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Mechanotransduction of human pluripotent stem cells cultivated on tunable cell-derived extracellular matrix

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ABSTRACT

Cell-derived matrices (CDM) are becoming an attractive alternative to conventional biological scaffolding platforms due to its unique ability to closely recapitulate a native extracellular matrix (ECM) de novo. Although cell-substrate interactions are recognized to be principal in regulating stem cell behavior, very few studies have documented the acclimation of human pluripotent stem cells (hPSCs) on pristine and altered cell-derived matrices. Here, we investigate crosslink-induced mechanotransduction of hPSCs cultivated on decellularized fibroblast-derived matrices (FDM) to explore cell adhesion, growth, migration, and pluripotency in various biological landscapes. The results showed either substrate-mediated induction or inhibition of the Epithelial-Mesenchymal-Transition (EMT) program, strongly suggesting that FDM stiffness can be a dominant factor in mediating hPSC plasticity. We further propose an optimal FDM substratum intended for long-term hPSC cultivation in a feeder-free niche-like microenvironment. This study carries significant implications for hPSC cultivation and encourages more in-depth studies towards the fundamentals of hPSC-CDM interactions.

1. Introduction

Human pluripotent stem cells (hPSCs) are traditionally cultivated on feeder cells such as mouse embryonic fibroblasts (MEFs) but there has been a progressive move away from the use of feeders because they can introduce xenogeneic contaminants or other undefined factors into the culture systems [1]. For this reason, Matrigel® (Corning, Corning, NY) is one of the most widely used extracellular components for feeder-free culture of hPSCs but it suffers from limited customization and inherent heterogeneity as it is derived from Engelbreth-Holm-Swarm mouse sarcomas which may also contain unwanted xenogeneic materials [2]. While recent advancements continue to demonstrate synthetic and biological alternatives ranging from nanopatterns [3], hydrogels [4], and single ECM components such as vitronectin [5], another caveat is that unlike cellular secretions, these platforms are unable to recapitulate a complex, organized, and heterogeneous mixture of macromolecules reminiscent of native microenvironments.

The extracellular matrix (ECM) is the non-cellular component of tissues and organs composed of various macromolecules secreted by cells to serve as a natural scaffold with structural integrity and biological cues. While its specific composition and function may vary tremendously depending on the type of particular tissue, it is mainly composed of two interlocking macromolecules known as proteoglycans, such as glycosaminoglycans (GAGs), and fibrous proteins, such as collagen, vitronectin, elastin, fibronectin, and laminin [6]. In essence, it is a highly dynamic structure that is constantly being remodeled and subjected to a myriad of post-translational modifications as it plays a pivotal role in the

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biophysical and biochemical dialogue between various cells [7]. It possesses a wide range of distinct properties that depend on the inhabitant cells but it is widely known that homeostasis, cell adhesion, cell-to-cell communication, and differentiation are fundamental functions of the ECM [8].

There has been a growing interest in engineering cell-derived matrices (CDM) to create a biomimetic microenvironment that resembles a native niche. Early studies have shown that the biophysical and biochemical cues can be reserved after the decellularization process, which makes the generated matrix a naturally derived culture substratum for stem cell adhesion, proliferation, and differentiation [9,10]. Substrate features are of particular interest because it has a profound impact on stem cell lineage specification. For example, substrate stiffness is an essential characteristic by which cells sense the external forces and subsequently respond to the environment in an appropriate manner. Our previous study demonstrated the effects of mechanotransduction on the fate of human mesenchymal stem cells (hMSCs) and recent reports continue to highlight the importance of cell-ECM interactions in regulating stem cell fate [11,12]. However, even though hPSCs possess enormous potential in regenerative therapy, little attention has been paid to some fundamental aspects of hPSC-CDM interactions partly due to the lack of proper tools.

Hence, we prepared a natural ECM analog that is engineered de novo from *in vitro* cultured fibroblasts to explore the acclimation of hPSCs cultivated on pristine and altered decellularized fibroblast-derived matrices (FDM). Mechanotransduction was conducted via non-cytotoxic crosslinking to modulate FDM rigidity to various degrees. Both substrate types were utilized to investigate cell adhesion, growth, migration, and pluripotency. This study shows that simple yet effective physiological controls can dictate hPSC-ECM dynamics in a closely recapitulated natural environment. Based on our findings, we further propose an optimal FDM platform intended for feeder-free and long-term hPSC cultivation (up to 14 days).

2. Materials and methods

2.1. Preparation of fibroblast-derived extracellular matrix (FDM)

To prepare FDM, NIH3T3 mouse fibroblasts (ATCC, Manassas, VA) or human foreskin BJ1 fibroblasts (ATCC) were seeded at the density of 2×10^4 cells/cm^2 on either plastic dishes or gelatin-coated glass in 12-well plates, and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin streptomycin (P/S; Thermo Fisher Scientific). After 1 week, confluent cells were washed twice with phosphate buffered saline (PBS; Thermo Fisher Scientific), then decellularized by incubation with a detergent solution containing 0.25% Triton X-100 and 10 mM NH_4OH (Sigma-Aldrich Co., St. Louis, MO) in PBS at 37 °C. The samples were then treated with 50 U/ml DNase I and 2.5 μL/ml RNase A (Thermo Fisher Scientific) for 2 h at 37 °C. The decellularized samples were gently washed with PBS three times and the resulting FDM was used immediately or stored at 4 °C prior to use. Major FDM components are identified by immunofluorescence of mouse anti-fibronectin (FN; Santa Cruz Biotechnology, Inc., Dallas, Texas) and mouse anti-laminin (LN; Santa Cruz Biotechnology, Inc.). Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) and Alexa Fluor 594-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Inc., West Grove, PA) were used as a secondary antibody.

2.2. Preparation of crosslinked FDMs

The prepared FDM was then treated with different amounts of genipin (GN; Wako Chemicals USA, Richmond, VA) to induce a crosslinking of the FDM. Briefly, a stock solution of 5% (w/v) GN was prepared in dimethyl sulfoxide (DMSO) and diluted to different concentrations (0.5, 1, and 2%) with PBS. GN solution was added to FDM, and the samples were incubated at room temperature for 4 h to allow the crosslinking of FDM itself. The crosslinked FDM (X-FDM) was then washed five times with PBS and stored at −4 °C before use. The X-FDM was named as follows: Gx0.5, Gx1.0 and Gx2.0, respectively. Gx0 represents the non-crosslinked or pristine FDM.

2.3. Quantitative analysis of ECM proteins

To quantify the ECM constituents of the FDM and X-FDMs, fibronectin (FN), laminin (LN), collagen type I (Col I), and vitronectin (VN) were selected. Each sample in 6-well plates was incubated overnight with the following primary antibodies: mouse anti-FN (Santa Cruz Biotechnology, Inc.), mouse anti-LN (Santa Cruz Biotechnology, Inc.), rabbit anti-Col I (Abcam, Cambridge, UK), and rabbit anti-VN (Santa Cruz Biotechnology, Inc.), respectively and followed by the treatment of goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (HRP; Santa Cruz Biotechnology, Inc.). After several washing, the samples were added with 1 ml of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific) and incubated for 20 min. The reaction was stopped using 0.16 M sulfuric acid, after which 100 μL aliquots were taken for the absorbance measurements at 405 nm (Multiskan Microplate Reader, Thermo Fisher Scientific). For the comparison, the fluorometric readouts are converted to the protein levels that are normalized to the surface area (9.6 cm^2) of one well per each sample.

2.4. Characterization of FDM and X-FDMs: SEM and AFM

The surface morphology of FDM and X-FDM was observed using a scanning electron microscope (SEM; Model S-3000 N, Hitachi). Bio-atomic force microscope (Bio-AFM; NanoWizard II, JPK Instruments, Germany) equipped with an inverted optical microscope (Nikon) was also used in a liquid contact mode to investigate the surface topography and morphology of FDM and X-FDM, respectively. In addition, the AFM-nano indentation technique was employed to measure the Young's modulus (E) of each sample, as described previously [13]. A 10 μm diameter SiO_2 particle attached to PT.SiO2.AU.SN10 cantilevers (Novascan Technologies, Ames, USA) with a spring constant of 0.01 N/m was utilized. Based on the force spectra curves, E was calculated using Hertz's contact model in JPK data processing software (v3.3.25). The Poisson ratio of cells was set to 0.5. Approximately 5 indentations on each sample ($n = 3$, each group) at different regions were carried out for E measurement.

2.5. Cell culture

Various cell types are used: human pluripotent stem cell line; H9 (WiCell Research Institute, Madison, WI) [14], mouse embryonic fibroblasts (MEFs), and primary MEFs (Orient Bio Inc., Seongnam, Korea). H9-hPSCs were cultured in 80% DMEM: Nutrient Mixture F-12 (DMEM/F12; Thermo Fisher Scientific, Waltham, MA) supplemented with 20% KnockOut™ Serum Replacement, 1% P/S (Thermo Fisher Scientific), 1% Minimum Essential Medium Non-Essential Amino Acids (MEM-NEAA; Thermo Fisher Scientific), 0.1% beta-mercaptoethanol (β-ME;