Microsphere-Mediated Delivery of Retinoic Acid within Embryoid Bodies Directs Embryonic Stem Cell Differentiation

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Introduction. Embryonic stem cells (ESCs) are a promising cell source for tissue engineering and regenerative medicine applications due to their capacity for both infinite self-renewal and differentiation to all somatic cell types. However, for ESCs to transition from bench-top to clinical applications, methods for reliable and efficient differentiation to a targeted cell type must be established. Differentiation of ESCs is commonly induced through the formation of multicellular spheroids termed embryoid bodies (EBs), which recapitulate many aspects of embryonic development. Methods for directed differentiation generally rely on addition of soluble factors to EB media, which does not allow for spatial and temporal coordination of signaling events present in normal development, and may result in heterogeneous differentiation patterns. The purpose of this work is to investigate the ability for biodegradable polymer microspheres containing differentiation factors to be incorporated within the interior of EBs and release morphogenic factors within the EB microenvironment to direct ESC differentiation.

Materials and Methods. Poly(lactic-co-glycolic acid) (PLGA) microspheres incorporating a fluorescent dye or retinoic acid (RA) were fabricated using a single emulsion, solvent evaporation technique. Fluorescent microspheres were coated with gelatin and mixed with mouse ESCs at three microsphere:cell ratios (1:2, 2:1 and 5:1) and at three different rotary speeds (25, 40 and 55 rpm). The degree of incorporation of microspheres within EBs was assessed using fluorescent microscopy and spectroscopy. Additionally, ESCs were mixed with microspheres encapsulating RA under rotary suspension conditions to form embryoid bodies, and EBs with no and unloaded microspheres were also formed. Soluble RA (0.1 μM) was added to EBs (with no microspheres) from days 2-6 as a control. After 7, 10 and 14 days, EB samples were taken for gene expression analysis using quantitative PCR, and EBs were fixed in 10% formalin and paraffin embedded for histological examination.

Results. Fluorescent microscopy revealed that microspheres were incorporated into EBs under all mixing conditions examined, however slower rotary speeds and higher microsphere:cell ratios resulted in a greater degree of incorporation. Spectroscopic analysis of lysed EBs confirmed this observation, as EBs formed at 25 rpm with 5:1 microsphere:cell ratio showed the highest level of microsphere incorporation. After 7 days of differentiation, approximately 50% of EBs containing RA-loaded microspheres began to display multiple cystic regions, as revealed by hematoxylin and eosin staining. After 10 days of differentiation, approximately 30% of RA microsphere-containing EBs

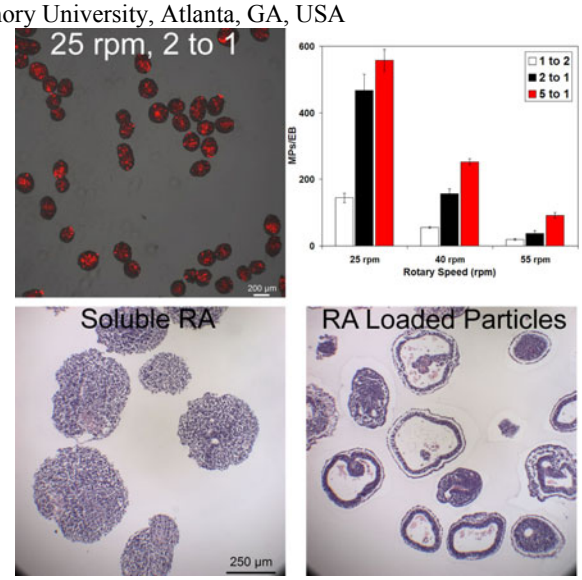

Figure 1. Fluorescent image (overlayed on phase image) of Cell Tracker Red labeled microparticles incorporated within EBs formed at 25 rpm (A); Quantification of EB incorporation as a function of rotary speed and microparticle:cell ratio (B); H&E staining of day 10 soluble RA-treated (C) and RA-loaded microsphere treated EBs (D).

exhibited a large cystic region comprised of a pseudo-stratified columnar epithelial layer of cells surrounded by a primitive endoderm layer, while the other treatment groups showed few or no large cystic regions. Expression of Fgf5, a primitive ectoderm marker, increased in RA MP EBs between days 4 and 10, corresponding to cystic EB formation and appearance of the columnar morphology. Additionally, enhanced expression of α-fetoprotein (AFP), a primitive endoderm marker, was observed in RA microsphere-containing EBs relative to the other treatment groups after 10 and 14 days of differentiation. Immunohistochemical staining for AFP confirmed its presence in the outermost cell layer of RA microsphere EBs, while soluble RA, unloaded microsphere and untreated controls exhibited much less positive staining. Together, this data indicates that RA microspheres within EBs produce cystic EBs, containing an outermost endoderm layer enveloping a primitive ectoderm pseudo-stratified columnar epithelium.

Conclusion. Polymer microspheres containing differentiation factors can be readily incorporated within EBs, and the degree of incorporation can be modulated by altering initial mixing conditions. Microsphere-mediated presentation of RA to differentiating ESCs within EBs resulted in drastic morphological and gene expression changes, with the predominant appearance of a pseudo-stratified epithelial layer in a majority of EBs. This provides evidence that local release of differentiation factors within EBs is a novel means to efficiently direct differentiation of ESCs for regenerative medicine applications.