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Differential Single Cell Responses of Embryonic Stem Cells Versus Embryoid Bodies to Gravity Mechanostimulation

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The forces generated by gravity have shaped life on Earth and impact gene expression and morphogenesis during early development. Conversely, disuse on Earth or during spaceflight, reduces normal mechanical loading of organisms, resulting in altered cell and tissue function. Although gravity mechanical loading in adult mammals is known to promote increased cell proliferation and differentiation, little is known about how distinct cell types respond to gravity mechanostimulation during early development. In this study we sought to understand, with single cell RNA-sequencing resolution, how a 60-min pulse of 50 *g* hypergravity (HG)/5 kPa hydrostatic pressure, influences transcriptomic regulation of developmental processes in the embryoid body (EB) model. Our study included both day-9 EBs and progenitor mouse embryonic stem cells (ESCs) with or without the HG pulse. Single cell t-distributed stochastic neighbor mapping shows limited transcriptome shifts in response to the HG pulse in either ESCs or EBs; this pulse however, induces greater positional shifts in EB mapping compared to ESCs, indicating the influence of mechanotransduction is more pronounced in later states of cell commitment within the developmental program. More specifically, HG resulted in upregulation of self-renewal and angiogenesis genes in ESCs, while in EBs, HG loading was associated with upregulation of Gene Ontology-pathways for multicellular development, mechanical signal transduction, and DNA damage repair. Cluster transcriptome analysis of the EBs show HG promotes maintenance of transitory cell phenotypes in early development; including EB cluster co-expression of markers for progenitor, post-implant epiblast, and primitive endoderm phenotypes with HG pulse but expression exclusivity in the non-pulsed clusters. Pseudotime analysis identified three branching cell types susceptible to HG induction of cell fate decisions. In totality, this study provides novel evidence that ESC maintenance and EB development can be regulated by gravity mechanostimulation and that stem cells committed to a differentiation program are more sensitive to gravity-induced changes to their transcriptome.

Keywords: mechanotransduction, embryoid bodies, embryonic stem cells, single cell RNA sequencing, development

Introduction

Differentiation, stem cell fate decisions and developmental patterning are regulated by a variety of spatiotemporal influences. The mechanical environment, defined by unit gravity on Earth, is one such influencer, modulating cell–cell and cell–matrix interactions via physical strain sensing through integrin and cadherin adhesion receptor signaling. These physical strains are then converted into cell signaling and transcriptional responses by a process termed mechanotransduction [1–3]. In a whole organism the tissue mechanical environment can be regulated by intrinsic factors such as blood pressure, muscle contraction, and others, or by extrinsic factors such as gravity weight-bearing and posture. Also, of importance are the cyclic nature of these normal loading and unloading processes.

On Earth, mammalian tissues normal hydrostatic pressures due to vascular activity can vary in the range of

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0.2–16 kPa, while pressures can go as high as 5–6 MPa in weight-bearing bone and cartilage and to 18 MPa while running or jumping [4]. However, the calculated hydrostatic pressure in a standard static mammalian laboratory cell culture (with a fluid medium with density of 1 kg/m^3 and height of 1 cm, at 1 *g*) is only about 0.1 kPa, well below normal tissue physiological mechanical loading conditions.

To simulate physiologically relevant ranges of mechanical loads in cell cultures, various strategies have been used including applying cyclic stretch [5], or pulses of hydrostatic pressure [6], and hypergravity (HG) [7], that often result in enhanced cellular proliferation and differentiation effects. In the unique environment of space, free fall-induced weightlessness can modulate the intrinsic factors of tissue mechanical environment leading to reduced mechanical loads in tissue niche environments [8]. Modeling the effects of microgravity on Earth has been attempted with a multitude of strategies; however, many of these strategies in an effort to replicate free fall, generate non-physiologic fluid shear and rotational loading to the cells [9]. The interesting observation that traditional static cell culture plating results in pressure loading well below the physiologically relevant range may provide a unique path for cellular level modeling of the microgravity free fall environment.

Embryonic stem cells (ESCs) are primary stem cells that can be turned toward specific fates by exclusive modulation of mechanical environment [10,11]. However, these methods often induce ESC differentiation to a single cell type, removing the cultures from physiologic relevance. Embryoid bodies (EBs) are a three-dimensional (3D) model of embryo development inclusive of all pre-implant cell lineages and germ layers within 7–12 days, providing a physiologically relevant platform for regenerative medicine and developmental studies [12,13]. Investigations of 3D EB models exposed to mechanical perturbation are rare, and rarer still are transcriptomic characterization of mechanotransduction regulation of development.

The most studied physical stress relevant to the EB model is fluid shear stress [14], imparted by spinner flasks [15] or microfluidic channels [16,17], or rotating wall vessels [9] used in their culture. Generally, these studies have shown that fluidic shear stress promotes growth and maturation; however, this general observation has been refined as the study of mechanical regulation of EBs has expanded. A 2010 study presented data that controlling the speed of hydrodynamic stimulation changes viability of the cells within the spheroid [18]. More direct stretch mechanical loading increases angiogenesis within the EB and increases the localization of beating cardiomyocyte foci [19]. Dynamic cyclic stretch to EBs encourages differentiation toward mesodermal lineages with the resulting terminal cell type selectable by manipulating the compressive and tensile strains [20].

As positive mechanical loading corresponds to increased differentiation commitment, reduced mechanical strain can be hypothesized to result in the maintenance of embryonic potency markers. Hanging drop and suspension EB cultures found increased apoptotic cell accumulations and irregular morphology in comparison to rotary fluid shear EB culture [21], although these may be explained by decreased mass transport around and inside EB. EBs space flown in low earth orbit for 15 days under microgravity conditions retained self-renewal markers and showed inhibition of ESC differentiation and germ layer lineage markers, however, upon return to earth-normal 1 g loading, the EBs demonstrated increased ability to readily differentiate into beating cardiomyocytes [22,23]. These results mirror findings that EBs derived from ESCs exposed to fluidic shear stress before aggregation are primed for differentiation, resulting in increased endothelial markers and spatial organization [24].

Mechanically regulated engineerability of developmental models has application in tissue engineering and regenerative medicine as self-organization and patterning are necessary for functional repair or replacement of tissue deficits. An intermediate step for development of many organoid models is induced pluripotent stem cell aggregation into EBs before additional chemical treatment [25], thus transcriptomic information from the EB stage could help improve fidelity of organoids and the research for which they are utilized. However, lack of a thorough understanding of transcriptomic regulation within difficult-to-access EB spheroids has hindered the development of the field.

Novel single cell transcriptomic approaches are beginning to bridge this deficit with recent work describing preimplantation embryos and in vitro EBs [26], germ layer developmental trajectory [27,28], and organoid efficacy assessment [29,30]. The use of single cell resolution has enabled detection of transient cell populations existing between established embryo staging, providing further insight into developmental programs, lineage decision omics and has exposed potential new cell classifications as targets for directed differentiation [27,28]. Our investigation utilizes single cell transcriptomics technology to probe how the absence of mechanical perturbation, similar to microgravity unloading, alters developmental programing in the EB model compared to a single pulse of physiologically relevant loading by HG.

Materials and Methods

EB culture and gravity mechanostimulation

Clonal C57BL/6J ESCs (ATCC SCRC-1002) were seeded at 3E6 cells in a 10 cm^2 tissue culture dish coated with 0.1% gelatin (Fig. 1A). The mESCs were expanded for two passages in mESC medium (Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum, 4 mM *L*-glutamine, 1 × nonessential amino acids, 1 mM sodium pyruvate, 1% antibiotic solution [penicillin/streptomycin], trace beta-mercaptoethanol, and 10 ng/mL leukemia inhibitory factor [LIF]). The medium was changed daily and cells were passaged every 48 h using 0.25% trypsin solution and re-seeded at 3E6 cells per dish before reaching experimental maturity (Fig. 1A, C). After the second expansion, a subset of the mESCs (6E6 cells per dish) were transferred to an ultra-low adhesion 10 cm^2 tissue culture dishes and LIF removed from the culture media to allow cells to aggregate and form EBs (Fig. 1B). Half of the media was changed daily and cell aggregation was inspected using a Leica DMi1 inverted light microscope.

The EBs were cultured for 5-days before further processing. On the fifth day, EBs were seeded onto 0.1% (w/v) collagen coated platinum-treated silicon membranes and allowed to adhere and outgrow for 4 days (Fig. 1D). After the 4 days, the EB cultures and control mESC cultures were exposed to a 1-h HG pulse of 50 *g* in a cell culture 1 ft-diameter centrifuge. The acceleration level of 50 *g* used in this study with a 1 cm medium column above adherent cells

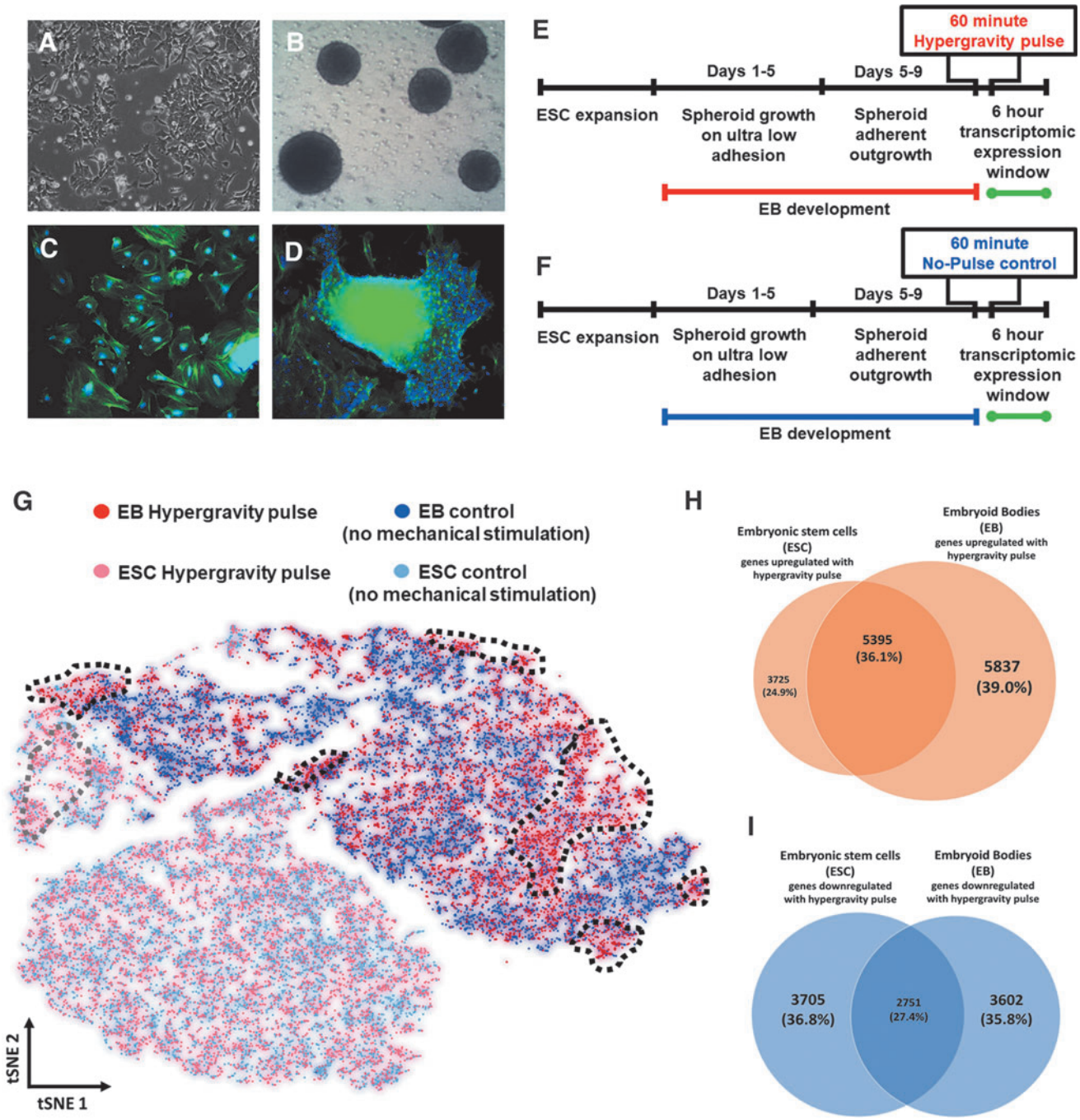


FIG. 1. Sixty-minutes 50 *g*/5 kPa hydrostatic pressure loading pulse induces limited transcriptomic shift in ESC or EB model. Brightfield images of cultured mouse ESCs **(A)** and non-adherent day-5 EBs **(B)** show morphologic differences from the actin cytoskeleton (*green*) and DAPI nuclear (*blue*) florescent images of post-HG pulse ESCs **(C)** and day-9 adherent EBs **(D)**. Experimental design for EB development and 60-min HG loading **(E)** and no-pulse control **(F)**. **(G)** tSNE mapping show the HG pulse cause limited positional shifts for either the ESC or EB cells. However, the HG-EBs do show islets (positional shifts indicated by dashed encirclement) indicating mechanotransduction is more pronounced in later developmental states of cell commitment. 36.1% of all upregulated genes **(H)** and 27.4% of downregulated genes **(I)** are shared between ESCs and EBs. DAPI; EB, embryoid body; ESCs, embryonic stem cells; HG, hypergravity; tSNE, t-distributed stochastic neighbor.

results in a calculated hydrostatic pressure of about 5 kPa corresponding to a mid-range physiological value for mammalian non-load bearing tissues ($P = \rho gh$, where ρ is the density of the medium in kilogram per cubic meter, g is gravity acceleration in meter per second squared, and h is the depth of the liquid in meter). Centrifugation was performed using a custom modified (vented lid) Eppendorf 5804 1 ft diameter rotor centrifuge with multi-well plate adapters inside an environmental chamber with 5% carbon dioxide, 37°C, and 90% humidity. After mechanostimulation, the cultures were continued for a further 6 h, allowing for transcriptomic response under static 1 *g* conditions.

Single cell library preparation, sequencing, analysis, and visualization

The EB and ESC cultures were dissociated into single cell suspension and prepared for 10×Genomics RNA sequencing according to the Gene Expression Profiling v2 chemistry protocol (10×Genomics). The four conditions evaluated were mESC control and HG mechanical pulse stimulated and EB control and HG mechanical pulse stimulated (respectively ESC_C, ESC_HG, EB_C, EB_HG). After Illumina sequencing, the samples were demultiplexed and analyzed using 10×Genomics CellRanger software v4.0 to produce barcode, gene expression, and library identification matrices. These datasets were then assessed for clustering by Seurat and for developmental trajectory by Monocle 2 and 3 to produce our data visualizations in t-distributed stochastic neighbor (tSNE), uniform manifold approximation, and projection (UMAP) statistical and Pseudotime relationship mapping methods. Additional details in Supplemental Methodologies (Supplementary Data).

Results

1/3 of mechanoregulated genes are shared between ESCs and day 9 EBs

Single cell tSNE mapping of ESCs and EBs show that a 60-min HG pulse results in minimal deviation in cellular transcriptomic signature (Fig. 1G). Cell mapping shows that most ESC cells share general overlap between the HG pulse (red) and unstimulated controls (blue); However, HG pulse EB cells have areas in the tSNE space largely populated by HG pulse cells with minimal EB non-pulse control cells (Fig. 1G—area largely populated by HG pulse cells indicated by dashed encirclement). Assessing mechano-induced (Fig. 1J) or -suppressed (Fig. 1K) differentially expressed (DE) genes shared between ESCs and EBs show only ~1/3 DE genes have common mechanoregulation. In response to HG pulse EBs show more upregulated genes than ESCs while ESCs and EBs have similar numbers of downregulated genes but fewer are shared.

Gene ontology pathway analysis demonstrates HG pulse correlates differentiation and developmental programs to increased mechanotransduction signaling pathways

When single cell expression information was assessed by Gene Ontology (GO) Consensus Pathway Analysis (CPA) [31] the DE genes were found to be related to developmental pathways, mechanotransduction, and genomic stress/repair mechanisms. Figure 2 presents the GO-CPA mapping and expression heatmaps for the highest DE genes from each pathway. The pathways presented are highly connected, indicating organized response to mechanical loading (Fig. 2A). HG pulse EBs DE genes show organized upregulation compared to no-pulse EBs expression while the HG pulse ESC cells show upregulation of many genes within the pathways but many also have no expression change, indicating cellular response to loading but not organized change in the population transcriptome (Fig. 2B–D). Genes associated with angiogenesis are the exception as ESCs cultured under HG pulse show organized upregulation of genes while the EBs do not.

HG pulse increases EB growth markers in extra-embryonic tissue cell types and pre- and post-implant epiblasts

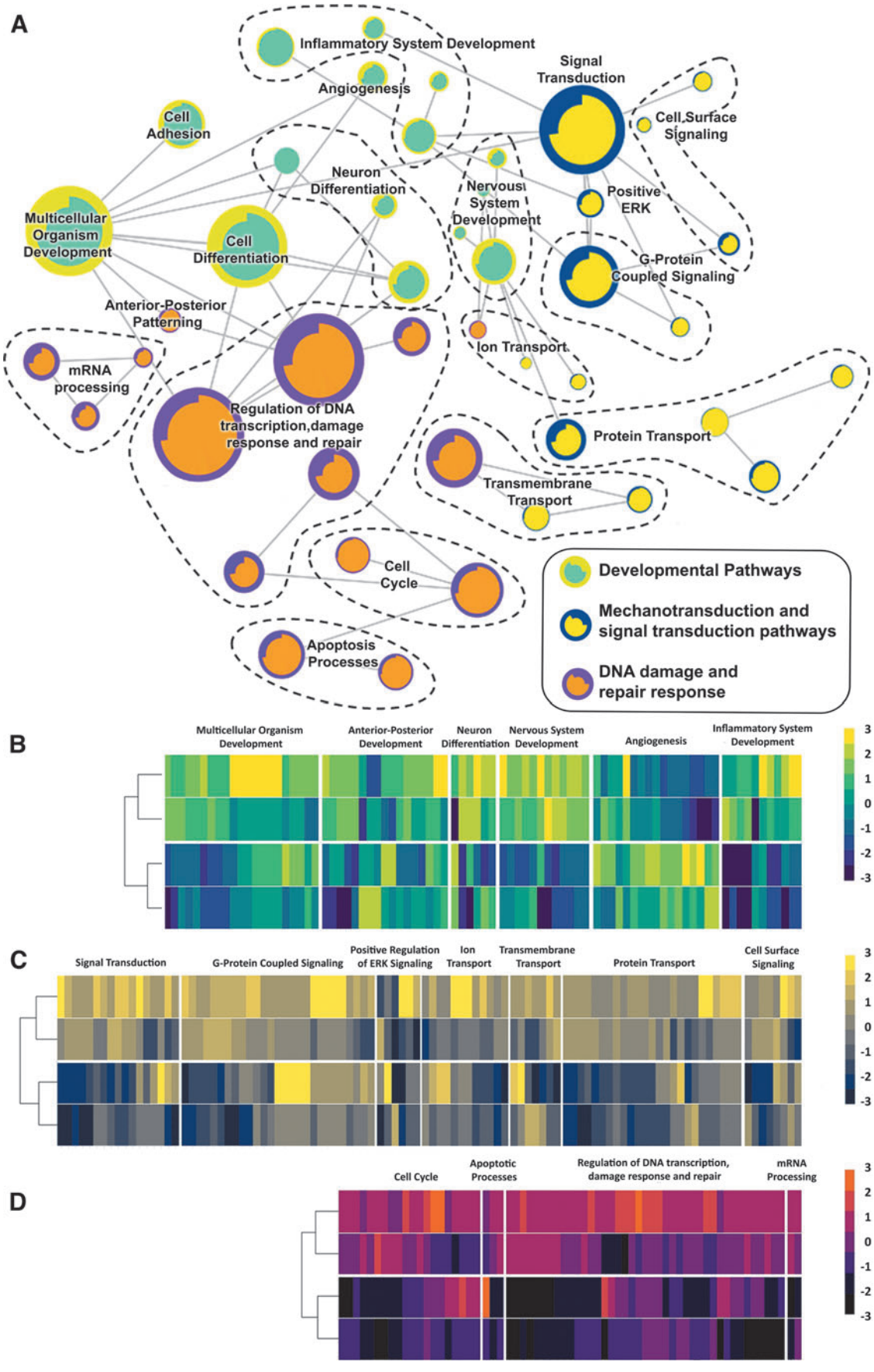
EB UMAP clustering shows little deviation in mathematical clustering due to HG pulse stimulation (Fig. 3A). Nine clusters are identified in both libraries with minimal mapping divergences. ESCs, pre- and post-implantation lineage, primitive endoderm, germ cells, and extra-embryonic cell general positions are identified within the UMAP space. Within the UMAP reference frame, the application of HG pulse changes the location of the extra-embryonic cells and cluster 9 is suppressed (indicated by grey boxes). Extra-embryonic cells are responsible for regulating implantation and development of the amnion and placental tissue, which are the first adherent cells affected by direct mechanical stimulation and the gene markers for the trophoblasts (cluster 1 markers include *Efna1*, *Dab2*, *Pparg*, and *Krt18*) and extraembryonic ectoderm (cluster 9—markers include *Tfap2a*, *Hand1*, *Cdx2*, *Krt7*, and *Wnt6*) have increased expression with HG pulse.

Figure 3B visualizes representative marker genes of potency, ESC, primitive endoderm, pre- and post-implantation epiblast lineages. Cluster-specific assessment show high expression of the potency marker *Oct4/Pou5f1* in all EB clusters irrespective of loading condition; However, clusters 5–9 show deviation in the level and normality of the *Oct4* expression with HG pulse. Clusters 5–7 deviate toward greater expression with HG pulse, suggesting maintenance of potency features within these clusters. Clusters 8 and 9 have reduced expression, suggestive of stem/progenitor cell commitment to specialization.

HG pulse increases transcriptomic signature of transitory cells

ESC marker *Esrrb* is expressed in clusters 3 and 4 independent of HG pulse while clusters 5 and 7 express *Esrrb* exclusively under conditions of HG pulse. These data suggest maintenance of ''stemness'' transcriptomic qualities in cells of clusters 5 and 7 in response to HG pulse. Mapping

FIG. 2. Pathway analysis shows HG pulse induces organized upregulation of multicellular organism developmental, mechanotransduction, and DNA stress/repair pathways. **(A)** CPA results show three main GO pathways differentially regulated by hypergravity pulse stimulation in developmental model: *green-blue* coloring labels developmental pathways; *navy-yellow* coloring labels mechanotransduction and signal transduction pathways; and *magenta-orange* coloring label DNA damage and repair mechanism pathways (see legend). **(B–D)** Expression mapping for key genes in representative nodes of each of the three key regulatory processes upregulated by mechanical stimulation in the developmental model. **(B)** Genes key to developmental pathways, **(C)** genes key to mechanotransduction and signal transduction, and **(D)** are genes key to DNA damage and repair mechanisms. Results demonstrate that HG stimulation induces upregulation of many pathway genes in both EB and ESC models. However, the EBs respond with the greatest number of upregulated genes per pathway and greatest upregulation of individual genes in response to hypergravity pulse. CPA, consensus pathway analysis; GO, gene ontology.


A
Inflammatory System Development
Angiogenesis
Signal Transduction
Cell Surface Signaling
Cell Adhesion
Neuron Differentiation
Nervous System Development
Positive ERK
Multicellular Organism Development
Cell Differentiation
G-Protein Coupled Signaling
Anterior-Posterior Patterning
Ion Transport
mRNA processing
Regulation of DNA transcription,damage response and repair
Protein Transport
Transmembrane Transport
Cell Cycle
Apoptosis Processes
Developmental Pathways
Mechanotransduction and signal transduction pathways
DNA damage and repair response
B
Multicellular Organism Development
Anterior-Posterior Development
Neuron Differentiation
Nervous System Development
Angiogenesis
Inflammatory System Development
3
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C
Signal Transduction
G-Protein Coupled Signaling
Positive Regulation of ERK Signaling
Ion Transport
Transmembrane Transport
Protein Transport
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Regulation of DNA transcription, damage response and repair
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and expression of Pre-Implant epiblast marker *Tfap2c* show expression in no-pulse control EB cells of clusters 4, 5, and 8. Co-expression of ESC marker *Esrrb* and Pre-Epiblast marker *Tfap2c* in cluster 4 indicate a transitory phenotype between ESC and Pre-Implant epiblast. The application of HG pulse induces co-expression of *Esrrb* and *Tfap2c* in clusters 5 and 7. This transcriptomic shift shows that HG pulse increases cells with ESC/Pre-Implant Epiblast transitory phenotype. In the non-pulse control EB cells clusters 5 and 8 express *Tfap2c* without corresponding ESC marker expression, defining these cells as non-transitory Epiblasts.

Post-implant epiblast marker *Dnmt3b* shows co-expression with pre-implant epiblast clusters 5 and 8. HG pulse results in additional co-expression of *Dnmt3b* and *Tfap2c* in cluster 7 indicating increased pre- to post-implant epiblast transitory cells. *Dnmt3b* is expressed in clusters 2, 3, 5–8 of both libraries, implying the majority of cells at day 9 EB culture are post-implant epiblast transcriptionally identified cells, which is consistent with published literature [13,26]. *Dmnt3b* is expressed in cluster 9 of the no-pulse control EB cells but not the HG pulse cluster 9 cells, linking post-implant epiblasts to the extra-embryonic tissue and suggesting a branch point in the developmental program that is hidden in the single cell trajectory, by mechanism of silencing or acceleration through the developmental program by the HG pulse.

Primitive endoderm marker *Npl* is present in cluster 6 of the no-pulse control cells and cluster 8 of the HG pulse EBs. The HG pulse cluster 8 identified primitive endoderm cells that show co-expression of pre- and post-implant epiblast markers. Location within the UMAP coordinate system demonstrates that HG pulse reduces the transcriptomic separation of pre-implant and primitive endoderm cells (distance indicated by dashed arrows in Fig. 3). These data show that early commitment lineages are regulated by the mechanical environment, and that specifically HG pulse stimulation causes transition of cells through the early developmental program.

HG pulse introduces unique cell signatures but does not cause divergence from developmental trajectory in germ layer lineages

To more closely examine how HG pulse influences cell transitory transcriptomic signature, Monocle 3 mapping assigned pseudotime scale to the non-pulse control EB UMAP and the HG pulse EB UMAP (Fig. 4A). The pseudotime mapping shows that the developmental tracing for the EB libraries is similar with HG pulse advancing pseudotime distance between the ESCs (cluster 3 cells identified as the start of the trajectory) and the terminal cells (ectoderm lineage). Monocle trajectory mapping was done to more directly identify developmental relationship of cells within the UMAP space (Fig. 4B). The developmental trajectory identifies several branch points unique to the HG pulse EB cells (Fig. 4C, grey boxes).

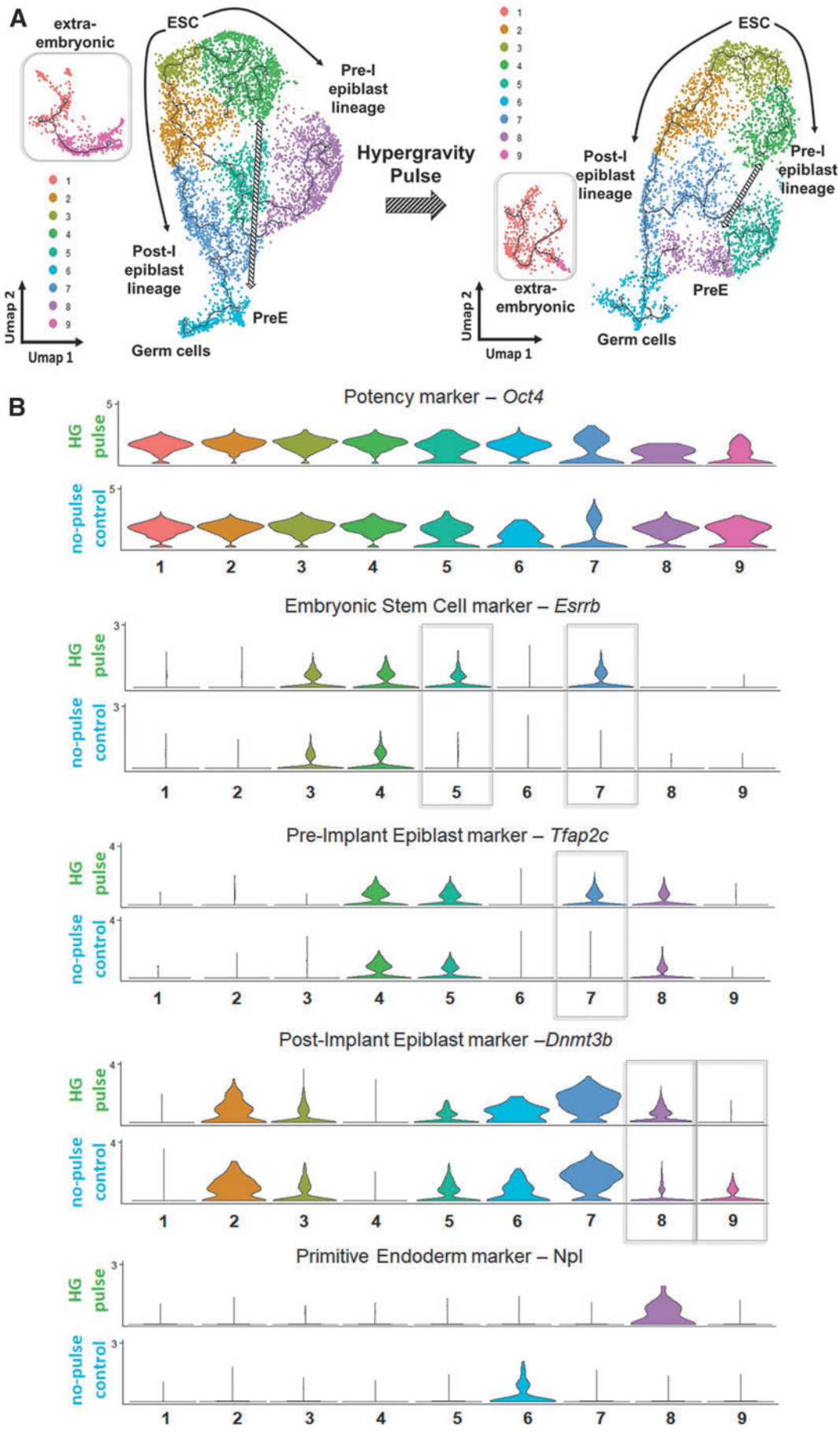
Programmatically the first branch of cells to diverge from the main trajectory pathway, labeled as branch point 5 in the HG pulse trajectory map, was identified as senescent ESC (senESC) cell type due to high expression of ESC markers *Oct4/Pou5f1*, *Esrrb*, *Zfp42*, and *Tcf15* in addition to higher expression of senescence marker *Cdkn2a*/P16. Branch point 1 cells present transcriptional signatures related to cephalocaudal folding (Ceph-Caud), showing co-expression of genes *Atp4a*, *Foxj1*, *Nkx2*, *Ctnnb1*, and *Wnt*s, descriptive of foregut formation. At branch point 3, cells are transcriptomically identified as Primitive Streak (PS), which are transitory cells with transcriptomic signature similar to post-implantation epiblasts (*Dnmt3b*, *Kif1a*, *Fgf5*), markers consistent with developmental signaling (*Wnt*s, *Ctnnb1*, *Fgf8*, *T*, *Psmb8*) and the of the three germ layer lineages (*Spink1*, *Apob*, *Postn*, *Sox11*, *Pdgfc*). These transcriptomically identified cells are positioned along the trajectory before the post-implant epiblast and endoderm branch point, again supporting that HG pulse encourages maintenance of diversity of transcriptional states by retention of cell markers associated with transitory stages.

Discussion

Investigations of mechanical unloading during spaceflight in microgravity have demonstrated the maintenance of ''stemness'' in ESCs, developmental EB spheroids, and lineage specific stem cells [22,23,32,33]. In contrast positive dynamic loading studies have shown that mechanical stimulation induces upregulation of proliferative and specialization gene pathways via independent molecular mechanisms [5]. Our study demonstrated that a 60-min HG pulse in day 9 EBs results in limited divergence in cell type assignment and transcriptomic signature from traditional static culture EBs. Embryo development studies varying gravity environment in murine models have shown that stage of gestation when the gravity disturbance is applied is critical. NASA-NIH.R1, NASA-NIH.R2 studies assessed rat breeding pairs on-orbit finding that on-orbit breeding resulted in no viable pups; However, there were signs of early stage pregnancies in a small portion of the females [34].

Conversely, studies of 2 *g* HG during gestation did not interfere with mating or gestation [35] but 3 *g* resulted in no viable pupping. Another study conducted by Schenker and Forkheim, on early pregnancy mice found that spaceflight exposure resulted in failed gestation of pre-implantation

FIG. 3. HG pulse elicits greater expression of developmental markers but reduces cluster expression exclusivity in favor of more transitory cell phenotype. **(A)** UMAP clustering identifies nine clusters for both HG pulse and no-pulse control EBs. Two clusters identify as extra-embryonic tissues (*gray boxes*) and seven clusters identify along the three germ layer developmental program. General cell type locals are indicated for progenitor ESCs, pre- and post-implant epiblasts (pre-I and post-I epiblast respectively), PrE, and germ cell transcriptomic signatures. HG pulse reduces the separation of pre-implant epiblasts and primitive endoderm cells (indicated by *hashed arrow*). **(B)** Cluster-specific expression of developmental cell type markers show HG pulse increases occurrence of multiple cell type marker expression within a cluster, indicating more transitory phenotypes with HG pulse loading. PrE, primitive endoderm; UMAP, uniform manifold approximation and projection.


A
extra-embryonic
ESC
Pre-I epiblast lineage
Post-I epiblast lineage
PreE
Germ cells
Hypergravity Pulse
Umap 2
Umap 1
B
Potency marker – Oct4
Embryonic Stem Cell marker – Esrrb
Pre-Implant Epiblast marker – Tfap2c
Post-Implant Epiblast marker –Dnmt3b
Primitive Endoderm marker – Npl
HG pulse
no-pulse control

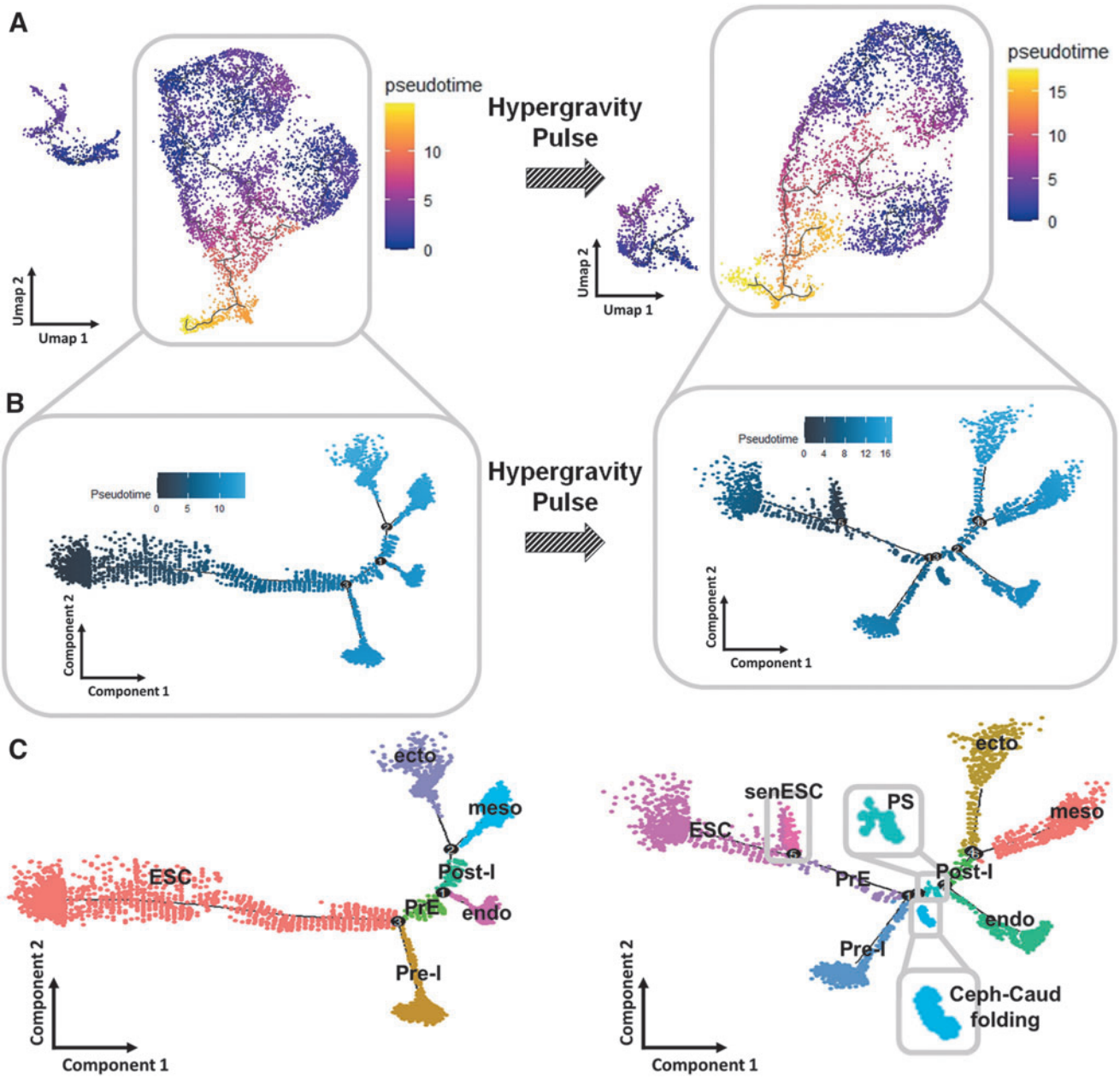


FIG. 4. Developmental trajectory of EBs stimulated with HG show more commitment to lineage differentiation and unique branching points responsible for cell fate decisions. **(A)** UMAP dimension colored for pseudotime scale show HG pulse advances cell commitment to developmental differentiation. **(B)** EB HG pulse and non-pulse cells mapped in the pseudotime coordinate system show the developmental trajectory of the non-extra-embryonic cells (*gray boxes* indicate cell carried into developmental trajectory analysis). **(C)** Phenotype identified cellular staging for progenitor ESCs (ESC), pre- and post-implant epiblasts (Pre-I and post-I respectively), PrE, and the Endo, Meso, and Ecto germ layers. Unique branching points for populations of senESC, cephalocaudal folding cells (Ceph-Caud), and PS cells. These phenotypes could not be segregated in the non-pulse control EBs; however, these are likely still present as the developmental trajectory of the EBs are not altered between control and HG pulse EBs. HG pulse moves the PrE cluster closer to ESCs along the developmental trajectory compared to control, indicating maintained progenitor potency in cells committed to later differentiation states. Ecto, ectoderm; Endo, endoderm; Meso, mesoderm; PS, primitive streak; senESC, senescent ESCs.

embryos, however mid- or late stage embryos completed gestation without significant developmental deviation [36,37]. Assessment of the developing embryos for molecular mechanism of failed early-stage gestation identified decreased DNA repair mechanisms and increased identification of DNA damage response (DDR). These data correspond with findings of Lei et al., which found that blastocyte formation and quality were compromised by spaceflight by mechanisms similar to ground based low dose radiation [38]. Our investigation established that a pulse of HG loading increased DNA repair and apoptotic processes. These studies collectively suggesting a potential gravity

regulated DDR pathway that may play a role in regulating how developmental cells respond to DNA damage, critical for the cell transition from pre- to post-implantation and gestation success.

Our single cell study evaluated the transcriptomic change associated with a single physiologically relevant pulse of mechanical loading and demonstrated the load-unload dynamics result in increased developmental progression. Similarly, a recent parabolic flight study evaluated repeated micro- to hyper-gravity periodic loading [39] finding ESCs exposed to the 31 parabolas elicited downregulation of proliferation and cell cycle genes and upregulation of developmental/differentiation genes. Additionally, ESCs recovered from the parabolic gravity loading then cultured as EBs for a further 12 days showed elevated Gene Ontology/ Kyoto Encyclopedia of Genes and Genomes pathways associated with skeletal, respiratory, blood vessels, liver, and nervous system and suppression of cardiac development pathways.

Blaber et al., collected 15 day EBs on-orbit for gene expression profiling, finding inhibition of terminal germ layer lineage markers and decrease in mechanotransduction signaling and membrane spanning proteins [22]. These data providing further evidence that absence of normal mechanical loading may restrict developmental potential. Together these complementary studies provide strong evidence that gravity loading regulates mammalian developmental processes and stem cell specialization. It is possible, however, that synergistic effects between mechanical unloading during spaceflight and space radiation, as reported by Blaber et al., may also contribute to the observed effects. Future investigations should address this potential synergy by combining mechanical loading with particle accelerator-simulated relevant space radiation doses and dose rates.

Finally, the single cell approach we used also allowed for mapping of the developmental program in a pseudotime coordinates system that models and orders temporal relationships between various gene expression states of cells in a population. Mapping EB development of the HG and unstimulated controls show cell populations are susceptible to ''branching'' cell fate decisions in response to gravity loading. Embryogenesis cell fate decisions are highly choreographed in response to cell–cell, cell–matrix, and cytokine signaling [40–42]. Because these cell fate decisions play a decisive role in execution of the developmental program, single cell may allow for more precise transcriptomic mapping of cell fate decisions based on transcriptomic similarity between cells [43,44].

Utilizing such methods to assess embryogenesis, investigations have found specificity of cell lineage tracing such as primordial germ cell tracing to pre-implant epiblasts [27] and definition of mid- to late-PS transitory transcriptomic signature [28]. Our single cell assessment of HG trajectory demonstrates that a positive gravity load induces branching of an inactive pluripotent population of ESC signature positive cells (Oct4+, Tcf15+, Gbx2+, etc.) with little to no cell cycle transcriptomic activity (cyclin/CDK complexes [45]), distinct from the starting ESC cell population. Our study also visualized branching points for cephalocaudal folding gene expression and PS signature cells between pre- and post-implant epiblast transition.

Conclusions

This study offers a novel assessment of how in vitro ESC maintenance and EB development can be regulated by a positive pulse of gravity loading at single cell resolution. Specifically, we show that a 60-min 50 *g*/5 kPa HG pulse resulted in cells during early development maintaining transitory phenotypes, and having enhanced expression of germ layer lineage markers. This study further defines the power of gravity loading in regulating stem cell self-renewal and differentiation by identification of transcriptomic patterns in stem cells and developmental processes, which can aid in understanding how gravity can be leveraged to elicit regenerative responses; benefiting in vitro platforms for organoid tissue culture, and regenerative medicine.

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Author Disclosure Statement

The principal investigator (PI) holds a NASA civil servant Research Scientist position that is similar in nature to a university tenured faculty position that does not depend on the opinions expressed by the scientist. In addition, the NASA funding supporting this work was externally competed based on external scientific peer review, and not intramural review and/or funding that might influence the findings. All other authors were either supported by the PI grant funding or by entities other than NASA. As such, no real competing financial interests exist.

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Supplementary Material

Supplementary Data

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