THE ROLE OF GRAVITY MECHANOTRANSDUCTION IN REGULATING STEM CELL TISSUE REGENERATIVE POTENTIAL AT THE SINGLE CELL EXPRESSOME LEVEL

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Background and Experimental Rationale

- Gravity is an omnipresent force on Earth, and all living organisms have evolved under the influence of constant gravity.
- Mechanical forces generated by gravity are potent modulators of stem cell based tissue regenerative mechanisms, inducing cell fate decisions and tissue specific commitment.

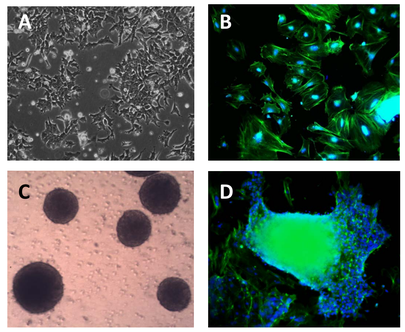


Figure 1. Embryonic Stem Cells (panels A and B) are a fundamental proliferative stem cell model for study of development and regeneration research. Embryonic Stem Cell derived Embryoid Bodies (panels C and D) serve as a model of complex differentiation pathways, recapitulating a normal embryo development in utero.

Fluorescent staining of cells. Blue – DAPI, nucleus; green – actin, cytoskeleton. (A) ESCs imaged using phase contrast at 20X and (B) ESCs florescent labeled at 40X. (C) Brightfield EBs at 4X and (D) EBs florescent labeled at 10X.

- A novel mechanical unloading investigation assessed the formation, morphology, and gene expression of embryoid bodies (EB), a transitory cell model of early differentiation. After 15 days of spaceflight, the mechanotransduction-null EB cells showed upregulated proliferative mechanisms while differentiation cues were silenced (Fig. 2).

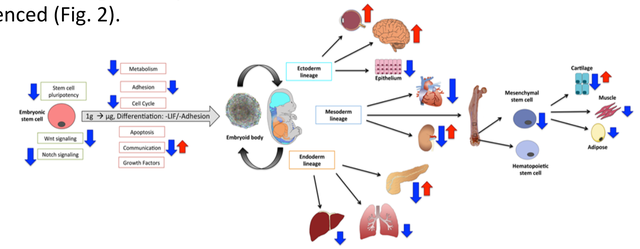


Figure 2. Mouse embryonic stem cells differentiated into embryoid bodies (EBs) and exposed to spaceflight for 15 days, exhibited significant down-regulation of multiple terminal lineage markers. Specifically, over 90% of genes expressed in ground controls related to differentiation of terminal lineages indicating that microgravity suppresses stem cell differentiation and regeneration capacity [1]

Hypothesis

Gravity mechanotransduction regulates stem cell tissue regenerative processes by modulating stem cell proliferation and differentiation fates at specific cell cycle stages

Methods

- Clonally-derived ESCs were plated on a collagen matrix and expanded for 36 hours.
- EBs were aggregated from ESCs. After formation, the EBs were transferred to a collagen matrix culture dish and given 4 days to allow implantation and outgrowth.
- Both cultures were then subjected to either a 60 min 50g centrifugal pulse of gravity mechanotransduction, or no stimulation.
- Six hours post-stimulation, we used a 10X Genomics Chromium/Single Cell controller to generate bar-coded single cell Illumina libraries and sequenced ESC and EB expressome libraries for 5,000 1g control cells, and 5,000 cells pulse-stimulated with 50g, simulating running/jumping bone marrow hydrostatic pressures

Results – Mechanoregulation

- Cluster distribution of the individual libraries generated by single cell sequencing illustrate heterogeneity and subpopulations within the cultured cells (Fig. 3).
- Clusters are generated by the graph-based hierarchical method.
- Clustering identifies like cell by expression profiling
- Our cursory assessment identified cluster specific cells positive for Nanog, SCF, Oct4, Sox2 and PCNA within the ESC and EB cultures.

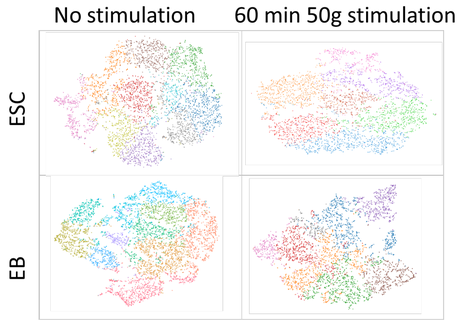


Figure 3. t-SNE visualization of individual mESCs and EBs to identify subpopulations. Colors denote corresponding clusters..

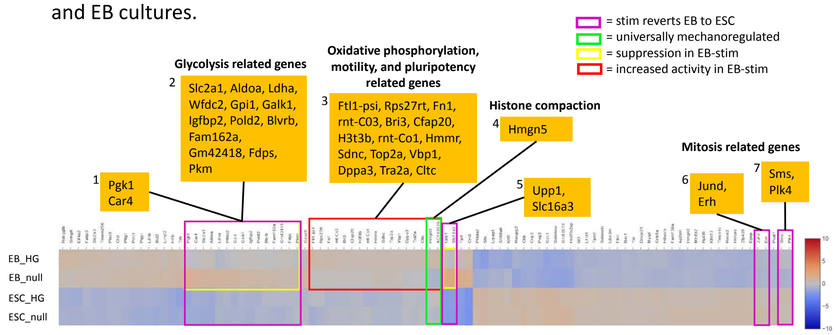


Figure 4. Heatmap of most significantly up- and don-regulated genes between the 4 conditions.

- The segregation of expression profiles is most evident between the ESC culture and EB cultures. Comparatively, expression changes due to mechanical regulation are less impactful; however, EBs demonstrate significant mechanotransduction in genes related to cell motility, ECM development and proliferation related chromosome condensation.
- Interestingly, mechanical stimulation of the early differentiation EBs depresses several genes back toward ESC levels (Fig 4 box 2). The majority of these genes are related to proliferative regulation and glycolysis metabolic regulation.

- Mechanical stimulation in the EBs causes suppression, compared to unstimulated EBs, of genes associated with glycolysis (Slc2a1, Aloda, Ldha, Gpi1, and Pkm) while simultaneously increasing the expression of genes associated with mitochondrial oxidative phosphorylation and the electron transport chain (mt-Co3, mt-Co1, and Sdhc).

Pgk1 – glycolysis enzyme
Mechanoregulated suppression in EB cells

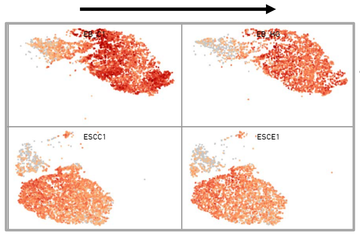
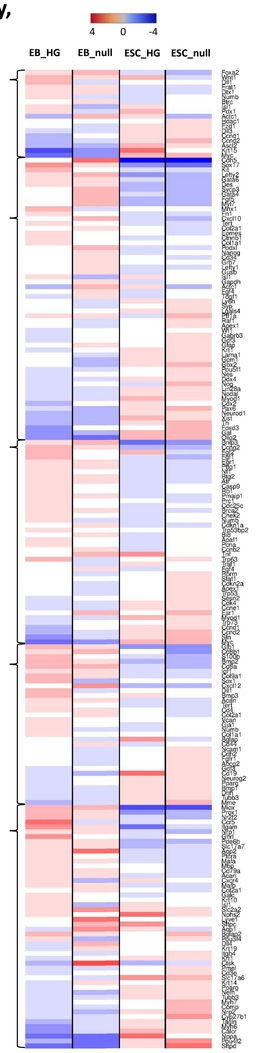


Figure 5. Pgk1 population relative expression for all 4 groups .

- Interesting for many of the same genes the opposite regulation is seen for the mechanostimulated ESCs compared to unstimulated ESCs (Fig.4inserts 1, 5, 6, and 7).
- Pgk1, a gene responsible for conversion of glucose to pyruvate upon entering cellular respiration, has elevated population expression in the early differentiation EBs compared to the ESCs. Mechanical stimulation depressed Pgk1 in the EB cells.

Results – Aggregated Libraries
Expression of key genes associated with pluripotency, lineage commitment, and cell cycle

Heatmap of genes associated with Stem Cell Pluripotency, Lineage Differentiation, and the Cell Cycle



Stem cell specific and cell signaling markers

Stem cell and related signaling markers are inversely expressed after commitment to early differentiation (ESC -> EB). Mechanical loading regulates several genes involved in signaling induction of embryogenesis folding (Wnt1) and differentiation (Foxa2, Actc1, Ccnd2, krt15).

Fig. 5. Lefty2 expression profile for all 4 conditions.

Marker of Pluripotency

- Pluripotency is reduced with commitment to early differentiation (ESC->EB).
- Mechanical stimulation in EB depresses genes associated with exit from the pluripotent cell state; returning gene expression toward those of undifferentiated ESCs.

p53 and cell cycle regulation markers

- Cell cycle inhibitors (TRP53, CDKN1a, CDKN2a) are suppressed by mechanical stimulation. This suppression lessens mitotic restrictions and encourages proliferation.

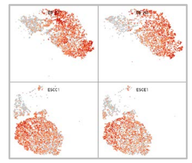


Fig. 6. TRP53 expression

Early lineage commitment

- Mechanical stimulation only encourages increased expression of early lineage commitment markers in the EB cells. Indicating that mechanical stimulation can only influence differentiation commitment after transition from totipotency.

Figure 7. Myc expression.

Terminal differentiation markers

Mechanical stimulation in differentiation committed cells upregulates terminal markers. However, single cell sequencing reveals that with mechanical stimulation a sub-population of EB cells returns to expression profiles representative of a earlier pluripotent state.

Conclusion

Single-cell RNA-seq technologies are revolutionizing the field of molecular biology, providing the capacity to go beyond global gradation analysis of gene activity to single cell expression within a population.

Our results suggest that gravity mechanostimulation elicits increased cell proliferation and differentiation, and that these effects at the single cell expressome level are most notable on a specific cell-cycle clusters.

1.Blaber, Elizabeth A et al. "Microgravity Reduces the Differentiation and Regenerative Potential of Embryonic Stem Cells" *Stem cells and development* vol. 24,22 (2015): 2605-21.