upon the colony size and morphology. All cultures were supplemented with fresh medium containing 30 ng/ml of bFGF (Life Technologies) daily.

2.8. Population doubling time

Population doubling time (PDT) of HUES9 cells grown on Matrigel, MEF, and PAm_6-co-PSS_2 hydrogel was calculated using the equation below [15]:

$$\mathrm{PDT(h)} = \frac{(T2 - T1)}{3.32*(\log N2 - \log N1)}$$

where $T1$ and $T2$ represents days 3 and 5, respectively; $N1$ and $N2$ are the number of cells at $T1$ and $T2$, respectively. The number of cells at each time point was counted using TC10™ Automated Cell Counter.

For PDT measurements, HUES9 cells were cultured as single cells by enzymatically splitting the cells using Accutase. The cell count was carried out after 3 and 5 days of culture to calculate the population doubling time.

2.9. Immunocytochemistry

Immunofluorescent staining was performed using the following primary antibodies: OCT4 (1:200; Santa Cruz), NANOG (1:200; Santa Cruz), SOX17 (1:200; R & D systems), SMA (1:500; R & D systems), and NESTIN (1:50; BD Biosciences). The following secondary antibodies were used: goat anti-rabbit Alexa 647 (1:400; Life Technologies), donkey anti-mouse Alexa 546 (1:250; Life Technologies), and donkey anti-goat Alexa 546 (1:250; Life Technologies). For immunofluorescent staining, cells were fixed in 4% PFA for 5 min at 4 °C, followed by 10 min at room temperature. Immediately before staining, the cells were permeabilized with 0.2% (v/v) Triton X-100 and blocked with 1% (w/v) BSA and 3% (w/v) nonfat dry milk for 30 min. Cells were stained with primary antibodies diluted in 1% BSA overnight at 4 °C, washed 3 times with TBS, and incubated with secondary antibodies for 1 h at 37 °C. The nuclei were stained with Hoechst 33342 (2 μg/ml; Life Technologies) for 5 min at room temperature. Imaging was performed using an automated confocal microscope (Olympus Fluoview 1000 with motorized stage and incubation chamber).

2.10. RNA isolation and quantitative PCR

RNA isolation was carried out by using TRIzol (Invitrogen), and treated with DNase I (Invitrogen). Reverse transcription was performed by using qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR was carried out by using TaqMan probes (Applied Biosystems) and TaqMan Fast Universal PCR Master Mix (Applied Biosystems) on a 7900HT Real-Time PCR machine (Applied Biosystems). Taqman gene expression assay primers (Applied Biosystems) listed in Supplementary Table S3 were used. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as $C_t^{target} - C_t^{18s}$. All experiments were performed with three biological replicates.

2.11. FACS analysis

HPSCs were dissociated with Accutase. The cells were re-suspended in buffer (2% FBS/0.09% sodium azide/DPBS; BD Biosciences) and stained directly with Alexa 647 conjugated Tra-1-81 (Biolegend) or Alexa Fluor 647 mouse IgM,K isotype