Immobilized Sonic Hedgehog Increases Mesodermal Commitment of Mouse Embryonic Stem Cells

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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, which present the surface marker VEGFR-2 (Flk-1).[4,5] Embryoid Body (EB) formation is a common differentiation method; however, heterogenous sizes and cell lineages complicate analysis.[5] We hypothesize that exposing the EBs to Shh-conjugated dynabeads (DBs) will yield more homogenous mesodermal-progenitor populations, and circumvent the diffusional limitations associated with incubation of EBs with soluble Shh.

Methods: R1 mESC (A. Nagy, Toronto Canada) were expanded on 0.1% gelatin treated flasks without feeder cells in ES Knockout medium (Invitrogen) as described previously.[6] R1 between passages 18-25 were used in the experiments.

Shh was purchased with a 6x histidine tag on the N-terminus (R&D Systems). The Shh protein was localized to DBs with an antibody against the histidine tag (Invitrogen). The dynabeads were incubated with the Shh on a rotisserie rotator for 3 hours at 4°C. Binding efficiency was determined by ELISA.

The Shh-conjugated DBs were co-cultured with the mESC in Aggrewell plates (Stem Cell Technologies) without LIF supplementation to allow aggregation into EBs (**Figure 1**).

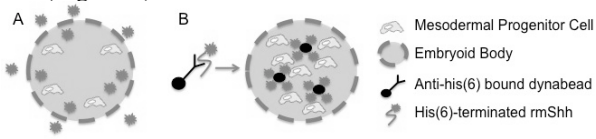

Figure 1: (A) Soluble Shh is restricted to the EB periphery. (B) Shh-bound dynabeads distribute uniformly within the EB interior.

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

Immunohistochemistry was performed to visualize Shh distribution in EBs. EBs were dissociated into single cells in Accumax (Invitrogen) for FACS analysis to compare the percentage of SSEA-1 positive (pluripotent) cells and Flk-1 positive (progenitor) cells. Trypan blue was used to determine the impact of the culture conditions on cell viability.

Results: Shh attachment to DBs was confirmed by ELISA. Approximately 50% of the starting concentration of Shh was detected on the DBs following the 3-hour incubation. Shh release from the DBs was also determined over the course of 7 days. An initial burst release was observed after 1 day, with detectable amounts of Shh still present by day 7 (data not shown). No difference in viability was detected after DB exposure compared to control conditions (data not shown).

Following EB formation in Aggrewell plates, the distribution of Shh confirmed by immunohistochemistry. EBs with Shh-immobilized DBs showed increased Shh in the EB interior (**Figure 2B**). In contrast, soluble Shh was only distributed to the periphery of the EB (**Figure 2A**).

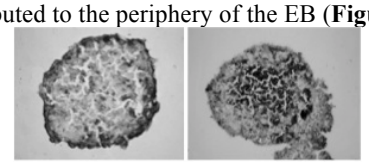

Figure 2: (A) Localization of Shh to outer EB layer, 20X. (B) Localization of Shh-immobilized beads within the EB, 20X.

FACS was performed on day 4 to quantify the percentage of cells that were SSEA-1+ and Flk-1+ following exposure to Shh-immobilized DBs (Shh-DBs). The percentage of Flk-1+ cells increased five-fold over controls (control EB) in larger EBs (1000 R1/EB), while the percentage increased two-fold in smaller EBs (500 R1/EB) (**Figure 3**).

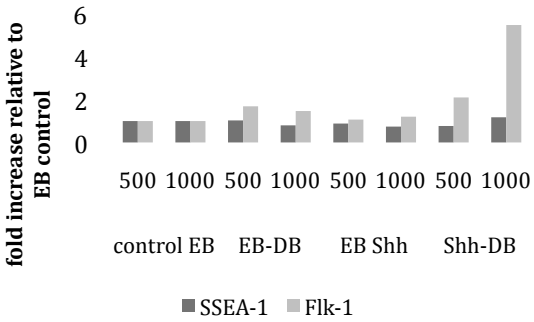

Figure 3: Percentage of SSEA-1+ and Flk-1+ cells on Day 4. Control EB: EBs cultured without DBs or Shh. EB-DB: EBs cultured with unloaded DBs. EB Shh: EBs exposed to soluble Shh. DB-Shh: EBs cultured with Shh-loaded DBs. EBs were prepared at 500 R1/EB (500) or 1000 R1/EB (1000).

Conclusions: Exposure of EBs to immobilized Shh increases the percentage of mesodermal progenitor cells compared to soluble delivery alone. Future studies will examine the effect of Shh-conjugated DBs on the expression of ectodermal and endodermal markers. The impact of this method on cardiomyocyte generation will also be examined.

References:

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