Cell Mechanosensitivity to Extremely Low Magnitude Signals is Enabled by a LINCed Nucleus

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Abstract

A cell’s ability to recognize and adapt to the physical environment is central to its survival and function, but how mechanical cues are perceived and transduced into intracellular signals remains unclear. In mesenchymal stem cells (MSC), high magnitude substrate strain (HMS, ≥2%) effectively suppresses adipogenesis via induction of FAK/mTORC2/Akt signaling generated at focal adhesions [1]. Physiologic systems also rely on a persistent barrage of low level signals to regulate behavior [2]. Exposing MSC to extremely low magnitude mechanical signals (LMS) suppresses adipocyte formation [3] despite the virtual absence of substrate strain (<0.001%) [2], suggesting that LMS-induced dynamic accelerations can generate force within the cell. Here we show that MSC response to LMS is enabled through mechanical coupling between the cytoskeleton and the nucleus, in turn activating focal adhesion kinase (FAK) and Akt signaling followed by FAK-dependent induction of RhoA. While LMS and HMS synergistically regulated FAK activity at the focal adhesions, LMS-induced actin remodeling was concentrated at the perinuclear domain. Preventing nuclear-actin cytoskeleton mechanocoupling by disrupting LINC (Linker of Nucleoskeleton and Cytoskeleton) complexes inhibited these LMS-induced signals as well as prevented LMS repression of adipogenic differentiation, highlighting that LINC connections are critical for sensing LMS. In contrast, FAK activation by high magnitude strain (HMS) was unaffected by LINC decoupling, consistent with signal initiation at the focal adhesion.

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Author Contributions

G.U.: Concept/design, collection/assembly of data, data analysis/interpretation, manuscript writing, final approval of manuscript; W.T., B.S., Y.S., M.S., C.T.R., S.J. and K.B.: data analysis/interpretation, final approval of manuscript; Z.X., S.M. and G.B.: collection/assembly of data; J.R.: concept/design, financial support, data analysis/interpretation, manuscript writing, final approval of manuscript.
(FA) mechanosome. These results indicate that the MSC responds to its dynamic physical environment not only with “outside-in” signaling initiated by substrate strain, but vibratory signals enacted through the LINC complex enable matrix independent “inside-inside” signaling.

Keywords
Mesenchymal stem cells; Vibration; Strain; Nucleus; Nesprin; FAK; Akt; RhoA

Introduction
Since the beginning of life, mechanical cues have guided cell fate and function. The role of mechanical signaling in defining cell fate is evidenced in the pluripotent mesenchymal stem cells (MSC) that regenerate and repair tissues [2, 4]. Lineage guidance of MSCs relies in part on physical cues derived from the environment [5]. Strain of bone and muscle during daily activity suppresses adipogenesis [6] while promoting osteogenesis [7] and myogenesis [8]. In vitro, when cells are attached to an extracellular matrix (ECM), mechanical cues derived from substrate deformation or quality (e.g., stiffness and topology) can be transmitted through focal adhesion (FA) connections to initiate signal pathways that cause reorganization of cytoskeletal structure [9, 10] and allow auto-modulation of signal strength transmission to the nucleus [11]. Mimicking exercise, in vitro application of high magnitude substrate strain (HMS, ≥2%) effectively suppresses adipogenesis via induction of FAK/mTORC2/Akt signaling generated at FAs [1]. Physical signals that regulate biologic functions, however, do not necessarily need to be large to be influential. Physiologic systems ranging from hair cells responding to sound in the cochlea [12] to circadian rhythms of Drosophila [13] rely on a persistent barrage of low magnitude, high frequency signals. Moreover, application of high frequency, low magnitude signals (LMS) copy high impact exercises to improve musculoskeletal function [14, 15], decrease adipose encroachment in the bone marrow in vivo [2, 16] and augment MSC osteogenesis [17] while decreasing adipogenesis [3] in vitro. In contrast to HMS signaling that depends on matrix strain, the mechanism by which LMS is perceived and induces relevant signaling pathways in cells is not clear.

Despite their physiologic relevance, little is known about how very small signals, such as LMS, are perceived at the cell or nuclear level to control function [18]. LMS creates a complex local loading environment that depends on many factors including frequency, amplitude and viscosity [19]. Peak strains generated by LMS are on the order of ~1–2 micro strain (µε) [20] while cell responses linked to HMS are usually applied at ≥0.000µε [9, 21, 22], suggesting that substrate deformation does not contribute to the LMS response. Current literature addressing how cells sense vibration is focused on computational analyses or correlations that do not directly speak to the mechanisms responsible for the cell response to vibration [23–26]; indeed research has been largely limited to functional outcome assays describing cell osteogenesis, adipogenesis, proliferation or tissue/organism level response [27–55].
Remodeling its cytoskeleton in response to the surrounding physical environment allows the cell to actively participate in the mechanoregulation of cell fate and function [56]. Not only does internal cell tension driven by RhoA activity directly modulate cell differentiation [57] but mechanically-guided cytoskeletal remodeling alters signal transmission [58]. For example, HMS induced remodeling of the MSC actin cytoskeleton, enhances connections through new FA complexes and results in amplification of mechanically generated signals in response to repeated force [1] thus more efficiently suppressing MSC adipogenesis. Scaling the same analogy to the organismal level, shorter but repeated exercise durations have shown to be more effective in improving glycemic control compared to a single longer duration [59]. Not surprisingly, repeated exposure to LMS was also more effective than a single bout in controlling MSC lineage decisions [3], presumably due to adaptive remodeling of cell structure[17, 55].

Ultimately, mechanical force influences the nuclear transcriptional machinery via physical and biochemical means [60–62]. The mechanical connection between the cytoskeleton and the nucleus is transferred via the LINC complex [63, 64]. LINC-associated giant isoforms of Nesprin-1 and Nesprin-2 bind actin filaments through their N-termini [65, 66] and SUN proteins via their C-terminal KASH (Klarsicht, ANC-1, Syne Homology) domains [67]. LINC complexes form a filamentous network on the nuclear surface [68], perhaps akin to focal adhesions [69, 70], where force can dynamically alter LINC mediated mechanocoupling between nucleus and cytoskeleton [71, 72]. LINC mediated force has been shown to regulate nuclear structure and function [73, 74]. Pathologic alterations in the nuclear structure, including lamin mutations common to progeria and depletion or dislocation of giant Nesprins, disrupt LINC connections [64, 75] to interfere with cellular processes including proliferation [76], migration [64] and differentiation [77]. Recently, forces applied via Nesprin bound magnetic beads were shown to cause phosphorylation of the Lamin/LINC binding partner Emerin resulting in increased nuclear stiffness [78].

The cytoplasmic cytoskeleton, connected to substrate via peripheral focal adhesions, spans through F-actin stress fibers to attach to LINC on the outer nuclear membrane, in this way transmitting forces from outside the cell inwards [79]. In smooth muscle cells, dissection of a single apical actin stress fiber generates a force of 65nN on the nucleus [80]. Switching between weak and strong LINC-actin coupling states can generate up to 40nN force differentials which are an order of magnitude larger than the cytoskeletal forces required to initiate F-actin assembly and signaling (~10–50pN) [81]. This suggests that forces generated by LINC-actin coupling alone should be able to generate sufficient internal force from inside the cell to initiate signaling events. The positive correlation between rate of acceleration and LMS response [23] suggests the possibility that the nucleus might participate in LMS-induced signaling as a passive structural element by virtue of its denser/stiffer nature. We previously explored this possibility in silico and found that LMS-induced accelerations caused relative nuclear motions that were 100 to 1000 times larger than those generated by LMS-induced fluid shear stresses [26]. Supportive of the hypothesis that nucleus might participate in the sensing of vibratory signals, Sun1−/− mice gradually become deaf [82], thus strengthening the notion that LINC may be important for vibrational sensing, including sound.
Here, using biochemical and imaging techniques, we approach the question of how LMS generates signaling, considering whether LMS and HMS utilize same signaling mechanisms to initiate cells response. We address whether LMS or HMS are perceived in the same way and, more specifically, ask if LMS directed signaling and regulation of MSC differentiation require LINC facilitated mechanical coupling between the nucleus and cytoskeleton.

**Experimental Design**

MSCs were seeded at 100k/well in 6-well polystyrene plates (LMS) or in Bioflex Collagen-I coated silicone plates [3] (HMS, LMS or LMS+HMS). LMS was applied one time (1X), and repeated after a 2h rest period (2X) in the form of high frequency low magnitude vibration of 0.7g (1g = Earth’s gravitational field) at 90Hz for 20min at RT. HMS was applied as a uniform uniaxial strain of 2% at 0.17Hz for 20 min at RT. First, we studied the LMS-induced FAK phosphorylation (p-FAK, Tyr397) events by a time course study to test if 1X LMS served to augment the second (2X) LMS. We then investigated the cellular adaptations following 1X LMS by FA isolation and RhoA activation assays. We further tested if FAK phosphorylation was necessary for the RhoA activity via PF573228 (3µM) pretreatment. We then asked if activating RhoA alone via LPA (Lysophosphatidic acid 30µM) also amplifies subsequent LMS response. Alternatively, we also tested if HMS and LMS work synergistically to amplify each other using combinations of LMS+HMS. Role of the cytoskeleton in facilitating LMS-induced FAK activation was tested by disrupting the actin and microtubule cytoskeletons as well as cellular tension via pretreatment of Cytochalasin-D (0.2µM), Colchicine (1µM) and Y27632 (10µM). We used immunofluorescence to determine if LMS causes rearrangement of the actin cytoskeleton.

To test whether LINC mediated mechanocoupling of nucleus and cytoskeleton was required for LMS mechnoresponse, we measured LMS induced FAK and Akt activation as well as modulation of MSC adipogenesis after the nuclear envelope LINC complex was disrupted by siRNA treatment of SUN1&2 [63] or by overexpression of a dominant negative form of Nesprin KASH domain [64]. A role of Emerin in LMS signaling was queried using a targeting siRNA. Finally, to identify differences in proximal signaling due to LMS and HMS, mechanically activated Akt was quantified by blocking FAK activity or use of siRNA targeting the FAK co-modulator Fyn [21].

**Results**

**Repeated LMS exposures generate RhoA-reinforced cell structure to augment mechanically-induced FAK activity**

We investigated LMS mechanotransduction, asking if it differed from that induced by HMS where substrate strain triggers FAK activation [21] at FA mechanosomes [83, 84]. To test if FAK situated at FAs was involved in LMS signaling we performed a detailed time course experiment. As illustrated in Fig.1a, Group 1 is subjected to a single LMS (0.7g, 90Hz, 20min ) and p-FAK measured 2hr after (prior to 2nd LMS), Group 2 is subjected to LMS and p-FAK measured immediately. Group 1+2 received both LMS treatments with p-FAK measured after the 2nd LMS. Experiments were timed such that all the samples were collected at the same time. p-FAK increased 3.4-fold (p<0.001) immediately after LMS but,
shown in Fig.1b, returned to baseline levels after 2h (p<0.001) where it was not significantly elevated compared to control. A prior application of LMS, however, augmented a second LMS (1+2) by 2-fold compared to single application alone (p<0.001). This suggests generation of an amplification mechanism in response to the first LMS.

We previously showed that HMS triggered cytoskeletal adaptations amplify signaling responses to subsequent mechanical challenges [1]. To elucidate the amplification mechanism resulting from the first LMS signal, we asked if, similar to HMS [9], LMS induced focal adhesions and RhoA activity. Immediately before the second LMS application (group 1, 2h after LMS) there were more focal adhesions as measured by western blot analysis of substrate attached FAs [9, 85] against vinculin, paxilin and t-Akt (Fig.1c). We also observed increased RhoA activity 2 hours after the first application of LMS (Fig.1d). These finding suggest that similar to HMS [1], LMS increases cytoskeletal remodeling and strengthens FA substrate connections. Importantly, p-FAK activity was required for LMS activation of RhoA; pharmacologic inhibition of FAK (PF573228, 3µM) prevented LMS-induced RhoA activation (Fig.1e).

To confirm that the LMS signal response could be enhanced by an increased cytoskeleton, we delivered LMS after treatment with LPA, which increases actin bundling through RhoA activation (Fig.S1). LPA increased basal p-FAK by 3-fold and a single LMS application further increased p-FAK by 2-fold (p<0.001, Fig.1f). Two-way ANOVA showed that both LPA and LMS significantly affect the outcome (p<0.001) but no significant interaction was detected (p=0.102).

As both HMS [9] and LMS (Fig.1c) elicited increased focal adhesions and an LPA-induced cytoskeletal served to amplify LMS signaling (Fig.1f), we asked if LMS induced cytoskeletal change could synergistically amplify FAK signaling situated at the focal adhesions. Depicted in Fig.1g, MSCs were treated with one (1X) or two (2X) bouts of LMS followed by HMS. While a single HMS (2% uniaxial strain, 0.17Hz, 20 minutes) and 2X LMS induced comparable elevation of p-FAK relative to control (2.1-fold each, p<0.001), pretreatment with LMS augmented the HMS response. Pretreating MSC’s with 2X LMS before HMS yielded the largest p-FAK response compared to control (3.5-fold, p<0.001); this response was higher than both HMS alone (60% p<0.001) and that following a single LMS pretreatment (32% p<0.01). Synergistic regulation of p-FAK activity through LMS and HMS (p<0.001, two-way ANOVA) supports that LMS treatment results in more robust cytoskeleton terminating in FAs, where HMS is known to initiate signaling in MSCs [21].

**LMS induced p-FAK requires an intact actin cytoskeleton that reorganizes at the perinuclear domain**

To further implicate the cytoskeleton in the response to LMS, we asked if an intact cytoskeleton was required for LMS activation of FAK. MSCs treated with the actin polymerization inhibitor cytochalasin D (CyD, 0.2µM) had diminished basal and LMS-induced p-FAK (Fig.2a, p<0.001) but a small LMS response was still measurable (p<0.05). In contrast, inhibiting the RhoA effector protein ROCK (Y27632, 10µM) to deplete cytoskeletal tension prevented response to LMS (Fig.2b, p<0.001). The microtubule-specific
inhibitor colchicine (Colc, 1µM), in contrast, did not impair LMS-activation of FAK (Fig. 2c).

Uniaxial strain induces actin stress fibers perpendicular to loading direction [86, 87] while laminar fluid flow induces parallel stress fibers that span the entire cell length [88, 89]. We tested if LMS induced actin remodeling. 1h after LMS, actin remodeling was concentrated at the perinuclear domain, as shown in Fig.2d with increases in both basal and apical surfaces indicating a unique cytoskeletal adaptation arising in the absence of substrate strain [3, 20] (see Fig.S2&S3). Observer blinded counting of cells after LMS treatment showed that twice as many cells displayed visible perinuclear actin fibers (Fig.2e, p<0.01). We identified the cell for positive perinuclear remodeling if there was i) a distinct bright actin ring around the nuclear rim or ii) if there was a distinct and bright accumulation of short actin stress fibers that coincide with the nuclear position.

### Decoupling nucleus from cytoskeleton by inhibiting LINC function prevents LMS signaling

The unique LMS-induced perinuclear actin remodeling suggests the presence of LMS generated force at the nucleus. We hypothesized that the LINC scaffold (Fig.3a) might support the LMS response by providing mechanical coupling between the nucleus and the actin cytoskeleton. Shown in Fig.3b, MSCs treated with a targeted siRNA (siSUN) to deplete SUN nuclear envelope proteins eliminated the LMS-induced FAK response (p<0.01) and decreased basal p-FAK levels (48%, p<0.05). Similarly, overexpression of a dominant negative KASH domain of Nesprin (DNKASH, Fig.S4) that competes for SUN protein binding, ablated the LMS-induced p-FAK response (p<0.001) (Fig.3c). These results imply that decoupling the nucleus from the actin cytoskeleton interferes with the ability of the cell to respond to vibratory LMS signals.

As expected [63], depleting both SUN1 and SUN2 proteins in MSCs disrupted Nesprin-2 localization to the nuclear envelope (Fig3d, Fig.S5b) and decreased Nesprin-2 signal intensity along the major axis of the nuclear envelope (Fig.S5c, 43%, p<0.01). To confirm that our siRNA strategy to disrupt LINC was effective, we showed that siSUN decreased MSC migration (Fig.S7a). Overexpressing DNKASH fragment similarly displaced Nesprin-2 from the nuclear envelope (Fig3e, Fig.S6b), leading to reduced Nesprin signal localization (Fig.S6c, 45%, p<0.01). The expression of DNKASH in a homogenous cell population was ensured by puromycin selection of transfected cells prior to experiments (Fig.S7b).

As activation of Akt is a well-accepted response to mechanical force and is required for the MSC lineage response to HMS [9, 21] we next evaluated the ability of LMS to activate Akt. Similar to FAK activation, LMS activated Akt by 3-fold (p<0.01) and a repeated LMS treatment further amplified the Akt phosphorylation (46%, p<0.05) to 4.4-fold (p<0.001, Fig.3f). Importantly, preservation of LINC connectivity between the actin cytoskeleton and the nucleus was critical: in cells treated with siSUN or overexpressing DNKASH, LMS failed to activate Akt (Fig.3g–h).

Recently, nuclear stiffness was shown to be modulated after force applied by Nesprin bound beads, an effect dependent on phosphorylation of the LINC binding partner Emerin (EMD)
To test whether Emerin played a role in LMS induced p-FAK activation, we depleted Emerin using siRNA (siEMD) and found that the signal response to LMS was unaffected (Fig.4a). Further, probing for p-FAK in isolated nuclear membranes yielded no LMS-induced activity (Fig.4b), suggesting that FAK activation occurs at a distance from the nucleus. As such, this data suggests that the stiffer and denser cell nucleus might passively participate in the sensation of vibratory signals by virtue of nuclear-cytoskeletal coupling dependent on the LINC complex.

**LINC complex function is required for LMS repression of adipogenic differentiation**

To further elucidate the LINC requirement for LMS-induced mechanical signaling, we tested whether LINC function was necessary for the LMS regulation of MSC adipogenesis. When compared to control siRNA (siCtrl), limiting LINC function by siRNA knockdown of both SUN 1 and 2 proteins (siSUN) diminished the transcriptional expression of SUN proteins while increasing the adipocyte specific markers Adiponectin (APN, 287% \( p<0.001 \)) and Fatty acid binding protein 4 (AP-2, 315%, \( p<0.001 \)) after five days culture with adipogenic medium, quantified by PCR (Fig.5a). Immunoblot analysis confirmed increases in adipogenic protein levels of APN (Fig.5b, 250%, \( p<0.001 \)) and AP-2 (Fig.5c, 150%, \( p<0.01 \)) in SUN knockdown groups. Similarly, cells treated with the dominant negative form of Nesprin KASH domain (DNKASH) demonstrated increases in both gene expression and protein levels of adipogenic markers (Fig.S8a–b).

Treatment with LMS (0.7g, 90Hz, 20 min twice daily separated by 2h) for five days was sufficient to repress adipogenic markers APN (Fig.5d, 42%, \( p<0.001 \)) and AP-2 (Fig.5e, 43%, \( p<0.01 \)) in MSCs treated with control siRNA. Importantly, consistent with our finding that LINC functionality is required for LMS-induced mechanoregulation of MSCs, LMS repression of adipogenic differentiation was abrogated when LINC complex was disrupted through siSUN knockdown. DNKASH groups also displayed a restrained LMS effect to decrease adipogenesis (Fig.S8c). Compared to knockdown experiments (Fig.5a–c and Fig.S8a–b), during LMS experiments the adipogenic response due to siSUN and DNKASH treatments was less robust (Fig.5d–e& Fig.S8c); adipogenic differentiation might be compromised by the increased handling required for the LMS application protocol where the cultures are removed from optimal incubator conditions into room temperature.

**HMS signal generation does not require functional LINC**

We showed that HMS and LMS synergistically increased FAK signaling (Fig.1g) suggesting a common amplification mechanism through regulation of cell structure. We then asked if HMS, which inhibits adipogenesis via signals initiated at focal adhesions [1], also required LINC connections or whether the peripheral FA mechanosome was sufficient. Application of HMS increased p-FAK in cells overexpressing DNKASH (1.8-fold, \( p<0.05 \), Fig.6a) or treated with siSUN (1.7-fold, \( p<0.01 \), Fig.6d) indicating that nuclear-cytoskeletal coupling was not critical. Akt signaling was also tested and showed that HMS increased p-Akt despite disruption of nuclearcytoskeletal tethers by either DNKASH (2.3-fold, \( p<0.01 \), Fig.6b) or siSUN (1.7-fold, \( p<0.01 \), Fig.6e). As such, HMS-induced FAK and Akt signaling did not require mechanical coupling between the nucleus and cytoskeleton.

*Stem Cells. Author manuscript; available in PMC 2015 June 08.*
We considered if different mechatransduction mechanisms might be invoked by high and low magnitude mechanical signals. Interestingly, while proximal FAK activity by HMS was required for subsequent Akt activation (Fig.6c), it was not required for Akt phosphorylation due to LMS (Fig.6f, p<0.01), emphasizing that different adaptive strategies may result in discrete signaling mechanisms. Further evidence that distinct proximal mechanisms differentiate HMS and LMS was the different requirement for the FAK co-regulator, Fyn. Recruitment of Fyn to the FA mechanosome was essential for HMS Akt activation (Fig.6g) [21], while Fyn depletion did not prevent LMS-induced Akt phosphorylation (Fig.6h, 1.8-fold, p<0.05).

**Discussion**

Both low and high magnitude mechanical signals influence fate selection of mesenchymal stem cells, activating signals that can be tuned to build musculoskeletal tissues and suppress adiposity [2, 6, 7]. Here, we asked if the LINC complex was involved in transducing relevant regulatory signals. We show that LINC is necessary to allow MSC to respond to low magnitude high frequency signals that induce FAK and Akt phosphorylation and lead to adipogenic repression, but that LINC is not necessary for responses to HMS transmitted at the FA mechanosome. Furthermore, we show that, similar to the ability of the MSC to reorganize its actin cytoskeleton to amplify responses at the FA mechanosome [1, 9], LMS also generates cytoskeletal change. This cytoskeletal reorganization involves not only maturation of the FA mechanosome, which serves to synergistically enhance HMS signaling, but also accrual of a perinuclear actin structure that connects structures of the cytoplasm to the inner nucleus via the LINC complex.

The perinuclear accumulation of F-actin induced by LMS suggests the presence of force at the boundary of the nucleus and the cytoplasmic cytoskeleton. Importantly, mechanically decoupling the nucleus from the cytoskeleton by inhibiting LINC connections disabled the LMS response. Considering the integral function of LINC in providing functional connections between the nucleus and focal adhesions [70], our findings imply that LMS-induced FAK activity is enabled by the LINC-connected nucleus. Although we did not show a direct a physical connection between FAK activity and LINC or measure the level of forces required in this response, limitations of the current study, our results strongly demonstrate the importance of the LINC complex in the cellular response to LMS. Further, our previous in silico findings suggest that in response to LMS, the nucleus is capable of generating sufficient mechanical deformation within the cell to serve as a passive, force generating element [26]. Moreover, the lack of FAK activity at the nuclear envelope, and the insensitivity of LMS-induced FAK to knockdown of Emerin (a nuclear envelope protein and LINC partner [90] shown to participate in nuclear stiffness [78]) suggests that FAK signaling is largely due to LMS actions that require connections between the nucleus and the cytoplasmic cytoskeleton. In contrast, HMS-induced FAK and Akt signaling relies exclusively on large substrate deformations and is unaffected by LINC disruption. Finally, our results indicate that in addition to the LINC requirement for LMS-induced signaling, a requirement for proximal Fyn signaling also differed between LMS and HMS, reinforcing that LMS and HMS utilize divergent signaling modalities.
An alternative physical mechanism by which LMS might initiate signaling is via LMS-induced fluid shear stress. Computational studies revealed that when vibrated at high frequency and low magnitude (30-100Hz, 0.1-1g) the relative velocity of solid bodies submerged in the fluid environment can generate fluid shear up to 2Pa [19, 24] in highly viscous environments like bone marrow (400cP [91], water is 1cP). In the current study, LMS-induced fluid shear was a function of surface strain (~1.4µε, Fig.S9a) as the vertical well motion limited the lateral fluid sloshing (Fig.S9b). Our previously validated simulation model [25] predicted a peak velocity differential of ~0.00004m/s (Fig.S9c) corresponding to a LMS-induced fluid shear of 0.0008Pa (0.008dyn/cm²), a level that while comparable to fluid shear required for maintenance of LINC-bound actin cap structures (0.001-0.005Pa), [72] is two orders magnitudes below that to which bone cells respond [92]. Moreover, this level of shear is insignificant compared to fluid shear generated during handling plates (identical for controls and LMS), particularly when considering the potency of oscillating fluid shear diminished at the higher frequencies [93]. While we cannot exclude the possibility that LMS-induced accelerations works synergistically with fluid shear, we previously did not detect such interactions under much higher shear (5Pa) where cell responses were associated more strongly with the acceleration magnitude than the LMS-induced fluid-shear [17, 23, 26]; altogether this suggests that the low level shear generated by LMS is unlikely to be responsible for FAK and Akt activation and subsequent cytoskeletal reorganization as well as repression of adipogenesis.

Differentiation of stem cells requires complex interactions of multiple signaling pathways. We previously showed that adipogenic commitment of MSCs was largely regulated by reduced βcatenin signaling, and that both HMS and LMS impact this pathway during repression of adipogenic commitment [3, 94, 95]. Interestingly, LINC function also affects βcatenin signaling [76]. Here we demonstrated that limiting LINC functionality by knockdown of SUN1 and SUN2, as well by expressing the dominant negative form of Nesprin KASH domain, increased MSC adipogenesis (Fig.5a–c & Fig.S8a–b). In agreement with previous work from our laboratory, we found that application of LMS repressed adipogenesis in MSCs in control groups, but when LINC was dysfunctional due to interference with SUN or Nesprin, LMS failed to prevent adipogenesis (Fig.5&Fig.S8). This indicates that interfering with LMS activation of FAK, Akt or RhoA, all early events leading to preservation of βcatenin signaling, will also interfere with LMS regulation of MSC differentiation.

In sum, cell signaling in response to extremely low magnitude mechanical signals requires LINC complex coupling between the nucleus and cytoskeleton and implicates the LINC interface as a mechanosensory site. We showed for the first time that LMS, an applied physical force that generates neither significant fluid shear (Fig.S9) nor strain [96], activates signaling events inducing FAK, Akt, RhoA and FA maturation which have been previously associated with HMS. For the first time we show, at the cellular level, that cytoskeletal adaptations and proximal signaling events initiated by low and high magnitude physical signals differ between LMS and HMS. The LMS-induced cytoskeletal adaptations and requirement for the LINC interface to support LMS-induced signaling not only provides new insights as to how cells respond to vibratory mechanical information but also shows
that perinuclear actin remodeling amplifies the mechanoresponse. Our findings further demonstrated that decreased connectivity between the cell nucleus and cytoplasmic actin impairs anti-adipogenic effects of LMS. With this in mind, it is interesting to consider that, resembling the gradual hearing loss in LINC deficient mice [82], alterations in nuclear-cytoskeletal connections associated with aging or laminopathies like Hutchinson-Gilford progeria [97] might cause a loss in LINC complex-dependent mechanosensitivity to vibratory signals. Such a decrease in mechanosensitivity could potentially contribute to reported failure of musculoskeletal tissues by limiting the accessible spectrum of mechanical information thereby interfering adaptation to functional loading [98]. Translating this to the clinic, physical or chemical interventions that modulate the LINC interface might have the potential to enhance the mechanical sensitivity of an otherwise unresponsive cell population. Finally, our data demonstrate that cells utilize a multitude of strategies to sense and respond to mechanical signals. While the perception of high magnitude stimuli appears to rely on signaling at the substrate/membrane interface, extremely low magnitude mechanical stimuli are detected through the physical connections between nucleus and the cytoskeleton.

Materials and Methods

mdMSC Isolation

mdMSC from 8–10 wk male C57BL/6 mice were prepared after Peister et al.[99]. Tibial and femoral marrow were collected in RPMI-1640, 9% FBS, 9% HS, 100 µg/ml pen/strep and 12µM L-glutamine. After 24 h, non-adherent cells were removed by washing with phosphate-buffered saline and adherent cells cultured for 4 weeks. Passage 1 cells were collected after incubation with 0.25% trypsin/1 mM EDTA × 2 minutes, and re-plated in a single 175-cm² flask. After 1–2 weeks, passage 2 cells were re-plated at 50 cells/cm² in expansion medium (Iscove modified Dulbecco’s, 9% FBS, 9% HS, antibiotics, L-glutamine). mdMSC were replated every 1–2 weeks for two consecutive passages up to passage 5 and tested for osteogenic and adipogenic potential, and subsequently frozen.

Application of LMS and Strain

Vibrations were applied to mdMSCs at peak magnitudes of 0.7g at 90Hz for 20min at RT [3]. Controls were sham handled. Unless stated otherwise, LMS was applied as two 20 min bouts separated by 2h rest. Uniform 2% biaxial strain was delivered at 10 cycles per minute for 20 min to mdMSCs using the Flexcell FX-4000 system (Flexcell International, Hillsborough, NC).

Cell Culture and Pharmacological Reagents

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA). Culture media, trypsin-EDTA, antibiotics, and Phalloidin-Alexa-488 were from Invitrogen (Carlsbad, CA). KU63794, PF573228, Y27632, Colchicine and Cytochalasin D were purchased from Sigma Aldrich (St. Louis, MO). Lysophosphatidic Acid (LPA) was purchased from Cayman chemicals (Ann Arbor, MI). mdM-SCs [94] were maintained in IMDM with FBS (10%, /v/v) and penicillin/streptomycin (100µg/ml). For phosphorylation and RhoA activity, seeding density was 10,000 cells /cm² and 2,500 cells /cm² for immunostaining experiments. All the groups were cultured for 48h before beginning

Stem Cells. Author manuscript; available in PMC 2015 June 08.
experiments and were serum starved over-night in serum free medium. LPA (30µM) was added 2h prior to LMS. All other pharmacological inhibitors were added 1 hour before either LMS or Strain at the following concentrations: Cytochalasin D (0.2µM), Y27632 (10µM), Colchicine (1µM), PF573228 (3 µM) and KU63794 (2µM).

For adipogenic differentiation experiments, marrow derived MSCs were plated at a density of 100,000 cell/well into 6-well culture plates and treated with either with siRNA against SUN-1 and SUN-2 (siSUN) or with pCDH-EF1-MCS1-puro-mCherry-Nesprin-1αxKASH (DNKASH) plasmids using 1µg DNA per 100,000 cell (proper controls were used for both treatments). 18h after the transfection, growth medium was replaced with adipogenic medium containing 0.1 µM dexamethasone and 5µg/ml insulin. Cultures were incubated for five days with or without LMS treatment (2x20 min per day separated by 2h).

Overexpression and Knockdown Sequences

pCDH-EF1-MCS1-puro-mCherry (mCherry control) and pCDH-EF1-MCS1-puro-mCherry-Nesprin-1αxKASH (DNKASH) plasmids were kindly provided by Dr. Lammerding [100]. mdMSCs were transfected using 1µg DNA per 100,000 cells using LipoD293 transfection reagent (SignaGen Laboratories, Rockville, MD) according to manufacturer’s instructions. 72h after the initial transfection, stably transfected cells were selected using 10µg/ml puromycin. For transiently silencing specific genes, cells were transfected with gene-specific small interfering RNA (siRNA) or control siRNA (20 nM) using PepMute Plus transfection reagent (SignaGen Labs) according to manufacturer’s instructions. Strain or LMS were applied 72 hours after initial transfection. The following Stealth Select siRNAs (Invitrogen) were used in this study: negative control for SUN-1 5′-GAAATCGAAGTACCTCGAGTGATAT-3′; SUN-1 5′-GAAAGGCTATGAATCCAGAGCTTAT-3′; negative control for SUN-2 5′-CACCAAGACTCGGAAGATCTCTTCA-3′; SUN-2 5′-UAAAGCGCCACAAACAGUGUCACUC-3′; negative control for Fyn 5′-GCCUCGUACAGAAGAAACGCCGAAU-3′; Fyn 5′-CACCAGAGGCTAGAATCCTCTTACTCA-3′; SU-2 5′-CACCAAGACTCGGAAGATCTCTTCA-3′; negative control for Emerin 5′-CAACCUUACUCG-GGUUACUAGGUG-3′; Emerin 5′-CAACAUCCCUAUCCGGCCUAUUGUG-3′.

Isolation of focal adhesions

Cells were incubated with TEA-containing low ionic–strength buffer (2.5 mM triethanolamine [TEA], pH 7.0) for 3 minutes at room temperature, 1× PBS containing protease/phosphatase inhibitors. A Waterpik (Fort Collins, CO) nozzle held 0.5 cm from the plate surface at ~90° supplied the hydrodynamic force to flush away cell bodies, membrane-bound organelles, nuclei, cytoskeleton, and soluble cytoplasmic materials[85] so that residual focal adhesions could be isolated.

Isolation of nuclear envelope proteins

MSCs were plated on one-well (100cm², Greiner Bio-One, NC) at 10,000cell/cm². Nuclear envelope proteins were extracted using Minute Nuclear Envelope Protein Extraction Kit
(Invent biotech, Germany) according to manufacturer’s instructions using at least ten million cells per group.

**Real-time RT-PCR**

Total RNA was isolated by using the RNeasy mini kit (QIAGEN) and treated with deoxyribonuclease 1 to remove contaminating genomic DNA. Reverse transcription was performed with 1 µg RNA in a total volume of 20 µl per reaction. Real-time PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Inc., Hercules, CA). Twenty-five-microliter amplification reactions contained primers at 0.5 µm, deoxynucleotide triphosphates (0.2 mm each) in PCR buffer, and 0.03 U Taq polymerase along with SYBR-green (Molecular Probes, Inc., Eugene, OR) at 1:150,000. Aliquots of cDNA were diluted 5- to 5000-fold to generate relative standard curves with which sample cDNA was compared. Standards and samples were run in triplicate. PCR products from all species were normalized for the amount of 18S amplicons. Primer sequences as follows Adiponectin (APN, Forward: 5′-GCAGAGATGGCA CTCCTGGA-3′, Reverse: 5′-CCCTTCAGCTCCTGTCATTCC-3′), Fatty acid binding protein 4 (AP-2, Forward: 5′-CATCAGCGTAAATGGGGATT-3′, Reverse: 5′-TCGACTTTCCATCCCCACTTC-3′), Peroxisome proliferator-activated receptor gamma (PPARγ, Forward: 5′-GCTTATTTATGATGTGTGATC-3′, Reverse: 5′-GCATTGTGAGACATCCCCAC-3′) expressions were normalized to and 18S (Forward: 5′-GAACGTCTGCCCTATCAACT-3′, Reverse: 5′-CCAAGATCCACTACGAGCT-3′).

**Western Blotting**

Whole cell lysates were prepared using an radio immunoprecipitation assay (RIPA) lysis buffer (150mM NaCl, 50mM Tris HCl, 1mM EDTA, 0.24% sodium deoxycholate, 1% Igepal, pH 7.5) to protect the samples from protein degradation NaF (25mM), Na3VO4 (2mM), aprotinin, leupeptin, pepstatin, and phenylmethylsulfonylfluoride (PMSF) were added to the lysis buffer. Whole cell lysates (20µg) were separated on 9% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with milk (5%, w/v) diluted in Tris-buffered saline containing Tween20 (TBS-T, 0.05%). Blots were then incubated overnight at 4°C with appropriate primary antibodies. Following primary antibody incubation, blots were washed and incubated with horseradish peroxidase-conjugated secondary antibody diluted at 1:5,000 (Cell Signaling) at RT for 1h. Chemiluminescence was detected with ECL plus (Amersham Biosciences, Piscataway, NJ). At least three separate experiments were used for densitometry analyses of western blots and densitometry was performed via NIH ImageJ software.

**RhoA Activation Assay**

Purification of recombinant proteins and construction of the pGEX4T-1 prokaryotic expression constructs containing the Rho-binding domain (RBD) of Rhotekin has been described [101]. Briefly, expression of the fusion proteins in Escherichia coli was induced using isopropyl β-D-1-thiogalactopyranoside (100 µM) for 12–16 hours at RT. Bacterial cells were lysed in lyss buffer containing Tris HCl (50mM, pH 7.6), NaCl (150mM), MgCl2 (5 mM), dithiothreitol (1mM), aprotinin (10µg/ml), leupeptin (10µg/ml), and PMSF (1mM). Recombinant proteins were purified by incubation with glutathione-sepharose 4B
beads (GE Healthcare, Piscataway, NJ) at 4°C. Pull down of active RhoA, using glutathione-
S-transferase-RBD (GST-RBD) beads, was performed as described[102]. mdMSC cells
were lysed in buffer containing Tris HCl (50mM, pH 7.6), NaCl (500mM), Triton X-100
(1%, v/v), SDS (0.1%, v/v), sodium deoxycholate (0.5%, w/v), MgCl2 (10mM),
orthovanadate (200µM), and protease inhibitors. Lysates were clarified by centrifugation,
equalized for total volume and protein concentration and rotated at 4°C for 30 minutes with
50µg of purified GST-RBD bound to glutathione-sepharose beads. The bead pellets
were washed in lysis buffer three times, followed by pelleting of the beads by centrifugation
between each wash, and subsequently processed by SDS-polyacrylamide gel
electrophoresis.

Immunofluorescence
Following strain or LMS treatment, cells were fixed with paraformaldehyde. For Nesprin-2
staining, cells were incubated in anti-Nesprin-2 primary antibody solution (0.5%, v/v in
blocking serum) for 24h at 4°C, followed by secondary antibody incubation DyLight 649
AffiniPure Donkey Anti-Mouse IgG (Jackson Immuno Research Laboratories, West Grove,
PA) For actin staining, cells were incubated with phalloidin-conjugated Alexa Fluor-488
(Invitrogen).

Statistical analysis
Results were presented as mean ± SEM. Densitometry analyses were performed on at least
three separate experiments. Differences between groups were identified by one-way analysis
of variance (ANOVA) followed by Newman-Keuls post-hoc tests. Interactions between two
different treatments were evaluated using two-way ANOVA analysis. P-values of less than
0.05 were considered significant.

Acknowledgments
Technical expertise from Lisa Sharek was greatly appreciated. Funding by the National Institutes of Health is
gratefully acknowledged. This study was supported by EB014351, AR056655, AR042360, and GM029860.

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Figure 1. RhoA enhanced cytoskeleton modulates LMS-induced FAK phosphorylation

a) Schematic of time course experiment depicting different groups. Groups (1) and (2) were subjected to single LMS with or without a 2h rest, respectively. Group (1+2) was subjected to both LMS treatments.

b) LMS acutely increased FAK phosphorylation 3.4-fold (p<0.001), 2h later p-FAK was not significantly different than control. Re-application of LMS resulted in 7-fold increase, doubling the single LMS response.

c) Isolation of FAs 2hr after a single LMS treatment was subject to Western blot analysis; increased total vinculin, paxillin and Akt were consistent with increased FAs.

d) FA increase was accompanied by
RhoA activity and e) RhoA activity was dependent on initial LMS-induced FAK activity as FAK inhibitor PF573228 (PF, 3µM) prevented LMS-activated RhoA. f) RhoA activated with LPA (30µM) increased p-FAK 3-fold (p<0.05); following LPA, a single LMS application increased p-FAK response by 7-fold (p<0.001). The black bar on the representative western blot indicates that it has been cropped to change the sample positions. g) LMS amplified the HMS response: 1 and 2X LMS pretreatment synergistically augmented the HMS response by 30% (p<0.01) and 60% (p<0.01), respectively. * p<0.05, ¥ p<0.01, † p<0.001, against control and each other.
Figure 2. LMS signaling requires an intact actin cytoskeleton and induces actin re-arrangement at the perinuclear domain

Depolymerizing actin with a) CytochalasinD (CyD, 0.2µM) decreased both basal and LMS-induced p-FAK (p<0.001). b) Inhibiting RhoA activity with ROCK inhibitor Y27632 (10µM) ablated p-FAK due to LMS (p<0.001). c) Inhibiting microtubule polymerization with colchicine (Colc, 1µM) did not change the LMS response. d) LMS treatment induced actin rearrangement around cell nucleus 1h later (arrows). Nuclear straining (DAPI) was merged with actin images to clarify nuclear position. e) Quantification of cells exhibiting 
actin staining around perinuclear domain revealed that LMS increased this frequency from 17% (Control, n=250) to 36% (p<0.01, n=500). *p<0.05, ¥p<0.01, †p<0.001, against control and each other.
Figure 3. LINC mediated actin-nuclear connectivity is required for LMS signaling

a) Mechanically decoupling LINC from the cytoskeleton through inhibition of Nesprin localization to the nuclear envelope by either b) siRNA repression of SUN expression (both SUN-1&SUN-2) or c) overexpressing a dominant negative KASH domain (DNKASH) prevented LMS-induced FAK activation. d) siSUN and e) DNKASH treatments diminished Nesprin staining on the nuclear envelope (p<0.001, n=25, Fig.S5c&6c), visibly reducing the actin connectivity around nucleus. DNKASH and control plasmid groups were subjected to puromycin selection to ensure that cells express the desired constructs (Fig.S7b) f) MSC
lysates were probed for Akt phosphorylation (Ser 473). p-Akt was increased 3-fold (p<0.01) immediately after LMS and after 2hr p-Akt was reduced 42% (p<0.05) and was not different than control. Similar to FAK, a repeated LMS exposure after 2hr increased p-Akt 3.7-fold (p<0.001), 46% higher than a single LMS exposure (p<0.05). *p<0.05, ¥ p<0.01, † p<0.001, against control and each other.
Figure 4. Nuclear envelope protein Emerin does not contribute to LMS-induced FAK signaling

a) Marrow-derived MSCs treated with either control siRNA (siCtrl) or siEmerin (siEMD) were subjected to LMS and probed for FAK phosphorylation (Tyr 397). Application of LMS increased p-FAK equivalently in both siCtrl (3.6-fold, p<0.05) and siEMD (3.9-fold, p<0.01) groups. Densitometry analysis used data from at least three separate experiments.

b) Nuclear envelope proteins were isolated from whole cell lysates immediately after LMS and probed for possible FAK activation. Western blot analysis shows no LMS-induced p-FAK activity at the nuclear envelope. * p<0.05, ¥ p<0.01, † p<0.001, against control and each other.
Figure 5. LINC complex is required for LMS inhibition of adipogenesis
Marrow derived MSCs were plated at a density of 100,000 cell/well into 6-well culture plates and treated with siRNA against SUN-1 and SUN-2. 18h after the transfection growth medium was replaced with adipogenic medium containing 0.1µM dexamethasone and 5µg/ml insulin. a) After 5 days siSUN treated cells showed diminished expression of both SUN-1&2 (p<0.001) and significantly increased expression of known adipogenic markers APN and AP-2 (p<0.001). Densitometry analysis (n=4) showed increased protein levels of b) APN (p<0.001) and c) AP-2 (p<0.01). Treatment with LMS (0.7g, 90Hz, 20 min twice daily separated by 2h) decreased d) APN (p<0.001) and e) AP-2 (p<0.01) in siCtrl groups consistent with repressed adipogenesis. In siSUN treated cells, decoupling of cytoskeleton from nucleus limited LMS ability to reduce adipogenesis. * p<0.05, ¥ p<0.01, † p<0.001, against control and each other.
Figure 6. LINC is not required for HMS-induced signaling
HMS activation of p-FAK was preserved in both a) DNKASH (1.8-fold, p<0.05 and d) siSUN (1.7-fold, p<0.01) treated cells. HMS activated p-Akt in both b) DNKASH (2.3-fold, p<0.01) and e) siSUN treated cells (1.75-fold, p<0.01). c) FAK inhibitor PF573228 (PF, 3µM) inhibited strain-induced Akt activation (p<0.01), but f) PF did not inhibit LMS-induced p-Akt (2-fold, p<0.01). g) siRNA Fyn knockdown (siFyn) inhibited strain induced p-Akt compared to cells treated with a control siRNA. h) Akt response to LMS was
preserved in siFyn cells (1.8-fold, p<0.05). *p<0.05, ¥ p<0.01, † p<0.001, against control and each other.