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Mechanical Signal Influence on Mesenchymal Stem Cell Fate is Enhanced by Incorporation of Refractory Periods into the Loading Regimen

Buer Sen¹, Zhihui Xie¹, Natasha Case¹, Maya Styner¹, Clinton T Rubin², and Janet Rubin¹

Buer Sen: buer_sen@med.unc.edu; Zhihui Xie: zxie@med.unc.edu; Natasha Case: ncase@med.unc.edu; Maya Styner: mstyner@med.unc.edu; Clinton T Rubin: clinton.rubin@sunysb.edu; Janet Rubin: jrubin@med.unc.edu

¹Department of Medicine, University of North Carolina, Chapel Hill, NC, 27599

²Department of Biomedical Engineering, State University of New York, Stony Brook, NY, 11794

Abstract

Mechanical signals of both low and high intensity are inhibitory to fat and anabolic to bone in vivo, and have been shown to directly affect mesenchymal stem cell pools from which fat and bone precursors emerge. To identify an idealized mechanical regimen which can regulate MSC fate, low intensity vibration (LIV; < 10 microstrain, 90 Hz) and high magnitude strain (HMS; 20,000 microstrain, 0.17 Hz) were examined in MSC undergoing adipogenesis. Two × twenty minute bouts of either LIV or HMS suppressed adipogenesis when there was at least a 1 hour refractory period between bouts; this effect was enhanced when the rest period was extended to 3 hours. Mechanical efficacy to inhibit adipogenesis increased with additional loading bouts if a refractory period was incorporated. Mechanical suppression of adipogenesis with LIV involved inhibition of GSK3 β with subsequent activation of β -catenin as has been shown for HMS. These data indicate that mechanical biasing of MSC lineage selection is more dependent on event scheduling than on load magnitude or duration. As such, a full day of rest should not be required to “reset” the mechanical responsiveness of MSCs, and suggests that incorporating several brief mechanical challenges within a 24 hour period may improve salutary endpoints in vivo. That two diverse mechanical inputs are enhanced by repetition after a refractory period suggests that rapid cellular adaptation can be targeted.

Keywords

Mechanotransduction; Adipogenesis; Obesity; Osteoblastogenesis; β -catenin

1. INTRODUCTION

Exercise influences a range of physiologic systems, and is part of both prevention and treatment strategies for diseases including obesity and osteoporosis. Often examined within

Corresponding author: Buer Sen MD, buer_sen@med.unc.edu, Tel: 919.966.6744. Mail: 5032 Burnett Womack, CB #7170, University of North Carolina, Chapel Hill, NC 27599.

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Conflict of interest

Clinton Rubin declares an association with Marodyne Medical.

the context of the musculoskeletal and adipose systems, exercise in general - and mechanical signals in particular - are recognized to be anabolic to bone and muscle and inhibitory to formation of fat. Mechanically mediated influences on musculoskeletal and fat phenotypes are achieved not only through the resident cell population (osteocytes, adipocytes, myocytes), but also by biasing the differentiation of their common progenitor, the mesenchymal stem cell (MSC) (Krishnan et al., 2006; David et al., 2007; Menuki et al., 2008). Studies using high magnitude mechanical loading, a regimen that assumes that benefits of exercise respond to increasing intensity and duration, and that a single daily session is sufficient to maximize any beneficial response (Egan et al., 2010), show that mechanical signals are recognized directly by the MSC population, and serve to promote osteoblastogenesis and inhibit adipogenesis even under environmental conditions inducing fat formation (Sen et al., 2008). If we allow that assumptions about intensity, duration and daily repetition are incorrect, however, we may be able to improve design of mechanical regimens. The sensitivity of MSC to mechanical signals allows study of a single target where comparisons can be made between diverse types of mechanical input generated during loading, including both high and low intensity strain.

That non-strenuous loading such as standing or walking can impact aging and “disuse” provides evidence that neither magnitude nor duration is essential to mechanical challenge (Rubin and Rubin 2010). Even brief exposure to high frequency, small accelerations (30Hz/0.3g/20 min/day) can stimulate bone formation and inhibit adiposity (Rubin et al., 2001; Garman et al., 2007; Rubin et al., 2007; Humphries et al., 2009; Slatkowska et al., 2010; Wenger et al., 2010). Refining mechanical regimens to control MSC commitment should be an important goal to harness this non-pharmacologic strategy.

Mechanical biasing of MSC lineage emphasizes the reciprocal relationship between bone and fat formation: decreased adipogenesis due to PPAR γ haploinsufficiency results in enhanced bone acquisition (Akune et al., 2004). This osteoblast/adipocyte relationship relies on β -catenin, which modulates adipogenesis through attenuation of PPAR γ expression (Ross et al., 2000). Further, β -catenin is critical to mechanical restraint of adipocyte differentiation; mechanical inhibition of adipogenesis is prevented by β -catenin knockdown (Sen et al., 2009). Mechanical phosphorylation and inhibition of GSK3 β serves to preserve β -catenin (Armstrong et al., 2007; Sen et al., 2008) and also prolongs NFATc1 activation resulting in upregulation of COX2, which in turn promotes MSC commitment towards an osteochondroprogenitor lineage (Sen et al., 2009).

It is important to understand the types of mechanical signals recognized by MSC. We here investigate whether brief periods of Low Intensity Vibration (LIV, low strain/high frequency) can influence MSC commitment in a manner similar to High Magnitude Strain (HMS, high strain/low frequency), and if these mechanical effects could be enhanced by incorporating a refractory period between loading events.

2. MATERIALS AND METHODS

Experimental Design

MSC grown under media conditions which promote adipogenesis by 5–8 days were exposed to two distinct mechanical regimens. High magnitude strain (HMS) delivered as a 6h daily protocol was used as a starting point (Sen et al., 2008) for comparison with effects of low intensity vibration (LIV) to inhibit adipogenesis. To determine if modifications in delivery of mechanical regimens could influence efficacy, the duration and number of daily applications were altered, and further separated by a refractory period. Molecular pathways involved in the cellular response were compared between HMS and LIV.

Reagents

Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA). Culture media, trypsin-EDTA reagent, antibiotics, Lipofectamine 2000, reverse transcriptase, *Taq* polymerase and siRNA were from Invitrogen. Insulin and SB415286 were from Sigma-Aldrich. DKK-1 was from R & D Systems (Minneapolis, MN). RNA isolation kit was from Qiagen (Valencia, CA).

Cells and culture conditions

C3H10T1/2 embryonic MSCs were maintained in growth medium (α -MEM, 10% FBS, 100 μ g/ml penicillin/streptomycin) before plating 6,000–10,000 cells/cm² 2d before loading. Experimental media were adipogenic (0.1 μ M dexamethasone, 5 μ g/ml insulin, $\pm$ 50 μ M indomethacin) or multipotential (50 μ g/ml ascorbic acid, 1 μ M β -glycerophosphate, 10 nM dexamethasone, 10 nM all trans-retinoic acid, 5 μ g/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine). siRNA (100 nm) was transfected in serum-free OptiMEM overnight.

Key experiments were replicated in marrow derived MSC (mdMSC) generated from C57Bl/6 wild-type mice that readily undergo differentiation into multiple lineages (Case et al., 2010).

HMS: High Strain Magnitude

Uniform biaxial strain was applied to MSC plated on collagen-I coated silicone membrane plates using a biaxial deformation device (Schaffer et al., 1994; Sen et al., 2008), with all cultures remaining in the incubator. The loading regimen consisted of peak strains of 2.0% at 0.17 Hz.

LIV: Low Intensity Vibration

An amplified loudspeaker was used to generate a 0.7g peak, 90Hz sinusoidal accelerations through culture dishes (Figure 1AB). Approximate displacements of 0.04mm were required to deliver this mechanical regimen. Single element strain gages, with a sensitivity to detect $\pm$ 5 microstrain (0.0005%), were used to establish the magnitude of strain generated by LIV at the center of the culture dish. Accelerometer measurements were used to quantify the mechanical information delivered at the dish bottom. All dishes were outside incubator during loading either on the bench (control) or loudspeaker (LIV) such that - within an experimental series - all dishes were handled equivalently.

Protein Fractionation

After 1 $\times$ PBS wash, cell pellets were re-suspended in 0.33 M sucrose, 10mM Hepes, pH 7.4, 1mM MgCl₂, 0.1% Triton X-100 and placed on ice $\times$ 15 min. Cytoplasmic supernatant was collected and nuclear pellet re-suspended (0.45 M NaCl and 10mM Hepes, pH 7.4) for 15 min, re-pelleted and nuclear supernatant collected.

Real-time RT-PCR

Primers were PTHR1 forward, 5'-CAAGAAGTGGATCATCCAGG-3'; PTHR1 reverse, 5'-TAGTGGACACCGAAGAGTGG-3'; OC forward, 5'-CTGACCTCACAGATGCCAA-3'; OC reverse, 5'-GGTCTGATAGCTCGTCACTCAA-3'; 18S forward, 5'-GAACGTCTGCCCTATCAACT-3'; 18S reverse, 5'-CCAAGATCCAACCTACGAGCT-3'. Standards and samples were run in triplicate and normalized for 18S amplicons as in (Rubin et al., 2002).

Western blotting

Antibodies included those targeting active β -catenin (Upstate, Temecula, CA), total β -catenin (BD, Bedford, MA), total GSK3 β (Chemicon, Billerica, MA), aP2, PPAR γ 2, adiponectin, COX2, NFATc1 and tubulin (Santa Cruz, CA). Blotting performed as in (Sen et al., 2008). Densitometry was determined using NIH ImageJ, 1.37v.

Histochemical staining

After cell fixation in 2% formaldehyde, cytoplasmic triglyceride droplets were stained with oil-red-O.

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical significance was evaluated by two-way ANOVA or t-Test (GraphPad Prism). Densitometry data, where given, were compiled from at least 3 separate experiments.

3. RESULTS

Strain and acceleration generated by LIV

Strain at the bottom of the culture dish resulting from the 90Hz, 0.04mm displacement of the loudspeaker/stage/dish system was not detectable, despite a strain gage system capable of detecting less than ten microstrain (less than 0.0005% strain, or five microstrain, peak to peak; data not shown). The 90Hz accelerations measured at the dish bottom indicated a high fidelity sine wave (Fig 1C), with peak accelerations at approximately 0.7g. A Fast Fourier Transform of the LIV signal emphasized that >99% of the power was delivered at 90Hz (Fig 1D). Thus, strain at the dish bottom of the HMS system was at least 2000 $\times$ greater than the LIV system, while the frequency of the LIV system was 530 $\times$ higher.

Suppression of adipogenesis by high and low magnitude mechanical signals

Uninterrupted HMS delivered for 2 or 6 hours daily (0.17Hz; 1200 or 3600 cycles of strain, respectively) prevented acquisition of the fat phenotype over 7 days. The fat marker adiponectin was repressed >50% by 2 hours/day HMS, and near completely inhibited by 6 hours daily HMS (figure 2A, left panel). While HMS 40 min/day was still somewhat effective (figure 2A, right panel), 20 min/day failed to prevent fat differentiation. When the HMS regimen was bisected into two 20 minute periods each day, separated by at least 1 hour, adipogenesis was inhibited to a degree similar to that seen with 6 hour loading. Inhibition of adipogenesis was confirmed by decreased expression of the fat transcription factor, PPAR γ 2, which decreased more significantly when a 40 min daily regimen was divided into 2 $\times$ 20 minute sessions separated by a 1 hour refractory period. That twice daily 20 minute HMS were more effective than single treatments of 40 or 120 minutes suggested that a refractory period inserted between mechanical treatments enhanced responsiveness.

Daily treatments of either 20 or 40 min of LIV for 7 days failed to suppress adipogenesis. However, when the LIV regimen was divided into 2 $\times$ 20 minute bouts separated by > 1 hour, fat markers adiponectin, PPAR γ 2 and aP2 decreased (figure 2B,C), and development of lipid granules was prevented (figure 2D). While HMS is known to increase cytoplasmic β -catenin (Armstrong et al., 2007; Sen et al., 2008), increases in cytoplasmic β -catenin were measurable after LIV (figure 2C).

When cultures were grown in presence of indomethacin, which accelerates the rate of adipogenesis (Styner et al., 2010), twice daily LIV failed to inhibit adipogenesis, while HMS

was efficacious causing reductions in adiponectin, PPAR γ and increasing β -catenin (figure 2E).

Suppression of adipogenesis is dependent on the refractory period between loading events

Inhibition of adipogenesis by both HMS and LIV was enhanced by incorporating a refractory period between loading events. To further define the specifics of the refractory period, MSC were treated with 2 $\times$ LIV daily over 7 days, with mechanical bouts separated by either 1 or 3 hours. Repression of adiponectin protein (figure 3A, n=3) seen with 2 LIV treatments separated by a 1 hour rest period (decreased 30% $\pm$ 8%, p<0.05) was less effective than when LIV treatments were separated by 3 hours (70% $\pm$ 5% below control, p<0.01, n=3). Increasing the refractory period from 3 to 6 hours failed to further influence efficacy (figure 3B).

Increasing the number of LIV treatments from two to three bouts daily separated by 3 hours significantly enhanced mechanical inhibition of adipogenesis (figure 3C). Densitometry shows that 2 $\times$ LIV spaced at 9 hours reduced adiponectin by 40 $\pm$ 6%, p<0.05, while 3 $\times$ LIV spaced at 3 hours reduced adiponectin protein by 70% $\pm$ 2% compared to control (p<0.01).

LIV was also studied in mdMSC (Case et al., 2010), where adiponectin and lipid granules develop after 8 days under adipogenic conditions (Sen et al., 2009). Separation of 2 LIV bouts by 1 hour each day for 8 days was ineffective in reducing adiponectin (figure 3D left), but with the refractory period between loading bouts increased to 3 hours, LIV repressed adiponectin. The anti-adipogenic effect was further enhanced by increasing to three bouts daily, each separated by 3 hours (figure 3D right).

Mechanical signals enhance MSC responsiveness to BMP2

To evaluate whether mechanical signals promote osteogenesis, C3H10T1/2 MSC were grown in multi-potential medium and subjected to daily 2 $\times$ LIV separated by 3 hours for 8 days. LIV treatment enhanced PTHR1 expression by 33% $\pm$ 15% (p<0.001) (figure 4A) but did not significantly increase expression of osteogenic osterix and osteocalcin. To query whether mechanical signals might preserve multipotentiality, cells were cultured in adipogenic medium $\pm$ 2 $\times$ LIV separated by 3 hours for 7 days before switching to multipotential medium $\pm$ BMP2 100 ng/ml. LIV alone did not induce either PTHR1 or osteocalcin mRNA as compared to control (figures 4B and C). The addition of BMP2 for 2 days increased osteoblast phenotype in all cultures despite 7 day exposure to adipogenic medium; importantly, pre-treatment with the LIV regimen significantly enhanced response to BMP2, as shown by a 100% $\pm$ 26% (p<0.01) increase of PTHR1 expression and an increase of 50% $\pm$ 6% in osteocalcin (p<0.05) above that of control cultures.

LIV inhibition of adipogenesis requires β -catenin signaling via GSK3 β inhibition

The role of β -catenin in LIV efficacy was probed. siRNA targeting β -catenin (+siCat) or a siRNA scrambled control (–siCat) was added to MSC (Sen et al., 2009). When β -catenin was knocked down, LIV did not inhibit adipogenesis as shown by similar adiponectin and PPAR γ 2 in control and LIV treated cultures (figure 5A). The ability of the GSK3 β inhibitor SB415286 to prevent adipogenesis was also blocked by β -catenin knock-down (figure 5B). We next considered whether LIV's potential utilization of the GSK3 β / β -catenin pathway utilizes a Wnt autocrine loop. DKK1 added to cultures at doses that inhibit Wnt/Lrp signaling (Case et al., 2008; Sen et al., 2009), failed to block LIV's anti-adipogenic effect (figure 5C).

HMS inhibition of GSK3 β causes preservation and activation of β -catenin (Sen et al., 2009). In contrast to the HMS induced β -catenin activation (figure 2E), LIV did not increase β -catenin in whole cell lysates (figure 2C). Fractionating nuclear and cytoplasmic proteins, however, revealed that LIV did increase active β -catenin present in the nucleus (figure 5D).

HMS inhibition of GSK3 β also promotes nuclear accumulation of NFATc1, a transcription factor responsible for increased COX2 expression (Sen et al., 2009). In MSC treated with LIV (2 $\times$ 20 min separated by 3hours), consistent increases in COX2 expression were not seen. However, by fractionating nuclear and cytoplasmic proteins, LIV was shown to increase both nuclear NFATc1 and cytoplasmic COX2 expression (figure 5D), confirming an effect on GSK3 β . LIV had similar effects on NFATc1/COX2 in mdMSC (figure 5E).

Higher levels of GSK3 β inhibition are required to activate NFATc1 than β -catenin as showed by SB415286 dosing; in mdMSC, β -catenin activation was seen with SB415286 5 μ M, but NFATc1/COX activation did not appear until ≥ 10 μ M (figure 5F). To determine GSK3 β involvement in LIV effects on NFATc1/COX2, GSK3 β was knocked down by targeted siRNA. When GSK3 β was deficient, both nuclear NFATc1 and COX2 increased (figure 5G). COX2 induction by LIV requires GSK3 β as demonstrated by an absent COX2 response MSC when GSK3 β was silenced (lanes 3/4).

4. DISCUSSION

Mechanical signals provide regulatory information to prokaryotic and eukaryotic cells, catalyzing adaptive changes in morphology that accommodate new loading challenges. In humans, mechanical signals – most often considered in the context of exercise – affect a range of physiologic systems and are essential in achieving a robust musculoskeletal system, and central to a goal of slowing formation of adipose tissue. Despite general agreement that mechanical signals play a central role in defining fat and bone/muscle phenotypes (Engler et al., 2006; Lee et al., 2010), the ideal characteristics of an effective loading regimen, or the molecular cues which control the adaptive responses are poorly understood. Here we provide insights into the characteristics by which physical input controls MSC lineage allocation, and ultimately the formation of both fat and bone.

Extremely small bone strains (<0.003%), induced at a sufficiently high frequency (10–100Hz), are anabolic to bone (Rubin et al., 2001a). Importantly, mechanically mediated bone remodeling exhibits a strong interdependence of strain magnitude and cycle number, such that bone mass can be enhanced either with a few large strain events or 100,000's of extremely low magnitude strain signals (Qin et al., 1998). This leads to the hypothesis that bone structure depends as much on extremely low magnitude, high frequency strains arising during predominant activities (i.e., standing), as it does on rarer, large strain events generated during strenuous activity (Fritton et al., 2000). Recent evidence confirms that extremely small mechanical signals are both anabolic to bone (Rubin et al., 2001a; Slatkowska et al., 2010), and suppress the isocaloric formation of adipose tissue: growing mice subject to a daily LIV signal for 12 weeks acquired about 25% less fat than controls (Rubin et al., 2007b). The work presented here, with the suppression of adipogenesis by the LIV signal as achieved by negligible strain yet significant accelerations, suggests that bone matrix deformation may not be the exclusive agent of mechanotransduction in musculoskeletal tissues (Garman et al., 2007b).

The ability of both high and low intensity mechanical challenges to promote bone and decrease fat has been demonstrated in vivo, but difficulty in delivering physiologically relevant mechanical signals to cells in vitro has created hurdles in identifying molecular events that control these adaptive responses. With the loading systems described here it

becomes possible to compare the biologic responsiveness to spectral extremes of mechanical signals, and through controlled changes in loading schedules, to begin to optimize influential loading parameters. While prior studies have demonstrated the importance of cell or substrate distortion (Song et al., 2007), a similar anti-adipogenic response was here obtained with both high and low magnitude strain. Recent in vivo evidence suggests that some mechanical responses even occur in the absence of matrix strain, through acceleration rather than tissue loading (Judex and Rubin, 2010). As such the physical acceleration of a cell may represent a generic signal that transmits information via cytoskeletal or cell/matrix interrelationships, rather than requiring substrate deviation.

Independent of the magnitude of the regimen, mechanical control of MSC lineage allocation was markedly enhanced when the stimulus was separated into multiple applications separated by refractory periods of at least one hour, indicating that scheduling of events was at least as important as input duration. However, under extreme adipogenic constraints, only the HMS regimen effectively inhibited adipogenesis, suggesting that there is, ultimately, a critical combination of signal parameters necessary to combat environmental cues. Nevertheless, application of LIV three times daily, while adhering to a 3 hour refractory period between bouts, markedly increased the ability of this extremely low intensity mechanical challenge to inhibit fat formation. Interestingly, incorporation of a rest cycle between individual loading cycles has also been shown to enhance cell response, and represents another parameter which can be optimized to maximize mechanical response (Srinivasan et al., 2002; Batra et al., 2005). In sum, within a 24 hour period, brief but repetitive loading challenges can develop an adaptive response greater than that achieved in a single extended daily bout.

Although the LIV signal is several orders of magnitude smaller than the mechanical challenge of HMS, both inputs are transduced through the GSK3 β locus. Both HMS (Sen et al., 2009), and LIV as shown here, suppressed adipogenesis through processes involving β -catenin: when β -catenin was deficient, LIV was unable to restrain adipogenesis. As β -catenin activation by LIV treatment paralleled effects of pharmacological GSK3 β inhibition it was not surprising that LIV also activated NFATc1 and increased COX2. Although LIV induced signaling events are not as great in magnitude as those resulting from HMS, LIV appears to bias MSC from the adipocyte pathway using similar signaling pathways.

While mechanical induction of osteogenic genes in MSC has been reported (Li et al., 2004; David et al., 2007), mechanical inhibition of MSC adipogenesis per se does not reciprocally promote osteogenesis: neither HMS (Sen et al., 2008) nor LIV induced typical osteogenic genes (Runx2, osterix or osteocalcin). However, BMP2 induction of PTH receptor and osteocalcin was enhanced after pretreatment with LIV, suggesting that biochemical and biophysical factors combine to enhance osteogenesis. As both HMS and LIV stimulate bone formation in animal models (Rubin and Lanyon 1987; Rubin et al., 2001a; Garman et al., 2007; de Oliveira et al., 2010), it is not surprising that mechanical signals enhance the effect of other environmental factors.

In sum, both high-magnitude/low-frequency and low-magnitude/high-frequency challenges influence the fate of MSC, repressing adipogenesis and protecting their capacity to respond to osteogenic stimuli. Our data suggest that magnitude is not the dominant determinant of efficacy. Further, that the MSC response to mechanical signals is markedly augmented by incorporating a refractory period of at least 1 hour between loading events, indicates that the scheduling of mechanical signals is perhaps as important as the signals themselves. Additional bouts within 24 hours further build upon the response. This ability of the MSC pool to “reset” its sensitivity to a loading challenge suggests that the impact of mechanical regimens, whether strenuous or subtle, might be effectively delivered with several brief

periods daily, rather than a single extended session. As such, multiple daily bouts of mechanical signals could be leveraged in cases of rehabilitation and recreation, particularly in the context of the degradation of the musculoskeletal system that accompanies both aging and functional compromise.

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Figure 1. LIV instrumentation

A. The LIV signal is delivered by setting the culture dishes on a polyethylene stage, driven exclusively in the Z-direction by an amplified loudspeaker. **B.** The speaker:stage:dish system is driven vertically with 0.7g 90Hz sinusoidal accelerations/decelerations, creating a standing wave at the fluid surface. **C.** Acceleration measurements from each of the four stages, measured using a 3DOF accelerometer at the corners and center of the dishes filled with 2ml of media, indicated a high-fidelity 90Hz sinusoidal waveform through the plates. **D.** A FFT of the acceleration showed that >99% of the power was delivered at 90Hz. No strain was detectable (<10 microstrain peak to peak) at the bottom of the well (data not shown). A full characterization of the mechanical environment at the bottom of the dish as well at the cell surfaces, as performed by finite element modeling, indicates very little shear strain or fluid flow at the cell surface (Uzer et al., 2010).

Figure 2. Mechanical signals prevent adipogenesis of MSC

C3H10T1/2 MSC grown in adipogenic medium $\pm$ mechanical input for 7d (A–D). All experiments were performed with similar results more than 3 $\times$. **A.** HMS (0.17Hz) applied daily for 2 or 6 hours (left panel); or in 20 min increments for 20, 40 min, or 2 $\times$ 20 min with 6 hour spacing (right panel); immunoblots for adiponectin (APN), PPAR γ and tubulin were performed as shown. **B.** LIV applied for 20 min 1 $\times$ or 2 $\times$ daily with 6 hour spacing (left panel), or for 20, 40 min, or 2 $\times$ 20 min with 6 hour spacing (right panel). **C.** 2 $\times$ 20 min daily LIV with 6 hour spacing with measurement of fat markers (APN, aP2, PPAR γ) and β -catenin level. **D.** After LIV treatment, cultures showed reduced Oil-Red-O lipid staining. **E.** MSC grown in a highly adipogenic medium express adipocyte phenotype by 4 days; cultures were treated with 2 $\times$ 20 min LIV or HMS with 6 hour spacing. Immunoblots show that fat markers are reduced with HMS treatment, and that active β -catenin is preserved, but LIV is ineffective in altering MSC fate.

Figure 3. Mechanical efficacy is improved by increasing spacing and number of treatments C3H10T1/2 cells (A – C) or mdMSC (D) in adipogenic medium for 7d were treated with LIV. **A.** Daily 2× 20 min LIV with 1 or 3 hour spacing for 7 days before protein assessment of APN and tubulin loading marker. Densitometry of adiponectin bands from 3 separate experiments in series is shown on right side. Difference from control is shown by single asterisk (*) at $p < 0.05$, and double (**) at $p < 0.01$. **B.** Daily LIV 2× 20 min with 3 or 6 hour spacing shows that 3 hours is maximal spacing, as APN decreased similarly with the longer refractory period. **C.** Daily 20 min LIV with 3 hour spacing between 2 or 3 LIV treatments shows that increasing LIV exposure from 2 to 3 x/day increases the inhibition of APN; densitometry of 3 experiment series with 3 hour spacing, (*) = $p < 0.05$, and (**) = $p < 0.01$. **D.** Daily LIV 2 × 20 min with 1 or 3 hour spacing (left panel) or LIV daily at either 2 or 3 × 20 min treatments with 3 hour (right panel) shows increasing efficacy with spacing and increasing treatment number.

Figure 4. LIV promotes an osteogenic response

Real Time RT PCR was performed with mRNA. Figures represent compiled data from at least 3 experiments. **A.** C3H10T1/2 grown in multipotential medium $\pm 2 \times 20$ min LIV daily with 3 hour spacing for 8days, (***) = $p < 0.001$. **B and C.** MSC were cultured in adipogenic medium $\pm 2 \times 20$ min LIV daily with 3 hour spacing for 7 days then changed to BMP2 (100ng/ml) in multipotential medium; mRNA analyzed at 9 days. 2 way ANOVA shows (a) = different from CTL BMP, $p < 0.05$; (b) = different from LIV BMP, $p < 0.001$; (c) = different from CTL+BMP, $p < 0.01$.

Figure 5. β -catenin and NFATc1 are activated by LIV

A–B, MSC grown in adipogenic medium were treated with β -catenin knock down (+siCat) or scrambled siRNA (0). The next day, LIV (2x20 min, 3 hour spacing) was begun for 7 days. **A.** LIV treatment did not reduce APN and PPAR γ proteins when β -catenin was deficient. Similarly, **B** shows SB415286 (20 μ M) did not prevent adipogenesis with deficient β -catenin. **C.** DKK1 (50ng/ml) did not prevent efficacy of 2x spaced LIV daily. C3H10T1/2 (**D**) and mdMSC (**E**) in adipogenic medium treated with 2 x 20 LIV (3 hour spacing) show active β -catenin and NFATc1 in nuclear fraction increased by LIV (right 2 lanes of each blot, see PARP nuclear marker). Cytoplasmic COX2 (+LDH marker) was increased by LIV. **F.** mdMSC $\pm$ GSK3 β inhibitor, SB415286 x 4 hours as indicated. Nuclear β -catenin rises at 5 μ M, while NFATc1 requires at least 10 μ M. **G.** 1d after GSK3 β knock down (siGSK3 β , 100nm), MSC were exposed to LIV (2 x 20, 3 hour spacing): LIV increased nuclear NFATc1 and cytoplasmic COX2. GSK3 β knockdown increased NFATc1 but prevented LIV induced increase in NFATc1 and COX2.