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Modulation of Bone Marrow Primary Cell Osteoblastogenesis and Cell Senescence by Mechanical Stimulation.

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Cell and animal studies conducted onboard the International Space Station and formerly the Shuttle flights have provided groundbreaking data illuminating the deleterious biological response of bone to mechanical unloading. Specifically CDKN1A/p-21 a cell senescence protein, was found to be upregulated in osteoprecursor cells of the femur during 15-day spaceflight, leading to the working hypothesis that CDKN1A/p-21 plays a role in inhibition of bone formation via mechanical regulation. To evaluate this hypothesis, utilizing a p-21 knockout mouse-line and relevant wildtype control, we cultured femoral bone marrow primary cells under unloaded (static) and cyclically stretched loading through a 30 day osteoblastogenesis protocol. Morphologic evaluation of the cultures demonstrated that mechanical stretching aligned the cells and increased the presence of defined focal adhesion expressing talin, integrin αvβ3, and PTK2 protein tyrosine kinase 2, also known as focal adhesion kinase (FAK) in both mouse strains. In corroboration with previous investigations of cell survival signals relation to FAK, our study found that with greater concentration of focal adhesions via stretch stimulation the live cell percentage was significantly higher than the unloaded controls (p-21 knockout line: +49.70%, p*=0.009, wildtype control: +18.14%, p*=.01). Also evaluated was the mineralization and ECM secretion capability of the differentiating cells. Von Kossa staining has shown that in the p-21 knockout cells unloaded cells produce more matrix that the stretch stimulated, however the matrix is unorganized presenting in sporadic nodules covering approximately 30% of the culture area at day 14 (n=6 wells) while the stretch stimulated cultures have less mineralization content the surface area containing mineralized matrix is greater (~68% at day 14). Q-PCR evaluation of the p-21 knockout cells revealed that canonical (β-catenin cascade) and non-canonical wnt11 and downstream planar cell polarity (wnt/PCP) pathway molecule RAC1 are prevalently upregulated with mechanical stimulation. Immunofluorescence for β-catenin and RAC1 showed co-localization at the nuclear membrane of the p-21 knockout cells but not the wildtype (n=1) suggesting that molecular communication via the canonical and wnt/PCP pathway are initiated by mechanical loading and experience regulation along the signaling cascade by CDKN1A/p-21. Future investigations will further elucidate this relationship and provide causal data demonstrating mechanical loading's modulatory effect on p-21 expression change.

MODULATION OF BONE MARROW PRIMARY CELL OSTEOBLASTOGENESIS AND CELL SENESCENCE BY MECHANICAL STIMULATION.

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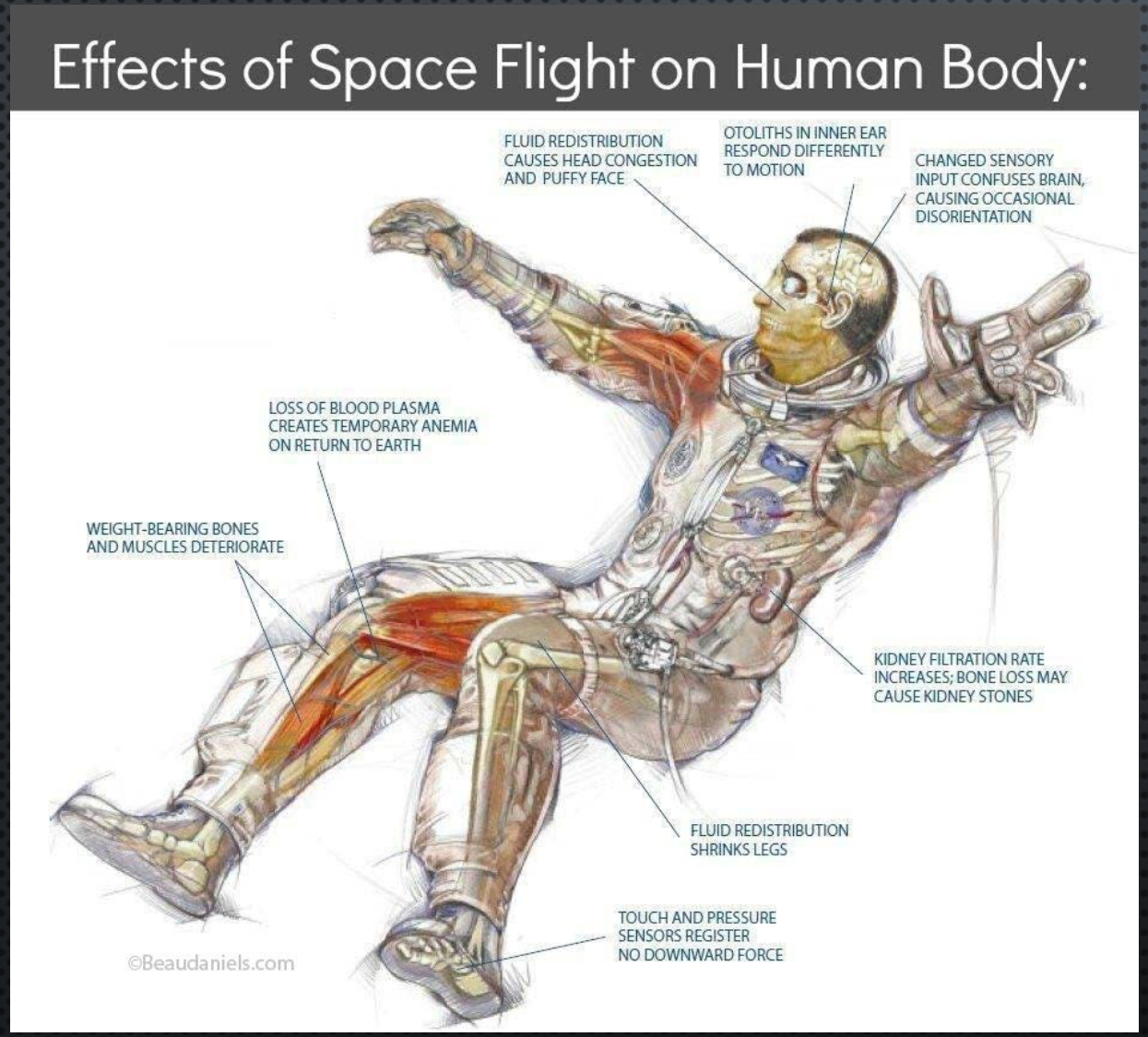
SPACEFLIGHT: BAD FOR BIOLOGY… YEAH, YEAH

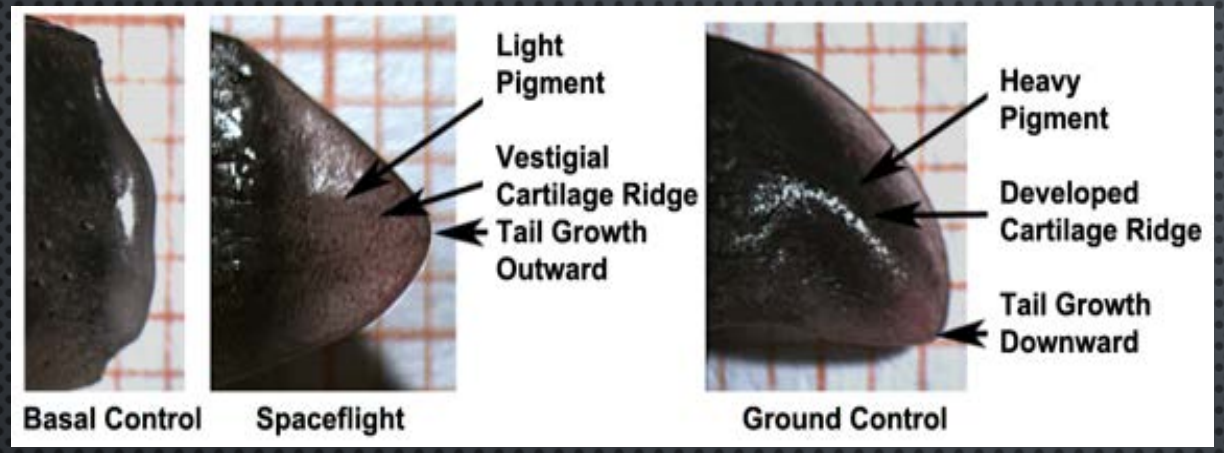


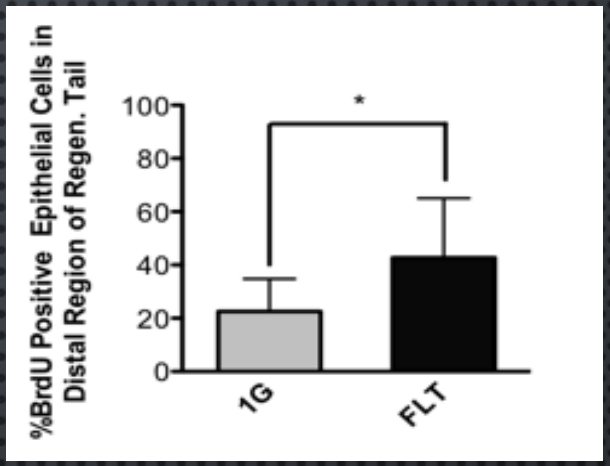
- SPACEFLIGHT HAS BEEN SHOWN TO HAVE DETRIMENTAL EFFECTS ON MECHANISMS OF HOMEOSTASIS IN THE HUMAN BODY.

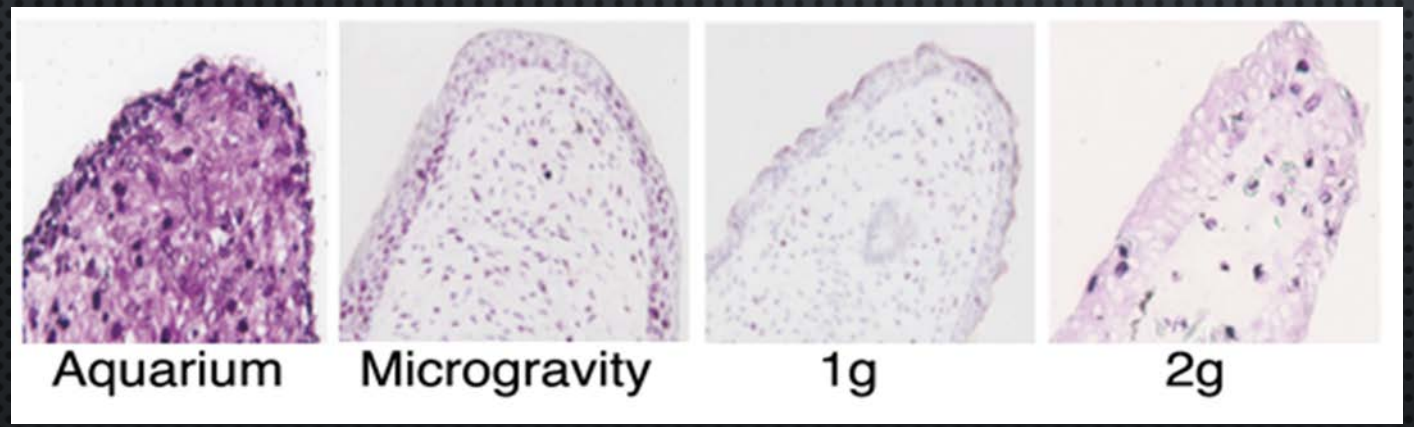
- TISSUES WITH HIGH CELL TURNOVER FROM **STEM CELL** POPULATIONS LIKE **IMMUNE** CELL POPULATIONS, **CARDIOVASCULAR** CELLS, **INTESTINAL** CELLS, **BLOOD** AND **BONE** MSCS AND HPCS ARE VULNERABLE TO DECREASED PRIMARY CELL PROLIFERATION DURING AND POST FLIGHT.

Image credit: NASA Human Research Program

WHOLLY REGENERATIVE SPECIES:
AMPHIBIANS: SPANISH RIBBED NEWT FLOWN ON FOTON M2 & M3







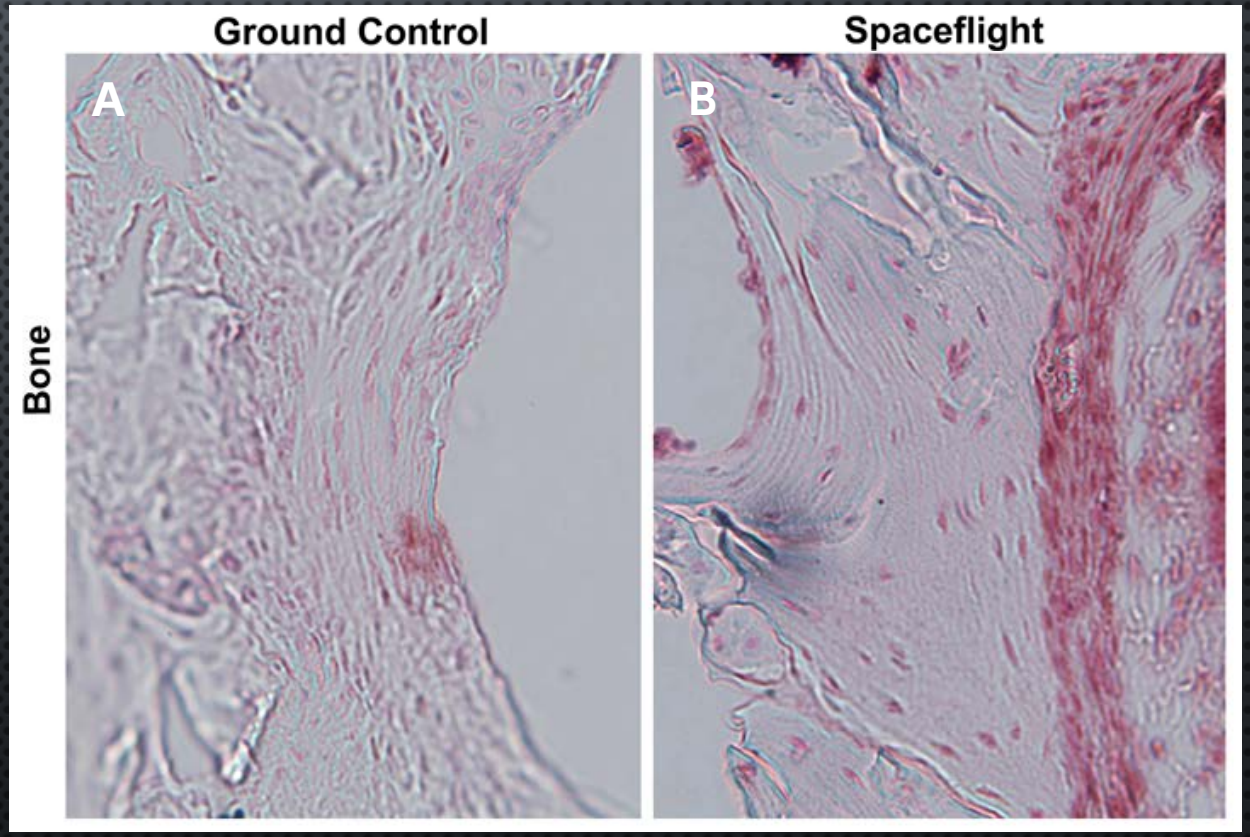
Cellular analyses of BrdU nuclear incorporation show regenerative deficit is related to blastema stem cells maintaining stemness longer, and failing to fully differentiate (more BrdU incorporation in microgravity)

Unloading in microgravity interferes with stem cell-based tissue regeneration in the newt model, and suggested that the critical step affected was the transition between proliferative stem cell populations and differentiated cells and tissues.

MICROARRAY ANALYSIS OF SPACEFLIGHT MOUSE **BONE MARROW** CELLS SHOW DOWN-REGULATION OF KEY PATHWAYS RELATED TO TISSUE REGENERATION

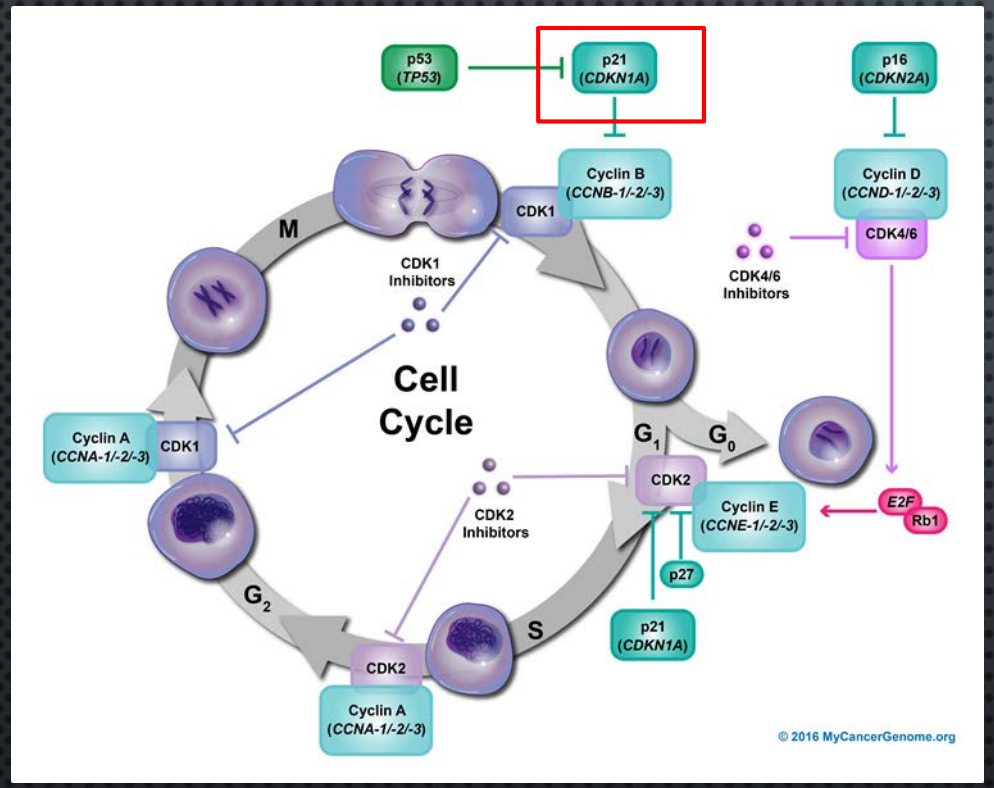
Modified image: myBioscience.com

Of specific interest CDKN1A/p21 is a **modulator of cell cycle progression** showed **elevated expression** on spaceflight samples.



Blaber, E. A., et al. "Mechanical unloading of bone in microgravity reduces mesenchymal and hematopoietic stem cell-mediated tissue regeneration." Stem cell research 13.2 (2014): 181-201.

CDKN1A/P21 ROLE IN CELL CYCLE AND WHAT HAPPENS WHEN ITS KNOCKED OUT

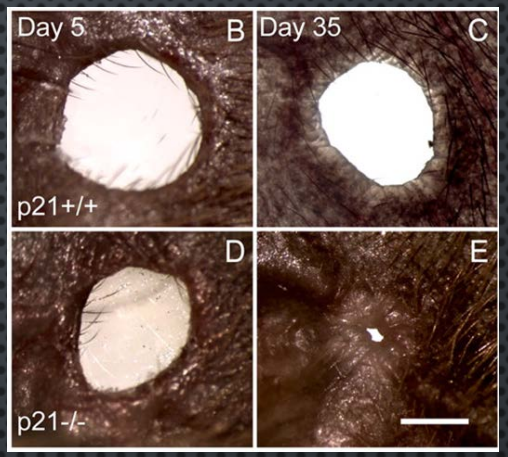


Modified image: mycancergenome.com

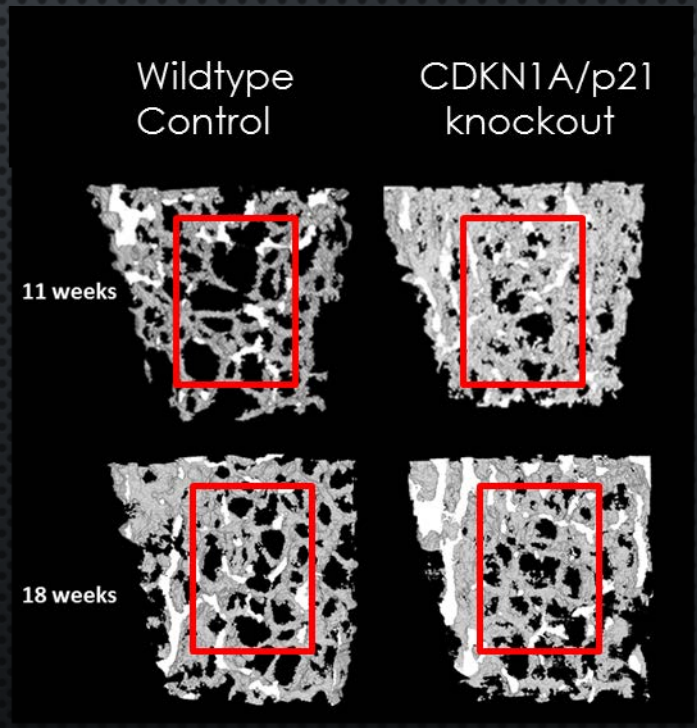
P21 INCREASED PRESENCE INTERRUPTS PROGRESSION THROUGH EARLY G1 PHASE.

Image credit: NASA Human Research Program

Mice genetically modified to not express CDKN1a/p21exhibit regenerative abilities, heightened wound healing and repair response similar to that of embryonic stem cells and amphibians with regenerative abilities

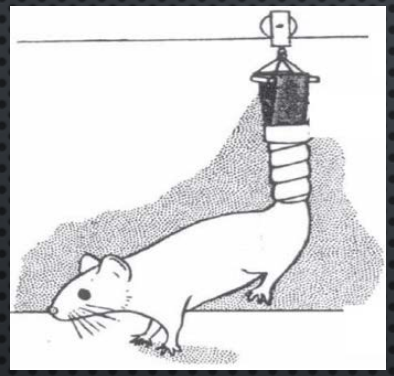


- In juvenile skeletal development **greater trabecular bone volume** is seen at 18 weeks in the CDKN1A/p21 knockout mice.
- Trabecular volume decrease between 11 weeks and 18 weeks is due to **osteoclastic bone resorption**, as not affected by p21

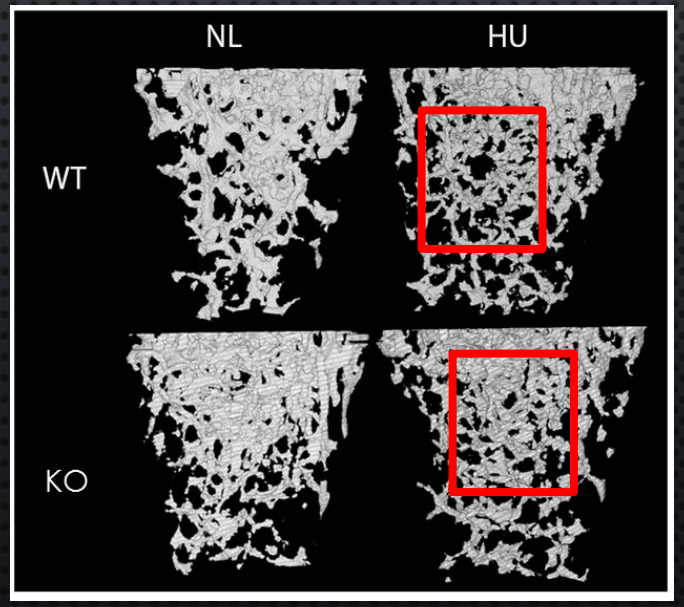


GROUND BASED SPACEFLIGHT SIMULATORY EXPERIMENTS: BONE STRUCTURE ANALYSIS AND MARROW OSTEOBLASTOGENESIS

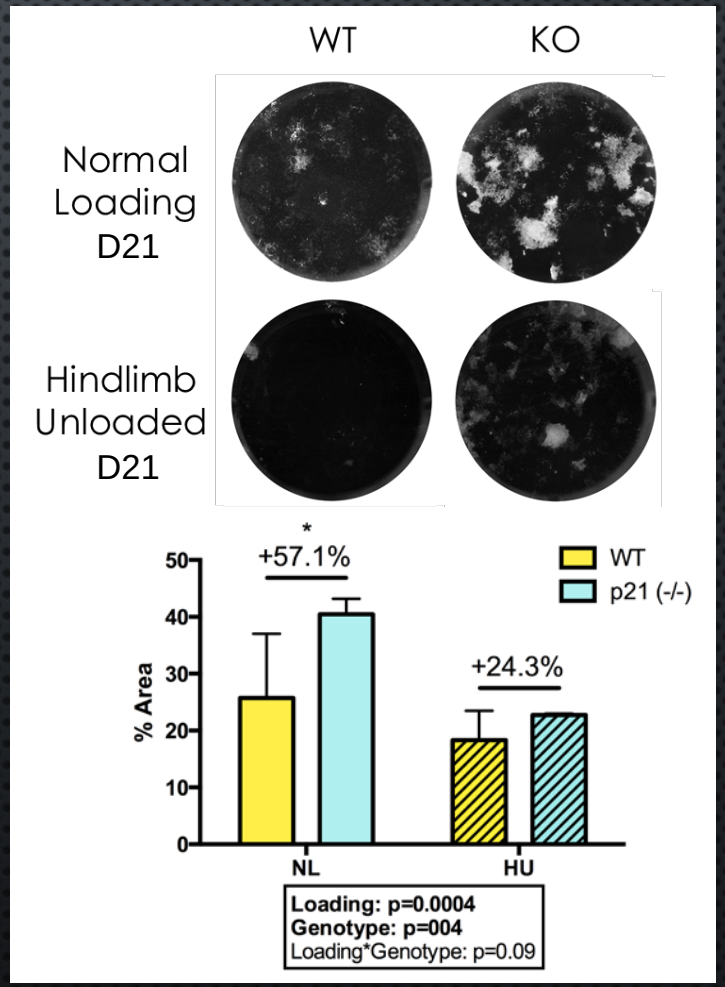
HINDLIMB UNLOADING (HU) IS AN ESTABLISHED GROUND BASED MODEL OF SPACEFLIGHT EFFECTS



MICROCT ANALYSIS OF THE TRABECULAR BONE OF THE FEMUR OF CDKN1A/P21 KNOCKOUT AND RELEVANT CONTROL MICE SHOW THE KNOCKOUT MICE MAINTAIN OSTEOBLASTIC BONE VOLUME DURING **UNLOADING.**



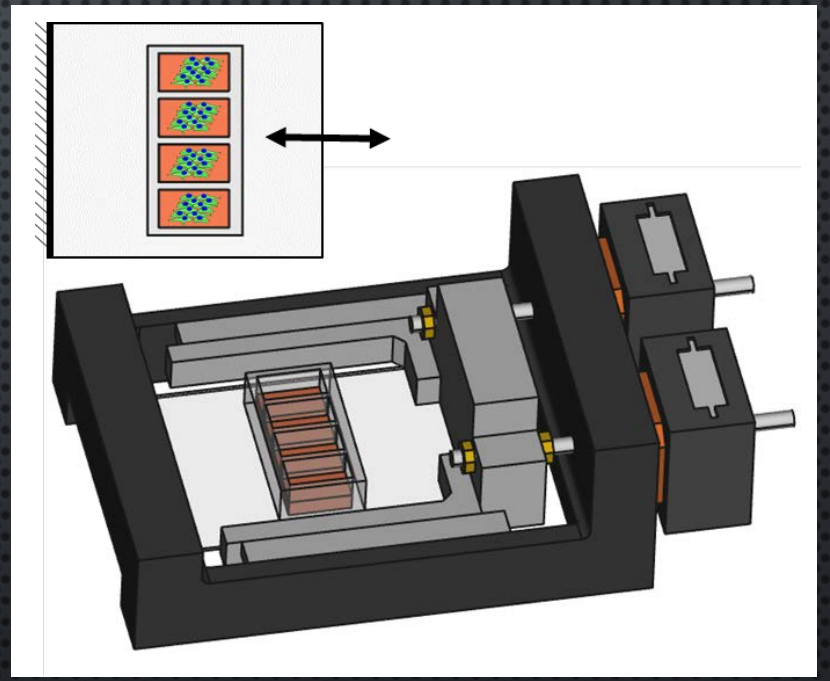
BONE MARROW FLUSH ISOLATED CELLS FROM THE HINDLIMB UNLOADED MICE DEMONSTRATE REDUCED MINERALIZATION FROM THE NORMALLY LOADED CONTROLS. HOWEVER, THE KNOCKOUT MOUSE CELLS BETTER MAINTAIN THE ABILITY TO FORM MINERAL NODULES AFTER UNLOADING.



Question: If ***mechanical unloading in microgravity negatively influences proliferation and/or differentiation of adult stem cells required for normal tissue repair and regeneration, will mechanical stimulation during osteoblastogenesis of bone marrow primary cells positively regulate proliferation and/or differentiation?***

EXPERIMENTAL PLAN

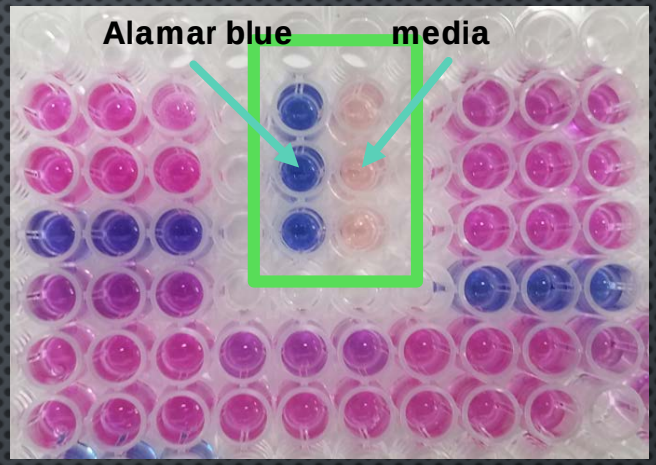
- Isolate bone marrow primary and MSC populations from CDKN1A/p21 and culture under exaggerated stretch loading.
  - CAVIAT: Wildtype controls are in Progress
- Assess proliferation, metabolism and cell network morphology
- Evaluate cultures for functions of functioning osteoblast cells.



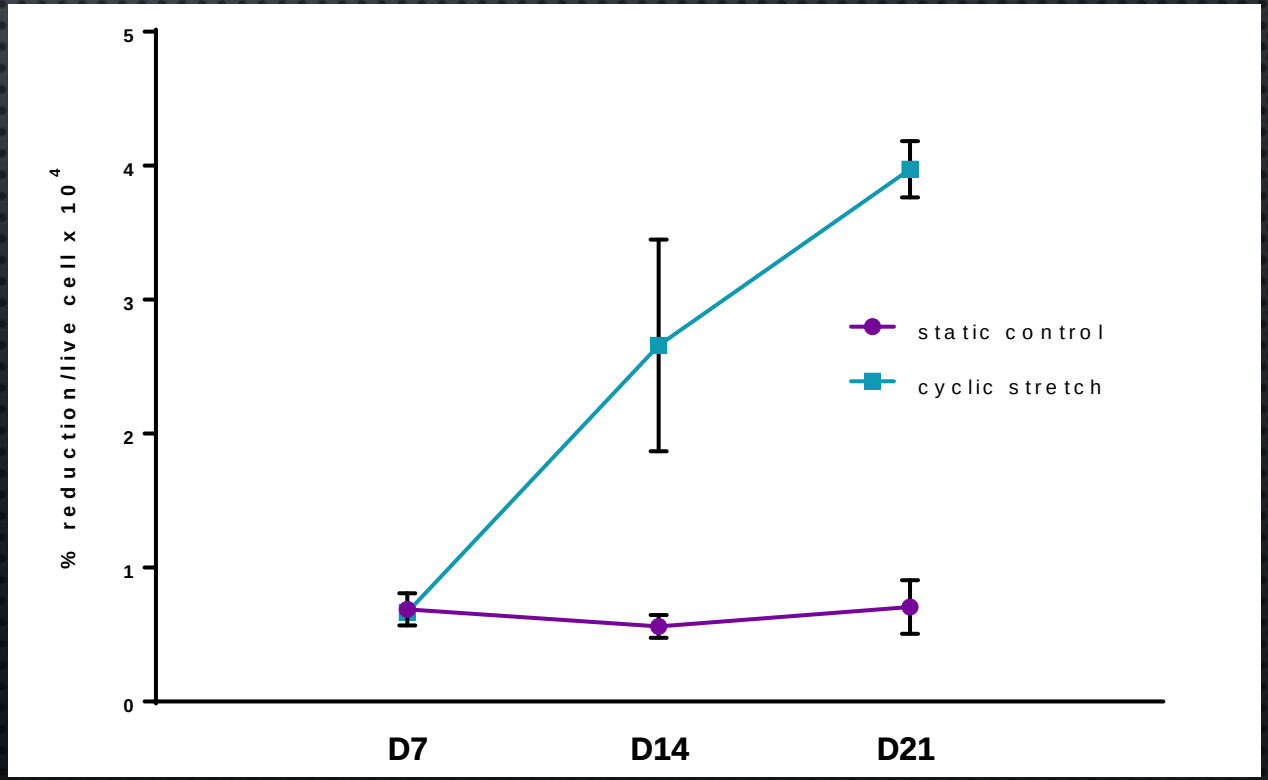
Event	**Cell Isolation and Plating**	**Start stretch culture**	**Time Point 1**	**Time Point 2**	**Time Point 3**
Day	D0	D10	D17 – D7 post-stretch	D24– D14 post-stretch	D31 – D21 post-stretch

PROLIFERATION AND METABOLISM

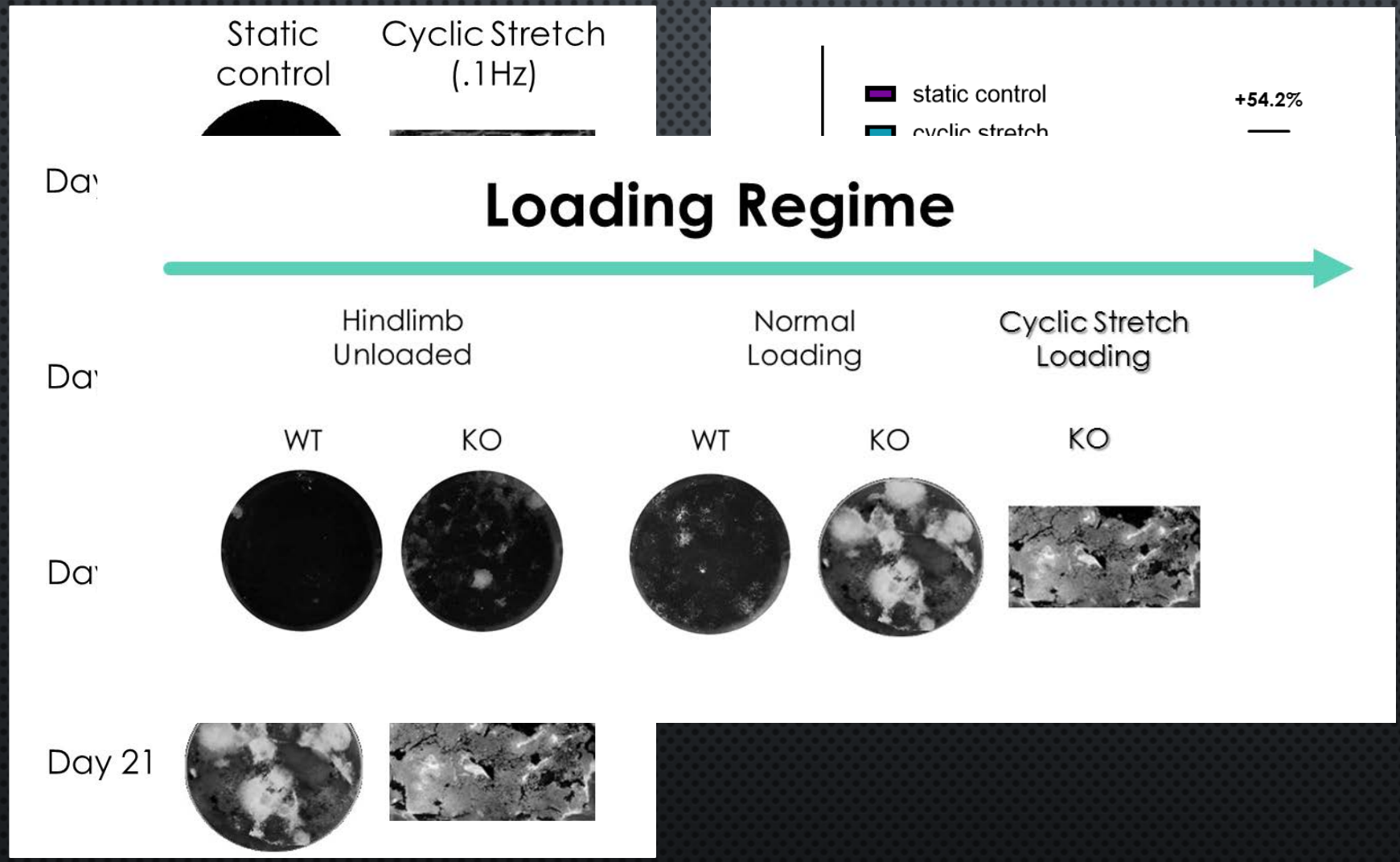
Time Point	Average Total cell count		Average Live cell count	
	Stretch	Static	Stretch	Static
D7	2.50E+05	2.57E+06	2.18E+05	6.80E+05
D14	1.91E+05	2.35E+06	1.12E+05	5.16E+05
D21	1.64E+05	2.63E+06	9.06E+04	4.66E+05



- Total Static cell counts are an order of magnitude higher than stretch: however percent live cell count is higher for the stretch cultures.
- %reduction of Alamar Blue shows increasing trend in the cyclic stretch cultures.
- However when normalized for live cell count it is clear that the



BONE MINERALIZATION



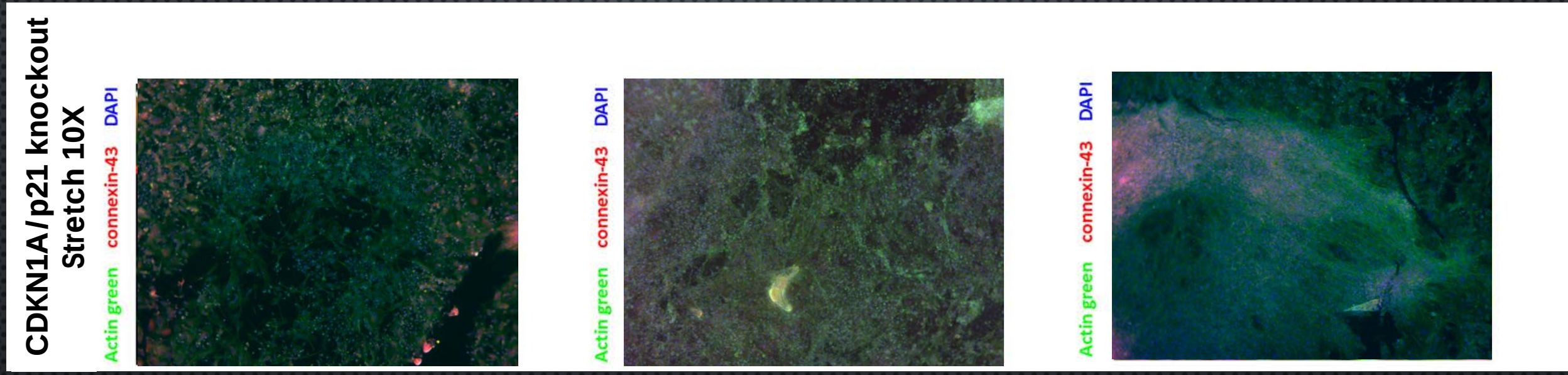
Chronologic Changes in mineralization

- Mineral area coverage
- Nodule formation
- Aligned matrix

Loading induced changes in mineralization

- Mineral area coverage
- Nodule formation
- Aligned matrix

EXTRA: NOT COMPLETED
CELL MORPHOLOGY ASSESSMENT



- AT 10X MAGNIFICATION CDKN1A/P21 KNOCKOUT CULTURES PRESENT **UNIFORM CELL COVERAGE** AND **ALIGNING ACTIN NETWORKS AND CELL INTERCONNECTIVITY** EVEN AT EARLY TIME POINTS.
- WHEN VISUALIZED AT 40X THE CDKN1A/P21 KNOCKOUT CULTURES HAVE AN EVEN MORE **OBVIOUS ALIGNMENT AND DEVELOPED MATRIX WITH HIGHLY INTERCONNECTED CELLS AND SHARED FOCAL ADHESION SITES**.

STUDY CONCLUSIONS AND CONTINUING EFFORTS

- The inclusion of mechanical strain on a primary cell population during osteoblast differentiation increases cellular vitality (metabolism per live cell), mineralization, and functional structural organization (actin alignment).
- Relevant wildtype cyclic stretch experiments are in progress to complete the study and further probe CDKN1A/p21's role in mechanical loading induced intracellular signaling.
- Further investigation into the mechanotransductive signaling pathways responsible for encouraged differentiation is ongoing.
- Main conclusion: Always work left to be done…

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