Guided Differentiation of Mouse Embryonic Stem Cells with Protein-Immobilized Beads

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Statement of Purpose: Myocardial infarction (MI) accounts for approximately 1 million deaths per year in the United States.[1] While stem cell delivery strategies have shown promise, cells are not retained at the site of injection.[2] Embryonic stem cells (ESC) are currently being investigated for their potential use in MI repair due to their pluripotency. However, pre-differentiation of ESCs prior to injection *in vivo* is critical to prevent tumor formation.[3] The Sonic hedgehog (Shh) signaling cascade has been shown to drive differentiation of mouse ESCs (mESC) towards mesodermal progenitor fates.[4] Our group has demonstrated that aggregation of mESCs into embryoid bodies (EB) can direct differentiation into cardiovascular cell types.[5] In this study, we presented mESC in EBs with Shh-conjugated dynabeads to direct early mesodermal commitment of the mESCs.

Immunohistochemistry and FACS analysis were used to analyze the concentration of mesodermal progenitors. Our hypothesis is that presentation of Shh on dynabeads to cells within the EB will enhance more uniform differentiation towards mesodermal progenitor cells *in vitro*. This method of Shh presentation will overcome the diffusional limitations associated with incubation of EBs with soluble Shh.

Methods: R1 mESCs (A. Nagy, Toronto, Canada) were expanded in ES Knockout culture medium (Invitrogen) as described previously.[5] Briefly, Mouse Embryonic Fibroblasts (MEFs; ATCC) were cultured to confluence on gelatin-treated flasks and mitotically inactivated with Mitomycin-C (Sigma). MESCs were expanded on the MEF feeder layers for 24-48 hours in the presence of 1000 U/ml recombinant LIF (Millipore) to prevent aggregation into EBs. Prior to differentiation, the mESCs were cultured on gelatin-coated flasks with LIF for 48 hours to remove the MEF.

Shh was purchased with a 6x histidine tag on the N-terminus (R&D Systems). The Shh protein was localized to dynabeads with an antibody against the histidine tag (Invitrogen) at various concentrations (1-10nM). The Shh-conjugated dynabeads were co-cultured with the mESC in Aggrewell plates (Stem Cell Technologies) without LIF supplementation to allow aggregation into EBs (**Figure 1**).

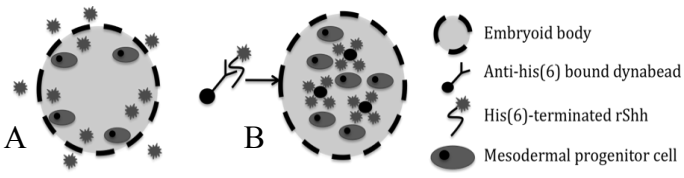

Figure 1: Model of Shh presentation to Embryoid Bodies. (A) Soluble Shh is restricted to the EB periphery. (B) Shh-bound dynabeads increased differentiation of ESC into mesodermal progenitor cells, and distribute uniformly.

Control groups were presented with soluble Shh in the culture medium. EBs were cultured for 72 hours in the Aggrewell plates, then transferred to low-adherence culture plates.

Immunohistochemistry was performed to visualize Shh distribution in EBs. Following 12 days in 2D culture and EB dissociation, expression levels of undifferentiated and differentiated cell markers were quantified by FACS analysis. Flk1 (VEGF-R), a downstream receptor in the Shh pathway, serves as a marker for mesodermal progenitor cells. Yields of Flk1+ cells were compared to those of SSEA-1 and OCT-4, which are present on undifferentiated ESC.

Results and Discussion: The distribution of Shh-immobilized dynabeads was confirmed by immunohistochemistry. Recombinant Shh was uniformly distributed within the core of the EB (**Figure 2**). Soluble Shh was only distributed to the periphery of the EB. This suggests that Shh can be presented to the interior of EBs using dynabeads, increasing the number of cells exposed to the protein.

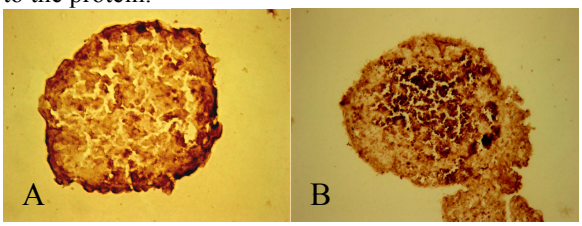

Figure 2: (A) localization of Shh to the outer, endodermal layer of the EB, 20X; (B) localization of Shh-immobilized proteins within the EB, 20X

FACS analysis was used to calculate the number of cells positive for undifferentiated and differentiated markers. By day 12 in 2D culture, there was a relatively high concentration of cells positive for SSEA-1 (9.0%) and OCT-4 (39.5%). Only intermediate expression levels of markers for differentiated cell types (CD31, 4.0% and VE-cadherin, 14.9%) were calculated in comparison. This demonstrates differentiation within EBs is heterogenous, and Shh presentation has the potential to drive differentiation more uniformly.

Conclusions: Shh-immobilized beads support uniform Shh presentation to a higher concentration of cells within the EB compared to soluble Shh. FACS analysis demonstrates the potential of these cells to differentiate into mesodermal progenitor cells. This method for pre-differentiation allows us to yield high concentrations of mesodermal progenitor cells that can be used for *in vivo* repair of MI.

References:

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