Topical: Stem Cell-Based Tissue Regenerative Health in Space

Author:
Eduardo A. C. Almeida, Ph.D.,
NASA Ames Research Center,
Space Biosciences Research Branch
Tel. 1(650)6041772
e.almeida@nasa.gov

Co-author:
Cassandra M. Juran, Ph.D.,
Blue Marble Space Institute of Science
at NASA Ames Research Center
cassandra.m.juran@nasa.gov

ABSTRACT: The maintenance of healthy adult tissues in mammals requires a complex homeostasis of molecular, cellular, tissue, and metabolic processes which are fundamentally different from the development and aging processes that bookend life. Cellular homeostasis in the adult requires molecular maintenance and repair of non-dividing cells such as cardiomyocytes and neurons, but also stem cell-based tissue regeneration via direct replacement of cell loss, such as in the blood, immune system, bone, skin, liver, intestine, and other tissues. Because stem cell-based tissue regenerative health requires constant proliferation and differentiation of stem cell progenitors in the bone marrow, and other adult stem cell niches, it is uniquely sensitive to the stresses of spaceflight including exposure to space radiation and mechanical unloading in microgravity. A key central hypothesis in this field is that those spaceflight stress factors can have profound negative effects on long-term tissue regenerative health mediated by adult stem cells, and that unmitigated, they may lead to premature tissue aging and functional failure. Specifically, it is thought that mechanical unloading due to lack of weight-bearing in space reduces mitogenic mechanotransduction necessary to promote adult stem cell proliferation and differentiation, and that space radiation can also lead to activation of cell cycle arrest mechanisms, further reducing adult stem cell proliferation. These hypotheses are being tested in low earth orbit (LEO) using a variety of cellular and whole organism tissue model systems, suggesting that spaceflight consistently interferes with stem cell tissue regenerative processes such as in mammalian embryoid bodies, regenerating newt tails, and mouse bone marrow hematopoietic and osteoprogenitor cells. Furthermore, potential molecular mechanisms integrating both space radiation and mechanical disuse via oxidative stress and the cell cycle inhibitor *Cdkn1a* are now under study using single cell (scRNAseq) expressome analysis of bone marrow osteoprogenitors, both under stretch loading, and spaceflight conditions including in various mouse transgenic null backgrounds relevant to these mechanisms. Future work in the area of tissue regenerative health in space for the coming decade should seek to understand the responses the various tissue regenerative stem cell niches in humans and relevant model organisms, and how they respond to long-term exposure to the space environment. Special emphases of future work should be on how regenerative deficits in whole-organism stem cell niches may lead to tissue degeneration and premature aging, and on the long-term proliferation and differentiation of stem cell derived tissue organoid models in the deep space environment outside of LEO.

BACKGROUND: In recent years a concept of stem cell regenerative health in space has been proposed (1, 2) in which adult stem cell-based tissue regenerative homeostasis is recognized as a key factor in health maintenance. Specifically the topical area of stem cell regenerative health studies how the fate of adult stem cells leading to quiescence, self-renewal, or proliferation and differentiation are regulated by intrinsic and external factors such as mechanical stimulation from physical activity and unloading associated with injury, age-related decreased mobility, oxidative stress, and in the case of astronauts in space, microgravity and radiation exposure (3). A current broad model of mammalian tissue regenerative processes now acknowledges mechanical loading from physical activity under earth's gravity field as the most common tissue regenerative stimulus, and factors as disuse, as well as oxidative status, such as from various stresses including radiation exposure, as major factors suppressing adult stem cell-regenerative processes that require extensive progenitor proliferation and differentiation. As individuals age, intrinsic biological clock replicative senescence is also a major factor limiting the ability of adult stem cells to self-renew and supply progenitors for the course of proliferation and differentiation required to repair and renew damaged or disuse atrophied tissue. A particularly well-characterized regenerative stem cell and precursor reservoir that we focus our studies on, is the bone marrow compartment of long bones, where mesenchymal and hematopoietic stem cells give rise to differentiation lineages that generate osteoblasts, osteoclasts, osteocytes, red blood cells, all the immune cells, chondrocytes, adipocytes, et cetera. The bone marrow compartment mechanical environment in mammals is thought to play an important role in regenerative health, as it is subjected to wide

ambulation-related cyclical hydrostatic pressure changes as the organism walks, runs and jumps, varying in intensity and periodicity. Because of this, cells adherent to bone surfaces such as mesenchymal lineage-derived osteoprecursors, osteoblasts and osteocytes are subjected to bone surface mechanical stretch and compression deformations, such as those associated with elastic bending of long bones. The current understanding of the tissue-level stem cell regenerative processes in the context of the mechanical environment has led investigators to focus on how mechanosensing and mechanotranduction pathways affect the course of cell cycle progression in stem cell self-renewal, and in activation of proliferation and differentiation programs leading to tissue regeneration. Specifically, one of the most striking examples of mechanical regulation of stem cells have demonstrated the ability of mechanical signals from physical interactions with the matrix, in the absence of established biochemical inductors, to prompt tissue lineage specific differentiation from pluri- or multi-potent cell populations in vitro (4-6). In vivo, stem cells are often protected from direct mechanical stimulation within carefully regulated localized niche environments, which cater to the maintenance of stem cell pluripotency (7, 8). Biophysical stresses have been reported both as promoting niche maintenance or as disruptors, eliciting signaling that promotes stem cell self-renewal or initiates transition from a state of quiescence to active proliferation and subsequent differentiation (9).

In vivo mechanoregulation studies conducted in the field of exercise physiology have established that loading stressors activate satellite pools of progenitor cells in musculoskeletal, cardiac, neural, and fibroblastic tissues, modulating proliferation, subsequent differentiative path commitment and terminal tissue growth or repair (10, 11). Fracture healing in bone is a high turnover regenerative process dependent on mechanical conditions. A large bone deficit study determined that ambulation loading, after an initial fracture stabilization of 4 weeks, significantly increase regenerate bone volume and improved mechanical properties of the developing bone (12). Collectively, these studies demonstrate that physiologically relevant mechanical loading has regenerative and tissue building effects.

Conversely, studies of unloading disuse from a compilation of post-operative recovery, bed rest, and aging studies have provided evidence that unloading results in catabolic unbalancing of regenerative stem cell homeostatic mechanisms. This imbalance results in muscle wasting, reduction of bone mass, immune suppression, and the development of anemia (13-17). However, it is important to recognize that an innate limitation of these disuse studies is the omnipresence of gravity meaning that a fully loading-null experiment cannot be accurately conducted on Earth.

Spaceflight in LEO offers a unique platform for unloading studies as investigations are conducted in the microgravity environment, in which a constant state of free-fall counteracts the still present but slightly diminished gravitational pull of Earth. To probe the absence of Earth normal gravity loading effects on regenerative cellular processes as a response to spaceflight, tail regeneration of the wholly regenerative newt (Pleurodeles waltl) was evaluated during the Foton M2 and Foton M3 missions (18, 19). Regeneration in the newt begins with accumulation of highly proliferative cells at the injury site and the formation of a blastema and terminates with functional reformation of the deficit tissue. Spaceflight newts developed a smaller regenerating tail outgrowth with fewer differentiated pigment, muscle and cartilage cells, and a greater number of proliferating BrdU-positive cells. Numerous organismal, cellular, and transcriptomic investigations conducted during spaceflight support this finding suggesting dysregulation in stem cell commitment to tissue specific cell lineages. Thus, spaceflight appears to affect the regenerating newt by interfering with stem cell-based tissue regeneration at the transition between proliferation and differentiation.

To probe the effects of spaceflight on proliferation and differentiation of somatic and adult progenitor stem cells, a study of bone marrow cells conducted in spaceflight environment compiled a molecular census of proliferation and differentiation related genes from pelvic ilia isolated bone marrow cells of 15 day space flown mice (20, 21). Results of the census demonstrated that spaceflight suppressed pro-osteogenic growth and proliferation genes and identified a cell cycle inhibitor gene Cdkn1a, which has regulatory influence in proliferation and osteogenic development (22, 23), as experiencing significant upregulation in the spaceflight bone marrow heterogeneous cell population. The p21/CDKN1a protein is a potent cyclin-dependent kinase inhibitor that functions as a check/stop for stem cell and progenitor proliferation and exit from the cell cycle for differentiation, senescence, or apoptosis (24). Interpretation of these studies' collective findings implies that p21/CDKN1a may serve as a molecular lynchpin for integrating space radiation and microgravity effects and inducing reduced regenerative capacity.

Despite being known as a radiation response gene, when *Cdkn1a* is deleted in mice, the knockout mouse presents a regenerative phenotype, with the ability to regenerate toward restoration of severed digits and closure of ear punches without scaring (25, 26). The mechanism of wound regeneration has some similarity to the mechanisms of newt regeneration, with regeneration being initiated by rapid onset of high cell turnover and formation of a blastema-like structure. In the ear punch model, cellular proliferation replaces the tissue removed by the excision and subsequently, the cells transition to differentiate into appropriate populations, which lay down new matrix. By the end of the regeneration process, the ear punch is sealed, and new scar-less cartilage tissue has replaced the deficit. Another important phenotypic feature of the *Cdkn1a* knockout strain is deletion does not significantly increase the rate of tumor formation (27).

When transcriptomic analysis of osteogenesis in the *Cdkn1a* gene knockout mouse model is examined at single cell resolution cyclic stretch mechanical stimulation, of bone marrow stem cells in vitro, results in advancement of progenitor cells to early proliferating osteoblast population (28). This transition increases the proliferating population and cell number in the cyclic stretch samples. Additionally, *Cdkn1a* -null cells, when cultured through a 21-day osteoblast differentiation protocol, were found to have greater proliferative regenerative capacity, maintaining a high level of proliferation even after passaging compared to their wildtype counterparts. The single cell resolution data also demonstrates early to late osteoblast commitment is elevated in the *Cdkn1a*-null cells and coupled mineral matrix quantification corroborates the transcriptome data showing better mineral quality in the null cultures. Collectively, these data show that cells at different stages of osteogenesis respond differentially to mechanical loading, features that had not been resolved until the utilization of scRNAseq transcriptomic technologies.

NOVEL HIGH-RESOLUTION TRANSCRIPTOMIC TECHNIQUES FOR INVESTIGATION OF STEM CELL-BASED TISSUE REGENERATIVE HEALTH IN SPACE: Recent analyses of bone marrow tissue scRNAseq public NASA GeneLab datasets from the NASA Rodent Research Reference Mission – 2 (RRRM2) (https://genelab.nasa.gov/node/675), as well as our own NASA Rodent Research 10 (RR-10) mission are starting to reveal much greater insights into how spaceflight affects stem cell-based tissue regenerative health. This approach using the 10X Genomics scRNAseq technologies allows, for instance, the investigation of how aging influences the regenerative potential of the femoral bone marrow stem cell populations after spaceflight live animal return and recovery (RRRM2), versus bone marrow collected and cryopreserved by astronauts on orbit (RR-10). Preliminary analyses of single cell-segregated bone marrow osteogenic lineage clusters, using the single cell filters from our previously published in vitro osteogenic single cell analysis (28),

finds for instance that the (RRRM2) ground control samples have higher expression than spaceflight of periostin (POSTN), 2.75-fold higher in young juvenile mice and 4.5-fold higher in mature mice (Figure 1).

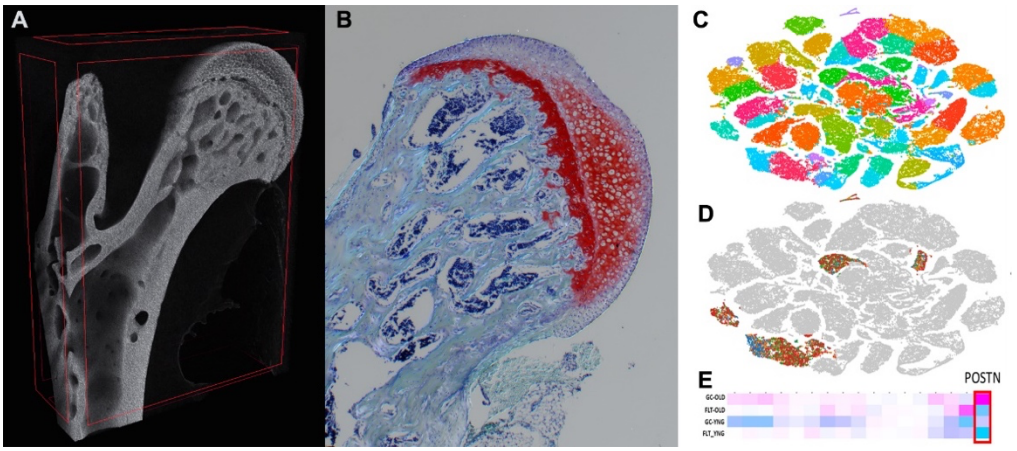

Figure 1: Single cell clustering of >90,000 femoral bone marrow cells from the publicly available NASA GeneLab RRRM2 spaceflight experiment scRNAseq dataset. A) Femur microCT showing bone marrow compartment stem cell niche B) Histological section of the proximal femur showing bone marrow cells in dark blue (hematoxylin) and cartilage in red (Saffranin O) C) ScRNAseq clustering of bone marrow cells of spaceflight, ground control, old, and young cells after return to Earth from 30 days in space. D) Osteogenic cells identified by gene marker panel. E) Top differentially expressed genes within the osteogenic populations of aging and juvenile spaceflight live animal return and ground control mice.

In bone tissue, POSTN is preferentially expressed by periosteal single osteoblasts in response to mechanical stimulation or parathyroid hormone with our data providing an example of how with scRNAseq analyses can dissect age, gravity field, and specific cell type-dependent individual cell regulation of osteogenesis within the highly complex bone marrow tissue regenerative stem cell niche.

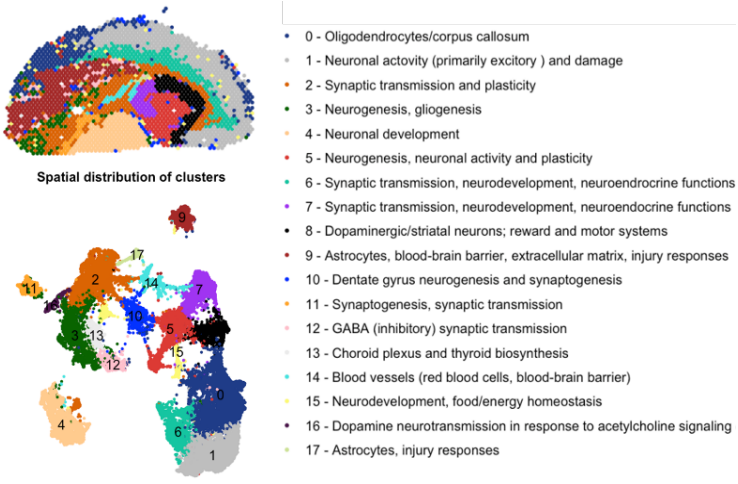

Figure 2: Spatial mapping of brain cell type clusters and mathematical

In addition to scRNAseq, spatial high-resolution transcriptomic approaches in tissue sections developed by 10X Genomics with the Visium technology is enabling more detailed analysis of regenerative structure-function responses to the stresses of spaceflight including in assessing the responses of specific cell types in brain histological sections to spaceflight, aging, and radiation, and mechanical loading. Collaborative assessment of Rodent Research – 3 (RR-3) mission brain (https://giacomellolabst.io/rr3-brain-shiny/) and NASA GeneLab shows differential expression of metabolic and neurogenesis genes, plus up-regulation of a multitude of lipid and mitochondrial dysfunctional pathways (Figure 2).

Tissue degeneration and failure of normal stem cell-based regeneration and homeostatic maintenance during spaceflight are thus increasing concerns for the preservation of astronaut health. The novel high-resolution, high-content single cell and spatial transcriptomic studies outlined above provide early evidence that spaceflight factors including microgravity and space radiation can interfere with the delicate

balance of adult tissue regenerative homeostasis via disruption of cellular transition from a proliferative state to a functional differentiative state, and strongly suggest this is an important topical focus area for the next decade of space biological research.

PROPOSED DECADAL STUDIES: Although the study of tissue regenerative health in space has significantly progressed in the last decade with the first pioneering studies of tissue regenerative stem cell niches in whole organisms and stem cell cultures, this is only true for LEO. Unfortunately, the effects of long-term deep space exposure on stem cell-based tissue regenerative health remain largely unknown. The overarching basic scientific question in space tissue regenerative health with the potential to influence the course of human exploration of the solar system is whether the highly radiation- and microgravity-sensitive adult regenerative stem cell pools can survive long-term deep space travel until normal gravity and radiation shielding are restored. Specifically, we also need to understand how the length of exposure to the deep space environment will affect which specific stem cell niches, and tissue regenerative processes, and whether this may lead, to premature regenerative aging and irreversible senescence. Finally, we need to understand how real (not accelerator-simulated) deep space radiation dose-rates and cumulative exposures affect stem cell niches in vivo. Because stem cells and early progenitors are few and highly localized in tissue niches and microenvironments, such as the bone marrow and intestinal crypts, most conventional studies have been unable to definitively resolve spaceflight responses of stem cells involved in tissue regeneration. Novel single cell mRNA seq, and tissue spatial transcriptomics of space-flown mice such as NASA's RR-10, RRRM2, and RR-3 are starting to break new ground for niche compartments such as in bone marrow and the brain respectively, but still these investigations only study exposure to the radiation-protected LEO environment, and often with the confounding effects of live organism return to earth, exposing them to reentry vibration and hypergravity, plus lengthy readaptation to 1g before tissue harvest. An exception to the live animal return is the recent NASA RR-10 mission in which for the first-time, astronauts collected and cryopreserved bone marrow stem cells in space for scRNAseq analyses both in WT and *Cdkn1a* null mice on ISS. Going forward we need to conduct studies in which mammalian model organisms are exposed to deep space for realistic extended exploration periods with tissue harvest or preservation conducted in space, such as in a Gateway station location. However, to enable deep space studies of tissue regenerative health in vivo a whole new set of technical capabilities needs to be developed, including smaller deep space rodent habitats, streamlined biological experimentation methods, and sample collection capabilities on Gateway, plus cold storage for return to earth for analysis. Despite the fact that whole animal in vivo experiments are the most informative studies to understand tissue regenerative health, cell science studies are also an important option, providing simpler in-vitro models of tissue systems and stem cell derived organoids (1), that can offer early fully autonomous cell culture experimental capabilities in exploration missions without animal habitat capabilities.

CONCLUSION: Current spaceflight experiments with stem cells in LEO are already revealing that tissue regenerative heath is a serious concern for conducting long-term deep space exploration. To resolve this concern and determine to what extent tissue regenerative health will be affected in deep space, we need to enable experimentation in this environment with novel compact and automated model organism habitats, and tissue culture facilities. In addition, we need to leap forward in utilizing novel single cell and spatial transcriptomic, plus epigenomic, high-content analyses to obtain both the single cell/niche resolution and large-scale data to support safe exploration and development of deep space. The experimental answers to these science questions in regenerative health should be used for designing rational data- and facts-based engineering solutions in radiation shielding and disuse countermeasures for exploration outside of LEO.

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