Embryonic Stem Cell Differentiation is Modulated by Incorporation of Biomaterials within Embryoid Bodies

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Statement of Purpose: Controlled differentiation of embryonic stem cells (ESCs) remains one of the primary obstacles to their use in regenerative therapies. ESCs are commonly differentiated by the formation of embryoid bodies (EBs), which are aggregates grown in suspension culture. EB formation results in heterogeneous differentiation, although recent efforts to incorporate biomaterial microparticles (MPs) within EBs for delivery of soluble factors show promise for homogeneous directed differentiation (*1*). Stem cell-biomaterial interactions have been previously studied through stem cell encapsulation (*2*), however, biomaterial interactions are limited to exterior cells and little is known about the potential effects of biomaterials incorporated within EBs on differentiation. The objective of this study is to analyze ESC differentiation within EBs in response to incorporated material delivery vehicles fabricated from synthetic and natural degradable and non-degradable biomaterials.

Methods: PLGA, gelatin and agarose MPs were produced using a single oil-in-water or water-in-oil emulsion. EBs were formed using forced aggregation of 1.2E6 ESCs and MPs into 400x400um polydimethylsiloxane microwells. Average MP incorporation per EB was assayed over a range of MP-to-ESC seed ratios. After formation, EBs were removed from microwells and further cultured on a rotary orbital shaker at 40 RPMs for up to 14 days. The gross effects of MPs on EB development were analyzed through time lapse microscopy of EB formation and histological analysis through 14 days of culture. Gene expression of early germ lineage markers was analyzed through qRT-PCR and protein expression was evaluated using whole-mount immunofluorescent staining. One way ANOVA with post-hoc Tukey analysis was performed for gene expression statistical analysis.

Results: PLGA (5.2 $\pm$ 2.8 um), gelatin (5.1 $\pm$ 2.9 um), and agarose (4.5$\pm$ 1.7 um) MPs were fabricated with similar diameters. The incorporation of gelatin MPs linearly increased as the MP-to-ESC seed ratio increased, whereas the incorporation of non-adhesive materials (PLGA, agarose) saturated at a ratio of 4:1 MPs-to ESCs (Figure 1A). MP-to-ESC seed ratios of 1:4 for gelatin and 4:1 for PLGA and agarose resulted in an average incorporation of 125 MP/EB and thus, these seeding ratios were selected for further studies to normalize for the number of different MPs incorporated within EBs. The effects of increased MP incorporation, were analyzed using gelatin MPs incorporated at a MP-to-ESC ratio of 2:1 which corresponded to an average incorporation of 800 MP/EB. No differences were observed in the kinetics of EB formation with any of the incorporated MPs.

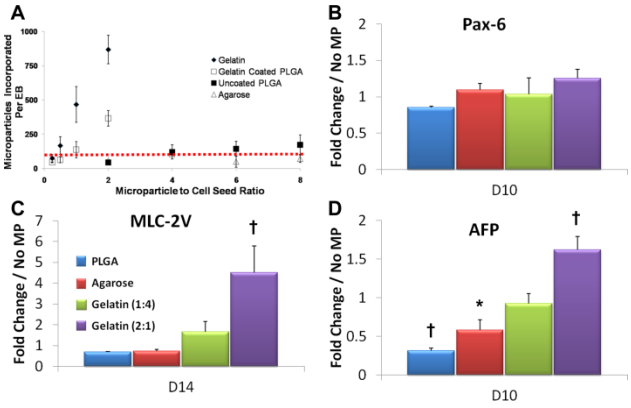

Figure 1. (A) MPs incorporated per EB as a function of MP to Cell seed ratio. (B-D) Gene expression analysis of EB with MPs. Results are normalized to expression of EBs with no MP incorporated. * = $p < .05$, † = $p < .005$ n = 3.

Likewise, EBs from each group were of similar size and internal cell density as determined by histological analysis. Gene expression analysis of alpha-fetoprotein (endoderm), MLC-2V (cardiac mesoderm), and Pax-6 (neuroectoderm) was performed on samples of day 4,7,10 and 14 EBs. Pax-6 expression was not affected by material incorporation and did not increase significantly beyond ESC expression levels for any time point assayed (Figure 1B). MLC-2V expression at day 14 was significantly increased ($p< .005$) in gelatin 2:1 EBs (Figure 1C). AFP expression at day 10 was significantly decreased for EBs with PLGA ($p< .005$) and agarose ($p<.05$) while expression increased in gelatin 2:1 EBs ($p< .005$) (Figure 1D). To further investigate differences observed in mesoderm and endoderm gene expression, whole-mount immunofluorescent staining for alpha-sarcomeric actin and AFP was performed. Alpha-sarcomeric actin staining was observed in large discontinuous areas across gelatin 1:4 EBs but only in small pockets within untreated and agarose EBs and not at all in EBs with PLGA and gelatin 2:1. AFP staining indicated that EBs with gelatin 2-1 had diffuse AFP staining while positive staining was localized to the periphery of EBs for the other materials.

Conclusions: These results indicate that biomaterials incorporated within EBs can modulate differentiation of ESCs as demonstrated differences in gene and protein expression of early germ lineage markers.

References:

1) Carpenedo RL, *Biomaterials* **2009,** 30, (13), 2507-15.
(2) Benoit DS, *Nat Mater* **2008,** 7, (10), 816-23.