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Differential effects of acellular embryonic matrices on pluripotent stem cell expansion and neural differentiation

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ABSTRACT

Extracellular matrices (ECM) derived from pluripotent stem cells (PSCs) provide a unique tissue microenvironment that can direct cellular differentiation and tissue regeneration, and rejuvenate aged progenitor cells. The unlimited growth capacity of PSCs allows for the scalable generation of PSC-secreted ECMs. Therefore, the derivation and characterization of PSC-derived ECMs is of critical importance in drug screening, disease modeling and tissue regeneration. In this study, 3-D ECMs were generated from decellularized undifferentiated embryonic stem cell (ESC) aggregates (AGG), spontaneously differentiated embryoid bodies (EB), and ESC-derived neural progenitor cell (NPC) aggregates. The capacities of different ECMs to direct proliferation and neural differentiation of the reseeded mouse ESCs and human induced pluripotent stem cells (iPSCs) were characterized. Proteomic analysis by liquid chromatography −tandem mass spectrometry (LC-MS/MS) revealed protein expression profiles that reflected distinct niche properties for each tested ECM group. The reseeded mouse ESCs and human iPSCs responded to different types of ECMs with different cellular phenotypes. Cells grown on the AGG-ECM displayed high levels of pluripotent markers Oct-4 and Nanog, while the cells grown on the NPC-ECM showed increased expression of neural marker β-tubulin III. The expression levels of β-catenin were high for cells grown on the AGG-ECM and the EB-ECM, but reduced in cells grown on the NPC-ECM, indicating a possible role of Wnt/β-catenin signaling in the cell-matrix interactions. This study demonstrates that PSC-derived ECMs can influence stem cell fate decisions by providing a spectrum of stem cell niche microenvironments during tissue development.

1. Introduction

The microenvironment of stem cells plays an essential role in directing cellular differentiation and tissue morphogenesis. A critical component of the stem cell microenvironment, or “niche”, is the extracellular matrix (ECM), which not only provides adhesion sites for the cells, but also sequesters growth factors and interacts with the cells through intrinsic signaling pathways [1–3]. Stem cells secrete distinct profiles of ECMs that coordinate the signaling cascade during self-renewal and lineage commitment. These ECMs provide a spectrum of signaling networks corresponding to the specific tissue development stage that are absent in the ECMs derived from somatic cells [4–6]. For example, mesenchymal stem cell (MSC)-derived ECMs preserve the stem cell niche, which maintains the long-term re-population ability of hematopoietic stem cells [4]. In addition, ECMs derived from “young” stem cells (*i.e.*, MSCs isolated from young donors) can rejuvenate aged progenitor cells, indicating the importance of niche properties during tissue aging and regeneration [7]. These native cell-derived ECMs become the unique biomaterials that can serve as the substrates, scaffolds, growth factor carriers, and even the bioinks in 3-D printing for various *in vitro* culture and *in vivo* transplantation studies [5,8,9].

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), secrete a large

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amounts of ECM components, such as fibronectin, laminin, collagen type IV, and glycosaminoglycans [10–12]. ECM proteins are found to be involved in several signaling pathways (*e.g.*, BMP, TGF-β, FGF, Nodal, and Wnt) that affect tissue development through the binding of the ligands (*e.g.*, integrins) that trigger intracellular signaling [13]. PSC-deposited ECMs also contain numerous growth factors such as Lefty and Cerberus, which may direct the survival and function of the re-populated cells [14–17]. Additionally, ECMs can sequester the heparin-binding fibroblast growth factors (FGFs), which can activate mitogen-activated protein kinase and extracellular-signal-regulated kinase signaling and stimulate the proliferation and survival of adult myogenic progenitors, MSCs, and human ESC-derived neural progenitors [4,15,16]. Moreover, matrix components such as biglycan, syndecan and glypican are able to modulate Wnt/β-catenin signaling through frizzled receptor and its co-receptors such as low-density-lipoprotein receptor-related protein (LRP6) [18,19]. Compared to somatic cell-derived ECMs, PSC-derived ECMs have embryonic origins, a broad capacity to direct stem cell fate decisions, and the scalability for ECM derivation [6,20–23]. However, the signaling capacity of PSC-derived ECMs to act as the embryonic microenvironment is not well understood.

To further explore the signaling capacity of PSC-derived ECMs based on our previous study [22,24], three types of ECMs were derived from decellularized undifferentiated ESC aggregates (AGG), spontaneously differentiated embryoid bodies (EB), and ESC-derived neural progenitor cell (NPC) aggregates. The derived ECMs were re-populated with mouse ESCs and human iPSCs, and the capacity of these three ECMs to influence pluripotency and neural differentiation of the reseeded cells was investigated. Proteomic analysis revealed the distinct composition of the three ECMs, which partially explained the different cellular response to each type of ECM. Additionally, canonical Wnt signaling for the cells grown on different ECMs was assessed to determine the influence of cell-matrix interactions on intracellular signals. Our results indicate that the embryonic niche and the differentiated niche provided by tissue context-dependent ECMs derived from PSCs elicit distinct signaling responses, impacting stem cell fate decisions. This study has significance in biomaterials design with applications in directing cellular differentiation, rejuvenation of progenitor cells, and tissue regeneration.

2. Materials and methods

2.1. Undifferentiated mouse ESC culture

Murine ES-D3 cells (American Type Culture Collection, Manassas, VA) were maintained on 0.1% gelatin-coated 6-well plates as previously reported [22]. The expansion medium was composed of Dulbecco's Modified Eagle's Medium (DMEM), 10% ESC-screened fetal bovine serum (FBS, Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) (all from Life Technologies, Carlsbad, CA), and 1000 U/mL leukemia inhibitory factor (LIF, Millipore, Billerica, MA).

2.2. Generation of undifferentiated aggregates, spontaneous EBs, and NPC aggregates

Undifferentiated aggregates were generated by seeding cells from ESC monolayers into Ultra-Low Attachment (ULA) 6-well plates (Corning Incorporated, Corning, NY) at 1×10^6 cells per well in growth media containing LIF [22]. The aggregates were cultivated for 4 days and used to derive ECM scaffolds. For spontaneous EB formation, 1×10^6 ESCs were seeded in ULA plates in 3 mL of differentiation medium (DMEM plus 10% FBS without LIF). The EBs were cultivated for 4 days and used to derive ECM scaffolds. To generate NPC aggregates, ESCs were seeded at 1×10^6 cells in 3 mL of DMEM-F12 plus 2% B27 serum-free supplement (Life Technologies) [25,26]. After 4 days, 1 μM *all-trans* retinoic acid (RA) (Sigma–Aldrich, St. Louis, MI) was added to enrich neural lineage. After an additional 4 days, the resulting NPC aggregates were collected to derive ECM scaffolds.

2.3. Decellularization to generate ECM scaffolds

Decellularization of ESC-derived aggregates was performed as previously described [22]. Briefly, about 600–1000 undifferentiated aggregates (AGG), spontaneously differentiated embryoid bodies (EB), or ESC-derived neural progenitor cell (NPC) aggregates were distributed into each of 1.5 mL microcentrifuge tubes and treated with sterile 1% Triton X-100 (Sigma) for 30 min. After the treatment, the samples were spun at 18,000 g for 2 min, rinsed twice with phosphate buffered saline (PBS), and incubated with 2000 unit/mL DNAse I (Sigma) for 30 min. The samples were then centrifuged at 18,000 g for 2 min and rinsed with PBS prior to subsequent experiments. About 90 μg proteins of acellular matrices were generated per million of day 0 seeded cells for aggregate formation. The morphology and topography of the ECM scaffolds have been characterized previously [22,24].

2.4. Undifferentiated human iPSC culture

Human iPSK3 cells were derived from human foreskin fibroblasts transfected with plasmid DNA encoding reprogramming factors OCT4, NANOG, SOX2 and LIN28 (kindly provided by Dr. Stephen Duncan, Medical College of Wisconsin) [27,28]. Human iPSK3 cells were maintained in mTeSR serum-free medium (StemCell Technologies, Inc., Vancouver, Canada) or knockout serum replacement (SR) medium supplemented with FGF-2 (40 ng/mL) on 6-well plates coated with Geltrex (Life Technologies) [29]. The SR serum-free medium contains 80% DMEM, 20% knockout SR, 1 mM L-Glutamine, 0.1 mM β-mercaptoethanol, and 0.1 mM nonessential amino acids. The cells were passaged by Accutase dissociation every 5–6 days and seeded at 1×10^5 cells per cm^2 in the presence of 10 μM Y27632 (Sigma) for the first 24 h.

2.5. Cell reseeding and cultivation on the decellularized ECMs

Undifferentiated ES-D3 cells were seeded at 0.5×10^5 per mL on the decellularized ECMs in ULA 24-well plates as previously described [22]. The cells self-assembled into aggregates and were associated with ECM scaffolds during the culture (Supplementary Figure 1). The cells were cultivated for 4 days for short-term study and three passages for long-term study in growth media without LIF (a suboptimal growth condition of ES-D3 cells to assess cell fate decisions). The cells were counted at each passage, assessed by alkaline phosphatase (ALP) assay, bromodeoxyuridine (BrdU) assay, and pluripotent marker expression. The supernatants were measured for glucose and lactate levels. For neural differentiation, the cells after three passages were replated on Geltrex-coated surface in neural differentiation medium (DMEM/F-12 and 2% B27) for 7–8 days and assessed for neural marker expression. For human iPSK3 cells, about 1×10^5 per mL were seeded on the decellularized ECMs in the presence of Y27632. The cells were cultivated in SR media plus FGF-2 (40 ng/mL) for 3 passages. During the culture, hiPSK3 cells self-assembled into aggregates associated with ECM scaffolds. For early stage neural differentiation, the cells were cultured in DMEM/F-12 plus B27 medium for 5 days (with 1 μM RA). For late stage differentiation, the RA-treated cells were replated on Geltrex-coated surface in DMEM/F-12 plus B27