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A microparticle approach to morphogen delivery within pluripotent stem cell aggregates

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ABSTRACT

Stem cell fate and specification is largely controlled by extrinsic cues that comprise the 3D microenvironment. Biomaterials can serve to control the spatial and temporal presentation of morphogenic molecules in order to direct stem cell fate decisions. Here we describe a microparticle (MP)-based approach to deliver growth factors within multicellular aggregates to direct pluripotent stem cell differentiation. Compared to conventional soluble delivery methods, gelatin MPs laden with BMP4 or noggin induced efficient gene expression of mesoderm and ectoderm lineages, respectively, despite using nearly 12-fold less total growth factor. BMP4-laden MPs increased the percentage of cells expressing GFP under the control of the Brachyury-T promoter as visualized by whole-mount confocal imaging and quantified by flow cytometry. Furthermore, the ability to localize MPs laden with different morphogens within a particular hemisphere of stem cell aggregates allowed for spatial control of differentiation within 3D cultures. Overall, localized delivery of growth factors within multicellular aggregates from microparticle delivery vehicles is an important step towards scalable differentiation technologies and the study of morphogen gradients in pluripotent stem cell differentiation.

1. Introduction

Transfer of pluripotent stem cells from adherent monolayer to three-dimensional suspension culture of cell aggregates, referred to as embryoid bodies (EBs), is frequently used to promote differentiation towards cell types of all three germ layers [1–3]. In many instances, limiting differentiation within aggregate cultures to a particular cell type has proven difficult, but the addition of specific morphogenic growth factors to the culture medium often enhances the production efficiency of desired cell types. For example, the addition of bone morphogenetic protein 4 (BMP4) to the culture medium at early time points after aggregate formation enhances mesoderm differentiation by activation of the transcription factor Brachyury-T through SMAD 1/5/8 signaling, a mechanism conserved in mouse and human development [4,5]. BMP4 signaling can also be inhibited to promote ectoderm differentiation by the addition of noggin, which directly binds BMP4, or other small molecule inhibitors of the SMAD pathway [6,7].

Due to the three-dimensional nature of multicellular aggregates, inherent barriers exist to the free diffusion of molecules throughout the aggregate and we, in addition to several other labs, have demonstrated diffusion limitations encountered with several different types of molecules [8–10]. Therefore, concentration gradients of molecules created throughout cell spheroids may be, at least in part, responsible for the general difficulty in controlling the homogeneity of differentiation within three-dimensional aggregates. The challenge of precise dosing control is further compounded by the fact that, in terms of scalability, growth factor delivery methods have not kept pace with recent advances in stem cell technologies allowing for scalable formation and culture of homogeneous pluripotent stem cell aggregates [11–14].

We present here a scalable method for integrating biomaterial, microparticle (MP)-based growth factor delivery vehicles that, because the growth factor is delivered from within the aggregate itself, is independent of the volume of the medium in the culture vessel and can circumvent barriers to diffusion of molecules from

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the culture medium throughout the aggregate. Delivery of BMP4 and noggin from gelatin-based MPs was separately tested to direct early pluripotent lineage commitment in three-dimensional aggregates. The ability to localize MPs laden with different morphogens within a particular hemisphere of multicellular aggregates was also investigated as a method to spatially control differentiation within a model of mammalian development. MP delivery of growth factors does not require medium manipulation for directed differentiation and is an important step towards scalable differentiation of pluripotent stem cells for cell biomanufacturing and tissue engineering purposes.

2. Methods

2.1. Fabrication and loading of gelatin microparticles

Microparticles (MPs) of gelatin type B (G9391, Sigma–Aldrich, St. Louis, MO) were generated using a water-in-oil emulsion method and fluorescently labeled as previously described [2]. Heparin sodium salt (CalBiochem, San Diego, CA) was conjugated to MPs after MP formulation in the following manner: EDC and S-NHS (Thermo Scientific, Waltham, MA) were added to heparin at 10:1 and 25:1 M ratios respectively, relative to heparin dissolved in 800 μL activation buffer (0.1 M MES, 0.5 M NaCl, pH 6.0) and allowed to react for 15 min at room temperature to modify the carboxyl groups of heparin to amine reactive S-NHS esters. The EDC/NHS reaction was quenched with 20 mM 2-mercaptoethanol and the activated heparin was added to 400 μL of MP in PBS at a 5:1 M ratio of heparin to gelatin and agitated for 4 h at 37 °C. Prior to cell culture, MPs were treated in 70% ethanol for a minimum of 30 min before washing 3× with ddH_2O. Each MP batch was lyophilized and stored at −20 C until further use. Growth factor solutions were added to lyophilized MPs at 5 μL/mg overnight at 4 C to allow for rehydration of the MPs and uptake of the growth factor. Growth factors were added at either 50 or 125 ng/mg of MPs. After growth factor loading, all of the loaded MPs were suspended in differentiation media (500 μL) and then 10 μL of the solution was counted on a hemocytometer to determine the concentration of MPs.

2.2. Albumin release from MPs

Bovine serum albumin (Sigma–Aldrich, St. Louis, MO) was labeled with AlexaFluor 555 using EDC/S-NHS chemistry. Free dye was removed from the protein solution using an Amicon Ultra-15 centrifugal filter unit with a 30 kDa cutoff (Millipore, Billerica, MA). Labeled BSA was loaded into gelatin MPs as described above at a 1 mg/mL loading concentration.

2.3. Cell culture

Undifferentiated D3 ESCs were maintained on 0.1% gelatin-coated tissue culture dishes in DMEM media supplemented with 15% fetal bovine serum, 2 mM L-glutamine (Mediatech), 1X MEM non-essential amino acid solution (Mediatech), antibiotic/antimycotics (Mediatech), 0.1 mM β-mercaptoethanol (MP Biomedicals, LLC), and 10^3 U/ml leukemia inhibitory factor (LIF) (Millipore, Billerica, MA). Mouse Brachyury-T GFP cells (E14.1, 129/Ola) [15] were maintained on 0.5% gelatin coated petri dishes in a humidified 5% CO_2 atmosphere using modified serum-free maintenance media and base differentiation media. The defined media was composed of a DMEM/F12 (50/50) (Thermo Scientific) media supplemented with N2 (Gibco) and 50 μg/mL BSA (Millipore) in a 1:1 combination with B27 (Gibco) supplemented Neurobasal™ medium (Gibco) with 100 U/ml penicillin, 100 g/mL streptomycin, 0.25 g/mL amphotericin (Mediatech) and 2 mM L-glutamine (Mediatech) [16]. Media was routinely exchanged in ESC cultures every 1–2 days, and cells were passaged every 2–3 days as needed before reaching 80% confluency. ESGRO complete basal medium (Millipore) was used for all differentiation cultures.

2.4. Aggregate formation and culture

ESCs were trypsinized into a single cell suspension and aggregates were formed by forced aggregation in AggreWell™ 400 inserts (Stem Cell Technologies, Vancouver, CA) [13]. Briefly, 1.2×10^6 cells in 0.5 mL of ESGRO complete basal medium were added to each insert, containing approximately 1200 wells, and centrifuged at 200× *g* for 5 min to cluster cells in the wells. Gelatin MPs were incorporated within EBs using a second centrifugation of the culture plates after addition of 200 μL of a MP solution. In all cases, the MP:cell seed ratio was 1:3. After 24 h of culture, cell aggregates were removed from the wells using a wide-bore pipette and transferred to suspension culture on a rotary orbital shaker (40 rpm) to maintain the homogeneity of the aggregate population and prevent EB agglomeration. In the case of soluble growth factor addition, BMP4 (10 ng/mL) or noggin (50 ng/mL) was added during the initial 24 h of formation, again when transferred to suspension culture, and then supplemented every other day when the spent medium was exchanged until day 4 of EB culture. In some cases, EBs were plated onto 0.1% gelatin-coated culture vessels at day 7 of differentiation to allow attachment and EB cell spreading. After attachment, spent medium was exchanged every other day.

For EB merging studies, after 24 h of initial aggregate formation, one population of EBs (population A) was added to a second distinct EB population (population B) formed in a separate microwell insert. After an additional 24 h of culture, EBs from the two populations would merge to form single larger aggregates in the individual microwells. EBs from population A were added at a 1:2 ratio (A:B) to decrease the probability of adding more than one EB from population A to microwells containing fully formed aggregates from population B.

2.5. Spheroid morphology analysis

At days 4 and 7 of differentiation, EBs were collected from rotary culture, fixed in 10% formalin for 30 min, and suspended in Histogel (Richard–Allan Scientific, Kalamazoo, MI). The samples were then embedded in paraffin and cut into 5 μm-thick sections (MICROM HM 310, Global Medical Instrumentation Inc., Ramsey, MN). After the sections were deparaffinized, they were stained with hematoxylin and eosin (H&E). Histological samples were imaged via a Nikon 80i upright microscope equipped with a SPOT Flex camera (15.2 64 MP Shifting Pixel, Diagnostic Instruments).

2.6. Confocal microscopy

The presence of GFP expressing cells within EBs was analyzed using a LSM 510 NLO confocal microscope (Zeiss, Thornwood, NY). EBs were removed from suspension culture, fixed in 4% paraformaldehyde, and stained with Hoechst (1:100) before imaging on glass slides. Visualization of GFP signal was performed using an argon laser with a 488 nm excitation filter and a 510 emission filter.

2.7. Gene expression analysis

RNA was extracted from spheroids after 4 days of differentiation with the RNeasy Mini kit (Qiagen Inc, Valencia, CA). RNA was converted to complementary DNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and analyzed using real time PCR (MyIQ cycler, BioRad). Forward and reverse primers for *Oct4*, Brachyury-*T*, *Pax6*, *Flk1*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) were designed with Beacon Designer software and purchased from Invitrogen (Table 1). Gene expression was calculated with respect to expression levels of EBs without MPs using the Pfaffl method [17].

2.8. Flow cytometry

Spheroids were rinsed thoroughly with PBS and dissociated to a single cell suspension with 0.25% trypsin-EDTA and trituration for 10 min. The single-cell suspension was rinsed 3 times in PBS and pelleted at 1000 rpm for 5 min between rinses. Flow cytometry was performed with an Accuri C6 cytometer (Accuri Cytometers, Ann Arbor, MI), with a minimum of 20,000 events per sample collected within the FSC/SSC gate for live cell populations ($n = 3$ independent experimental samples per condition). Heparin–gelatin MPs, gelatin MPs, and undifferentiated Brachyury-T GFP cells alone were used for to establish appropriate gates and compensation. Within the FSC/SSC gate, polygonal gating was used on the (FSC)/FL-1 (480 nm excitation; 530 ± 15 nm emission) plots to limit 1% of undifferentiated negative control population via FlowJo software (Tree Star, Inc., Ashland, OR). The whole cell population was gated to include less than 2% heparin–gelatin and 5% gelatin MPs (Supplemental Fig. 4).

2.9. Statistical analysis

Unless otherwise indicated, all data are reported as mean ± standard error for a minimum of triplicate experimental samples. All data was normalized to a Gaussian distribution using a Box–Cox power transformation before statistical analysis. Statistical significance was assessed using student's *t*-test or one-way ANOVA with Tukey's post hoc analysis after verifying variance equality from Levene's equality of variances test. *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Morphogen delivery via incorporated MPs

Gelatin MPs were incorporated within embryonic stem cell aggregates using forced aggregation within microwells at a 1:3 (MP:cell) seeding ratio. The number of gelatin MPs incorporated was below the level at which material incorporation was previously observed to alter differentiation within stem cell aggregates [2]. Fluorescently labeled MPs were easily identifiable within the

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