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Megakaryocytes are mechanically responsive and influence osteoblast proliferation and differentiation

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Abstract

Maintenance of bone mass and geometry is influenced by mechanical stimuli. Paradigms suggest that osteocytes embedded within the mineralized matrix and osteoblasts on the bone surfaces are the primary responders to physical forces. However, other cells within the bone marrow cavity, such as megakaryocytes (MKs), are also subject to mechanical forces. Recent studies have highlighted the potent effects of MKs on osteoblast proliferation as well as bone formation *in vivo*. We hypothesize that MKs are capable of responding to physical forces and that the interactions between these cells and osteoblasts can be influenced by mechanical stimulation.

In this study, we demonstrate that two MK cell lines respond to fluid shear stress in culture. Furthermore, using laser capture microdissection, we isolated MKs from histologic sections of murine tibiae that were exposed to compressive loads *in vivo*. *C-fos*, a transcription factor shown to be upregulated in response to load in various tissue types, was increased in MKs from loaded relative to non-loaded limbs at a level comparable to that of osteocytes from the same limbs. We also developed a co-culture system to address whether mechanical stimulation of MKs in culture would impact osteoblast proliferation and differentiation. The presence of MKs in co-culture, but not conditioned media, had dramatic effects on proliferation of preosteoblast MC3T3-E1 cells in culture. Our data suggests a minimal decrease in proliferation as well as an increase in mineralization capacity of osteoblasts co-cultured with MKs exposed to shear compared to co-cultures with unstimulated MKs.

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Keywords

megakaryocyte; fluid shear; mechanotransduction; laser capture microdissection

1.0. Introduction

Skeletal mass and geometry are strongly influenced by mechanical cues. The basic concept of “form follows function” has been supported by numerous *in vivo* studies demonstrating an adaptive response of bone to applied force [1-5]. Osteocytes are thought to be the principal cell in bone responsible for the cellular conversion of mechanical information to biochemical signaling (also known as mechanotransduction) because of their broad distribution and location embedded within the bone matrix. *In vitro* studies have demonstrated osteocyte-like cell lines to be mechanoresponsive [6-8], and *in vivo* studies confirm the physiologic importance of these cells in response to mechanical unloading [9]. Though osteocytes are ideally situated for exposure to physical stimulus, a variety of other cell types within bone may also be positioned to sense and respond to mechanical perturbations, particularly those that reside within the marrow cavity.

The mechanical environment of the marrow cavity is not well-characterized but is likely influenced by several factors. The intramedullary space is pressurized (~3 kPa) due to downstream venous resistance [10], and studies have demonstrated increases in intramedullary pressure (ImP) due to impact loading, muscle contraction, or externally applied loads [11-14]. Experimentally altering ImP has been shown to elicit potent anabolic effects in a model of disuse [15]. These changes in ImP can also be driven by muscular contraction and can lead to prevention of disuse osteopenia in the trabecular compartment, as well [16]. Other models of alterations in ImP, such as use of venous tourniquet or venous ligation, have elicited similar results [17, 18].

Cells within the marrow cavity may also be subject to fluid shear forces. Marrow is a viscous fluid [12], and computationally, its viscosity has been shown to be a critical factor in the shear stresses that develop within vertebral trabecular bone subject to high frequency vibrations [19]. Arterial blood flow within marrow might be a significant contributor, as well. Medullary blood flow can be directly increased by muscular stimulation [20], however reported changes in blood flow as a result of exercise are less striking [21].

Given their location near osteoblasts, osteoclasts, and precursor cells for both of those lineages, bone marrow hematopoietic cells may also play a role in bone homeostasis. Recent studies have begun to highlight effects of “accessory cells” on osteogenic differentiation of progenitor cells [22-24]. A specific role for megakaryocytes (MKs) in regulating bone mass was demonstrated in gene targeted mice lacking either the GATA-1 or NF-E2 transcription factors necessary for full MK maturation. These mice accumulate immature MKs in the marrow and develop a dramatically high bone mass phenotype with age [25]. Furthermore, *in vitro*, both osteoblast proliferation and differentiation of mesenchymal progenitors is enhanced by the presence of MKs [25, 26]. This effect requires cell-cell contact between the MKs and the osteoblast lineage cells [25] and evidence suggests this effect may be mediated by gap junctions [27] or integrins [28]. In addition, MKs have been shown to effect

osteoclasts. The addition of MKs to pre-osteoclast culture inhibits the formation of osteoclasts [29, 30]. Furthermore, the presence of MKs can impair the bone-resorbing function of osteoclasts [29].

Physiologically, MKs represent a very small percentage of the bone marrow milieu, approximately 0.02-0.05% of the total nucleated cells in bone marrow [31]. The frequency of MKs has been shown to be similar across different age groups (neonates and adults) when adjusted for cellularity [32]. The physiologic effects of this low frequency cell type on bone homeostasis are still unclear, though the effects seen in culture as well as in mice with elevated cell numbers are marked. Some evidence suggests that the elevated cell number in these mouse models may be driving the dramatic bone phenotype. Kacena, et al, demonstrated that both wild-type and GATA-1 deficient MKs have similar effects on co-cultured osteoblasts, suggesting that the bone phenotype associated with the GATA-1 deficient mice was not due to altered function of the MKs but rather the elevated number of MKs [25]. However, mouse models with elevated MK number due to overexpression of thrombopoietin, a potent inducer of MK proliferation and differentiation, possess a different skeletal phenotype, in which there is increased bone, but the bone is osteosclerotic and myelofibrotic [33, 34]. This suggests that there may be other factors beyond cell number which regulate MKs' effects on OBs. Interestingly, traditional bone anabolic agents, such as estrogen, have also been shown to lead to increased MK numbers, both *in vitro* and clinically. Estrogen treatment also regulates MK expression of factors that impact bone homeostasis, such as an increase in osteoprotegerin and decrease in RANKL. [35-37].

The location of MKs within the marrow, and subsequently the mechanical cues that they may be subject to physiologically, depends upon the differentiation state of the cell. Hematopoietic stem cells from which MKs are derived are believed to reside at osteoblast surfaces [38, 39]. Immature MKs are localized to the marrow stroma. MKs have been observed in complexes with marrow stromal cells isolated from bone marrow, suggesting a physical association between these cell types *in vivo* [40]. Mature MKs are most frequently observed at the abluminal surface of marrow sinusoids and extend cytoplasmic processes into the sinusoid in association with the formation of platelets [41, 42].

The understanding of how these cells respond to fluid shear is still limited. Eldor, et al. demonstrated that, like other marrow constituents, MKs will adhere more readily to an ECM-coated slide in the presence of shear than in quiescent conditions [43]. De Bruyn, et al. similarly demonstrated that, under turbulent fluid conditions, Rap1, a small GTPase, is activated, which facilitates the adhesion of MKs to fibrinogen via $\alpha\text{IIb}\beta 3$ integrin [44]. Mature MKs in contact with sinus endothelial cells are exposed to low but constant levels of shear from blood flow, estimated to be approximately 0.13-0.41 Pa [42]. Shear forces on these mature cells in contact with collagen-IV-rich vascular matrix are thought to trigger the formation of platelets [42, 45].

Based on these observations, we hypothesize that MKs respond to the mechanical environment induced by physiologic loading of bone and, furthermore, this response could impact downstream effects that MKs have on osteoblast behavior. In order to address this hypothesis, we evaluated the responsiveness of MKs to controlled mechanical stimuli both

in culture and in an *in vivo* model. Subsequently, we addressed the functional implications of MK mechanosensitivity by assessing whether physical stimulation of MKs in culture can directly impact their effects on osteoblast proliferation and differentiation.

2.0. Methods

Our approach to assessing mechanoresponsiveness of MKs was two-pronged, utilizing both *in vitro* and *in vivo* techniques. Subsequently we investigated the effects of load on MK-osteoblast communication using a co-culture model.

2.1. Mechanoresponsiveness of MKs in culture

2.1.1. Cell Culture—Two separate megakaryocytic cell lines were studied to assess effects on cells at different stages of maturation – the Meg-01 megakaryoblastic cell line and the K562 erythromyeloblastic progenitor cell line differentiated to mature megakaryocytes by treatment with phorbol 12-myristate 13-acetate (PMA). Meg-01 cells (ATCC; Manassas, VA) were maintained in RPMI + 10% fetal bovine serum + 1% Penicillin-Streptomycin. K562 cells (a kind gift from Dr. Laurie McCauley) were grown in IMDM + 10% fetal bovine serum + 1% Penicillin-Streptomycin [46]. To induce megakaryocyte differentiation, K562 cells were cultured in the presence of 10 nM PMA (Sigma-Aldrich; St. Louis, MO) for 3 days prior to utilization in fluid shear experiments [46, 47].

2.1.2. Fluid Shear Loading—All fluid shear protocols and devices were developed in our laboratory [48] and have been utilized in other studies [49]. Glass slides were either coated with 0.1% gelatin (Sigma-Aldrich; St. Louis, MO) for Meg-01 cells or 5 $\mu\text{g}/\text{cm}^2$ fibronectin (Sigma-Aldrich; St. Louis, MO) for K562 cells. Following substrate coating, plates were rinsed with PBS and serum-free media, and cells were then seeded at a density of 1×10^6 cells/plate in DMEM + 10% fetal bovine serum + 1% Penicillin-Streptomycin. After 24 h in culture, the media was changed to serum-free conditions for approximately 16 hours. There was no observable difference in cell morphology based on evaluation using optical microscopy (data not shown).

Plates were inserted into a parallel plate flow chamber device developed in our laboratory based on work by Frangos [50] and Jacobs [51]. Flow was induced by a series of syringes attached to a linear slide. The motion of the slide was controlled by a motor programmed to flow rates that were calculated to engender different magnitudes of shear stress, 0.5, 1, or 2 Pa, based on the laminar flow equation. This range of shear stresses has been shown to elicit a mechanoresponse in previous work using pre-osteoblastic cells [48], and is also predicted to be within the physiologic range of shear forces in marrow due to low magnitude high frequency loading based on computational models [52]. Fluid shear was imparted using a “pulse-static” waveform at 0.033Hz (15s forward, 15s reverse, with 0.2s transitions). Sham controls were included to capture the potential effect of handling of the plates on subsequent gene expression. Sham control plates were inserted into the chamber and immediately removed and placed into a sterile 10 cm culture dish with fresh serum-free media for the duration of the 30 minute experiment. Untouched (UT) samples were also harvested.

2.1.3. RNA extraction and transcript quantification using real-time PCR—

Immediately following treatment, cells were rinsed with PBS and lysed in Buffer RLT (Qiagen; Valencia, CA) + β -mercaptoethanol, homogenized using Qiashredder columns, and stored at -70°C until further processing. RNA was isolated using the RNeasy kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. RNA was quantified and 0.5–1 μg of RNA was reverse transcribed using Superscript III reverse transcriptase and primed by random hexamers. For a given experiment, the same amount of RNA was reverse transcribed across all groups. Real-time PCR was performed and amplification of gene products was determined by the incorporation of SYBR Green fluorescent nucleic acid stain (Invitrogen; Carlsbad, CA). 18s rRNA was chosen as an internal control based on its common use as a control in *in vitro* [53] and *in vivo* [54] loading studies. Normalized changes in expression of *c-fos* and COX-2 in response to load relative to untouched controls were calculated using the $C(t)$ method [55]. All primer sequences are reported in Table 1. Data is expressed in terms of mean $\pm$ SD values from four (Meg-01) or two (K562) independent experiments and samples of similar treatments were run in duplicate (Meg-01) or triplicate (K562).

2.1.4. Statistics—For gene expression studies, a one-way ANOVA with a Tukey's test post-hoc was used to determine statistical significance across groups. Statistical significance was determined at $p < 0.05$.

2.2. Mechanoresponsiveness of MKs in vivo

2.2.1. Tibial loading model—A model for application of axial compressive load on a mouse tibia was developed based on previous studies [56, 57]. The right tibia of 8-week-old male C57Bl6 mice from a colony maintained in-house was subject to axial loading for 10 minutes at a frequency of 4 Hz and peak load of approximately 9N, corresponding to roughly 2000 μe on the medial surface at the mid-diaphysis based on strain gauging (data not shown). Animals were euthanized either 30 ($n=5$) or 60 ($n=5$) minutes following the completion of load. These times were selected based on studies demonstrating maximal increase in *c-fos* expression in osteocytes in response to applied mechanical stimulation *in vivo* [58, 59].

2.2.2. Tissue Processing and Histology—Loaded tibiae, along with their non-loaded contralateral control, were harvested and decalcified in 20% EDTA in RNase-free water for 2 days at 4°C . Specimens were rinsed in RNase-free water and subsequently fixed in methacarn, a fixative composed of 60% methanol, 30% chloroform, and 10% acetic acid. These processing conditions were based on previously published work on techniques for processing bone in preparation for laser capture microdissection [60] as well as preliminary studies conducted to confirm that RNA integrity was optimally preserved when tissue was fixed following decalcification. Following fixation, specimens were rinsed in 100% ethanol and cryopreserved in 30% sucrose in PBS at 4°C overnight prior to embedding in Tissue-Tek OCT (Sakura Finetek; Torrance, CA). Blocks were frozen by placing them on an aluminum block super-cooled in liquid nitrogen and stored at -70°C until sectioned.

Cryosectioning was performed in a Hacker-Bright OTF cryostat. Blocks were placed in the cryostat approximately 30 minutes prior to sectioning to allow them to equilibrate to the chamber temperature of -20°C . Sections were cut using a tungsten carbide blade and mounted onto a PEN Membrane Glass Slide (Applied Biosystems; Foster City, CA) and stored at -70°C until needed. Slides were stained using the HistoGene Staining Kit (Applied Biosystems; Foster City, CA) according to the manufacturer's protocol. Special care was taken to use RNase-free reagents when staining slides. Following dehydration in xylenes, slides were air-dried and taken directly to be microdissected.

2.2.3. Laser Capture Microdissection—Microdissection was performed using an Arcturus Veritas system (Applied Biosystems; Foster City, CA). In brief, a CapSure Macro LCM cap (Applied Biosystems; Foster City, CA) for osteocytes or a CapSure HS LCM cap for MKs coated with a thin “transfer film” was placed above a given region of interest. A UV cutting laser was utilized to trace the cells to be captured, either areas of bone encompassing primarily osteocytes or individual MKs. An infrared laser was then pulsed through the cap, melting the transfer film onto the cells of interest, thereby bonding the cells to the cap. The cap was directly visualized to confirm the completeness of the capture. In the event that extraneous material was captured onto the cap, it was removed from the cap by ablating the material with the cutting laser, or else carefully removed using a Post-itTM note (3M; St. Paul, MN). For osteocytes, regions of cortical bone encompassing approximately 2500 cells from the metaphyseal region were captured across two serial slides from a given limb. One hundred MKs, selected based on their unique morphology (Figure 3a), were captured from two serial slides from a given limb. Individual cells were captured with relatively little excess material (Figure 3b). MKs were captured from four animals harvested 30 minutes following loading. Following capture, either 10 μl (for MKs) or 50 μl (for osteocytes) of Extraction Buffer (Applied Biosystems; Foster City, CA) was added directly to the surface of the cap and incubated at 42°C for 30 minutes. The caps were then centrifuged at $800 \times g$ for 2 minutes to collect cell lysate, and samples were then stored at -70°C until further processing.

2.2.4. RNA extraction and transcript quantification using real time PCR—RNA was extracted using the PicoPure Extraction Kit (Applied Biosystems; Foster City, CA) based on the manufacturer's protocol. Following RNA elution, 9.5 μl of RNA was reverse transcribed using Superscript III reverse transcriptase and primed by random hexamers. Real-time PCR was performed and amplification of gene products was determined by the incorporation of SYBR Green fluorescent nucleic acid stain (Invitrogen; Carlsbad, CA). Beta-2-microglobulin ($\beta 2\text{m}$) was chosen as an internal control based on evidence that it also serves as a reliable control for *in vivo* mechanical stimulation studies [61]. Normalized changes in expression of *c-fos* relative to $\beta 2\text{m}$ were calculated using the $C(t)$ method [55]. All primer sequences are reported in Table 2.

2.2.5. Statistics—For *in vivo* loading studies, a Student's t-test was performed to compare changes in loaded vs. non-loaded gene expression for a given cell type. Statistical significance was determined at $p < 0.05$.

2.3. Mechanoresponsiveness of MKs in culture: Effects on Osteoblast Behavior

2.3.1. Co-culture methods—Plates were coated with 0.1% gelatin (Sigma-Aldrich; St. Louis, MO) for 1 h at room temperature. Following substrate coating, plates were rinsed with PBS and serum-free media, and cells were then plated in DMEM + 10% fetal bovine serum + 1% Penicillin-Streptomycin. Meg-01 cells were seeded either at a density of 100,000 cells/plate for the UT condition or 250,000 cells/plate for sham and 1 Pa conditions. These were determined to yield approximately 50,000 cells remaining adherent to the plate following their respective treatment (data not shown). After 24 h in culture, the media was changed to serum-free conditions for approximately 16 hours. There was no observable difference in cell morphology based on evaluation using optical microscopy (data not shown).

Only one shear condition, a flow rate generating 1 Pa shear stress for 30 minutes, was selected since it still elicited a robust mechanoreponse but resulted in fewer cells shearing off the plate than the 2 Pa condition. Plates were also subject to sham treatment or harvested untouched. Conditioned media from each treatment group was also harvested for proliferation studies. Media was centrifuged to remove cellular debris, and fetal bovine serum and Penicillin + streptomycin were added and sterile filtered using a 0.22 μ m syringe filter. 50,000 MC3T3-E1 cells were then resuspended in conditioned media and plated in 6-well dishes.

Immediately following treatment, slides were placed in a sterile 10 cm dish and 1 mL of a 50,000 cells/mL suspension MC3T3-E1 clone 4 pre-osteoblastic cells in DMEM + 10% fetal bovine serum + 1% Penicillin-Streptomycin were added directly to the slide. After approximately 16 hours, 12 mL fresh DMEM + 10% fetal bovine serum + 1% Penicillin-Streptomycin was added. For plates to be used in mineralization studies, co-cultures were maintained with antibiotic-antimycotic in lieu of penicillin-streptomycin to minimize fungal contamination that was prone to develop in handled plates.

2.3.2. Assessment of proliferation—In order to specifically quantify proliferation of MC3T3-E1 within co-culture plates, we took advantage of the fact that these cell lines are derived from different species – MC3T3-E1 from mouse and Meg-01 from human. Therefore we used PCR using species-specific markers to amplify genomic DNA as a surrogate measure for cell number. After 3 days in culture, plates were rinsed with sterile PBS and stored at -70°C . Cells were harvested in 200 μ L PBS and genomic DNA extracted using the DNeasy Blood & Tissue kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. PCR was performed on the resulting genomic DNA using species-specific primers for cox-1 [62]. We confirmed that mouse primers specifically amplified MC3T3-E1 cells, and also that a linear relationship exists between C(t) value and $\log_2$ (DNA content) from serial dilutions of known quantities of DNA (data not shown). Data reflects three independent experiments with 2-4 plates per condition for each experiment.

2.3.3. Mineralization studies—After 3 days in culture, the media was replaced with differentiation media containing 10mM β -glycerophosphate (BGP) (Sigma-Aldrich; St.

Louis, MO) and 50µg/mL L-ascorbic acid (Sigma-Aldrich; St. Louis, MO). Media was replaced every three days, with fresh BGP and ascorbic acid being added fresh each time.

Plates were harvested at days 0 (prior to addition of differentiation media), 4, 9, and 14 following addition of differentiation media to assess changes in gene expression representative of osteoblast maturation. At the indicated day of harvest, cells were rinsed with PBS and stored at -70°C until further processing. Samples were lysed in Buffer RLT (Qiagen; Valencia, CA) + β -mercaptoethanol and homogenized using Qias shredder columns. RNA was isolated using the RNeasy kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. RNA was quantified and 1 µg of RNA was reverse transcribed using Superscript III reverse transcriptase and primed by random hexamers. Real-time PCR was performed and amplification of gene products was determined by the incorporation of SYBR Green fluorescent nucleic acid stain (Invitrogen; Carlsbad, CA). Expression of Runx2, osterix, osteocalcin, and bone sialoprotein were all normalized to β -actin. Primer sequences for gene expression studies (Table 3) were selected based on previously published work [63]. We also confirmed that these primers specifically detected gene expression from mouse-derived MC3T3-E1 and not the human-derived Meg-01 cells (data not shown). Data represents two independent experiments with 2-4 plates per condition per time point for each experiment.

2.3.4. Alizarin red S staining—At 21 days, plates were rinsed with sterile PBS and allowed to air dry. 50% ethanol was then added for 3 minutes, aspirated, and Alizarin red S stain added for 5 minutes. Plates were rinsed several times with ddH₂O to remove excess stain and allowed to air dry before scanning.

2.3.5. Calcium assay—Cell layers from 18- or 21-day cultures were scraped directly into 1.5 mL tubes and incubated in 0.5M HCl for 1 hr at room temperature. Samples were centrifuged for 10 min. at 6,000 x g and supernatant removed and stored at -20°C until assayed. Calcium content was determined using a QuantiChrom™ calcium assay kit (BioAssay systems; Hayward, CA) and optical densities from the colorimetric assay were read at 612 nm on a SpectraMax5 spectrophotometer (Molecular Devices; Sunnyvale, CA).

2.3.6. Statistics—For co-culture experiments, one-way ANOVA was used to assess differences across groups. For proliferation studies, MC3T3-E1 cultured in the presence of Meg-01 directly or conditioned media were compared to MC3T3-E1 only control to assess effect of treatment of MK on the resulting osteoblast proliferation. For gene expression studies, a one-way ANOVA was conducted across different treatment groups at the same timepoint to assess effect of MK treatment on osteoblast mineralization potential. In all experiments, statistical significance was determined at $p < 0.05$.

3.0. Results

3.1. Mechanoresponsiveness of MKs in culture

Meg-01 displayed a robust response to fluid shear after 30 minutes of loading, as indicated by increases in expression of *c-fos* (Figure 1a) and COX-2 (Figure 1b) genes, which are early responders to mechanical stimuli in a variety of cell types. The increase in *c-fos*

expression when subject to a 0.5 Pa fluid shear stress was similar to the baseline effects of the sham control. However, with both 1 Pa or 2 Pa shear, there was a statistically significant ($p < 0.05$) increase in *c-fos* expression relative to both UT and sham-treated control. Meg-01 exposed to 1 Pa or 2 Pa fluid shear stress also demonstrated significantly higher expression of COX-2 relative to UT controls (Figure 1b).

In PMA-treated K562 cells, *c-fos* expression was enhanced with fluid shear in all shear stress conditions compared to the UT control, but only cells exposed to 0.5 Pa displayed a statistically significant increase in *c-fos* expression compared to sham-treated controls (Figure 2a). Similarly, cells exposed to 0.5 or 2 Pa fluid shear stress demonstrated significantly greater expression of COX-2 relative to 18s rRNA when compared to UT controls (Figure 2b).

3.2. Mechanoresponsiveness of MKs in vivo

To explore if mechanical force alters gene expression of MKs *in vivo*, tibiae were loaded and specimens prepared for laser capture microdissection of MKs. Megakaryocytes and osteocytes were easily discriminated based on standard histomorphological characteristics (Figure 3). In tibiae harvested 60 minutes following one bout of loading, *c-fos* expression normalized to $\beta 2m$ was approximately 1.9-fold higher in osteocytes from loaded bones relative to non-loaded contralateral controls. However, 30 minutes following load, *c-fos* expression was upregulated approximately 2.8-fold in osteocytes within loaded relative to those within non-loaded limbs (Figure 4). A load-response was also confirmed in MKs captured from bones harvested 30 minutes following load. Expression of *c-fos* in this cell population was upregulated approximately 3.1-fold in loaded relative to non-loaded tibiae.

3.3. Mechanoresponsiveness of MKs in culture: Effects on Osteoblast Behavior

To determine if mechanical forces acting on MKs regulate their effects on osteoblast proliferation or differentiation, MC3T3-E1 cells were cultured in the presence of Meg-01 cells. Direct cell co-cultures, but not conditioned media, yielded a pronounced increase in proliferation of MC3T3-E1 relative to MC3T3-E1 only controls after 3 days in culture (Figure 5). The fold-change in mouse genomic DNA content in UT Meg-01 and sham treated Meg-01 containing co-cultures were significantly higher than MC3T3-E1 only controls. However, the corresponding quantity in 1Pa-treated Meg-01 containing co-cultures was not significant over MC3T3-E1 only plates (though there was still a greater-than four-fold increase). This suggests a negative trend in the enhancement of proliferation from Meg-01 cells exposed to fluid shear compared to UT or sham treated cells.

To assess effects of Meg-01 on MC3T3-E1 differentiation, we evaluated the expression of genes essential for osteoblastogenesis. Runx2 and osterix (Osx) are transcription factors that are critical in the early stages of osteoblast differentiation [64, 65]. Osteocalcin (OCN) and bone sialoprotein (BSP) are extracellular matrix proteins that are expressed later in differentiation [66, 67]. We utilized RT-PCR to detect changes in the expression of these genes normalized to levels of β -actin (Figure 6). Expression of all four genes increased over the course of the 14-day experiment (d0 – d14). However, the expression of each of these genes was diminished in MC3T3-E1 cultured in the presence of 1 Pa treated Meg-01 cells

compared to those cultured in the presence of UT or sham-treated Meg-01. Specifically, osteocalcin expression at d4 and BSP expression at d4 and d14 were found to be significantly lower in co-cultures with 1 Pa treated Meg-01 compared to those with sham-treated Meg-01 cells. A trend in *Runx2* expression was noted at d9 ($p=0.06$), BSP at d9 ($p=0.1$), *Osx* at d14 ($p=0.16$), and OCN at both d9 ($p=0.13$) and d14 ($p=0.15$) in co-cultures with 1 Pa-treated Meg-01 compared to those co-cultures with sham-treated cells.

Qualitative assessment of Alizarin Red S stained co-cultures indicated enhanced mineralization of MC3T3-E1 in the presence of mechanically-perturbed (i.e. either sham or 1 Pa treated) Meg-01 cells compared with co-cultures containing UT Meg-01 (Figure 7). This implies the handling of the sham treated specimens may be a sufficient “mechanical perturbation” to elicit some mechanoresponse in these cells. However, variation in the amount of Alizarin-staining nodules across two independent experiments also highlights the variability in the extent of mineralization in this co-culture system.

Quantitative determination of calcium content confirmed the staining patterns observed with Alizarin staining (Figure 8). Co-cultures of MC3T3-E1 with both sham-treated Meg-01 and Meg-01 exposed to 1 Pa shear contained significantly higher amounts of matrix-bound calcium than co-cultures with UT Meg-01.

4.0. Discussion

The purpose of these experiments was to determine whether MKs in the marrow cavity could play a role in sensing and responding to applied skeletal loads. MKs respond to controlled fluid shear forces in culture and, like osteocytes, respond to physical stimuli *in vivo*. To our knowledge, this is the first study that has assessed the mechanoresponsiveness of MKs in a physiologic context *in vivo*, as well as the first study to utilize laser-capture microdissection to investigate mechanically-induced changes in gene expression in any cell in response to applied loads in bone. For our *in vitro* studies, we adapted a parallel plate flow chamber previously developed in our laboratory to expose MKs to controlled fluid shear forces. Though the cell lines used can adhere to a matrix-coated substrate, they are not generally adherent cells and therefore the adhesion is transient and not robust. Our choice of substrate coatings, gelatin (denatured collagen) for Meg-01 cells and fibronectin for PMA-treated K562 cells, were found to enhance adhesion of these cells when exposed to fluid shear forces.

Though a variety of genes are differentially expressed in the presence of load, many studies have focused on a few commonly utilized markers of mechanoresponsiveness. The *c-fos* transcription factor in the AP-1 pathway has been shown to be upregulated in many cell types, including bone-lineage cells *in vitro* [68-70] and *in vivo* [7, 59], in response to physical stimuli. The results presented here demonstrate that MKs respond to load as evidenced by changes in expression of the mechanoresponsive gene, *c-fos*. The magnitude of this mechanoresponse in culture appears to depend on the differentiation state of the cell. The Meg-01 megakaryoblastic cell line demonstrated a more robust mechanoresponse than the more differentiated PMA-treated K562 cells. Similarly, undifferentiated K562 erythromyeloblastic progenitor cells responded more robustly than those treated with PMA

(data not shown). This may indicate that immature megakaryocytes are more sensitive to mechanical cues than mature cells. Additionally, cells at different maturation states may be subjected to different mechanical stimuli, and the subsequent response to these mechanical cues may also differ. Immature cells, thought to reside in closer proximity to osteoblastic precursor cells, could be exposed to shear stresses within a range between 0.5 - 5.0 Pa [52]. Mature MKs, residing at the abluminal surface of marrow sinusoids, may be physiologically exposed to blood-flow induced shear from association with the bone marrow sinusoids (~0.13-0.41 Pa) [42]. These more mature cells are thought to respond to fluid shear by increasing their adhesion (35,36) and releasing platelets (34,37) in response fluid shear. It is possible that the markers of mechanoresponsiveness selected for these studies may not be consistently regulated across cell types of different maturation states.

Osteoblastic and osteocytic cell lines exposed to fluid shear also demonstrate increases in expression of cyclooxygenase-2 (COX-2), a key component in the synthesis of prostaglandin [69, 71, 72]. Our results also demonstrate that COX-2 expression in MKs is less mechanoresponsive in the time course and load pattern examined than expression of *c-fos*. In contrast, osteoblasts subject to fluid shear for 30 minutes demonstrate roughly equivalent increases in *c-fos* and COX-2 relative to untouched baseline controls [69, 73]. One possible explanation for the apparent lack of COX-2 response might be the relatively short time course. Though 30 minutes of fluid shear was enough to induce a significant increase in COX-2 in MLO-Y4 osteocyte-like cells [71] and MC3T3-E1 pre-osteoblastic cells [72] in response to fluid shear, other studies suggest that COX-2 is maximally upregulated following 1 to 4 hours in other osteoblastic cell lines [69, 72]. Alternatively, COX-2 could be differentially regulated in MKs relative to osteoblast lineage cells.

Laser capture microdissection was utilized as a tool to assess gene expression *in vivo*. In response to 10 minutes of tibial axial compression, osteocytes captured from the metaphyseal region of the loaded limb express roughly 2.8-fold increase in *c-fos* relative to non-loaded limbs after 30 minutes and 1.9-fold increase after 60 minutes. The magnitude of this response is diminished compared to a study by Inaoka, et al., which, through use of Northern blotting, showed a maximum 4-fold induction in expression of *c-fos* normalized to actin following 1 hour of vertebral loading [58]. In an effort to specifically address the mechanoresponsiveness of MKs *in vivo*, we isolated individual MKs from loaded and non-loaded limbs using laser capture microdissection from bones harvested 30 minutes post-load, given the maximal response in osteocytes from this group. We determined that MK expression of *c-fos* was elevated approximately 3.1-fold in loaded relative to non-loaded limbs, comparable to the level of increased expression in osteocytes. This difference was statistically significant compared to the non-loaded control limbs. *C-fos* was the only gene evaluated in *in vivo* studies given the more robust changes noted than *cox-2* based on *in vitro* studies and given the challenge of obtaining sufficient RNA for multiple analyses when using laser capture microdissection. This is the first study to our knowledge that has assessed the response of these cells to an applied physical stimulus *in vivo*. It is important to note, however, that only more mature MKs were isolated based on their unique morphology that can easily be identified histologically. As discussed above, our *in vitro* results indicate that MK mechanoresponsiveness may depend on the maturation state of the cell. Therefore,

it is possible that the magnitude of changes in gene expression may differ if a more specific population of immature MKs had been isolated.

We have developed a system to address the hypothesis that mechanical stimulation of MKs will impact osteoblast behavior and confirmed that there is a substantial effect of the presence of MKs on osteoblast proliferation when in co-culture, but not when osteoblasts are cultured in the presence of MK-conditioned media. This is consistent with other studies that have demonstrated the importance of cell-cell contact, potentially through gap junctions or integrins, in the effects of MKs on osteoblast behavior [27, 28]. However, there is not a significant effect of mechanical stimulation of MKs on the resultant proliferative-inducing effects on MC3T3-E1. Our data suggests a small but non-significant decrease in MC3T3-E1 cell number, as determined by quantity of genomic DNA, in the co-cultures in the presence of 1Pa-stimulated Meg-01 compared with UT or sham-treated Meg-01.

The effect of mechanical stimulation of MKs on the differentiation of co-cultured MC3T3-E1, based on our findings, is perplexing. Alizarin Red staining and calcium assays suggest increases in mineralization of co-cultures with mechanically stimulated Meg-01 cells; however, this conflicts with gene expression data which suggest an impaired, or delayed, differentiation of MC3T3-E1 cultured in the presence of 1 Pa-treated Meg-01 compared with sham-treated controls. A definitive explanation to account for these two apparently contradictory findings remains elusive. Our findings may indicate that MKs could have effects on formation of mineralized nodules independent of the osteoblast transcriptional program. We observed that, oftentimes, there were more mineralized nodules formed in regions of the plate that were more densely occupied by MKs (Figure 9). It is important to note that MKs themselves express various bone matrix-associated proteins, such as BMPs 2, 4, and 6 [74], osteonectin [75], osteocalcin [76], and thrombospondins 1 and 2 [77] as well as crosslinking proteins such as factor XIII [78]. Though it is not clear what implications this might have on direct matrix formation, it highlights the potential complexity of the role of MKs on mineralization. Alternatively, the effects of mechanical stimulation may be temporally dependent and hence difficult to assess with the measures employed in this study.

Our results suggest that mechanotransduction in bone may not solely be a single cell phenomenon, but perhaps a more complex series of events involving communication between accessory cells, such as MKs, and their effector cells that can directly influence bone homeostasis. Though clinical implications are more distant, this work may elucidate new directions for development of therapeutics for treating bone metabolic diseases as well as enhanced techniques for bone regeneration and repair. By developing a deeper understanding of the mechanism by which accessory cells, such as MKs, act on bone metabolic processes, our perspective of potential therapeutic targets may be broadened.

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Abbreviations

(MK)	Megakaryocyte
(LCM)	Laser Capture Microdissection
(ECM)	extracellular matrix
(PMA)	phorbol 12-myristate 13-acetate
(UT)	untouched
(BGP)	β -glycerophosphate
(osx)	Osterix
(OCN)	Osteocalcin
(BSP)	Bone Sialoprotein

References

- [1]. Guldberg RE, Caldwell NJ, Guo XE, Goulet RW, Hollister SJ, Goldstein SA. Mechanical stimulation of tissue repair in the hydraulic bone chamber. *J Bone Miner Res.* 1997; 12:1295–302. [PubMed: 9258761]
- [2]. Torrance AG, Mosley JR, Suswillo RF, Lanyon LE. Noninvasive loading of the rat ulna in vivo induces a strain-related modeling response uncomplicated by trauma or periosteal pressure. *Calcif Tissue Int.* 1994; 54:241–7. [PubMed: 8055374]
- [3]. Turner CH, Akhter MP, Raab DM, Kimmel DB, Recker RR. A noninvasive, in vivo model for studying strain adaptive bone modeling. *Bone.* 1991; 12:73–9. [PubMed: 2064843]
- [4]. Rubin CT, Lanyon LE. Regulation of bone mass by mechanical strain magnitude. *Calcif Tissue Int.* 1985; 37:411–7. [PubMed: 3930039]
- [5]. Gross TS, Srinivasan S, Liu CC, Clemens TL, Bain SD. Noninvasive loading of the murine tibia: an in vivo model for the study of mechanotransduction. *J Bone Miner Res.* 2002; 17:493–501. [PubMed: 11874240]
- [6]. Klein-Nulend J, van der Plas A, Semeins CM, Ajubi NE, Frangos JA, Nijweide PJ, Burger EH. Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB J.* 1995; 9:441–5. [PubMed: 7896017]
- [7]. Kawata A, Mikuni-Takagaki Y. Mechanotransduction in stretched osteocytes--temporal expression of immediate early and other genes. *Biochem Biophys Res Commun.* 1998; 246:404–8. [PubMed: 9610372]
- [8]. Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. *J Bone Miner Res.* 2000; 15:209–17. [PubMed: 10703922]
- [9]. Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.* 2007; 5:464–75. [PubMed: 17550781]
- [10]. Wilkes CH, Visscher MB. Some physiological aspects of bone marrow pressure. *J Bone Joint Surg Am.* 1975; 57:49–57. [PubMed: 1123371]
- [11]. Bryant JD. The effect of impact on the marrow pressure of long bones in vitro. *J Biomech.* 1983; 16:659–65. [PubMed: 6643538]
- [12]. Bryant JD. On the mechanical function of marrow in long bones. *Eng Med.* 1988; 17:55–8. [PubMed: 3402646]
- [13]. Liu ZJ, Herring SW. Bone surface strains and internal bony pressures at the jaw joint of the miniature pig during masticatory muscle contraction. *Arch Oral Biol.* 2000; 45:95–112. [PubMed: 10716614]

- [14]. Zhang P, Su M, Liu Y, Hsu A, Yokota H. Knee loading dynamically alters intramedullary pressure in mouse femora. *Bone*. 2007; 40:538–43. [PubMed: 17070127]
- [15]. Qin YX, Kaplan T, Saldanha A, Rubin C. Fluid pressure gradients, arising from oscillations in intramedullary pressure, is correlated with the formation of bone and inhibition of intracortical porosity. *J Biomech*. 2003; 36:1427–37. [PubMed: 14499292]
- [16]. Qin YX, Lam H. Intramedullary pressure and matrix strain induced by oscillatory skeletal muscle stimulation and its potential in adaptation. *J Biomech*. 2009; 42:140–5. [PubMed: 19081096]
- [17]. Kelly P, Bronk J. Venous Pressure and Bone Formation. *Microvascular Research*. 1990; 39:364–375. [PubMed: 2362558]
- [18]. Bergula AP, Huang W, Frangos JA. Femoral vein ligation increases bone mass in the hindlimb suspended rat. *Bone*. 1999; 24:171–7. [PubMed: 10071908]
- [19]. Dickerson DA, Sander EA, Nauman EA. Modeling the mechanical consequences of vibratory loading in the vertebral body: microscale effects. *Biomech Model Mechanobiol*. 2008; 7:191–202. [PubMed: 17520305]
- [20]. Tavassoli, M.; Yoffey, J. *Bone Marrow: Structure and Function*. Alan R. Liss, Inc.; 1983.
- [21]. Gross P, Heistad D, Marcus M. Neurohumoral regulation of blood flow to bones and marrow. *American Journal of Physiology*. 1979; 237:H440–8. [PubMed: 495729]
- [22]. Aubin JE. Osteoprogenitor cell frequency in rat bone marrow stromal populations: role for heterotypic cell-cell interactions in osteoblast differentiation. *J Cell Biochem*. 1999; 72:396–410. [PubMed: 10022521]
- [23]. Rickard DJ, Kazhdan I, Leboy PS. Importance of 1,25-dihydroxyvitamin D3 and the nonadherent cells of marrow for osteoblast differentiation from rat marrow stromal cells. *Bone*. 1995; 16:671–8. [PubMed: 7669445]
- [24]. Eipers PG, Kale S, Taichman RS, Pipia GG, Swords NA, Mann KG, Long MW. Bone marrow accessory cells regulate human bone precursor cell development. *Exp Hematol*. 2000; 28:815–25. [PubMed: 10907643]
- [25]. Kacena MA, Shivdasani RA, Wilson K, Xi Y, Troiano N, Nazarian A, Gundberg CM, Bouxsein ML, Lorenzo JA, Horowitz MC. Megakaryocyte-osteoblast interaction revealed in mice deficient in transcription factors GATA-1 and NF-E2. *J Bone Miner Res*. 2004; 19:652–60. [PubMed: 15005853]
- [26]. Miao D, Murant S, Scutt N, Genever P, Scutt A. Megakaryocyte-bone marrow stromal cell aggregates demonstrate increased colony formation and alkaline phosphatase expression in vitro. *Tissue Eng*. 2004; 10:807–17. [PubMed: 15265298]
- [27]. Ciovacco WA, Goldberg CG, Taylor AF, Lemieux JM, Horowitz MC, Donahue HJ, Kacena MA. The role of gap junctions in megakaryocyte-mediated osteoblast proliferation and differentiation. *Bone*. 2009; 44:80–6. [PubMed: 18848655]
- [28]. Lemieux JM, Horowitz MC, Kacena MA. Involvement of integrins $\alpha(3)\beta(1)$ and $\alpha(5)\beta(1)$ and glycoprotein IIb in megakaryocyte-induced osteoblast proliferation. *J Cell Biochem*. 2010; 109:927–932. [PubMed: 20052668]
- [29]. Beeton CA, Bord S, Ireland D, Compston JE. Osteoclast formation and bone resorption are inhibited by megakaryocytes. *Bone*. 2006; 39:985–90. [PubMed: 16870519]
- [30]. Kacena MA, Nelson T, Clough ME, Lee SK, Lorenzo JA, Gundberg CM, Horowitz MC. Megakaryocyte-mediated inhibition of osteoclast development. *Bone*. 2006; 39:991–9. [PubMed: 16782418]
- [31]. Sun L, Hwang WY, Aw SE. Biological characteristics of megakaryocytes: specific lineage commitment and associated disorders. *Int J Biochem Cell Biol*. 2006; 38:1821–6. [PubMed: 16730215]
- [32]. Sola-Visner MC, Christensen RD, Hutson AD, Rimsza LM. Megakaryocyte size and concentration in the bone marrow of thrombocytopenic and nonthrombocytopenic neonates. *Pediatr Res*. 2007; 61:479–84. [PubMed: 17515875]
- [33]. Kakumitsu H, Kamezaki K, Shimoda K, Karube K, Haro T, Numata A, Shide K, Matsuda T, Oshima K, Harada M. Transgenic mice overexpressing murine thrombopoietin develop myelofibrosis and osteosclerosis. *Leuk Res*. 2005; 29:761–9. [PubMed: 15927672]

- [34]. Yan XQ, Lacey D, Hill D, Chen Y, Fletcher F, Hawley RG, McNiece IK. A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation. *Blood*. 1996; 88:402–9. [PubMed: 8695786]
- [35]. Bord S, Frith E, Ireland DC, Scott MA, Craig JI, Compston JE. Estrogen stimulates differentiation of megakaryocytes and modulates their expression of estrogen receptors alpha and beta. *J Cell Biochem*. 2004; 92:249–57. [PubMed: 15108352]
- [36]. Bord S, Frith E, Ireland DC, Scott MA, Craig JI, Compston JE. Synthesis of osteoprotegerin and RANKL by megakaryocytes is modulated by oestrogen. *Br J Haematol*. 2004; 126:244–51. [PubMed: 15238146]
- [37]. Bord S, Vedi S, Beavan SR, Horner A, Compston JE. Megakaryocyte population in human bone marrow increases with estrogen treatment: a role in bone remodeling? *Bone*. 2000; 27:397–401. [PubMed: 10962351]
- [38]. Taichman R, Reilly M, Verma R, Ehrenman K, Emerson S. Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br J Haematol*. 2001; 112:438–48. [PubMed: 11167845]
- [39]. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*. 2003; 425:841–6. [PubMed: 14574413]
- [40]. Cheng L, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) hematopoietic progenitor cells. *J Cell Physiol*. 2000; 184:58–69. [PubMed: 10825234]
- [41]. Lichtman MA, Chamberlain JK, Simon W, Santillo PA. Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. *Am J Hematol*. 1978; 4:303–12. [PubMed: 717392]
- [42]. Junt T, Schulze H, Chen Z, Massberg S, Goerge T, Krueger A, Wagner DD, Graf T, Italiano JE Jr, Shivdasani RA, von Andrian UH. Dynamic visualization of thrombopoiesis within bone marrow. *Science*. 2007; 317:1767–70. [PubMed: 17885137]
- [43]. Eldor A, Stromberg RR, Vlodavsky I, Hy-Am E, Koslow AR, Friedman LI, Levine RF. The effect of flow on the interaction of isolated megakaryocytes with subendothelial extracellular matrix. *Blood Cells*. 1991; 17:447–63. discussion 464–6. [PubMed: 1760556]
- [44]. de Bruyn K, Zwartkruis F, de Rooij J, Akkerman J, Bos J. The Small GTPase Rap1 is Activated by Turbulence and Is Involved in Integrin α IIb β 3-mediated Cell Adhesion in Human Megakaryocytes. *Journal of Biological Chemistry*. 2003; 278:22412–22417. [PubMed: 12690117]
- [45]. Dunois-Larde C, Capron C, Fichelson S, Bauer T, Cramer-Borde E, Baruch D. Exposure of human megakaryocytes to high shear rates accelerates platelet production. *Blood*. 2009; 114:1875–83. [PubMed: 19525480]
- [46]. Li X, Koh AJ, Wang Z, Soki FN, Park SI, Pienta KJ, McCauley LK. Inhibitory effects of megakaryocytic cells in prostate cancer skeletal metastasis. *J Bone Miner Res*. 2011; 26:125–34. [PubMed: 20684002]
- [47]. Tetteroo PA, Massaro F, Mulder A, Schreuder-van Gelder R, von dem Borne AE. Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. *Leuk Res*. 1984; 8:197–206. [PubMed: 6232432]
- [48]. Ominsky, MS. Effects of hydrostatic pressure, biaxial strain, and fluid shear on osteoblastic cells: Mechanotransduction via NF- κ B, MAP kinase, and AP-1 pathways. University of Michigan; 2003. Order No. 3096163 ProQuest Dissertations and Theses. p. 163–163. Retrieved from <http://search.proquest.com/docview/287952432?accountid=131239>. (287952432)
- [49]. Joiner DM, Tayim RJ, Kadado A, Goldstein SA. Bone marrow stromal cells from aged male rats have delayed mineralization and reduced response to mechanical stimulation through nitric oxide and ERK1/2 signaling during osteogenic differentiation. *Biogerontology*. 2012; 13:467–78. [PubMed: 22944913]
- [50]. Frangos JA, Eskin SG, McIntire LV, Ives CL. Flow effects on prostacyclin production by cultured human endothelial cells. *Science*. 1985; 227:1477–9. [PubMed: 3883488]

- [51]. Jacobs CR, Yellowley CE, Davis BR, Zhou Z, Cimbala JM, Donahue HJ. Differential effect of steady versus oscillating flow on bone cells. *J Biomech.* 1998; 31:969–76. [PubMed: 9880053]
- [52]. Coughlin TR, Niebur GL. Fluid shear stress in trabecular bone marrow due to low-magnitude high-frequency vibration. *J Biomech.* 45:2222–9. [PubMed: 22784651]
- [53]. Alford AI, Yellowley CE, Jacobs CR, Donahue HJ. Increases in cytosolic calcium, but not fluid flow, affect aggrecan mRNA levels in articular chondrocytes. *J Cell Biochem.* 2003; 90:938–44. [PubMed: 14624453]
- [54]. Maclean JJ, Lee CR, Alini M, Iatridis JC. Anabolic and catabolic mRNA levels of the intervertebral disc vary with the magnitude and frequency of in vivo dynamic compression. *J Orthop Res.* 2004; 22:1193–200. [PubMed: 15475197]
- [55]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods.* 2001; 25:402–8. [PubMed: 11846609]
- [56]. De Souza RL, Matsuura M, Eckstein F, Rawlinson SC, Lanyon LE, Pitsillides AA. Non-invasive axial loading of mouse tibiae increases cortical bone formation and modifies trabecular organization: a new model to study cortical and cancellous compartments in a single loaded element. *Bone.* 2005; 37:810–8. [PubMed: 16198164]
- [57]. Fritton JC, Myers ER, Wright TM, van der Meulen MC. Loading induces site-specific increases in mineral content assessed by microcomputed tomography of the mouse tibia. *Bone.* 2005; 36:1030–8. [PubMed: 15878316]
- [58]. Inaoka T, Lean JM, Bessho T, Chow JW, Mackay A, Kokubo T, Chambers TJ. Sequential analysis of gene expression after an osteogenic stimulus: c-fos expression is induced in osteocytes. *Biochem Biophys Res Commun.* 1995; 217:264–70. [PubMed: 8526921]
- [59]. Lean JM, Mackay AG, Chow JW, Chambers TJ. Osteocytic expression of mRNA for c-fos and IGF-I: an immediate early gene response to an osteogenic stimulus. *Am J Physiol.* 1996; 270:E937–45. [PubMed: 8764176]
- [60]. Shao YY, Wang L, Hicks DG, Ballock RT. Analysis of gene expression in mineralized skeletal tissues by laser capture microdissection and RT-PCR. *Lab Invest.* 2006; 86:1089–95. [PubMed: 16940962]
- [61]. Xing W, Baylink D, Kesavan C, Hu Y, Kapoor S, Chadwick RB, Mohan S. Global gene expression analysis in the bones reveals involvement of several novel genes and pathways in mediating an anabolic response of mechanical loading in mice. *J Cell Biochem.* 2005; 96:1049–60. [PubMed: 16149068]
- [62]. Parodi B, Aresu O, Bini D, Lorenzini R, Schena F, Visconti P, Cesaro M, Ferrera D, Andreotti V, Ruzzon T. Species identification and confirmation of human and animal cell lines: a PCR-based method. *Biotechniques.* 2002; 32:432–4. 436, 438–40. [PubMed: 11848419]
- [63]. Alford AI, Terkhorn SP, Reddy AB, Hankenson KD. Thrombospondin-2 regulates matrix mineralization in MC3T3-E1 pre-osteoblasts. *Bone.* 2010; 46:464–71. [PubMed: 19744582]
- [64]. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. *Osf2/Cbfa1*: a transcriptional activator of osteoblast differentiation. *Cell.* 1997; 89:747–54. [PubMed: 9182762]
- [65]. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell.* 2002; 108:17–29. [PubMed: 11792318]
- [66]. Hauschka PV, Reid ML. Timed appearance of a calcium-binding protein containing gamma-carboxyglutamic acid in developing chick bone. *Dev Biol.* 1978; 65:426–34. [PubMed: 680371]
- [67]. Chen J, Shapiro HS, Sodek J. Development expression of bone sialoprotein mRNA in rat mineralized connective tissues. *J Bone Miner Res.* 1992; 7:987–97. [PubMed: 1442213]
- [68]. Peake MA, Cooling LM, Magnay JL, Thomas PB, El Haj AJ. Selected contribution: regulatory pathways involved in mechanical induction of c-fos gene expression in bone cells. *J Appl Physiol.* 2000; 89:2498–507. [PubMed: 11090608]
- [69]. Pavalko FM, Chen NX, Turner CH, Burr DB, Atkinson S, Hsieh YF, Qiu J, Duncan RL. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. *Am J Physiol.* 1998; 275:C1591–601. [PubMed: 9843721]

- [70]. Granet C, Vico AG, Alexandre C, Lafage-Proust MH. MAP and src kinases control the induction of AP-1 members in response to changes in mechanical environment in osteoblastic cells. *Cell Signal*. 2002; 14:679–88. [PubMed: 12020768]
- [71]. Cheng B, Kato Y, Zhao S, Luo J, Sprague E, Bonewald LF, Jiang JX. PGE(2) is essential for gap junction-mediated intercellular communication between osteocyte-like MLO-Y4 cells in response to mechanical strain. *Endocrinology*. 2001; 142:3464–73. [PubMed: 11459792]
- [72]. Wadhwa S, Godwin SL, Peterson DR, Epstein MA, Raisz LG, Pilbeam CC. Fluid flow induction of cyclo-oxygenase 2 gene expression in osteoblasts is dependent on an extracellular signal-regulated kinase signaling pathway. *J Bone Miner Res*. 2002; 17:266–74. [PubMed: 11811557]
- [73]. Lee DY, Yeh CR, Chang SF, Lee PL, Chien S, Cheng CK, Chiu JJ. Integrin-mediated expression of bone formation-related genes in osteoblast-like cells in response to fluid shear stress: roles of extracellular matrix, Shc, and mitogen-activated protein kinase. *J Bone Miner Res*. 2008; 23:1140–9. [PubMed: 18333755]
- [74]. Sipe JB, Zhang J, Waits C, Skikne B, Garimella R, Anderson HC. Localization of bone morphogenetic proteins (BMPs)-2, -4, and -6 within megakaryocytes and platelets. *Bone*. 2004; 35:1316–22. [PubMed: 15589212]
- [75]. Kelm RJ Jr, Hair GA, Mann KG, Grant BW. Characterization of human osteoblast and megakaryocyte-derived osteonectin (SPARC). *Blood*. 1992; 80:3112–9. [PubMed: 1467517]
- [76]. Thiede MA, Smock SL, Petersen DN, Grasser WA, Thompson DD, Nishimoto SK. Presence of messenger ribonucleic acid encoding osteocalcin, a marker of bone turnover, in bone marrow megakaryocytes and peripheral blood platelets. *Endocrinology*. 1994; 135:929–37. [PubMed: 8070388]
- [77]. Kyriakides TR, Rojnuckarin P, Reidy MA, Hankenson KD, Papayannopoulou T, Kaushansky K, Bornstein P. Megakaryocytes require thrombospondin-2 for normal platelet formation and function. *Blood*. 2003; 101:3915–23. [PubMed: 12732502]
- [78]. Aeschlimann D, Mosher D, Paulsson M. Tissue transglutaminase and factor XIII in cartilage and bone remodeling. *Semin Thromb Hemost*. 1996; 22:437–43. [PubMed: 8989828]

Highlights

Expression of mechanoresponsive genes is upregulated in MKs subject to fluid shear.

MKs isolated from axially-loaded murine tibiae demonstrate a mechanoresponse.

MKs exposed to shear minimally decrease proliferation of co-cultured osteoblasts.

MKs exposed to shear affect mineralization capacity of co-cultured osteoblasts.

Figure 1. Meg-01 cells respond to fluid shear stress by increasing expression of *c-fos* and COX-2 RT-PCR was performed to detect changes in (A) *c-fos* and (B) COX-2 expression relative to 18s rRNA. Each treatment was performed in duplicate and data represents four independent experiments (mean $\pm$ SD). * $p < 0.05$ relative to untouched, ** $p < 0.05$ relative to sham treatment.

Figure 2. PMA-treated K562 cells respond to fluid shear stress by increasing expression of *c-fos* and COX-2

RT-PCR was performed to detect changes in (A) *c-fos* and (B) COX-2 expression relative to 18S rRNA. Each treatment was performed in triplicate and data represents two independent experiments (mean $\pm$ SD). *p<0.05 relative to UT, **p<0.05 relative to sham

Figure 3. Megakaryocytes (MKs) are easily identified histomorphologically for laser capture microdissection

(A) MKs were identified by their unique morphologic characteristics, namely large size and multi-lobed nucleus. (B) Individual cells were captured with relatively small amounts of extraneous material.

Figure 4. Osteocytes and MKs demonstrate a load response *in vivo* in a tibial loading model

Osteocytes and MKs were harvested from tissue sections utilizing laser capture microdissection. Data represents mean $\pm$ SD from n=5 (osteocytes) or n=4 (MKs) specimens. *p<0.05 relative to non-loaded value

Figure 5. Direct co-culture with Meg-01 cells stimulates proliferation of >MC3T3-E1, but this effect is less pronounced in the presence of Meg-01 cells exposed to fluid shear
Cells or conditioned media (CM) from UT, sham, or 1Pa treated Meg-01 were cultured with MC3T3-E1. Fold change in mouse genomic DNA content relative to MC3T3-E1 only controls after 3 days in culture. Experiments were repeated three times in duplicate. Data represents mean $\pm$ SD. * $p < 0.05$ compared to MC3T3-E1 only control

Figure 6. MC3T3-E1 expression of osteoblastic differentiation markers is decreased in the presence of co-cultured Meg-01 cells exposed to fluid shear relative to co-cultures with sham-treated Meg-01 cells

Expression of (A) Runx2, (B) osterix, (C) osteocalcin, and (D) bone sialoprotein were normalized to β -actin. Results for each treatment at a given timepoint are expressed relative to baseline expression for that treatment at d0. Data represents mean $\pm$ SD from two independent experiments with 2-4 samples per time point per experiment. ** $p < 0.05$ compared to MC3T3-E1 + sham-treated Meg-01

Figure 7. Alizarin Red S-stained co-cultures suggest enhanced mineralization of MC3T3-E1 in the presence of mechanically-perturbed Meg-01 cells compared with co-cultures containing untouched Meg-01 cells

Images represent duplicate wells from one experiment.

Figure 8. Calcium content of MC3T3-E1 co-cultures with sham, or 1Pa treated Meg-01 cells is elevated compared to co-cultures with UT Meg-01

Data represents mean $\pm$ SD from three independent experiments, with 2-5 plates for a given treatment per experiment. * $p < 0.05$ relative to No BGP treated control. ** $p < 0.05$ relative to UT.

Figure 9. Mineralized nodules in MC3T3-E1/Meg-01 co-cultures appeared to be more prevalent in areas more densely occupied by Meg-01 cells

Alizarin Red-positive nodules (black arrows) tend to be more prominent in regions where more Meg-01 cells (round cells, such as those marked by white asterisks) remain adherent (A) compared to regions less occupied by Meg-01 cells (B).

Table 1

Primer sequences for human-derived cell lines Meg-01 and K562.

Gene (human)	Forward	Reverse
<i>c-fos</i>	cgttgtgaagaccatgacag	tccgctggagtgatcagt
COX-2	caccctctactctggcatc	aacattctaccaccagcaa
18s rRNA	ctcaacacgggaaacctcac	atgccagagtctcttcgtt

Table 2

Primer sequences for laser capture microdissected samples from murine tibiae.

Gene (mouse)	Forward	Reverse
<i>c-fos</i> (osteocytes)	ggggacagccttcctacta	tggggataaagtggcacta
<i>c-fos</i> (MKs)	ggggacagccttcctacta	gacagatctgcgcaaaagtc
b2m	ctggctcttctgggtcttgt	cgttcttcagcatttggatt

Table 3

Primer sequences for co-culture mineralization studies

Gene	Forward	Reverse
Runx2	gtcagcaaagcttcttttcg	ttgttgctgttgctgttgtt
Osterix	tcttccatctgcctgactc	gtcagcgtatggcttctttg
Osteocalcin	cgtctgtctctctgacctc	tcacaagcagggttaagctc
Bone sialoprotein	ttccatcgaagaatcaaagc	tcgcagtcctcattttcttc
β -actin	aagagctatgagctgcctga	tggcatagaggtctttacgg