Matrix Rigidity-Modulated Cardiovascular Organoid Formation from Embryoid Bodies

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Statement of Purpose: Growing three-dimensional (3D) structures (organoids) anatomically and functionally resembling live tissues for use in drug discovery or organ transplantation has recently attracted a lot of attention. This study demonstrates that simple attachment of an embryoid body (EBs) to substrates with variable mechanical stiffness can significantly modulate cardiomyogenic/endothelial differentiation of embryonic stem cells (ES cells). In particular, matrix with stiffness similar to muscle tissue created a contracting, cardiovascular organoid vascularized by blood vessel-like endothelial lumens.. We believe the results of this study will be very useful for building *in vitro* 3D cell platforms used for both fundamental and applied bioscience studies.

Methods: The collagen gel was prepared by reconstituting bovine type I collagen solution with cold DMEM/F12. The final concentration of collagen was kept constant at 1.4 mg/ml. The resulting collagen gels were incubated at 37C and 5% CO2 for two hours before plating EBs. Separately, to prepare collagen-conjugated polyacrylamide gels with different elastic moduli, 3 mg/ml collagen solution was first incubated with 50 mg/ml poly(ethylene glycol) N-hydroxysuccinimide ester in ratio 10:1 at 4uC for two hours. Then, the collagen bound with acrylate PEG-NHS was mixed with stock solutions of 40% acrylamide and 2% N,N9-methylenebis(acrylamide), and finally with 10% ammonium persulfate (Sigma) and 10% tetramethylethylenediamine (TEMED, Fluka). The molar ratio between 2% N,N9-methylenebis(acrylamide) and acrylamide was varied from 0.0017 to 0.017. The resulting gel was further incubated in phosphate buffered saline (PBS) at room temperature overnight before plating EBs on the gel.

Results: Extended suspension culture of EBs for 23 days (Fig. 1) in the medium without serum led to formation of large areas of necrosis and less differentiated cell morphology, while keeping its original spheroidal shape. The EBs cultured on the gel with an elastic modulus (*E*) of 0.2 kPa and 6 kPa over 23 days also maintained 3D cellular organization in EBs,. In contrast, the EBs cultured on the gel with *E* of 40 kPa disrupted 3D structure and led to formation of a cell monolayer.

After 23 days of cell culture, EBs cultured on the gel with *E* of 6 kPa displayed continued contraction. The percentage of contractile EBs was almost 8-fold higher than that of EBs cultured in a suspended state. According to immunostainig for cardiomyocytes and endothelial cells, the EBc cultured on the gel with *E* of 6 kPa exhibited cardiac muscle-like tissue positively stained for an antibody to sarcomeric alpha actin. More interestingly, the cardiac muscle-like tissue was vascularized by endothelial tubes positively stained for the CD31 antibody (Figure 1). In contrast, the EBs cultured in a suspended state or those on the gel with *E* of 0.2 kPa showed only CD31-positive endothelial tubes. According to the gene expression analysis, genes encoding sarcomeric alpha actinin and cardiac troponin were transcribed at higher levels in EBs cultured on the gel with *E* of 6 kPa. Flow cytometry analysis also corroborated the results of gene expression analysis.

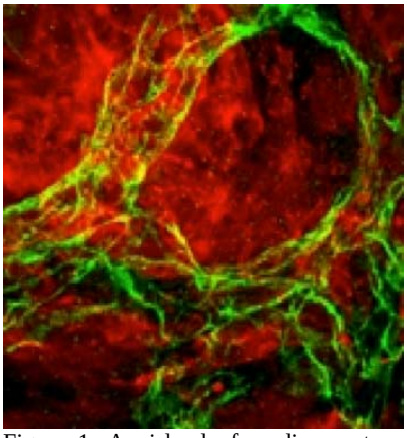

Figure 1. An island of cardiomycytes stained for sarcomeric alpha actinin (red) in close association with endothelium-lined channels stained for CD31 (green)

Further, examination of mitotic activity in cells comprising EBs showed higher share of proliferating cardiomyoblasts on a substrate with intermediate stiffness. The most significant reduction in proliferating cardiomyoblasts between days 15 and 23 occurred on the softest substrate.

Conclusions: Our results demonstrate that substrate stiffness can modulate the cardiomyogenic/endothelial differentiation within EBs and further quality of the resulting cardiovascular organoid. In particular, the hydrogel with stiffness similar to muscle tissue promoted cardiomyogenic differentiation of EBs, likely due to delayed exits from mitosis. It also supported vascularization of cardiac muscle tissue by endothelial tubes, to form vascularized cardiac organoid. Overall, the results of this study will greatly contribute to efforts to grow organ-like structures *in vitro* and development of new cellular assays for drug discovery and toxicity testing.

References: PLOS ONE | www.plosone.org 1 April 2014 | Volume 9 | Issue 4 | e94764