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Nanotopographical control for maintaining undifferentiated human embryonic stem cell colonies in feeder free conditions

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ABSTRACT

Recently emerging evidence has indicated surface nanotopography as an important physical parameter in the stem cell niche for regulating cell fate and behaviors for various types of stem cells. In this study, a substrate featuring arrays of increasing nanopillar diameter was devised to investigate the effects of varying surface nanotopography on the maintenance of undifferentiated human embryonic stem cells (hESC) colonies in the absence of feeder cells. Single hESCs cultured across gradient nanopattern (G-Np) substrate were generally organized into compact colonies, and expressed higher levels of undifferentiated markers compared to those cultured on the unstructured control substrate. In particular, hESC demonstrates a propensity to organize into more compact colonies expressing higher levels of undifferentiated markers towards a smaller nanopillar diameter range (D = 120–170 nm). Cell-nanotopography interactions modulated the formation of focal adhesions and cytoskeleton reorganization to restrict colony spreading, which reinforced E-cadherin mediated cell–cell adhesions in hESC colonies. Maintaining compact hESC colony integrity revealed to be indispensable for hESC undifferentiated state as the loss of cell–cell adhesions within spreading hESC on the control substrate exhibited morphological and gene expression signatures of epithelial-to-mesenchymal transition-like processes. Findings in this study demonstrated a feasible approach to screen the optimal nanotopographical cues for maintaining undifferentiated hESC colonies in feeder free conditions, which provides a platform for further investigations into developing hESC feeder free culture systems for the purpose of regenerative medicine.

1. Introduction

Stem cell fate is determined by a multitude of factors that constitute the complex and dynamic in vivo niche [1–3]. Attempts to mimic the cellular microenvironments to control various stem cells behaviors in vitro culture have relied on the use of soluble factors (e.g., growth factors, cytokines, small molecules, nutrients etc.). However, accumulating evidence has suggested that, in addition to the biochemical environments, the physical interactions between stem cells and the underlying basement membrane in the niche environment have been shown to be an important mediator for many cellular behaviors [4–8]. The basement membranes are extracellular matrices (ECM) that provide a substratum for anchorage-dependent stem cells to survive, proliferate and

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differentiate [7,9]. Topographical studies of the basement membranes reveal the presence of varying sizes of pores and elevations in nanoscale, which are created by intertwining fibrous proteins such as laminin and collagen [10,11]. For this reason, it has been proposed that stem cells' behaviors may intimately be influenced by varying nanoscale features.

Advances in nanotechnology, such as self-assembly and lithography, have led to creations of various types of nanoscale surface features to investigate the cell–nanotopography interaction on stem cell behaviors. Polydimethylsiloxane (PDMS) nanopatterned with line gratings force human mesenchymal stem cells (MSCs) to elongate and align with the gratings, which induces trans-differentiation into neuronal lineage cells [12,13]. In addition, it has been demonstrated that the fate of human MSCs is determined by the orderliness of nanopits arrays. Human MSCs cultured on disordered arrays of nanopits are induced to differentiate into osteogenic differentiation [14], while the nanopits arrays in a near-perfect square arrangement maintains human MSCs multipotency up to 8 weeks in culture [15]. A recent study has revealed that human MSCs cultured on nanoporous polystyrene surface exhibit an enhanced adipogenic differentiation compared to the control flat surface, whereas the reverse nanotopography (i.e., polystyrene surface featured with nanopillars) induces osteogenic differentiation of human MSCs [16].

In recent years, it is being established with concrete evidence that various behaviors of human embryonic stems cells (hESC) are also intimately influenced by the underlying nanotopography. Undifferentiated hESC are characterized by their compact colony integrity, and expressions of various surface proteins (such as SSEA4 and Tra-1-60) and transcription factors (such as OCT4 and SOX2) [17]. These pheno- and geno-types are maintained with the presence of mouse embryonic fibroblasts (MEFs) feeder cells in the majority of laboratorial settings. Studies have demonstrated that undifferentiated state can be modulated by nanotopography as the expression of OCT4, a transcription factor expressed in undifferentiated hESC, reveals to be sensitive to different nanoroughness and nanotopographical configurations [18,19]. Pluripotent hESC have been at the center of attention in regenerative fields due to the ability to differentiate into almost all types of cell in the body [20]. Evidence has indicated that hESC cell fate can be manipulated by different nanotopographical cues. For instance, elongation and alignment of single hESCs along the nanoimprinted gratings are observed following the cytoskeleton reorganization [21], which reveal to be a potent inducer of neuronal differentiation of hESC [22,23], while near square arrangements of nanopits (Nsq50) induces osteogenic differentiation of hESC without any soluble inducers [24]. Although the aforementioned studies indicate that applying different nanotopographical cues can control both hESC undifferentiated state and differentiation, it remains elusive whether the particular nanopatterns employed in previous studies are optimized. The concept of screening the optimal topography using a single topographical chip comprised of varying geometry and dimensions has only been limited to neuronal differentiation of hESC [25].

In this study, we have attempted to screen the optimal nanotopography for maintaining undifferentiated hESC colonies in feeder free conditions by fabricating gradient nanopattern (G-Np) substrate featuring a continuous gradient of nanopillar where its diameter increases from 120 to 360 nm with fixed 440 nm center-to-center distance over 35 mm with high fidelity.

2. Materials and methods

2.1. Fabrication of gradient nanopattern (G-Np) substrate

Ultrapure aluminum plates (99.999%, Goodfellow, UK) were polished electrochemically in a perchloric acid solution (perchloric acid:absolute ethanol = 1:4) at 20 V and 7 °C. Polished aluminum plates were anodized in phosphoric acid (DI water:methanol:phosphoric acid = 59:40:1) at 193 V and 0 °C for 12 h and etched out with chromic acid solution (9 g chromic acid and 20.3 mL phosphoric acid in 500 mL DI water). The well-ordered porous anodic aluminum oxides (AAOs) were anodized again in the same conditions for 4 h and were modulated with a gradually increasing dipping time in phosphoric acid solution using a tensiometer (operating speed 1.0 mm/min). A self-assembled heptadecafluoro-1,1,2,2-tetrahydrodecyl-trichlorosilane (HDFS) monolayer was coated onto the modulated gradient AAO molds. Each surface of AAOs was first hydroxylated by piranha solution (35% H_2SO_4:H_2O_2 = 7:3, v/v), and the self-assembly of HDFS monolayer on the surface was carried out using 3 mM water-free HDFS solution of n-hexane. PS samples with gradient nanopatterns were fabricated by thermally imprinting AAO molds containing gradient nanometer-scale pores using a nanoimprinting device, NANO-SIS™610 (Nano & Device, South Korea). PS polymer sheets of 700 mm^2 (35 mm × 20 mm) were heated at 130 °C for 10 min and pressed at 3 bar for 100 s. PS sheets were detached from the AAO molds at room temperature.

2.2. hESCs culture

The routine culture of H9 hESC (Wicell, WI) was performed as previously reported [17]. Briefly, small clumps of hESC colonies were dissected with a glass Pasteur pipette and were transferred on to Mytomycin-C (Sigma–Aldrich, MO) inactivated MEFs in DMEM/F12 (Gibco BRL, MD) supplemented with 20%(v/v) serum replacement, 100 mM β-mercaptoethanol, 1 mM L-glutamine, 1%(v/v) non-essential amino acid (all from Gibco) and 4 ng/mL bFGF (Invitrogen, NY). The cells were cultured for 6 days with a daily medium exchange. For single cell dissociation, hESC were incubated with 10 μM rho associated protein kinase (ROCK) inhibitor Y-27632 (Tocris, MO) for 1 h at 36 °C hESC colonies were then treated with Dispase (Gibco) at 36 °C for 5 min. The lifted hESC colonies were washed once with phosphate buffered saline (PBS, Sigma–Aldrich) and incubated with TrypLE™-select (Invitrogen) for 3 min at room temperature. The clumps were physically dissociated by a gentle pipetting and were centrifuged for 2 min at 900 rpm. The pallet was then resuspended in the hESC culture medium and filtered with a 5 mL Polystyrene Round-Bottom Tube with Cell-Strainer Cap (BD, NJ) to remove cell aggregates. Single hESCs were seeded at 5000 cells/cm^2 on the unstructured control and G-Np substrate in hESC medium supplemented with 10 μM Y-27632, which was removed after 2 days. To aid attachment of single hESCs, the control and G-Np substrates were coated with 0.1% gelatin (Sigma–Aldrich) for 20 min at room temperature prior to cell seeding. The culture was maintained for 4 days with a daily medium exchange. For antagonizing the Wnt and BMP4 signaling pathways, dissociated single hESCs were cultured in the basic hESC media supplemented with 10 ng/mL DKK-1 and Noggin (both R&D Systems, MN) on control substrate for 4 days.

2.3. Scanning electron microscope (SEM) imaging

hESC colonies cultured on G-Np for 4 days were twice washed with PBS, and pre-fixated with 4% paraformaldehyde for 4 h at room temperature. Pre-fixed hESC colonies were post-fixated with 1% Osmium tetroxide for 2 h and were dehydrated by exchanging of various concentrations of ethanol from 50% to 100%. Dehydrated hESC colonies were then washed three times with t-butyl alcohol and froze at −20 °C and were lyophilized for 1 day. Thoroughly lyophilized hESC colonies were coated with platinum for 5 min and were observed with scanning electron microscopy (FE-SEM, JSM6701, JEOL, Japan). All images were obtained with tilting of 40°.

2.4. Immunocytochemistry

Cells cultured on the control and G-Np substrate for 4 days were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The samples were then treated sequentially with permeabilizing (0.03% Triton-X in PBS for 10 min) and blocking solution (5% goat serum in permeabilizing solution for 30 min) at room temperature. The cells were then incubated with primary antibody Oct4 (Santa Cruz Biotechnology Inc., CA, 1:1000), E-cadherin (BD, 1:200), phalloidin (Invitrogen, 1:400), vinculin (Sigma–Aldrich, 1:100), vimentinn (abcam, 1:400) and β-catenin (BD, 1:400) in blocking solution overnight at 4 °C. The cells were then washed 3 times with PBS and incubated with either Alexa Flour 488- or 594-conjugated secondary antibodies (both from Invitrogen) for 2 h at the room temperature before rinsing with PBS for 5 min for 3 times. The cells were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, 1:1000) in PBS for 3 min at room temperature before acquiring fluorescence images using a fluorescence microscope (Nikon TE2000-U, Nikon, Japan).

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA extraction was performed using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. The RNA concentration was measured using a spectrophotometer (NanoDrop ND-1000; Thermo Scientific), and 1 μg of RNA was used for cDNA synthesis using the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's manual. Using SYBR Green PCR master mix (Invitrogen), 1 μL of the synthesized 1st strand cDNA was add to the master mix with the equal volume of primer pairs (Macrogen, Korea) in a total reaction volume of 20 μL. The reaction was performed using the ABI 7300 qRT-PCR system (Applied Biosystems, Carlsbad, Calif., USA). The primer pair sequences are listed in