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Growth of hollow cell spheroids in microbead templated chambers

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ABSTRACT

Cells form hollow, spheroidal structures during the development of many tissues, including the ocular lens, inner ear, and many glands. Therefore, techniques for *in vitro* formation of hollow spheroids are valued for studying developmental and disease processes. Current *in vitro* methods require cells to self-organize into hollow morphologies; we explored an alternative strategy based on cell growth in pre-defined, spherical scaffolds. Our method uses sacrificial, gelatin microbeads to simultaneously template spherical chambers within a hydrogel and deliver cells into the chambers. We use mouse lens epithelial cells to demonstrate that cells can populate the internal surfaces of the chambers within a week to create numerous hollow spheroids. The platform supports manipulation of matrix mechanics, curvature, and biochemical composition to mimic *in vivo* microenvironments. It also provides a starting point for engineering organoids of tissues that develop from hollow spheroids.

1. Introduction

Three-dimensional (3D) cell culture techniques vary the mechanical properties, geometry, and biochemical cues in a cell's microenvironment in order to influence processes such as differentiation, migration, and morphogenesis [1–4]. Under certain conditions, cells in 3D culture will exhibit *in vivo*-like physiological behaviors that are not observed using traditional cultures on flat, rigid substrates. As a result, 3D culture techniques are now widely adopted for fundamental cell biology studies [5,6] and for applied research in drug testing [7,8] and tissue engineering [9].

A common aim of 3D culture is to organize cells into certain configurations (e.g., tubes for vessels or layers for stratified tissues) [10,11]. In order to achieve a desired configuration, researchers can impart constraints on cell organization when creating their 3D system [12,13] and/or rely on the inherent ability of cells to self-organize [14]. To form vessels for example, previous studies either grew vascular cells in predefined channels, allowed the cells to self-organize into vascular networks, or combined defined channels with self-organization [15–18].

The configuration we are interested in is the hollow spheroid. Spheroidal vesicles are composed of a layer of cells surrounding a fluid-filled lumen and are frequently observed during development [19]. Examples include Rathke's pouch and the lens, otic, and renal vesicles, which are hollow precursors to the anterior pituitary, ocular lens, inner ear, and nephron, respectively. Cells organized into hollow spheroids are exposed to cues that they may not experience when organized as 2D monolayers, such as radial and circumferential stresses [20], asymmetric biochemical signals from the luminal and external sides of the sphere [4], and curvature that can alter cytoskeletal assembly and contraction [4,21]. Therefore, methods to create hollow spheroids are important for studying the development and physiology of the cells that compose them.

The prevailing methods for creating hollow spheroids rely on self-organization, especially with stem cells [22–24]. Complementary methods that predefine spherical geometries, analogous to the use of predefined channels for vessels, are limited. Soft lithography techniques can define the shapes of cavities in hydrogels for cell growth, but spherical chambers cannot be attained with these methods [25,26]. Several microfluidic and electrospraying techniques exist to encapsulate cells in solid or liquid core microbeads [27–30]. However, the beads were designed to generate solid microspheres and often used non-cell adhesive

Abbreviations: 3D, three-dimensional; LEC, lens epithelial cell; mT/nG, membrane Tomato/nuclear GFP; GelMA, gelatin methacrylate.

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materials (e.g., agarose and alginate). Here we describe a 3D culture technique that combines cell encapsulation in sacrificial microbeads with the formation of spherical chambers in hydrogel matrices. We demonstrate with lens epithelial cells (LECs) that the platform enables the directed formation of hollow, cell-lined spheroids.

2. Methods

2.1. Materials

Culture related reagents were purchased from Gibco including advanced DMEM/F-12, DMEM/F-12 with HEPES, 10x TrypLE, GlutaMAX, penicillin-streptomycin, fetal bovine serum (FBS), 100x B-27 supplement, 0.05% trypsin-EDTA, Hank's balanced salt solution with calcium and magnesium (HBSS), phosphate buffered saline (PBS), paraffin oil, LIVE/DEAD viability/cytotoxicity kit. Rat anti-E-cadherin antibody (Invitrogen, cat#13-1900) and fluorescently labelled phalloidins (Thermo-Fisher Scientific, Waltham, MA). Growth factor reduced Matrigel (Corning Life Sciences, Tewksbury, MA). TGF-β1 receptor inhibitor SB-431542 (Stemgent, cat#04-0010-10), and Rho-associated, coiled-coil containing protein kinase inhibitor Y-27632 (Selleck Chemicals, Houston, TX and ApexBio, Houston, TX). Gelatin type A from porcine skin 300 Bloom, dispase, methacrylic anhydride, dichlorodimethylsilane, and 3-(Trimethoxysilyl)propyl methacrylate (Sigma Aldrich, St. Louis, MO). Soybean lecithin and the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (TCI America, Portland, OR). Bovine serum albumin (BSA) (Research Products International, Prospect, IL). Normal goat serum and VECTASHIELD antifade mounting medium with 4′,5-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

2.2. Mouse strains

Mouse care and breeding were performed according to the Animal Care and Use Committee approved animal protocol (University of California Berkeley, Berkeley, CA, USA). Both wildtype mice and membrane tdTomato/nuclear GFP (mT/nG) transgenic mice in the C57BL/6 background were used. Double transgenic mT/nG mice were generated by mating mice expressing membrane-targeted tdTomato [31] (strain 007676, The Jackson Laboratory, Bar Harbor, ME) with mice expressing histone 2B-green fluorescent protein (H2B-GFP) fusion proteins [32] (strain 006069, The Jackson Laboratory).

2.3. Cell culture

Primary cultured lens epithelial cells (LECs) were harvested from adult mice. Eyes were removed from euthanized mice then the lenses were dissected out into advanced DMEM/F-12, washed in PBS, and transferred to trypsin-EDTA at 37 °C for 5 min with gentle shaking. The lenses were washed 3X with PBS then resuspended in Advanced DMEM/F-12. The lens capsules with attached LECs were then dissected away from the bulk mass of fibers and placed in 2U/mL dispase in HBSS (50 μl/capsule) at 37 °C for 30 min with gentle shaking. An equal volume of 10X TrypLE was then added and the capsules were incubated for an additional 5 min. The mixture was triturated by pipetting, added to advanced DMEM/F-12 (9 times the combined volume of dispase/TrypLE), and centrifuged at ~225 × g for 5 min. All but ~300 μl of supernatant were aspirated away, then the LECs were resuspended in growth media (advanced DMEM/F-12, 0.5X penicillin/streptomycin, 1X GlutaMAX, 2% FBS, 1X B-27, 5 μM SB-431452, and 10 μM Y-27632) and plated into 6 cm tissue culture dishes that were previously coated with 0.1 mg/ml Matrigel in DMEM/F-12. Typically, LECs from 2 to 4 lenses were used to seed each dish. Cells were grown at 37 °C in 5% CO_2, 95% relative humidity conditions and media was changed every 2–3 days.

2.4. Gelatin methacrylate synthesis

Gelatin methacrylate (GelMA) was synthesized similarly to as described previously [33,34]. 10 g of gelatin was dissolved in 100 ml of 100 mM Na_2HPO_4 at 50 °C. A total of 0.4 ml of methacrylic anhydride per gram of gelatin was added over the course of 3 h with stirring and the temperature was maintained at 50 °C. 1/6 of the total methacrylic anhydride was added every 30 min and the pH was maintained between 7.5 and 8.5 by dropwise addition of 4 M NaOH. The reaction was diluted in 100 ml of H_2O then subjected to dialysis with a 12–14 kDa molecular weight cut off membrane. The buffer was changed at least once daily over the course of one week. Dialysis was first against 1X PBS, then against 0.1X PBS, then against H_2O for the remainder of the week. The dialyzed GelMA solution was adjusted to pH ~7.5 with 7.5% w/v $NaHCO_3$ then frozen, lyophilized, and stored at −80 °C.

2.5. Cell encapsulation

Confluent monolayers of primary cultured, passage 0 LECs were detached using 1X TrypLE in PBS and resuspended in 12% w/v gelatin in DMEM/F-12 to a concentration of 5–7.5 × 10^6 cells/ml. 400–500 μL of cells in gelatin were then added to a 25 ml beaker containing 7 g of 0.2% w/w soy lecithin in paraffin oil to begin gelatin microbead synthesis. The beaker was held in a 37 °C water bath while being stirred with a magnetic stir bar at 300 RPM. After 4 min, the beaker was transferred to a room temperature water bath and after another 4 min it was transferred to an ice bath for 4 more minutes. Stirring was maintained at 300 RPM throughout. All subsequent centrifugation steps were performed at ~80 × g and ice cold solutions were used. The microbead emulsion was centrifuged for 3 min, the supernatant was pipetted away, then the microbeads were resuspended in 3 ml of paraffin oil. 6 ml of PBS was added and the mixture was centrifuged for 3 min. The oil layer was then removed, the remaining mixture was thoroughly mixed by pipetting, then centrifuged for 3 more minutes. The supernatant was pipetted away, the beads were resuspended in 10 ml of PBS, and then spun down for 1 min. The previous step was repeated and the beads were suspended in 5 mL of DMEM/F-12. The beads were filtered on a 100 μm nylon mesh to remove small beads and remaining beads were eluted off the mesh with 10 ml of DMEM/F-12 and then spun down for 1 min. Thereafter, 8 ml of the supernatant was removed and the remaining beads were used for encapsulation.

2.6. Microbead encapsulation and cell growth

GelMA was dissolved to 18% w/v in a 1.5 mg/ml solution of LAP in PBS. Microbeads (70 μl per 150 μl total volume), Matrigel (2.75 mg/ml), and PBS were mixed and held on ice. Molds were defined by sandwiching ~0.5 mm thick strips of Parafilm between a bottom glass layer and a top layer of parafilm or dichlorodimethylsilane-treated glass. The bottom glass layer was treated with 3-(Trimethoxysilyl)propyl methacrylate to allow covalent bonding of GelMA hydrogels for time course imaging. GelMA was mixed with the microbead/Matrigel mixture at a 2:1 vol ratio, pipetted into the molds, then immediately photopolymerized by 365 nm UV light for 45 s (4 mW/cm^2, UVP UVL-23RW). The top mold layers were then removed and the gels were immersed in DMEM/F-12 at room temperature. After 5 min the gels were moved