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The effects of electrospun substrate-mediated cell colony morphology on the self-renewal of human induced pluripotent stem cells

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ABSTRACT

The development of xeno-free, chemically defined stem cell culture systems has been a primary focus in the field of regenerative medicine to enhance the clinical application of pluripotent stem cells (PSCs). In this regard, various electrospun substrates with diverse physiochemical properties were synthesized utilizing various polymer precursors and surface treatments. Human induced pluripotent stem cells (IPSCs) cultured on these substrates were characterized by their gene and protein expression to determine the effects of the substrate physiochemical properties on the cells' self-renewal, *i.e.*, proliferation and the maintenance of pluripotency. The results showed that surface chemistry significantly affected cell colony formation via governing the colony edge propagation. More importantly, when surface chemistry of the substrates was uniformly controlled by collagen conjugation, the stiffness of substrate was inversely related to the sphericity, a degree of three dimensionality in colony morphology. The differences in sphericity subsequently affected spontaneous differentiation of IPSCs during a long-term culture, implicating that the colony morphology is a deciding factor in the lineage commitment of PSCs. Overall, we show that the capability of controlling IPSC colony morphology by electrospun substrates provides a means to modulate IPSC self-renewal.

1. Introduction

Induced pluripotent stem cells (IPSCs), derived from patients' somatic cells, hold promising potential in personalized regenerative medicine [1–3]. The capability of PSCs to self-renew and differentiate into multiple lineages makes them an ideal source for cell-based therapies by avoiding genetic mismatch and ethical issues associated with the use of embryonic stem cells (ESCs). Typical culture of IPSCs involves the use of animal-derived products, such as basement extracellular matrix (ECM) extracts from mouse carcinoma cells or mouse feeder cell layers. Because the essential biomolecular components and physiochemical cues for the expansion of human PSCs are not clearly defined, many researchers have depended on the use of animal-derived components, developed by phenomenological approaches, for long-term stem cell culture. However, the presence of possible immunogenic components or contaminants in these xeno-origin products limits the therapeutic use of IPSCs in human [4,5]. Significant batch-to-batch variability in those products also undermines reproducibility in the phenotypic characteristics of expanded stem cells [6]. Therefore, recent research in the field has focused on developing synthetic substrates for PSC culture [7–10].

Engineered synthetic substrates are advantageous over natural products due to the reproducibility and flexibility of the composition and structure. Their tunable material properties, including surface chemistry, topography, and stiffness, provide an opportunity to optimize the cellular microenvironment for the maintenance of IPSCs. Among many factors, the mechanical properties of scaffolds have been shown to be one of the main determinants for cellular behaviors in various stem cell types [11–13]. For example, mouse ESCs cultured on substrates with softer stiffnesses, similar to the intrinsic stiffness of the cells, maintain their pluripotency without the addition of exogenous leukemia inhibitory factor [14].

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Similarly, mesenchymal stem cells (MSCs) have also been shown to commit to a specific lineage when cultured on matrices with specific elasticity [11]. Changes in cell shape, cell-to-cell contacts, and focal adhesion points, regulated by physical characteristics of substrates, have all been shown to alter stem cell behaviors [15–17].

In this regard, electrospinning is a viable synthesis method to produce physiochemically distinctive substrates for cell culture systems. The advantages of electrospinning include its capability to produce ECM-resembling fibrous structures with controlled fiber dimensions (nanometer to micrometer) and fiber morphology (*e.g.*, cylindrical, ribbon-like, and pores on the fiber surface) using a diverse range of biocompatible polymers [18,19]. Another advantage is the simplicity of the process enabling scale-up for mass production. For this reason, many have shown the potential of electrospun fiber mats as cell culture substrates for various types of stem cells [20–24]. Surface-aminated polyethersulfone (PES) nanofibers enhanced the proliferation of human umbilical cord blood hematopoetic stem/progenitor cells and induced higher $CD34^+$ expression as compared to cells cultured on commercially available tissue-culture polystyrene (TCPS) [25]. The versatility of the technique has also allowed for the production of natural extracellular matrix-based electrospun substrates for adipose tissue-derived stem cell culture and expansion [26]. Recently, Hashemi et al. have also demonstrated the feasibility of using collagen-grafted electrospun substrates for maintaining pluripotency in mouse ESCs [27]. Put together, the flexibility of the electrospinning process to synthesize a wide range of fibrous structures provides an opportunity to develop xeno-product free culture systems for the expansion of various stem cell types. However, a more systematic approach is necessary to determine the optimal scaffold characteristics.

In this study, we hypothesized that the mechanical properties of the electrospun substrates would be one of the significant factors regulating stem cell self-renewal. We examined the differential development of IPSC colony on various electrospun fibrous substrates with systematically varied mechanical properties. Such differences in IPSC colony morphology was correlated to the cells' proliferation and the maintenance of pluripotency. Ultimately, we show that electrospun substrates provide an efficient platform for systematically dissecting and optimizing the physiochemical cues that mediate stem cell self-renewal.

2. Materials & methods

2.1. Substrate fabrication and morphological characterization

All reagents and products were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. Various fibrous substrates were synthesized by electrospinning using different precursor solutions including 8.5 wt.% poly(ε-caprolactone) (PCL) dissolved in 5:1 trifluoroethanol (TFE)-water, 11 wt.% polyethylene terephthalate (PET, Indorama Ventures, Spartanburg, SC) dissolved in 1:9 trifluoroacetic acid (TFA)-1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) (Oakwood Products Inc., West Columbia, SC), 5 wt.% poly(etherketoneketone) (PEKK, Oxford Performance Materials, South Windsor, CT) dissolved in HFP, or 4 wt.% polycarbonate-urethane (PCU, DSM PTG, Berkeley, CA) dissolved in HFP. A vertical configuration of an electrospinning setup was used with a tip-to-collector distance of 23 cm [28]. The applied voltage and flow rates were adjusted to form a stable Taylor cone for each solution [29,30]. As-spun samples were plasma-treated at 30 W for 5 min to improve the hydrophilicity of the substrates. The microstructure of electrospun fibers was observed under a Philips/FEI XL30 FEG scanning electron microscope (SEM).

2.2. Collagen conjugation and quantification

To examine the effects of biological surface modification, the plasma-treated substrates were further processed with collagen type I conjugation. The plasma-treated samples were subjected to a zero-length crosslinking agent, 100 mM N-hydroxysuccinimide (NHS)/N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC). After the chemical modification to ester linkages of the substrate, the samples were incubated in 1 mg/ml collagen type I in 0.01 M hydrochloric acid (HCl) overnight [31]. The amount of conjugated collagen on the electrospun substrates was quantified with a Sirius red colorimetric assay using a protocol similar to Zhu et al. [32]. Briefly, substrates conjugated with collagen were fixed in Bouin's fluid for 1 h at room temperature, washed with tap water, and air-dried overnight. The samples were then stained with Sirius red dye for 1 h under mild shaking. The dye was removed and each well was washed vigorously with 0.01 M HCl to remove unbound dye. Following washing, the substrate-bound dye was released using 0.1 M sodium hydroxide under mild shaking for 30 min, and optical density of the solution was measured at 490 nm using a plate reader (PerkinElmer, Waltham, MA). Substrates were dried overnight at room temperature and their mass measured. The surface area of the substrates was measured by Brunauer-Emmett-Teller (BET) method [33]. Collagen conjugation on each substrate was quantified to yield the amount of collagen per unit surface area. In addition, the electrospun fibers were immunofluorescently stained to reveal the uniform localization of collagen on the surface of the fibers.

2.3. Mechanical characterization of electrospun substrates

The mechanical properties of electrospun substrates were determined by atomic force microscopy (AFM) using a MFP-3D AFM (Asylum Research, Santa Barbara, CA). A modified silicon nitride tip (0.6 N/m) attached with a 20 μm diameter borosilicate sphere was used to measure the mechanical responses of electrospun fiber networks. At least three separate measurements were conducted on different locations per substrate at an indentation and retraction speed of 2 μm/s and a set trigger force of 20 nN. The load-displacement data were used to calculate the reduced Young's modulus using the Hertz model for spherical indenters [34].

2.4. IPSC culture

IPSCs were derived from BJ-2522 human neonatal foreskin fibroblast cells (ATCC) by transfecting the cells with OCT4, SOX2 and KLF4 retroviruses as previously described [35]. Additionally, the induced cells were transfected with a GFP-labeled OCT4 reporter [36]. The cells were adapted to Geltrex®-coated cell culture dishes (Life Technologies, Grand Island, NY) in mTeSR1 medium (StemCell Technologies, Vancouver, Canada) at 37 °C and 5% CO_2. The genetic stability was confirmed by karyotyping, and pluripotency of this stem cell line was verified by inducing differentiation to ectodermal, endodermal, or mesodermal lineage (Supplementary Figure S1). Electrospun substrates were sterilized with 70% ethanol for 1 h, air-dried overnight, and UV-sterilized prior to cell seeding. IPSCs were passaged from a tissue culture flask to electrospun substrates using 0.25% Trypsin-EDTA (Life Technologies) at a seeding density of 125,000 cells/cm^2. Rock inhibitor (10 μM, Reagents Direct, Encinitas, CA) was supplemented 1 h prior to cell harvesting and maintained for a day during substrate culture to improve cell attachment and survival. Cells cultured on Geltrex®-coated 25 cm^2 flasks at the same cell seeding density were used as controls. The IPSCs cultured on the substrates were maintained in mTeSR1, and medium was exchanged daily up to 12 days. To monitor stem cell proliferation and colony formation *in situ*, an OCT4-GFP transfected cell line was cultured on collagen-conjugated PCL or PCU substrates, and visualized every 4 h during culture using a Nikon BioStation CT with fluorescence and phase imaging.

2.5. Cell proliferation assay

Cell proliferation on different substrates was analyzed using alamarBlue® assay (Life Technologies) according to the manufacturer's protocol. Briefly, alamarBlue® was added directly to cell culture medium at 10 volume% and analyzed after 4 h. Fluorescence was measured at an excitation wavelength of 530 nm and an emission reading of 605 nm using a plate reader (PerkinElmer).

2.6. Cell colony morphological observation and image analysis

To examine cell colony morphology by SEM, samples were fixed in 10% formalin overnight and dehydrated as previously described [37]. Briefly, a sequential dehydration of 50%, 70%, 80%, 95%, and 100% ethanol, followed by 3:1, 1:1, and 1:3 Ethanol:Hexamethyldisilazane (HMDS, Ted Pella, Inc., Redding, CA) exchange was performed. After drying overnight, the samples were sputter coated with platinum–palladium prior to imaging under an SEM.

Cell colony morphology was also analyzed using immunofluorescence imaging. Samples were fixed with 4% paraformaldehyde for 30 min. To visualize colony morphology, the fixed cells were subjected to actin and nucleus staining using Alexa569-phalloidin (Life Technologies) and 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA), respectively. Alternatively, fixed cells were subjected to PAX6 (Developmental Studies Hybridoma Bank, Iowa City, IA) immunofluorescent staining to determine differentiation towards ectodermal lineage. The samples were observed under an epi-fluorescent microscope (Nikon Eclipse, Melville, NY) or a confocal microscope (Leica SP2, Buffalo Grove, IL).

Quantification and characterization of cell colony morphology were assessed using Imaris Bitplane 7.1.1 (Bitplane, South Windsor, CT). Using a surface area reconstruction, the sphericity (*i.e.*, ratio of the surface area of a sphere to the surface area of the object) was calculated for colonies cultured on different substrates [38]. At least five colonies per condition were analyzed.