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Combinatorial extracellular matrix microenvironments promote survival and phenotype of human induced pluripotent stem cell-derived endothelial cells in hypoxia

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ABSTRACT

Recent developments in cell therapy using human induced pluripotent stem cell-derived endothelial cells (iPSC-ECs) hold great promise for treating ischemic cardiovascular tissues. However, poor post-transplantation viability largely limits the potential of stem cell therapy. Although the extracellular matrix (ECM) has become increasingly recognized as an important cell survival factor, conventional approaches primarily rely on single ECMs for *in vivo* co-delivery with cells, even though the endothelial basement membrane is comprised of a milieu of different ECMs. To address this limitation, we developed a combinatorial ECM microarray platform to simultaneously interrogate hundreds of micro-scale multi-component chemical compositions of ECMs on iPSC-EC response. After seeding iPSC-ECs onto ECM microarrays, we performed high-throughput analysis of the effects of combinatorial ECMs on iPSC-EC survival, endothelial phenotype, and nitric oxide production under conditions of hypoxia (1% O_2) and reduced nutrients (1% fetal bovine serum), as is present in ischemic injury sites. Using automated image acquisition and analysis, we identified combinatorial ECMs such as collagen IV + gelatin + heparan sulfate + laminin and collagen IV + fibronectin + gelatin + heparan sulfate + laminin that significantly improved cell survival, nitric oxide production, and CD31 phenotypic expression, in comparison to single-component ECMs. These results were further validated in conventional cell culture platforms and within three-dimensional scaffolds. Furthermore, this approach revealed complex ECM interactions and non-intuitive cell behavior that otherwise could not be easily determined using conventional cell culture platforms. Together these data suggested that iPSC-EC delivery within optimal combinatorial ECMs may improve their survival and function under the condition of hypoxia with reduced nutrients.

Statement of Significance

Human endothelial cells (ECs) derived from induced pluripotent stem cells (iPSC-ECs) are promising for treating diseases associated with reduced nutrient and oxygen supply like heart failure. However, diminished iPSC-EC survival after implantation into diseased environments limits their therapeutic potential. Since native ECs interact with numerous extracellular matrix (ECM) proteins for functional maintenance, we hypothesized that combinatorial ECMs may improve cell survival and function under conditions of reduced oxygen and nutrients. We developed a high-throughput system for simultaneous screening of iPSC-ECs cultured on multi-component ECM combinations under the condition of hypoxia and reduced serum. Using automated image acquisition and analytical algorithms, we identified combinatorial ECMs that significantly improved cell survival and function, in comparison to single ECMs. Furthermore, this approach revealed complex ECM interactions and non-intuitive cell behavior that otherwise could not be easily determined.

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1. Introduction

Cardiovascular disease is the leading cause of death in United States, affecting more than eighty-five million people [1]. Stem cell-based therapies using autologous endothelial progenitor cells or mononuclear cells to treat myocardial infarction, critical limb ischemia, or other ischemic insults have shown only moderate benefits in clinical trials [2–6]. More recently, we and others have demonstrated that endothelial cells (ECs) differentiated from embryonic stem cells or induced pluripotent stem cells (iPSC-ECs) are another potent class of therapeutic cells for treatment of critical limb ischemia in pre-clinical animal models [7–11]. Given the infinite expansion capability of iPSCs, iPSC-ECs represent a promising therapeutic cell type for cell therapy and regenerative medicine.

Although stem cell delivery is a promising approach to treat cardiovascular diseases, numerous recent studies have demonstrated the critical limitation of low post-implantation cell survival, leading to reduced therapeutic potential of the cells [12,13]. Genetic approaches to enhance cell survival by induction of pro-survival factors such as Akt or hemoxygenase-1 have shown positive benefit [14–19]. However, due to concerns surrounding the safety of genetic modification, non-genetic approaches to improve cell survival are an attractive alternative.

In recent years, naturally derived extracellular matrix (ECM) proteins have been employed as cell delivery vehicles that provide pro-survival cues to the transplanted cells. Such ECMs include alginate [20], collagen [21], fibrin [22], hyaluronan [23], and chitosan [24]. However, a limitation of these ECMs is that they consist of single-factor ECMs, which overly simplifies the complex ECM milieu within biological tissues. In particular, ECs physiologically reside within an endothelial basement membrane that is comprised of numerous ECMs including laminin, collagen type IV, fibronectin, and heparan sulfate proteoglycans [25]. It is well-recognized that the complex ECM composition of the endothelial basement membrane provides important signaling cues for physiological maintenance of endothelial behavior and function [26]. However, so far there has been no study systematically quantifying the effects of chemically complex multi-component ECMs on endothelial survival and function under the condition of hypoxia (1% O_2) and reduced nutrients (1% fetal bovine serum), as is present in ischemic injury sites. This dearth of knowledge undermines the therapeutic potential of stem cell therapy for treatment of tissue ischemia and other cardiovascular diseases.

Under both physiological as well as pathological conditions, ECs interact with a milieu of combinatorial ECMs. Accordingly, we tested the hypothesis that combinatorial ECM interactions may be more effective than single-factor ECMs in improving iPSC-EC viability and function under hypoxia with reduced nutrient condition. Using a microscale high-throughput platform for simultaneous screening of iPSC-EC function and survival when cultured on hundreds of combinatorial ECMs, we demonstrate that multi-component ECM combinations such as collagen IV + gelatin + heparan sulfate + laminin (CGHL) and collagen IV + fibronectin + gelatin + heparan sulfate + laminin (CFGHL) show significant improvement over single-factor ECMs in maintaining phenotypic marker expression, as well as augmenting cell survival and function under the condition of hypoxia with reduced serum. Furthermore, this approach enables full-factorial analysis of interaction effects between ECMs, which may be critical for understanding how cells respond to complex microenvironmental cues.

2. Methods

2.1. Fabrication of ECM microarray slides

ECM microarrays were generated using single ECMs or multi-component mixtures of the following ECM proteins: collagen IV (C, mouse, Southern Biotech; Cat. No. 1250-04S), fibronectin (F, bovine, Sigma; Cat No. F1141), laminin (L, mouse, Life Technologies; Cat No. 23017-015), gelatin (G, bovine, Sigma; Cat No. G1393), heparan sulfate (H, mouse, Sigma; H4777), and Matrigel (M, mouse, BD Biosciences; Cat No. 356231) (Supp Fig. 1). Stock solutions (0.5 mg/ml) of each ECM combination were loaded into micro-well reservoirs. Using the OmniGrid Accent Microarrayer (Gene Machines), combinatorial ECMs were drawn from a reservoir using circular pins and spotted onto protein binding Schott H slides (Nexterion), forming individual circular "islands" that were 0.3 mm in diameter and 0.3 mm apart from neighboring islands. Each of the 63 single or multi-component (2-factor, 3-factor, 4-factor, 5-factor, and 6-factor) ECM compositions were printed onto slides with 6 replicates, which resulted in a total of 378 ECM islands. The total ECM concentration for each ECM combination was held constant at 0.5 mg/ml. For multi-component compositions, each individual ECM component was loaded in equal mass ratios as other ECMs (ie, 1:1, 1:1:1, etc). After covalent attachment of the ECMs, the microarray slides were air dried and transferred into vacuum sealed boxes and stored in the dark at 4 °C until used. Although Matrigel is composed of non-defined ECMs derived from Engelbreth-Holm-Swarm sarcoma, it was included for comparison to defined combinatorial ECMs because it has been frequently used for *in vivo* cell delivery applications [13].

Green 540 Reactive Fluorescence Dye (Arrayit) was used to reveal the amount of proteins attached to the slides after fabrication based on the intensity of fluorescence. Microarray slides were incubated in Green 540 Dye (1x) for 1 h, followed by washes with phosphate-buffered saline (PBS). Similar procedures were performed to quantify the amount of specific ECMs (laminin and fibronectin) using anti-laminin (Abcam) and anti-fibronectin (EMD Millipore) antibodies. Images were obtained using fluorescence microscope (Keyence, BZ-X710) at 4X objective. Quantification of fluorescence intensity was performed using Image J.

2.2. Generation and characterization of iPSC-ECs

Human iPSCs (HUF5 strain) were previously derived by retroviral-mediated transduction of Oct-4, Sox-2, Klf-4 and c-Myc in adult human dermal fibroblasts [27]. To generate iPSC-ECs, iPSCs were differentiated in the presence of vascular endothelial growth factor and bone morphogenetic protein-4 for two weeks as previously described [28]. Fluorescent activated cell sorting (FACS) for CD31 expression previously indicated >90% of the human iPSC-ECs expressed CD31 (Supp Fig. 2A and B) [7,28,29]. Immunofluorescence staining demonstrated that the cells express known endothelial markers such as von Willebrand Factor and could functionally take up acetylated low density lipoprotein (Supp Fig. 2C and D). Genetic, protein, and functional characterization of this strain of iPSC-ECs have been previously reported by us and others to confirm endothelial identity [28,30].

2.3. Cell seeding on ECM microarray slides

Prior to *in vitro* studies, ECM microarray slides were sterilized in 1X anti-mycotic solution (Life Technologies) for 30 min at 37 °C, followed by 3 washes in PBS. The iPSC-ECs were dissociated with Tryple Express (Life Technologies) and seeded on top of the slides at a density of 5×10^5 cells per slide in 5 ml EGM-2MV growth