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Supplemental Information

miR-208a-3p Suppresses Osteoblast

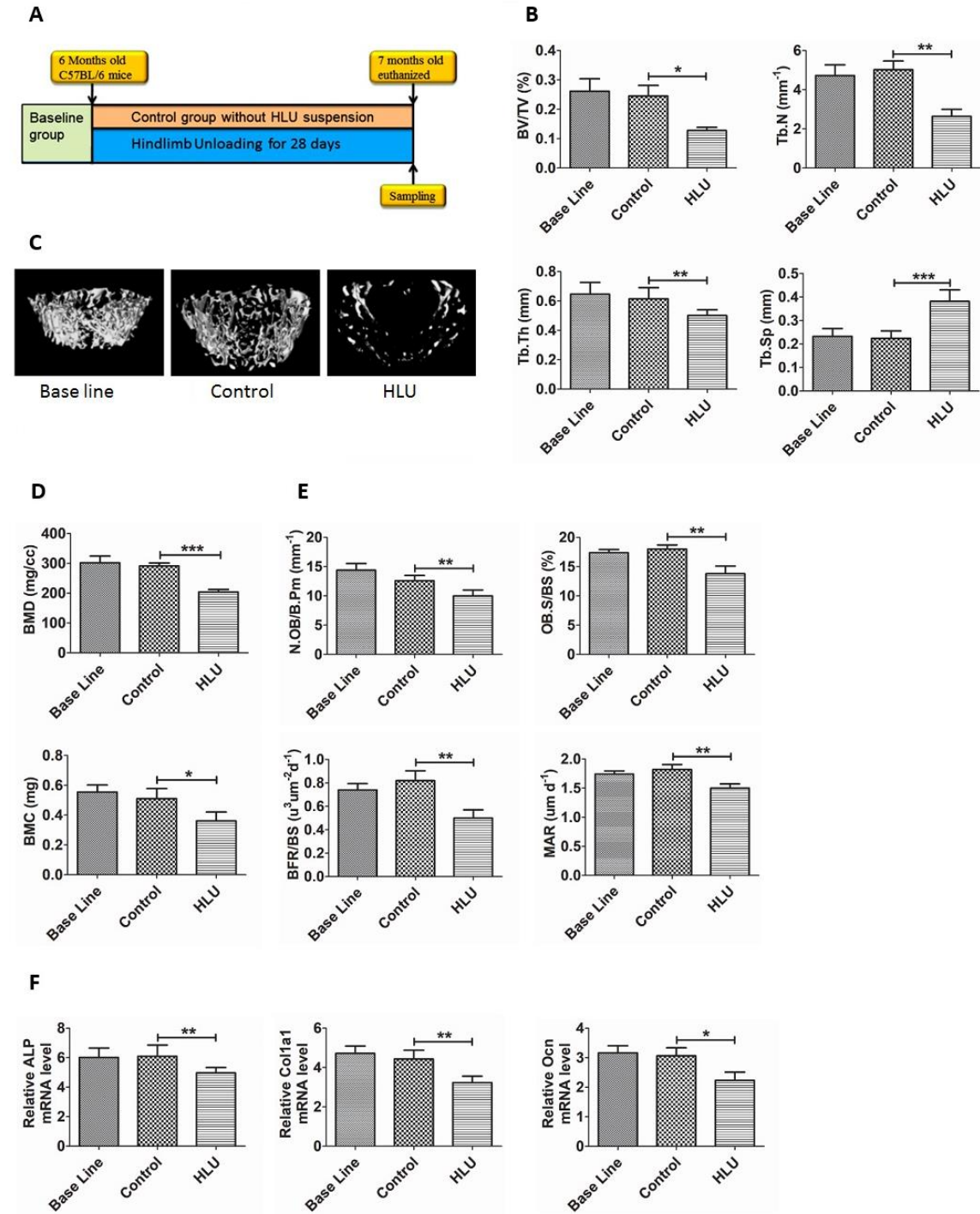
Differentiation and Inhibits Bone

Formation by Targeting *ACVR1*

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Supplementary Information

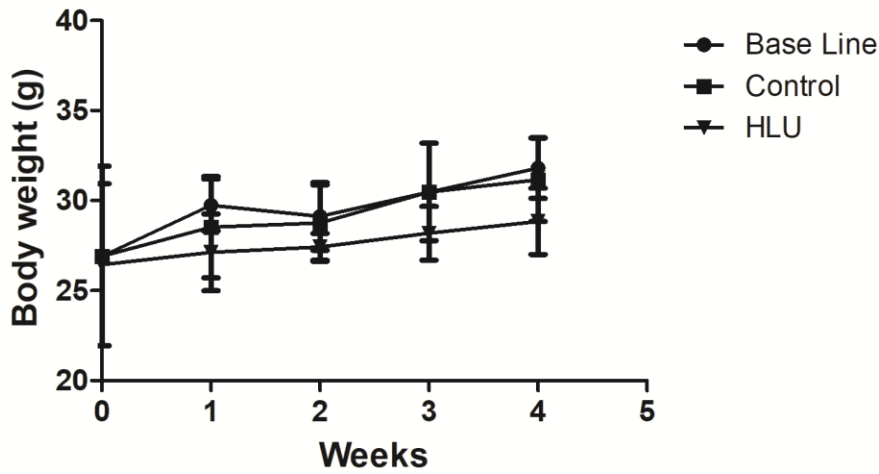
Supplementary Figure 1



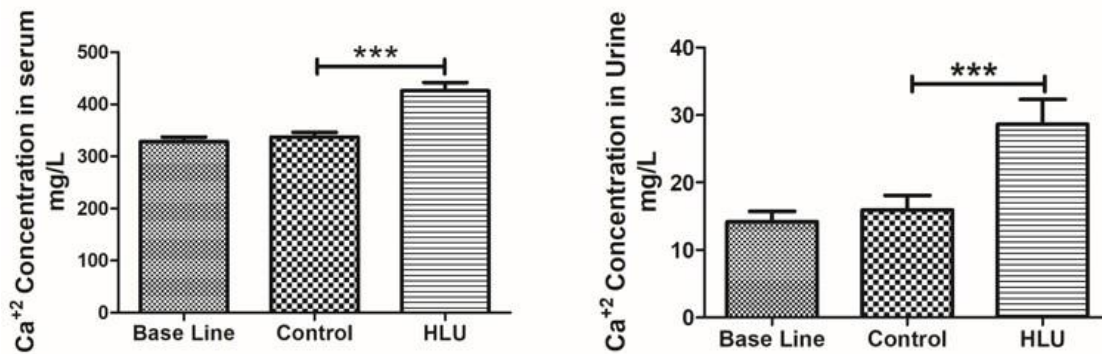
Supplementary Figure 1. Mechanical unloading increases bone resorption and inhibit bone mass *in vivo* (A-F). (A) Schematic diagram illustrating the experimental design (6-months-old, male mice n = 8). (B)

μ CT of the distal femur. Trabecular bone parameters include bone volume/tissue volume ratio (BV/TV), trabecular separation (Tb. Sp) trabecular number (TB. N) and trabecular thickness (Tb. Th). (C) Representative images are showing three dimensional trabecular reconstructive architecture in distal femurs of baseline, control and HLU mice. (D) μ CT measurements of bone mineral density (BMD) and bone mineral content (BMC). (E) Histomorphometric analysis of bone formation-related parameters (Ob.S/BS, MAR, BFR, and N.Ob/B.Pm) in baseline, control and HLU mice (F) qPCR analysis of bone formation-marker genes ALP, Col1a1 and OCN. All data were expressed as means \pm SD. Significance is noted at these thresholds: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA with a *post-hoc* test was performed. Statistical differences between two groups were determined by Student's *t* test. n=8 mice in each group.

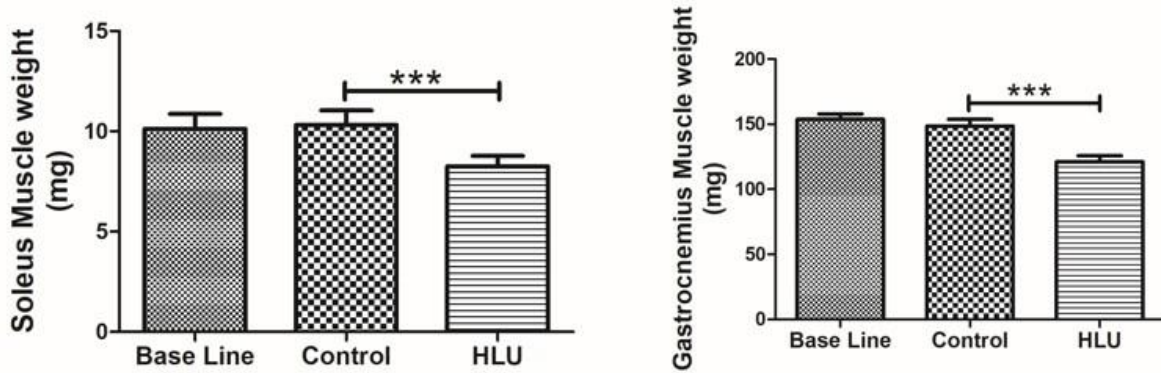
Supplementary Figure 2A



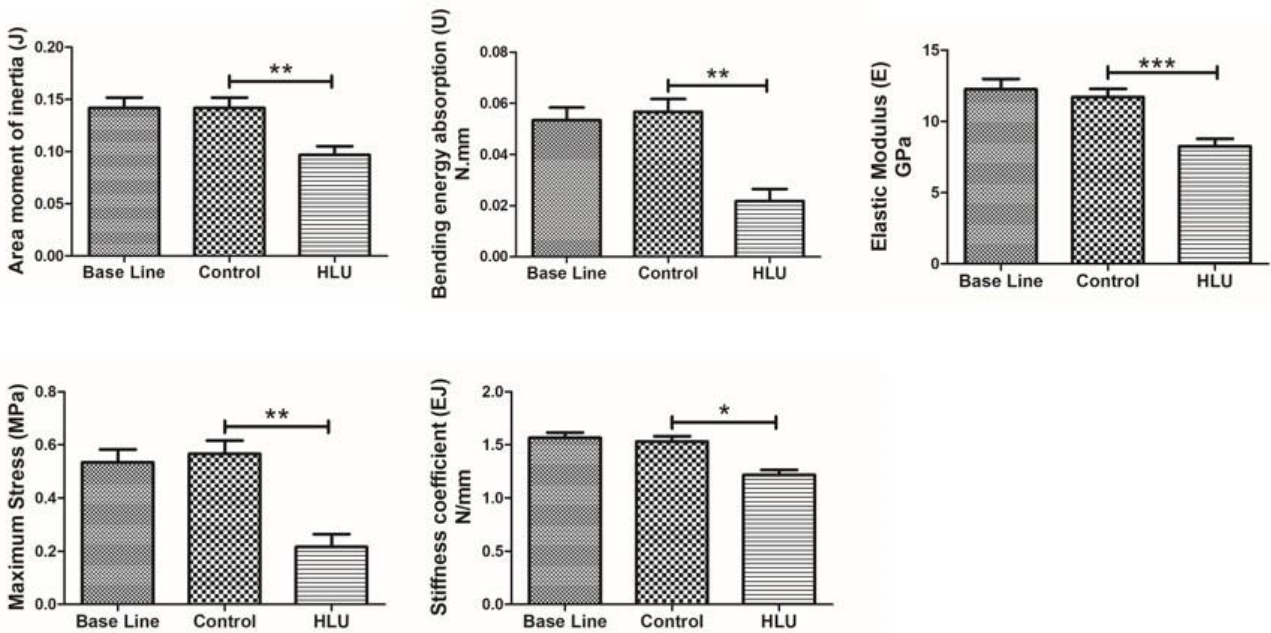
Supplementary Figure 2B



Supplementary Figure 2C



Supplementary Figure 2D



Supplementary Figure 2. Summary of physical and biochemical analysis. Mice of each group were weighed daily for the first four days and then every two days. Every three days, cages were cleaned. Feeders and bottles were refilled with food and water every day. Weights were presented with \pm SD for each condition of housing. Each experimental group was compared to the others $n = 6$ mice per group. Differences were found to be statistically significant using t test. (A) represents body weight (B) calcium analysis in serum and urine (C) Soleus and Gastrocnemius muscle weight (D) Effect of unloading on femoral strength as assessed by three-

point; Area moment of Inertia; Bending energy absorption; Elastic modulus; Maximum force; Stiffness coefficient. All data were expressed as means \pm SD. Significance is noted at these thresholds: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA with a post-hoc test was performed. Statistical differences between two groups were determined by Student's t test. n=8 mice in each group.

Supplementary Figure 3A

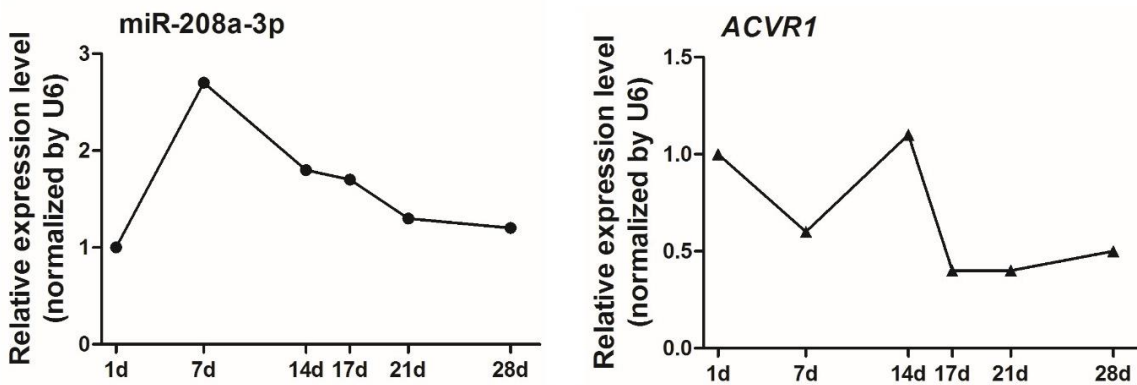
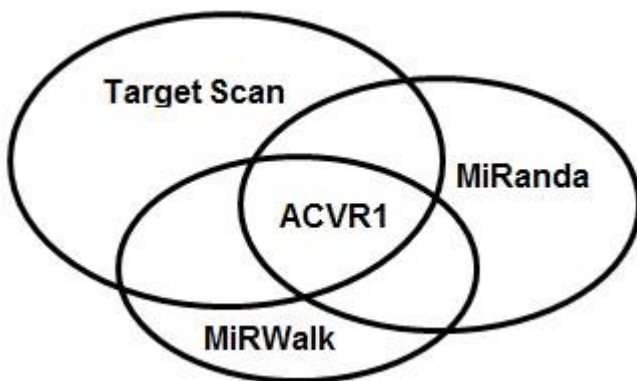


Figure 3. miR-208a-3p targets ACVR1 to functionally inhibit osteoblast activity *in vitro*. (A) Relationship between miR-208a-3p and ACVR1 mRNA level (determined by real-time PCR) during osteoblast mineralization in MC3T3-E1 cells.

Supplementary Figure 4A



Supplementary Figure 4B

Predicted consequential pairing of target region (top)		Seed	match	Pcr
and miRNA (bottom)				
Position 1101-11053 of ACVR1 3'UTR	5'-CGGCCUAAGGACAGUCUAGACUG-		8mer	0.87
mmu-miR-208	3' GCCGGAUGACGAGCAGAGAGCGC			

Supplementary Figure 4. Bioinformatics analysis predicted the relationship between miR-208a-3p and *ACVR1*. (A) Bioinformatics analysis by using miRNA target prediction software programs: TargetScan, miRanda, and miRWalk to screen for miR-208 targeting genes. (B) Bioinformatics analysis using TargetScan to predict relationship of miR-208 and its target gene *MACF1* and conserved site in UTR.

Supplementary Tables

Supplementary Table 1. MiRNAs primer sequence

microRNAs	Primer sequence (5'to3')
mmu-miR-30	CUUUCAGUCGGAUGUUUGCAGC
mmu-miR-33-5p	GUGCAUUGUAGUUGCAUUGCA
mmu-miR-96-5p	UUUGGCACUAGCACAUUUUUGCU
mmu-miR-103a	AGCAGCAUUGUACAGGGCUAUCA
mmu-miR-130b-5p	ACUCUUUCCUGUUGCACUACU
mmu-miR-137	ACGGGUAUUCUUGGGUGGAUAAU
mmu-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG
mmu-miR-148a-3p	UCAGUGCACUACAGAACUUUGU
mmu-miR-154-3p	AAUCAUACACGGUUGACCUAUU
mmu-miR-183-5p	UAUGGCACUGGUAGAAUUCACU
mmu-miR-208a-3p	AUAAGACGAGCAAAAAGCUUGU
Mmu-miR-365-3p	UAAUGCCCCUAAAAUCCUUAU
Mmu-miR-384-5p	UGUAAACAAUCCUAGGCAAUGU
mmu-miR-542-3p	UGUGACAGAUUGAUAAACUGAAA
U6-Forward	GTGCTCGCTTCGGCA GCA CATAT
U6-Reverse	AAAATATGGAA CGCTTCACGAA

Supplementary Table 2: mRNA primer sequence

Gene name	Primer sequence (5'to3')
<i>ACVR1</i> -forward	GCAACCAAGAACGCCTCAATC
<i>ACVR1</i> -Reverse	TTCCCGACACACTCCAACAG
<i>GAPDH</i> - Forward	TGCACCACCAACTGCTTAG
<i>GAPDH</i> - Reverse	GGATGCAGGGATGATGTTC
<i>ALP</i> - Forward	GTTGCCAAGCTGGGAAGAACAC
<i>ALP</i> - Reverse	CCCACCCCGCTATTCCAAAC
<i>Col 1a1</i> - Forward	GAAGGCAACAGTCGATTCACC
<i>Col 1a1</i> - Reverse	GACTGTCTTGCCCCAAGTTCC
<i>Ocn</i> - Forward	GAACAGACTCCGGCGCTA
<i>Ocn</i> - Reverse	AGGGAGGATCAAGTCCCG
<i>Runx2</i> - Forward	TGCACCTACCAGCCTCACCATAC
<i>Runx2</i> - Reverse	GACAGCGACTTCATTCGACTTCC
<i>BMP2</i> - Forward	CGAGACCTTCCAGATCACAGT
<i>BMP2</i> - Reverse	GGGAAGCAGCAACACTAGA
<i>Smad1</i> - Forward	ACGCTGCTCATCCCATAAT
<i>Smad1</i> - Reverse	AGTTCCGCGTCATCCTGATA
<i>Smad5</i> - Forward	AACCTGAGCCACAATGAACC
<i>Smad5</i> - Reverse	GTGGCATATAGGCAGGAGGA