Osteogenic transcription regulated by exaggerated stretch loading via convergent wnt signaling

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Background

Cell and animal studies conducted onboard the International Space Station and formerly the Shuttle flights have provided data illuminating the deleterious biological response of bone to mechanical unloading (**Figure 1**). Loss of bone mass and inherit microarchitecture is a feature similar to osteoarthritis, the causal mechanism of which has been highly researched. *In Vivo* down regulation of molecular intra- and inter-cellular signaling cascades has been demonstrated in osteoarthritis and unloading studies. Specific to osteocyte cells the canonical wnt and Connexin43 induced cAMP signaling cascades have been shown as critical regulators. However the intercellular communicative cues and mechanotranductive cascades responsible for osteogenic transcription and stem cell recruitment are still largely unknown.

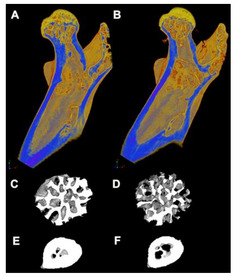


Figure 1. Proximal femur reconstruction from mice flown on 15-day STS-131 mission (B,D,F,) compared to ground controls (A,C,E). BV loss of 17% in flight samples.

Bone is a dynamic tissue undergoing constant remodeling and repair from stem cell precursors in the bone marrow (**Figure 2).** Osteocytes are believed to be responsible for the controlled regulation of cell activity in living bone. Thus how mechanical stimulation modulates biochemical activity of the osteocyte is a definitive factor in the study of bone biology and homeostasis maintenance.

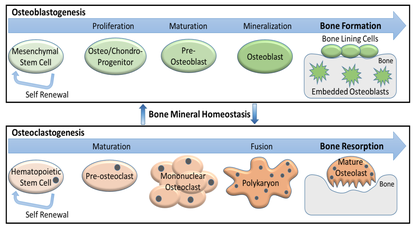


Figure 2. Bone mineral homeostasis is a balance between bone formation by osteoblasts and bone resorption by osteoclasts.

A significant feature of interest in mechanical regulation of bone biology is the mechanism of loading experienced by the cell modulates the cells reaction. Many of the previous osteocyte studies investigating response to loading have evaluated how the cells respond to fluid flow induced shear adjacent to cells in monolayer. This model however, is not representative of the critical osteocyte dendritic process activation within the canaliculus (Fig 3A). Thus our experiential design will utilize stretch loading, such that the cell process directly experience load to better represent the physiologic response of cells *in vivo* (Fig 3B).

In this investigation, MLO-Y4 osteocyte-like and MC3T3-E1 osteoblast-like cells (control cell) were culture under dynamic tensile conditions and evaluated for expression of CX43 and wnt-signaling proteins influential in driving cell-cell communication as well as stem cell recruitment and differentiation.

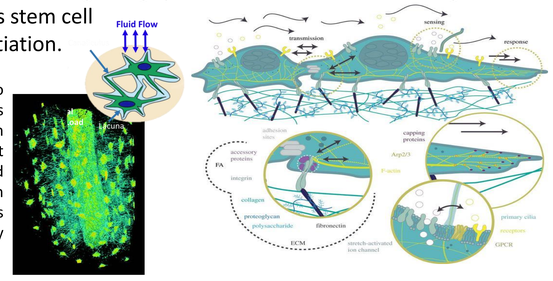


Figure 3. Cellular response to mechanical stimulation is dependent on the mechanism of cell deformation. Of import in this study is strain applied through the cell attachments, in contrast to fluid flow studies which are regulated by membrane strains.

Hypothesis

We hypothesize stretch loading induces gap junction and wnt11 choreographed convergent wnt signaling which regulates a cascade of molecular events terminating in osteogenic gene transcription.

Methods

MLOY4 osteocyte-like and MC3T3-E1 osteoblast-like cells were cultured in a custom designed biostimulator (**Figure 5**) and allowed to acclimate for 48 hours before 48 hours of stretch loading. Stretch loading was imparted by 2 Arduino controlled linear drive motors set to 0.1% tensile strain and 0.1Hz cyclic application. Measure of CX43 localization, cell number, metabolism, and phenotypic expression were taken at 10 minutes, 2, 12, 24 and 48 hours.

MLOY4 osteocyte-like cells | **MC3T3-E1 osteoblast-like cells**

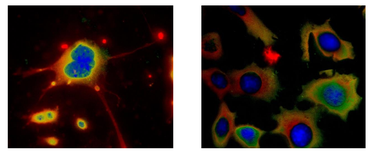


Figure 4. Experimental cell type morphology. MLOY4 represent critical dendritic cell processes inherent to functional osteocytes, while MC3T3-E1 pre-osteoblast cells present no such morphology.

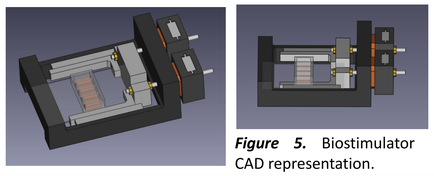


Figure 5. Biostimulator CAD representation.

Culture conditions were maintained at 5% CO_2 37°C and 90% humidity. Culture media was supplemented by 1% anti-anti, 10% FBS and changed every 48 hours such as to not interrupt the stimulation regime.

Results

Osteogenic Signaling Expression and Localization after Exaggerated Loading

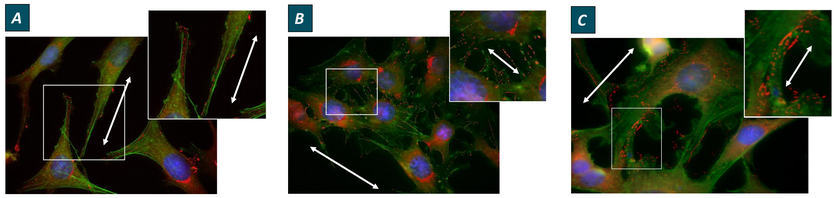


Figure 6: Florescence imaging demonstrating cell morphologies (actin – Green), connexin localization (CX43 primary antibody/2nd ary antibody 594 – Red) and counterstained with DAPI (nuclear –Blue). Figure **A) and insert** are MLOY4 osteocyte cells after 48 hours of stretch culture, **B) and insert** are MC3T3-E1 osteoblast cells, and **C) and insert** are co-cultured MLOY4 and MC3T3-E1. **A)** illustrates typical MLOY4 morphology with extended cell processes along the direction of stretch (indicated by white arrow) and localized CX43 presence at the process peripheries and nuclear envelope. While in **A)** and **C)** CX43 is highly expressed at membrane interfaces in **B)** the extent of localization is non-specific in osteoblast cells. Osteoblast-Osteocyte co-cultured cells **C)** demonstrate both increased CX43 presence within the cell and membranous localization inherit to mechanistic communication both intercellular and intracellular.

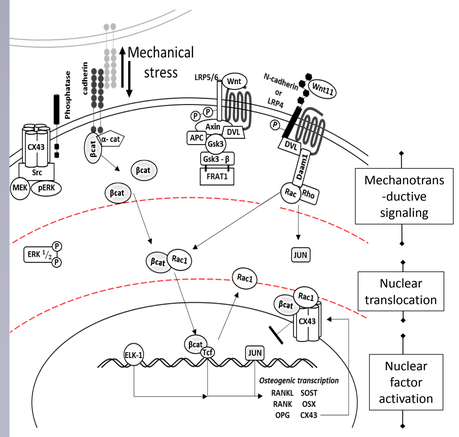
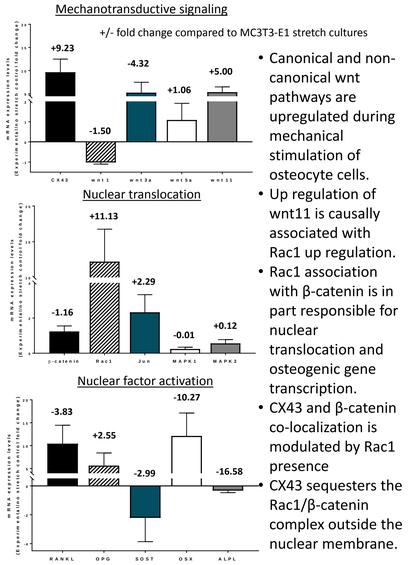


Figure 7. Overview of convergent wnt signaling regulation of osteogenic gene transcription pathways in response to stretch loading. CX43 signaling is increased by stretch loading. Co-localization of CX43 and β-catenin is coupled with wnt11/PCP pathway release of Rac1.



- Canonical and non-canonical wnt pathways are upregulated during mechanical stimulation of osteocyte cells.
- Up regulation of wnt11 is causally associated with Rac1 up regulation.
- Rac1 association with β-catenin is in part responsible for nuclear translocation and osteogenic gene transcription.
- CX43 and β-catenin co-localization is modulated by Rac1 presence
- CX43 sequesters the Rac1/β-catenin complex outside the nuclear membrane.

Results

Proliferation and Cellular Metabolic Activity with Exaggerated Loading

Cell number and metabolic activity of cultured MLOY4 cells demonstrate cellular viability. Stretch stimulation has a negative correlation with proliferation compared to unloaded controls. Cellular metabolism initially increases due to new stress applied to the cells however after acclimation metabolism reaches steady state after 24 hours.

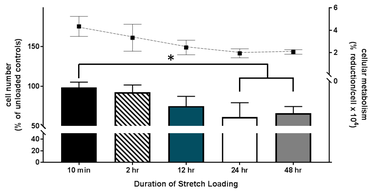


Figure 8. Analysis of MLOY4 viability during stretch stimulation. *p <0.05

Cellular Connectivity and Intercellular Regulation due to Exaggerated Mechanical Loading

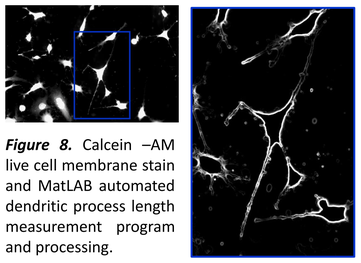


Figure 8. Calcein –AM live cell membrane stain and MatLAB automated dendritic process length measurement program and processing.

Table 1: Quantitative Assessment of MLOY4 Dendritic Process Length and Termination Junctions

	Length (μm)	Integer Representation of Shared Terminating Junctions per cell
Control (No Stretch)	3.19 ± 2.13	3
48 hours (0.1% strain, 0.1 Hz frequency)	6.50 ± 1.58	7

The self organization of osteocyte cells is a critical metric of the cell population function. MLOY4 cells cultured in unloaded conditions will form dense overlapping networks with short dendritic process lengths and little CX43 membrane localization. In vivo examination of osteocyte networks have been shown to be highly interconnected (**Figure 3**) and these connections are made between dendritic cell processes with lengths averaging 20-30μm. Within the osteon the cell processes are organized in the canaliculus network, the microarchitecture of which amplifies mechanical stress sensed by the processes which in turn lengthens the process. This supposition is supported by our measurement of MLOY4 dendritic cell process lengthening under stretch loading stimulation. Additionally, the shared terminating junctions were quantified and demonstrated greater MLOY4 population interconnectivity when cells were exposed to stretch loading.

Conclusion

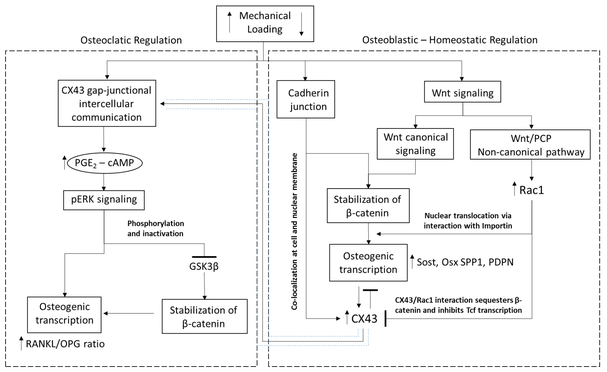


Figure X. Hypothetic Feedback regulation of osteogenic transcription in MLOY4 osteocyte cells due to mechanical loading. This theoretic feedback loop implies the critical importance of coupled wnt signaling control of CX43 intercellular communication channel.

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