Regulation of bone formation during disuse by miRNA

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Astronauts lose bone structure during long-duration spaceflight. These changes are due, in part, to insufficient bone formation by the osteoblast cells. Little is known about the role that small (~22 nucleotide), non-coding micro-RNAs (miRNAs) play in the osteoblast response to microgravity**. We hypothesize that osteoblast-lineage cells alter their miRNA status during microgravity exposure, contributing to impaired bone formation during weightlessness.** To simulate weightlessness, female mice (C57BL/6, Charles River, 10 weeks of age, n = 6) were hindlimb unloaded for 12 days. Age-matched and normally ambulating mice served as controls (n=6). To assess the expression of miRNAs in skeletal tissue, the right and left tibia of the mice were collected *ex vivo* and cleaned of soft-tissue and marrow. Total RNA was collected from tibial bone and relative abundance was measured for miRNAs of interest using quantitative real time PCR array looking at 372 unique and well-characterized mature miRNAs using the delta-delta Ct method. Transcripts of interest were normalized to an average of 6 reference RNAs. Preliminary results show that hindlimb unloading decreased the expression of 14 miRNAs to less than 1.4-2.9X control levels and increased the expression of 5 miRNAs relative to the control mice greater than 1-2-1.5X ($p<0.05$, respectively). Using the miRSystem we assessed overlapping target genes predicted to be regulated by multiple members of the 19 differentially expressed miRNAs as well as *in silico* predicted targets of our individual miRNAs. Our miRSystem results indicated that a number of our differentially expressed miRNAs were regulators of genes related to the Wnt-Beta Catenin pathway—a known regulator of bone health—and, interestingly, the estrogen-mediated cell-cycle regulation pathway, which may indicate that simulated weightlessness induced systemic hormonal changes that contributed to bone loss. We plan to follow up these findings by measuring gene expression of miRNA-regulated genes within these two pathways with the aim of furthering our understanding of the function of miRNAs in the skeletal response to spaceflight.

Background

1) Ground Flight

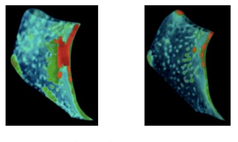


Figure 1: Ischium of spaceflight animals had a severe reduction in bone thickness after 15 days of spaceflight

Blaber et al., (2013)

2) Ground Flight

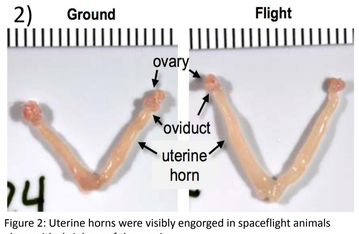


Figure 2: Uterine horns were visibly engorged in spaceflight animals along with shrinkage of the ovaries

Joseph Tash et al., (2010-2011, unpublished)

3) ERa / actin — Ground G1 G2 G3 G4 G5 G6 | Flight F1 F2 F3 F4 F5 F6

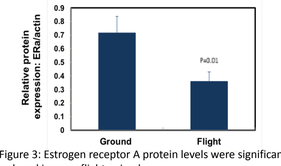


Figure 3: Estrogen receptor A protein levels were significantly reduced in spaceflight animals

Joseph Tash et al,(2010-2011, unpublished)

4) ESR1 vs. Runx-2 Abundance Levels

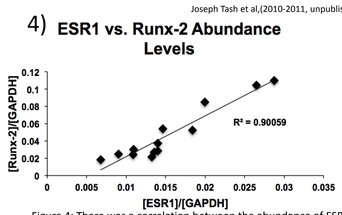


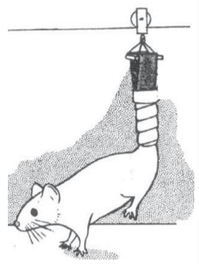
Figure 4: There was a correlation between the abundance of ESR1 and Runx2 in the femur bones of Day 12 HU mice suggesting that the pathways are possibly related or shared between bone and sex hormone receptor signaling molecules

Callewart et al., (2010) Trends Endocrinol Metab

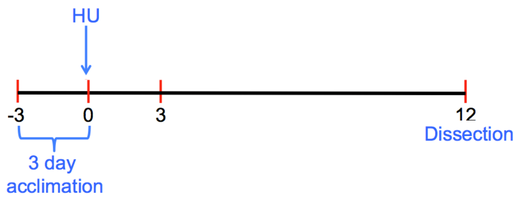
Methods

Experiment Design

- 12 female mice
  - 10 weeks of age
  - 6 sham, 6 hindlimb unloaded
- Hindlimb Unloading (HU)
  - For 12 days to simulate weightlessness
  - Dissections on day 12



Timeline



Sample Collection and Data Analysis

Dissected right and left tibiae, cleaned of soft-tissue and marrow → RNA isolation → cDNA conversion → PCR

- qPCR CT values above 33 were regarded as 'Not Detected'
- All p-values are calculated via a two-tailed T-test

miRNA Expression Results 12 Days HU

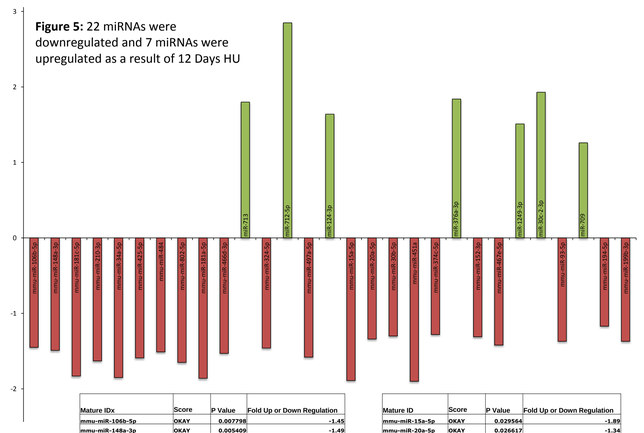


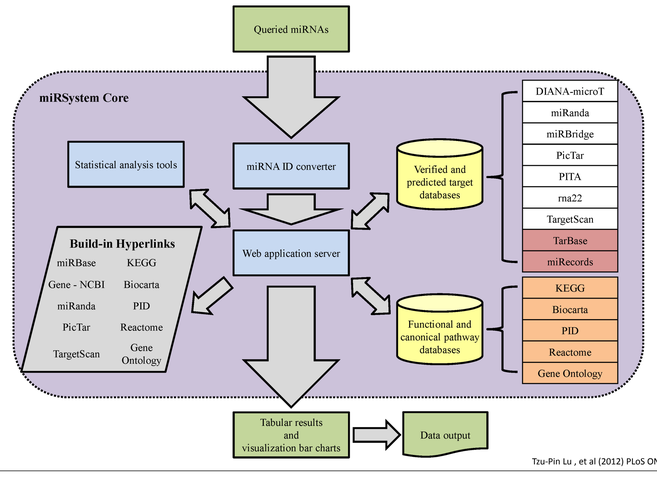
Figure 5: 22 miRNAs were downregulated and 7 miRNAs were upregulated as a result of 12 Days HU

Mature IDx	Score	P Value	Fold Up or Down Regulation
mmu-miR-106b-5p	OKAY	0.007798	-1.45
mmu-miR-148a-3p	OKAY	0.005499	-1.49
mmu-miR-181c-5p	OKAY	0.007311	-1.83
mmu-miR-210-3p	A*	0.003165	-1.63
mmu-miR-34a-5p	OKAY	0.000396	-1.85
mmu-miR-425-5p	OKAY	0.008681	-1.55
mmu-miR-484	OKAY	0.000281	-1.51
mmu-miR-802-5p	OKAY	0.02564	-1.65
mmu-miR-181a-5p	OKAY	0.009059	-1.86
mmu-miR-466d-3p	OKAY	0.011407	-1.53
mmu-miR-713	OKAY	0.018282	1.8
mmu-miR-324-5p	OKAY	0.010491	-1.46
mmu-miR-712-5p	A*	0.036654	2.85
mmu-miR-497a-5p	OKAY	0.047807	-1.58
mmu-miR-124-3p	OKAY	0.030324	1.64

Mature ID	Score	P Value	Fold Up or Down Regulation
mmu-miR-15a-5p	OKAY	0.029564	-1.89
mmu-miR-20a-5p	OKAY	0.026617	-1.34
mmu-miR-30b-5p	OKAY	0.047629	-1.3
mmu-miR-451a	OKAY	0.036223	-1.9
mmu-miR-374c-5p	OKAY	0.033358	-1.28
mmu-miR-376a-3p	OKAY	0.030808	1.84
mmu-miR-152-3p	OKAY	0.032255	-1.31
mmu-miR-467e-5p	A*	0.011567	-1.42
mmu-miR-1249-3p	OKAY	0.008713	1.51
mmu-miR-30c-2-3p	OKAY	0.026795	1.93
mmu-miR-93-5p	OKAY	0.007941	-1.37
mmu-miR-709	OKAY	0.016189	1.26
mmu-miR-194-5p	OKAY	0.030212	-1.17
mmu-miR-199b-3p	OKAY	0.045042	-1.37

Table 1: A*: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample, and is reasonably low in the other sample (< 30).These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result. This fold-change result may also have greater variations if p value > 0.05.

miRSystem Workflow



Tzu-Pin Lu , et al (2012) PLoS ONE; 7(8):e42390

Acknowledgements

This project was supported by a 2012 PECASE (JSA) NASA Space Biology. We thank the members of the Bone & Signaling Lab at NASA Ames for their camaraderie and collegial support.

In silico mirSystem Results

Differentially expressed miRNA Pathway Ranking

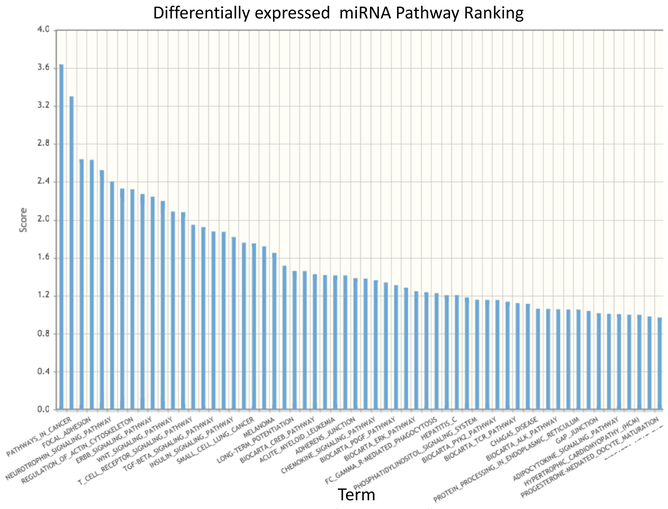


Figure 6: Molecular Pathways containing the highest number of genes targeted by differentially expressed miRNA based on tar...t prediction

Tzu-Pin Lu , et al (2012) PLoS ONE; 7(8):e42390

Table 1: Osteoblast Related Genes Targeted by miRNAs

Target Gene	Gene Description	Number of targeting miRNAs	Upregulated miRNAs	Downregulated miRNAs	Differenc e
Smad5	Mothers against decapentaplegic homolog 5	8	2	6	-4
Igf1r	Insulin-like growth factor 1 receptor	4	0	4	-4
Esr1	Estrogen receptor alpha	4	0	4	-4
Mapk1	Mitogen-activated protein kinase 1	4	0	4	-4
Tgfbr2	Transforming growth factor, beta receptor II	3	0	3	-3
Fos	FBJ osteosarcoma oncogene	3	0	3	-3
Fzd7	Frizzled class receptor 7	3	0	3	-3
Smad2	Mothers against decapentaplegic homolog 2	3	0	3	-3
Smad6	Mothers against decapentaplegic homolog 6	3	0	3	-3
Sostdc1	Sclerostin domain containing 1	3	0	3	-3
Bmpr2	Bone morphogenetic protein receptor, type II	3	0	3	-3
Fzd4	Frizzled class receptor 4	6	2	4	-2
Pthlh	Parathyroid hormone like peptide	2	0	2	-2
Dll1	Delta-like 1	2	0	2	-2
Klf10	Kruppel-like factor 10	4	1	3	-2
Lrp6	Low density lipoprotein receptor-related protein 6	3	2	1	-1
Notch1	Notch 1	1	0	1	-1
Lef1	Lymphoid enhancer binding factor 1	1	0	1	-1
Gsk3b	Glycogen synthase kinase 3 beta	8	4	4	0
Bmp2	Bone morphogenetic protein 2	2	1	1	0
Ap1g1	Adaptor protein complex AP-1, gamma 1 subunit	2	1	1	0

Table 2: Osteocyte Related Genes Targeted by miRNAs

Target Gene	Gene Description	Number of targeting miRNAs	Upregulated miRNAs	Downregulated miRNAs	Differenc e
Lin28a	Lin-28 homolog A	9	1	8	-7
Cd44	CD44 antigen	11	2	9	-7
Tcf71	Transcription Factor 7-like protein	9	1	8	-7
Tcf7l2	Transcription Factor 7 like 2	13	3	10	-7
Spp1	Secreted phosphoprotein 1	8	1	7	-6
Pthlh	Parathyroid hormone like peptide	7	1	6	-5
Pls1	Plastin 1	5	0	5	-5
Lifr	Leukemia inhibitory factor receptor	3	0	3	-3
Ffg23	Fibroblast growth factor 23	3	0	3	-3
Phex	Phosphate regulating endopeptidase homolog	9	3	6	-3
Sox9	SRY-box 9	7	2	5	+3
Ctnnb1	Catenin associated beta 1	4	1	3	-2
Sost	Sclerostin	1	0	1	-1
Mepe	Matrix extracellular phosphoglycoprotein	3	1	2	-1
apg	Capping protein, gelsolin-like	3	1	2	-1
Hyou1	Hypoxia upregulated 1	7	3	4	-1
Cdc42	Cell division cycle 42	7	3	4	-1
Npy	Neuropeptide Y	1	0	1	-1
Dstn/destrin	Destrin	3	1	2	-1
Dmp1	Dentin matrix protein1	1	1	0	+1
Osx	Sp7 transcription factor	1	1	0	+1
Capn1/calpain	Calpain 1	5	3	2	+1
Pdpn	Podoplanin	4	2	2	0

Tables 2&3: Target scoring based on methods in Plank MW et al. where *in silico* gene targeting was demonstrated to predict *in vivo* changes in gene expression for genes with the highest miRNA difference score.

Plank MW et al. (2015) PLoS ONE; 10(12): e0144810

Conclusions

- We successfully confirmed the first part of our hypothesis that Ob-lineage cells alter their miRNA status as a result of HU
- Future work will be directed towards the second half of the hypothesis looking at connecting the changes we see in miRNA to bone loss as a result of HU, and possibly the reproductive system

Future Directions

- *In vitro* work studying our differentially expressed miRNA
  - -Knockouts, and addition of mimics followed by qPCR and Western blotting
- *In vivo* study corroborating *in vitro* work utilizing miRNA knockouts and utilizing RNAseq