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Nano-on-micro fibrous extracellular matrices for scalable expansion of human ES/iPS cells

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ABSTRACT

Human pluripotent stem cells (hPSCs) hold great potential for industrial and clinical applications. Clinical-grade scaffolds and high-quality hPSCs are required for cell expansion as well as easy handling and manipulation of the products. Current hPSC culture methods do not fulfill these requirements because of a lack of proper extracellular matrices (ECMs) and cell culture wares. We developed a layered nano-on-micro fibrous cellular matrix mimicking ECM, named "fiber-on-fiber (FF)" matrix, which enables easy handling and manipulation of cultured cells. While non-woven sheets of cellulose and polyglycolic acid were used as a microfiber layer facilitating mechanical stability, electrospun gelatin nanofibers were crosslinked on the microfiber layer, generating a mesh structure with connected nanofibers facilitating cell adhesion and growth. Our results showed that the FF matrix supports effective hPSC culture with maintenance of their pluripotency and normal chromosomes over two months, as well as effective scaled-up expansion, with fold increases of 54.1 ± 15.6 and 40.4 ± 8.4 in cell number per week for H1 human embryonic stem cells and 253G1 human induced pluripotent stem cells, respectively. This simple approach to mimick the ECM may have important implications after further optimization to generate lineage-specific products.

1. Introduction

Human pluripotent stem cells (hPSCs), i.e., embryonic (hESCs [1]) and induced pluripotent (hiPSCs [2]) stem cells, hold great potential for industrial and clinical applications [3–5]. The establishment of efficient and robust three-dimensional (3D) hPSC culture systems is one of the main challenges in scaling up cell production required for regenerative medicine and drug discovery and for efficiently preparing a large amount of quality-controlled hPSCs for further processing to final products [3,5]. Most of the reported 3D culture systems (e.g., suspension culture systems [6–10], microcarriers [11,12], hydrogel [13] and micro-encapsulation [14,15]) have limitations related to insufficient cell growth, undesirable differentiation, difficulty to control cell aggregate size, scalability, and hydrodynamic shear stress [3,5], resulting in low quantity and/or low quality of cultured cells. Thus, there is a clear need for alternative and new 3D hPSC culture systems.

In an attempt to address the above issues, we hypothesized that the interaction between hPSCs and ECM is a key to hPSC self-renewal and preventing differentiation during culture. In living organisms, ECMs are components of the cellular microenvironment and form nanoscale fiber-and-mesh structures for effective interaction with surrounding cells to precisely regulate their functions

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and phenotypes, including cell proliferation and maintenance of stem cells [16]. Although adhesive molecules coated on conventional culture dishes have been widely used for a couple of decades, the ECM structure was not sufficiently considered, leaving room for the growth efficiency to be improved. Therefore, artificial ECMs for hPSC culture systems that (1) promote growth and prevent undesired differentiation, (2) allow large-scale culture, and (3) enable easy cell transplantation, should be developed.

To promote hPSC growth and prevent their differentiation, nanofibers have advantages over conventional culture substrate for increasing the cell–ECM interaction and mimicking the *in vivo* ECM-like structures using materials of interest [17–23]. Indeed, several research groups including ours have been able to develop nanofiber matrices that maintain hPSC self-renewal using simple materials [17,19] instead of sophisticated and expensive materials (e.g., recombinant ECMs [24–27] and synthetic polymers [28,29]). However, although many researchers have attempted to apply nanofiber matrices for culturing hPSCs, scaling up of the systems remains challenging because of their mechanical fragility.

Here, we developed a new type of artificial ECM consisting of layered nano- and microfibers, which we termed "fiber-on-fiber (FF)" matrix (Fig. 1A and B). While microfiber matrices have too wide a diameter to interact with cells and show low cell stimulation [17], they display better mechanical stability and flexibility than nanofiber matrices. The FF matrix combines the advantages of both nano- and microfibers, such as increased cell growth and mechanical stability. Thus, FF matrix allows culturing adherent cells using a method similar to conventional 2D cell culture, but is applicable to large-scale cell culture. Moreover, because of the mesh-like structure, soluble factors in the culture medium can easily penetrate an FF matrix and reach the cultured cells for efficient nutrient supply without stirring. Thus, in comparison with conventional culture systems applying microcarriers and hydrogels, the present method allows minimizing the mechanical stress for cells during culture.

2. Materials and Methods

2.1. Fabrication of pFF matrix

The FF matrix consists of a gelatin nanofiber layer and a microfiber layer. Polyglycolic acid (PGA) non-woven sheet (10 cm × 10 cm; Gunze Corp.) was used for microfiber layers (pFF). A microfiber sheet (50 mm × 25 mm) was placed at the center of a silicon wafer. Gelatin nanofibers were fabricated on the microfiber sheet by electrospinning a 10 wt% gelatin (Type A, from porcine skin; Sigma-Aldrich) solution in AA:EA:ultra-pure water (42:31:20 vol ratio) using a syringe equipped with a 23-gauge stain-less needle under the following conditions: distance = 10 cm, voltage = 11 kV, flow rate = 0.20 mL/h, and time = 7 min. The microfiber sheet with gelatin nanofibers was vacuum-dried at 37 °C overnight to evaporate remaining solvent and then cross-linked in a solution of 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide and 0.2 M *N*-hydroxysuccinimide in ethanol for 4 h, yielding FF matrix. The matrix was cut to 25 mm × 20 mm, rinsed with 99.5% ethanol, and vacuum-dried at 37 °C overnight to remove remaining solution. Prior to use for cell culture, the matrix was sterilized thrice with 70% ethanol, and dried.

2.2. hPSC culture

All experiments involving the use of hESCs were performed in accordance with the Guidelines for the Derivation and Utilization of Human Embryonic Stem Cells of the Ministry of Education, Culture, Sports, Science and Technology, Japan. H1 and H9 hESCs [1] and 253G1 hiPSCs [30] were obtained from WiCell and Riken Bioresource center, respectively, and routinely cultured on a gelatin nanofiber matrix on a glass slide (25 mm in diameter) as reported previously [17]. The cells were treated with non-enzymatic cell dissociation solution (Life Technologies) for 3 min at 37 °C and manually dissociated to the single-cell level by gently pipetting for

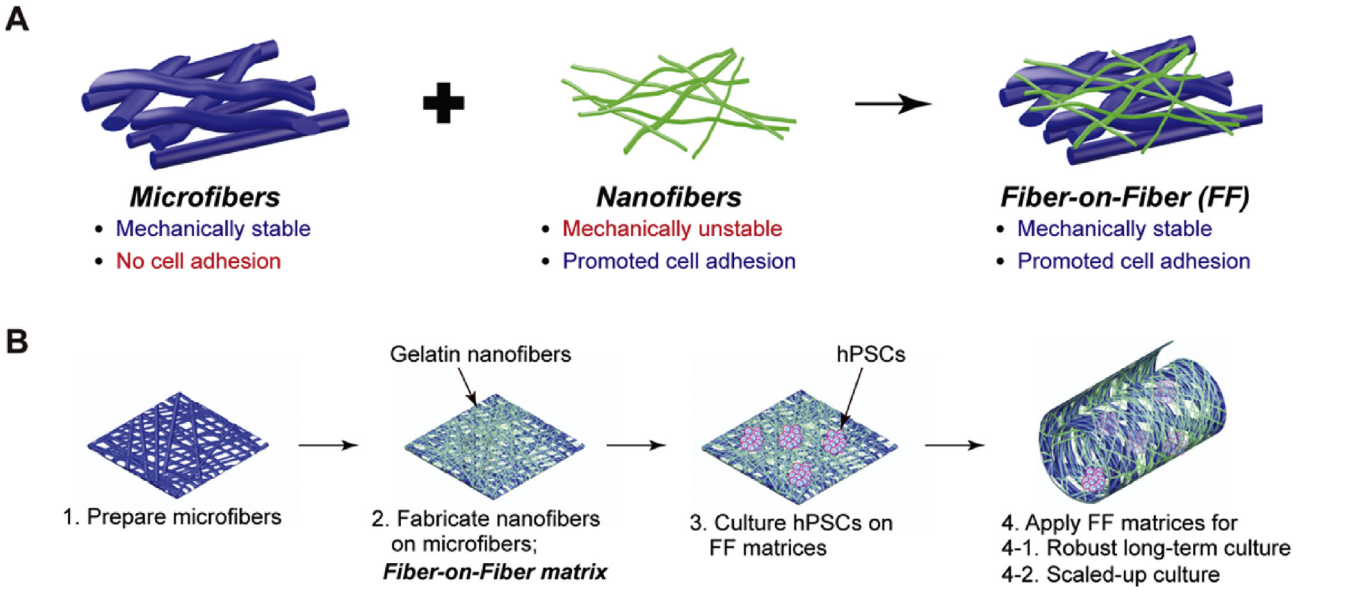

Fig. 1. FF matrix for large-scale culture of hPSCs. A) Conceptual illustration of an FF matrix. This matrix has a layered structure consisting of a nanofiber mesh on a microfiber sheet. The nanofiber matrix facilitates adhesion and growth of hPSCs with maintenance of pluripotency, but it is mechanically fragile. On the other hand, microfibers do not support hPSC adhesion and growth, but they have better mechanical stability. FF matrix combines the advantages of both nano- and microfibers. B) FF matrix fabrication procedure. Detailed procedures are shown in Supplementary Figs. S1 and S2.