Microsphere-Mediated Delivery of Retinoic Acid to Embryoid Bodies Induces Spatially Controlled 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 become clinically relevant, 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 objective of this work is to incorporate biodegradable polymer microspheres containing a small molecule morphogen within the interior of EBs in order to direct ESC differentiation in a homogeneous and temporally controlled manner.

Materials and Methods. Poly(lactic-co-glycolic acid) (PLGA) microspheres incorporating a fluorescent dye or retinoic acid (RA) were fabricated using a single emulsion 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 three different rotary speeds (25, 40 and 55 rpm). The degree of microsphere incorporation within EBs was assessed using fluorescent microscopy and spectroscopy. ESCs were mixed with RA microspheres at 40 rpm to form EBs, and EBs with no and unloaded microspheres were also formed. Soluble RA (0.1 μM) was added to EBs from days 2-6 as a control. After 2, 4, 7 and 10 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. Global gene expression analysis was performed using GeneChip Microarrays (Affymetrix). Day 10 RA MS EBs were further differentiated in serum-free media supplemented with BMP4 (10 ng/ml) or Noggin (200 ng/ml), and differentiation was assessed via whole-mount immunofluorescent staining and qPCR.

Results. Microspheres were incorporated into EBs under all mixing conditions examined, and lower rotary speeds and higher microsphere:cell ratios resulted in greater incorporation. After 7 days of differentiation, ~50% of EBs containing RA-loaded microspheres (RA MS EBs) began to display multiple cystic regions, and after 10 days, ~30% of RA MS EBs exhibited a large cystic region comprised of a pseudo-stratified columnar layer surrounded by an endoderm layer (Fig.1B), while the other treatment groups showed few or no large cystic regions (Fig.1A). Global gene expression analysis identified 1276 genes whose transcripts changed significantly between RA MS EBs and unloaded MS EBs, 480 of which increased in RA MS EBs, 796 of which decreased. The genes that increased in RA MS EBs were generally associated with early post- implantation mouse embryonic tissue, including visceral endoderm, epiblast, and primitive streak (Fig.1C). Temporal gene expression analysis using qPCR demonstrated that expression of *Oct4*, a transcription factor related to pluripotency, remained significantly higher in RA MS EBs after 7 days compared to untreated and unloaded MS EBs. Additionally, expression of *Fgf5*, an epiblast marker, was significantly enhanced in RA MS EBs after both 7 and 10 days of differentiation, correlating with the appearance of cystic RA MS EBs. OCT4 staining was restricted to the columnar, pseudo-stratified cell layer of RA MS EBs, while FOXA2, a visceral endoderm marker, was localized to the outermost, endoderm-like layer. The endoderm and epiblast cell layers were clearly discernable in SEM micrographs (Fig.1D). Additionally, preliminary results indicate that supplementation of low-serum media with BMP4 may enhance primitive streak induction in RA MS EBs. Together, these data indicate that RA microspheres incorporated within EBs induce synchronous differentiation of EBs into cystic spheroids containing spatially distinct epiblast and endoderm layers.

Conclusions. Polymer microspheres containing differentiation factors were controllably incorporated within EBs. Microsphere-mediated presentation of RA to ESCs within EBs resulted in the appearance of a bi-layered epithelium that phenotypically resembled the early primitive streak-stage embryo. These studies suggest that morphogen presentation mode (microsphere-mediated vs. soluble delivery) has a significant impact on stem cell differentiation. Ongoing studies are focused on directed differentiation of cystic RA MS EBs to mature cell types under defined media conditions.

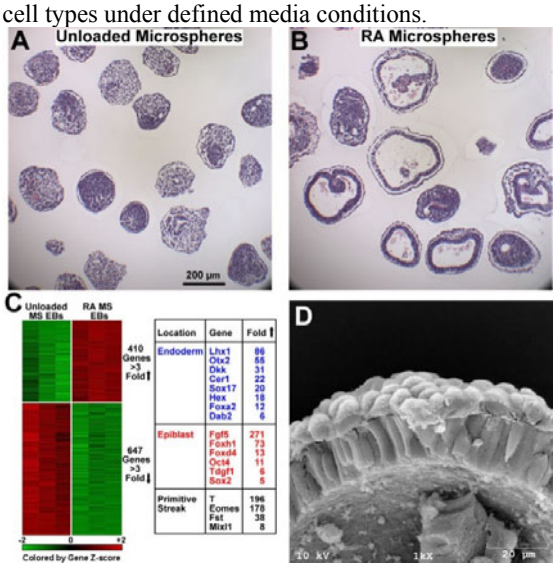

Figure 1. A) EBs containing unloaded microspheres contained small void regions, while B) RA MS EBs were frequently completely cystic after 10 days. C) Genes expressed early-streak mouse embryos were upregulated in RA MS EBs. D) Epiblast and visceral endoderm structures were visible in day 10 RA MS EBs.

Reference. Carpenedo RL. Biomater. 2009;30:2507-2515