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A biomimetic synthetic feeder layer supports the proliferation and self-renewal of mouse embryonic stem cells

Cristina López-Fagundo [a,b,1], Liane L. Livi [a], Talisha Ramchal [a,2], Eric M. Darling [a,b,c,d,*], Diane Hoffman-Kim [a,b,c,e,*]

[a] Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI 02912, USA
[b] Center for Biomedical Engineering, Brown University, Providence, RI 02912, USA
[c] School of Engineering, Brown University, Providence, RI 02912, USA
[d] Department of Orthopaedics, Brown University, Providence, RI 02912, USA
[e] Brown Institute for Brain Science, Brown University, Providence, RI 02912, USA

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ABSTRACT

Successful realization of the enormous potential of pluripotent stem cells in regenerative medicine demands the development of well-defined culture conditions. Maintenance of embryonic stem cells (ESCs) typically requires co-culture with feeder layer cells, generally mouse embryonic fibroblasts (MEFs). Concerns about xenogeneic pathogen contamination and immune reaction to feeder cells underlie the need for ensuring the safety and efficacy of future stem cell-based products through the development of a controlled culture environment. To gain insight into the effectiveness of MEF layers, here we have developed a biomimetic synthetic feeder layer (BSFL) that is acellular and replicates the stiffness and topography of MEFs. The mechanical properties of MEFs were measured using atomic force microscopy. The average Young's modulus of the MEF monolayers was replicated using tunable polyacrylamide (PA) gels. BSFLs replicated topographical features of the MEFs, including cellular, subcellular, and cytoskeletal features. On BSFLs, mouse ESCs adhered and formed compact round colonies; similar to on MEF controls but not on Flat PA. ESCs on BSFLs maintained their pluripotency and self-renewal across passages, formed embryoid bodies and differentiated into progenitors of the three germ layers. This acellular biomimetic synthetic feeder layer supports stem cell culture without requiring co-culture of live xenogeneic feeder cells, and provides a versatile, tailorable platform for investigating stem cell growth.

Statement of Significance

Embryonic stem cells have enormous potential to aid therapeutics, because they can renew themselves and become different cell types. This study addresses a key challenge for ESC use – growing them safely for human patients. ESCs typically grow with a feeder layer of mouse fibroblasts. Since patients have a risk of immune response to feeder layer cells, we have developed a material to mimic the feeder layer and eliminate this risk. We investigated the influence of feeder layer topography and stiffness on mouse ESCs. While the biomimetic synthetic feeder layer contains no live cells, it replicates the stiffness and topography of feeder layer cells. Significantly, ESCs grown on BSFLs retain their abilities to grow and become multiple cell types.

1. Introduction

Embryonic stem cells (ESCs) are pluripotent cells that are derived from the inner cell mass of the blastocyst and maintain the abilities of self-renewal and multilineage differentiation *in vitro* under defined conditions [1,2]. By harnessing and controlling the ability of these cells to differentiate into virtually all cell types found in the body, scientists hope to use these cells for

* Corresponding authors at: Department of Molecular Pharmacology, Physiology and Biotechnology, Brown University, 175 Meeting Street, Providence, RI 02912, USA.
E-mail addresses: clopezfagundo@gmail.com (C. López-Fagundo), liane_livi@brown.edu (L.L. Livi), talisha.ramchal@duke.edu (T. Ramchal), Eric_Darling@brown.edu (E.M. Darling), dhk@brown.edu (D. Hoffman-Kim).
[1] Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Switzerland.
[2] School of Medicine, Duke University, Durham, NC 27710, USA.

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myriad disease treatments. However, there are challenges that need to be overcome before such therapies can be utilized. Realization of the potential of pluripotent stem cells in regenerative medicine requires the development of well-defined conditions for long-term culture, growth, and directed differentiation. There is a need to produce ESCs on a large scale in a controlled manner, with well-characterized conditions free of foreign support cells and other unknown conditioning factors [3].

Mouse ESCs (mESCs) [1] and human ESCs (hESCs) [2] each have been established using similar procedures, through co-culture with mouse embryonic fibroblasts (MEFs) as feeder cells. The majority of stem cells are still maintained in co-culture with MEFs, as MEFs can provide the required environment for ESC self-renewal. However, the use of MEFs is less than ideal for several reasons, including limited usage due to early senescence, as well as the risk of xenogeneic contamination. Further, the presence of feeder layers complicates certain types of stem cell research, since collected data may reflect the combined response of stem cells and feeder layers [4].

Since MEFs present a highly supportive environment for stem cells, several studies have focused on exploring their characteristics. While some of the soluble or bound factors expressed by MEFs have been found [5], many cues are still unidentified. In addition, several efforts have tried to replace live MEFs with cell-dependent and cell-independent approaches [6]. Defined culture media formulation and defined surface coating approaches [7–9] have been explored. A variety of materials have been investigated including natural materials such as hyaluronic acid [10]. Recent strategies have focused on replacing biologically variable materials with synthetic materials in hopes of creating a more controllable, systematic and xeno-free platform for the growth and expansion of stem cells [9,11].

In addition, stem cells respond to substrate properties, including compliance and topography. Recently, materials of varying stiffnesses, including the range of 0.3–50 kPa, have been developed to explore stem cell self-renewal and differentiation [12–15]. In some of these studies, material patterning and topography, which have been shown to influence mesenchymal stem cell differentiation [16–19], have been investigated for their effects on embryonic stem cells as well [12,13].

To better understand how MEFs support stem cells, the goal of this study was to investigate the influence of MEF feeder layer topography and stiffness on the proliferation, self-renewal, and differentiation capabilities of mESCs. Here we have developed materials that replicate the combination of both the stiffness and the topography of MEF feeder layers. Using a replica molding technique that reproduces cell topography at the micro/nano-scale [20–22], we reproduced critical elements of the feeder layer that support mESCs, specifically by fabricating biomimetic synthetic feeder layers (BSFLs) that incorporate micro/nano-scale physical features with the shape, size, and stiffness of feeder layer cells. To evaluate whether the BSFLs can support stem cells, mESCs cultured on BSFLs were evaluated for their abilities to establish colonies, form embryoid bodies (EBs), and differentiate into the three germ layers.

2. Materials and methods

2.1. Culture of MEFs and their inactivation

Primary MEFs (Millipore PMEF-CLF strain CF-1 untreated, passage 3) were maintained in tissue culture flasks coated with 0.1% gelatin solution (Millipore, ES-006-B) in Dulbecco's Modified Eagle's Medium (DMEM, Millipore, SLM-220B) supplemented with 10% embryonic stem cell qualified fetal bovine serum (ES, FBS, Millipore, ES-009B), 100 μg/mL Penicillin, 100 μg/mL Streptomycin (Invitrogen), and 4 mM L-glutamine (Invitrogen). Cells were incubated at 37 °C with 5% CO_2, grown to confluence and passaged at 1:4. To mitotically inactivate MEFs, 10 μg/mL mitomycin C in PMEF media (Acros Organics) was added to confluent MEF cultures. Cultures were incubated for 2 h, and cells were rinsed 3 times with PBS for 10 min followed by the addition of fresh media. Mitotically inactivated MEFs were kept for up to 2 weeks at 37 °C with 5% CO_2 and were used as feeder layers for mESC cultures.

2.2. Mechanical and topographical characterization of MEFs

MEFs at a density of 100,000 cells/cm^2 were grown to confluence on gelatin-coated glass coverslips and assessed for their live-cell topographical features and elastic moduli using an MFP-3D-BIO atomic force microscope (AFM, Asylum Research). To facilitate conformal contact during testing, 5 μm diameter, borosilicate glass spheres were attached to the tip of AFM cantilevers with nominal stiffness values of 0.03 N m^{-1} (Novascan Technologies). Cellular elastic moduli were quantified using three techniques: single-cell indentation [23], force mapping [24], and force scanning [25]. The force scanning technique was also used to generate high-resolution (~15,000 points) topographical/elastic maps of live MEFs. Scan sizes ranged from 80 × 80 to 90 × 90 μm, typically incorporating 1–3 cells in a field of interest. Average MEF mechanical properties were determined by testing ten random sites via force maps (n = 100 indentations/site), in conjunction with single-cell indentations of confluent monolayers (n = 10 cells). Approach velocities of 15 μm/s were used for all indentation tests. Force-indentation data were fit with a modified, thin-layer Hertz model to extract elastic moduli as described previously [23–25]. Data are presented as mean ± standard deviation. Topographical scans were performed using spherical-tipped cantilevers at a scan rate of 0.3–1 Hz, resolution of 256 × 256, and set point force of 5 nN.

2.3. Fabrication of MEF templates

Cellular templates were fabricated as previously described by Bruder et al. [22]. Glass slides (75 × 50 mm, Corning) were cleaned with ethanol and deionized water (dH_2O) and then dried. Slides were oxygen plasma-etched (Harrick Plasma) for 1 min, coated with 0.1% gelatin solution, and 150,000 cells/cm^2 were plated. MEF cultures reached confluence in 2–3 days, at which point cells were fixed in 2% paraformaldehyde (PFA, Electron Microscopy Sciences) in 0.1 M phosphate buffered saline, pH 7.4 (PBS, Invitrogen) at room temperature for 20 min. PFA-fixed cells were subsequently post-fixed and dehydrated according to Bruder et al. [22] with procedures that use Karnovsky's fixative (Electron Microscopy Sciences) and ethanol (Sigma Aldrich), respectively. Templates were dried and sputter coated with gold-palladium at 20 mA for 3 min (Emitech K550 Sputter coater), resulting in a rigid cell template (Fig. 1A).

2.4. Fabrication of BSFLs on polyacrylamide substrates

To produce PDMS MEF impression replicas (IRs), Sylgard 184 elastomer base was mixed with Sylgard 184 curing agent (10:1 wt/wt) (Dow Corning) and centrifuged for 1 min at 2000 rpm to remove air bubbles. PDMS was cast over the fixed cell templates and baked for 4 h at 65 °C to cure. The cured PDMS was peeled from the template revealing a MEF cellular IR with cellular features indented in the PDMS. Freshly peeled PDMS IR was plasma etched for 1 min and immediately silanized through vapor deposition of tridecafluoro-1,1,2,2-tetrahydrooctyl (trichlorosilane) (Gelest, Inc.) for 2.5 h (Fig. 1B).