# Effect of Low-Magnitude, High-Frequency Vibration on Osteogenic Differentiation of Rat Mesenchymal Stromal Cells

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**ABSTRACT:** Whole body vibration (WBV), consisting of a low-magnitude, high-frequency (LMHF) signal, is anabolic to bone in vivo and may act through alteration of the lineage commitment of mesenchymal stromal cells (MSC). We investigated the effect of LMHF vibration on rat bone marrow-derived MSCs (rMSCs) in an in vitro system. We subjected rMSCs to repeated (six) bouts of 1-h vibration at 0.3*g* and 60 Hz in the presence of osteogenic (OS) induction medium. The OS differentiation of rMSCs under the loaded and non-loaded conditions was assessed by examining cell proliferation, alkaline phosphatase (ALP) activity, mRNA expression of various osteoblast-associated markers [ALP, Runx2, osterix (Osx), collagen type I alpha 1 (COL1A1), bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OCN)], and matrix mineralization. LMHF vibration did not enhance the OS differentiation of rMSCs. Surprisingly, the mRNA level of Osx, a transcription factor necessary for osteoblast formation, was decreased, and matrix mineralization was inhibited. Our findings suggest that LMHF vibration may exert its anabolic effects in vivo via mechanosensing of a cell type different from MSCs. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 29:1075–1080, 2011

**Keywords:** mesenchymal stromal cell; low-magnitude; high-frequency vibration; osteogenic differentiation; alkaline phosphatase; osterix

Osteoporosis, a disease characterized by progressive deterioration of bone tissue due to an imbalance in the breakdown and rebuilding of bone, leads to increased bone fragility and susceptibility to fracture. Current measures for the prevention and treatment of osteoporosis are primarily drug-based, which delay disease progression but do not fully restore the balance in bone resorption and formation. Based on the premise that bone is a dynamic and self-regulating organ capable of adapting its mass and morphology according to its mechanical environment, some researchers have turned to a biomechanical approach to treating osteoporosis. Of recent interest is a mechanical signal—low-magnitude, high-frequency (LMHF) vibrations—that produces anabolic responses in bone when applied to the entire body of subjects (termed whole body vibration or WBV).[1,2] Such responses increased bone formation in animal models, including young mice,[3] ovariectomized rats,[4] and rats subjected to hind limb disuse by tail-suspension.[5]

The mechanism by which LMHF vibration induces anabolic responses at the cellular level remains largely unknown. Recent in vivo studies suggest that LMHF vibration directs the lineage commitment of bone marrow mesenchymal stromal cells (MSCs) in favor of osteogenesis over adipogenesis.[6,7] MSCs can form bone in vitro under appropriate chemical cues,[8,9] a process shown to be modulated by mechanical signals. MSCs isolated from different sources, including bone marrow,[10–13] adipose tissue,[14] and calvariae,[15,16] were tested for their response to a variety of mechanical stimuli in vitro, including tensile strain,[10–12] fluid flow-induced shear stress,[13–15] and pressure.[16,17] Several osteoblast-associated markers of differentiating MSCs were upregulated by applied mechanical forces such as strain and fluid shear, possibly via activation of MAPK pathways.[10,12,18] However, the effect of LMHF vibration applied directly on MSCs has not been studied. Given the complex cellular heterogeneity in bone marrow, multiple cell types including MSCs may respond to LMHF vibration and account for the anabolic responses in vivo. Hence, in this study, we aimed to delineate the effect of LMHF vibration on MSCs in vitro.

We hypothesized that LMHF vibration enhances the osteogenesis of MSCs in vitro in the presence of osteogenic (OS) factors, leading to increased expression of osteoblastic markers and matrix mineralization. To test our hypothesis, we subjected rat MSCs to repeated bouts of the LMHF stimulus at a magnitude of 0.3*g* and a frequency of 60 Hz to mimic the vibration conditions used in animal and human studies.[1,5,19] We compared cell proliferation, alkaline phosphatase (ALP) activity, gene expression of certain transcription factors and matrix molecules, and their functional capacity to differentiate and form mineralized bone nodules between MSCs under LMHF stimulation and static controls.

## MATERIALS AND METHODS

### Bone Marrow Cell Isolation and Culture

Bone marrow cells were isolated from male Wistar rats (~4-week-old) as previously described[8] (see Supplementary Materials for details). After 6 days of primary culture, with medium changes every 2–3 days, cells of the first passage were seeded at a density of $5 \times 10^3$ cells/cm$^2$ for all experiments

Additional Supporting Information may be found in the online version of this article.

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(with the exception of the proliferation study, where cells were seeded at $1 \times 10^3$ cells/cm$^2$ to prevent inhibition of growth due to cell contact) in multi-well plates. Forty-eight hours later, cells were serum-starved overnight in α-MEM containing 10% antibiotics (AB) and 0.1% fetal bovine serum (FBS) to synchronize the cells.[12] After the first bout of vibration, cultures were maintained in OS medium (α-MEM containing 10% FBS, 1× AB, 10 nM dexamethasone, 50 μg/ml ascorbic acid, and 5 mM beta-glycerophosphate).

### LMHF Vibration System

A metal vibration plate was custom-made to contain two multi-well tissue culture plates as previously described.[20] The plate was attached to a shaker (ET-127, Labworks, Inc., Costa Mesa, CA) that delivered vertical accelerations. The amplitude, waveform, and frequency of the vibration provided by the shaker were controlled with VibeLab computer program (Labworks, Inc.). Peak-to-peak acceleration was measured at the center of the plate with a piezoelectric accelerometer (8632C5, Kistler, Amherst, MA), which output a voltage signal to the computer for feedback control between the desired and measured waveforms.

### Vibration Loading of Cell Cultures

After overnight serum-starvation to synchronize the cells, each well was completely filled with serum-free medium and sealed with gas permeable sealing film (Excel Scientific, Victorville, CA) immediately prior to vibration. This minimized fluid perturbation (and thus fluid shear stress) within the wells during vibration. Plates were placed securely onto the vibration plate and subjected to 60 Hz of sinusoidal vibrations at 0.3*g* for 1 h. Cells in the non-vibration group were placed on the same but stationary plate. After 1 h, cells in both the vibrated and non-vibrated group received fresh OS medium. Cultures received five more vibration loading bouts over 6 days (Fig. 1). Cell samples were collected immediately after the third consecutive bout of vibration (on days 2 and 6 and on days specified in Fig. 1).

### Proliferation Assay

The amount of DNA in the cultured cell samples was measured using CyQUANT® Cell Proliferation Assays Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, cell layers were rinsed 2× with phosphate buffered saline (PBS) and stored at −80°C overnight. Cells were then lysed with the kit's lysis buffer containing a nucleic acid-binding fluorescence dye. Using a microplate reader with excitation at 485 nm and emission detection at 530 nm, fluorescence measurements were made and compared against a standard curve of known cell number.

### mRNA Quantification

Quantitative PCR was used to measure the mRNA levels of several early to late osteoblastic markers, including the OS transcription factors runt-related transcription factor 2 (Runx2) and osterix (Osx), ALP, and bone matrix proteins such as collagen type I alpha 1 (COL1A1), osteopontin (OPN), and bone sialoprotein (BSP) and osteocalcin (OCN). See Supplementary Material for detailed methods and rat-specific primer sequences (Table S1).

### ALP Assay

For quantitative analysis of ALP activity, ALP was extracted and detected with SensoLyte *p*-nitrophenyl phosphate (*p*NPP) ALP assay kit (AnaSpec, Fremont, CA) according to the manufacturer's protocol. Briefly, cells were lysed using the kit's lysis buffer. Proteins were extracted by three rapid freeze–thaw cycles. Cell lysate was centrifuged for 15 min at 10,000*g* at 4°C. The supernatant was collected and combined with *p*NPP in a colorimetric reaction. Absorbance measurements at 405 nm were normalized to total protein content measured using BCA protein assay (Pierce, Rockford, IL). See Supplementary Material for ALP staining.

### Quantification of Matrix Mineralization

Two weeks after the first bout of vibration (day 14), cell layers were rinsed 2× with calcium-free PBS. To solubilize calcium from the matrix, the samples were incubated overnight at 4°C in 0.6 N HCl. The supernatant was collected, centrifuged to remove cell debris, and reacted with *o*-cresolphthalein complexone, which produced colorimetric changes proportional to calcium content.[21] Absorbance measurements at 570 nm were compared against a standard curve of known calcium concentrations and normalized to DNA content. See Supplementary Material for von Kossa staining.

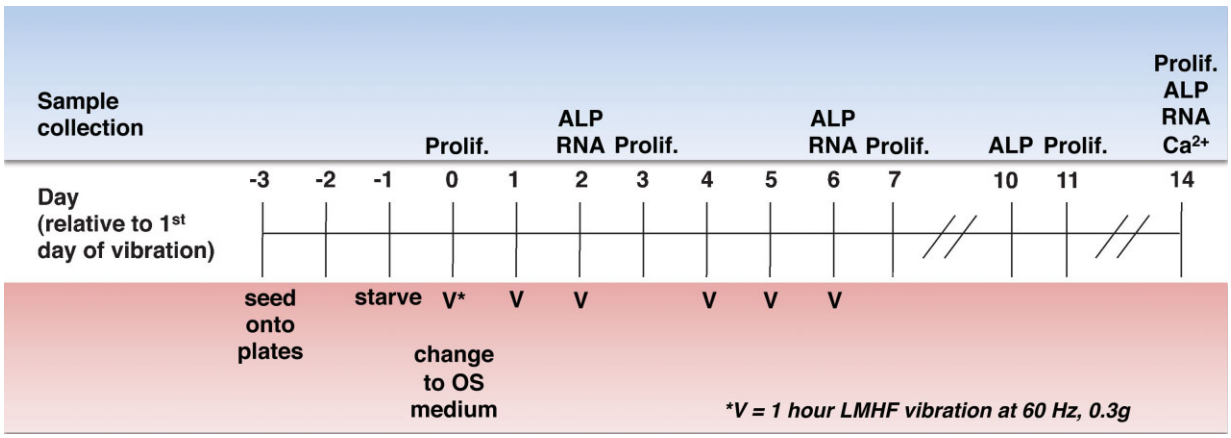


**Figure 1.** Cells were seeded at a density of 5,000 cells/cm$^2$ (or 1,000 cells/cm$^2$ for proliferation study). Two days later, they were serum-starved overnight, and subjected to 1 h of LMHF vibration at 0.3*g* and 60 Hz on day 0. After vibration, the cultures received and were maintained in OS medium. The vibration was repeated on days 1–2 and 4–6. Samples were collected for proliferation study on days 0, 3, 7, 11, and 14; for ALP activity on days 2, 6 (immediately after vibration) 10, and 14; mRNA analysis on days 2, 6, and 14; and for mineralization assay on day 14.

### Statistical Analysis

A two-tailed $t$-test was used to compare means between two groups. A significance level of $p < 0.05$ was employed. Experiments were repeated twice with cells pooled from two rats per experiment ($n = 6$ for all assays). Data are reported as mean ± standard deviation.

## RESULTS

### MSC Proliferation Was Not Affected by LMHF Vibration

On day 0, cell samples were collected immediately post-vibration to verify that vibration did not promote cell detachment from the culture plate. Both vibrated and non-vibrated cultures proliferated over the 14 days (Fig. 2A), and the proliferation rate did not differ significantly between groups at any tested time point (Fig. 2B).

### ALP Activity Was Not Altered by LMHF Vibration

The ALP levels expressed by rMSCs increased with time in both vibrated and non-vibrated groups in the presence of OS medium (Fig. 3). However, there was no significant difference in ALP activity due to LMHF vibration.

### RNA Level of Osx Was Inhibited by LMHF Vibration

In both groups, the temporal expression patterns of the examined osteoblastic markers were comparable to those found in previous studies[22,23] (Fig. 4A–F). The transcript levels of Osx, ALP, COL1A1, OPN, and BSP peaked at day 6, while that of Runx2 showed an increasing trend from days 2 to 14. OCN was detectable at days 2 and 6, but could not be quantified by qPCR due to its low expression levels. LMHF did not cause any significant change in the examined genes with the exception of Osx, which was decreased significantly on days 2 and 6 (Fig. 4G).

### Matrix Mineralization Was Decreased by LMHF Vibration

Cultures that received LMHF vibration loading contained a significantly lower amount of matrix mineralization normalized to the total number of cells (−24%) compared to the non-vibrated controls (Fig. 5A).

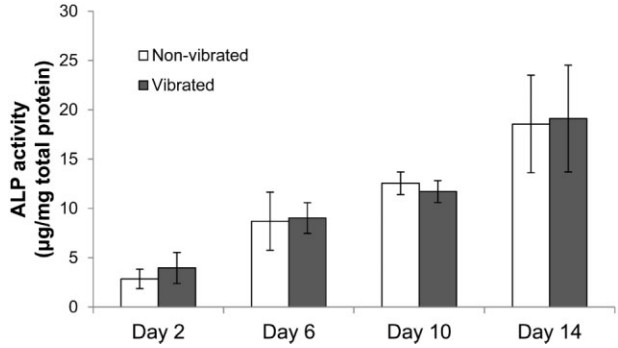


**Figure 3.** ALP activity increased from days 2 to 14. There were no significant differences in ALP activity levels between the vibrated and non-vibrated groups ($n = 6$).

Matrix mineralization in both cultures was confirmed by ALP/von Kossa staining (Fig. 5B,C).

## DISCUSSION

We investigated the OS effect of LMHF vibration applied directly to MSCs. Contrary to our expectation, LMHF vibration did not enhance the OS differentiation of rMSCs, as assessed by cell proliferation, ALP activity, mRNA levels of early to late markers of osteogenesis, and matrix mineralization. Specifically, LMHF vibration inhibited mRNA expression of Osx and decreased the amount of matrix mineralization. These results suggest that the direct application of LMHF vibration to MSCs does not produce OS effects.

LMHF vibration did not induce any changes in the proliferation of MSCs. Proliferation responses of MSCs under other mechanical stimuli were found in previous studies, from having no effect,[13] increasing proliferation,[24] to inhibiting cell growth.[12] Weyts et al.[25] noted that the osteoblastic response to stretching depends on the stage of osteoblast maturation; stretching in early osteoblastic cultures caused apoptosis, while in more differentiated cultures proliferation was stimulated. Thus, one explanation of our observation is that

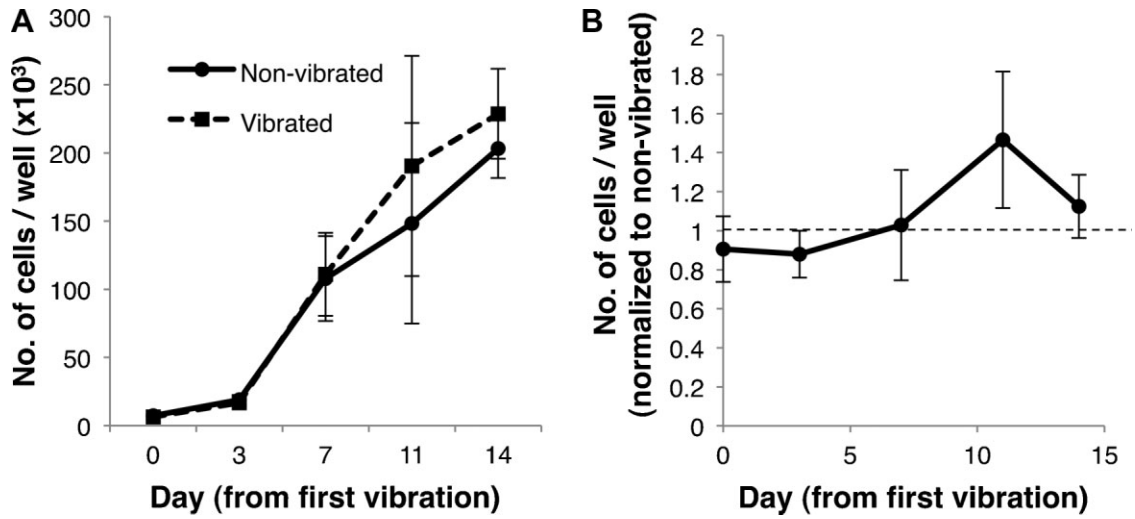


**Figure 2.** (A) Both vibrated and non-vibrated groups showed proliferation over the 14 days. (B) LMHF vibration did not affect proliferation rate ($n = 6$).

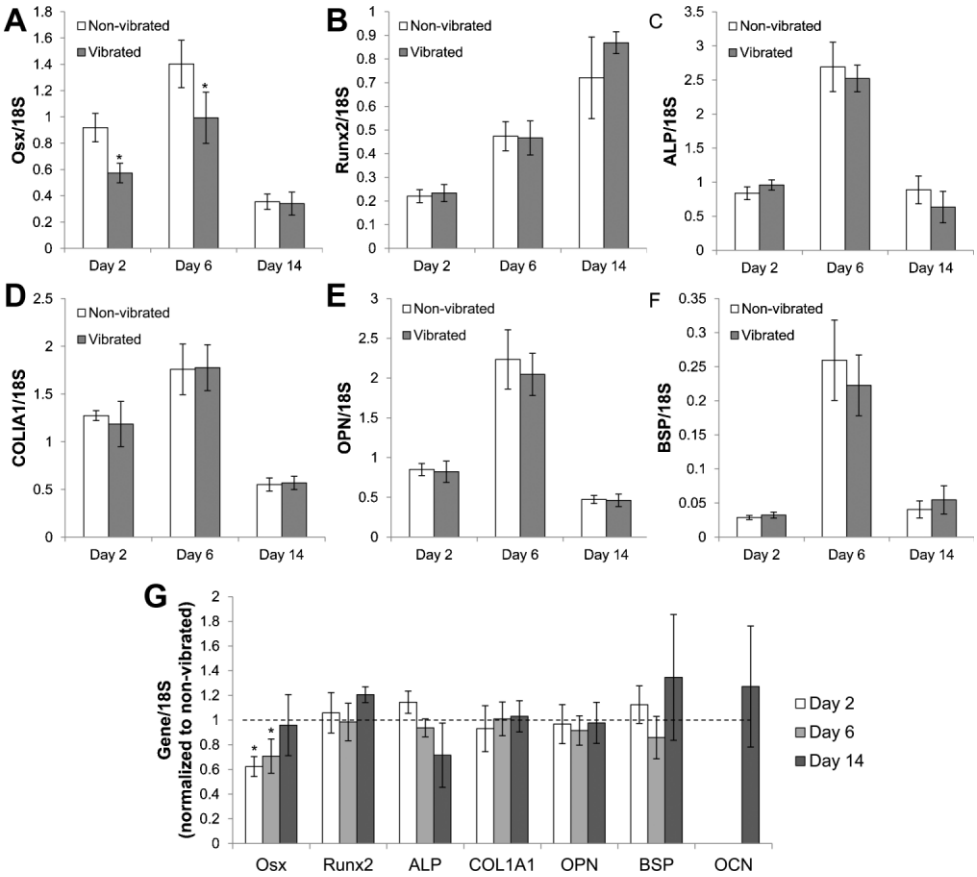


**Figure 4.** Expression levels of Osx (A), ALP (C), COL1A1 (D), OPN (E), and BSP (F) were highest on day 6, while Runx2 expression increased over 14 days (B). (G) Vibrated cultures exhibited a lower mRNA level of Osx on days 2 (−38%) and 6 (−29%). $^{*}p < 0.01$ compared to the non-vibrated control ($n = 6$).

the mechanical stimulation was applied in the early stages of OS differentiation (on days 0–6 of OS induction), and not at later stages when LMHF vibration may have proliferative effects.

Similarly, previous studies on the effect of mechanical stimulation on ALP activity, an early osteoblastic differentiation marker, have yielded mixed results.

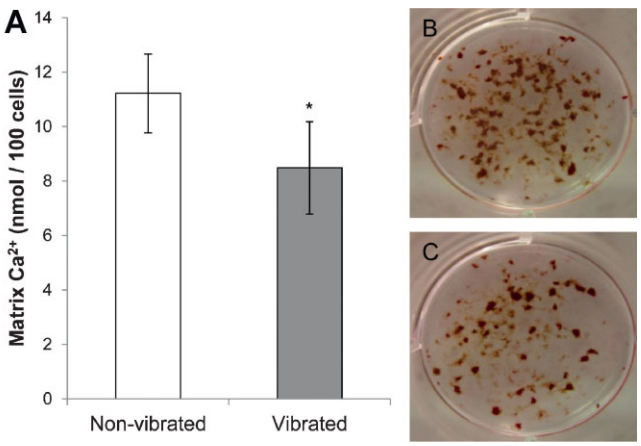


**Figure 5.** (A) Cultures that were subjected to LMHF vibration showed 24% lower matrix calcium deposition. $^{*}p < 0.05$ compared to the non-vibrated control ($n = 6$). Von Kossa/ALP staining of non-vibrated (B) and vibrated (C) cultures.

We showed that the amount of ALP activity was not altered by LMHF vibration, consistent with studies that applied strain to human MSCs[12] or fluid flow to rMSCs[13] but different from those that reported upregulation[16,26] or downregulation.[27] The differential functional responses of MSCs to various forms of mechanical stimulation underscore the important concept that cellular responses in mechanotransduction are unique to the specific mechanical stimulus.

We assessed the effect of LMHF on OS differentiation by measuring mRNA levels of early to late osteoblast-associated markers. The time course mRNA expressions corresponded well with those of Malaval et al. in the same culture system, indicating that the manipulation of the cultures during vibration did not affect OS differentiation. We did not observe any differential expression between vibrated and non-vibrated groups in the majority of the genes studied. We did, however, observe decreased mRNA level of Osx on days 2 and 6, which returned to control levels by day 14. Since Osx is crucial for osteoblast formation, the decrease in Osx expression may have contributed to the inhibited matrix mineralization observed in the vibrated cultures. Furthermore, as Osx acts downstream of Runx2 in the transcriptional control of bone formation,[28] we expected that Runx2 would also exhibit decreased expression under LMHF vibration. Although the unaltered level of Runx2

was unexpected, recent studies showed that Osx can be regulated via Runx-independent mechanisms,[29] indicating an incomplete understanding of the molecular mechanisms underlying Osx action in OS differentiation.

Our findings that LMHF vibration decreased Osx mRNA levels and matrix calcification but did not alter other indicators of osteoblast differentiation suggest that the signaling molecules mediating these observed effects may be ones other than those studied herein. Indeed, Simmons et al. showed that increased matrix mineralization as a result of cyclic strain applied to differentiating human MSCs is mediated via extracellular signal-regulated kinase signaling, but is independent of ALP stimulation. Furthermore, although widely used as an osteoblastic marker, ALP levels are independent of matrix calcification.[30]

Taken together, despite receiving repeated vibration stimulation, rMSCs showed limited to no response from gene to protein expression levels of early to late OS markers. This lack of response was unlikely due to a transient response that was not captured by the time points selected, as repetitive loading is able to amplify the cellular response elicited by a single bout of loading.[13] Also, it is improbable that potential differential effects between vibrated and non-vibrated groups were masked by the presence of dexamethasone, a potent osteoinductive agent. We chose to supplement cultures with 10 nM dexamethasone beginning from after the first bout of vibration loading to the end of each time point such that the culture protocol was consistent with previous studies.[10,12,13] To confirm that dexamethasone had not suppressed any OS effect of LMHF vibration, we repeated the same vibration protocol in the absence of osteoinductive supplements. Proliferation and mRNA expression of early OS markers showed no significant differences between vibrated and non-vibrated cells (results not shown), suggesting that LMHF vibration does not generate a baseline effect on the OS differentiation of rMSCs, even in culture medium deficient of a chemical osteoinductive agent.

Our results suggest that in vivo, cells may be responding to secondary mechanical stimuli induced by vibration, such as shear stress due to bone marrow movement,[31] as opposed to vibration itself. Alternatively, other mechanisms may exist by which WBV exerts its anabolic effect, possibly via a mechanosensor that is absent in our in vitro model. Animal studies suggest that alterations in the mechanical environment alter the cell fate of mesenchymal progenitors in the bone marrow.[6,7,32] However, the bone marrow is a heterogeneous compartment that houses hierarchical components of hematopoietic and mesenchymal cells. Thus, there may be heterotypic cellular interactions between the mechanosensor cell and the effector cells that are not immediately apparent in previous in vivo studies and in our in vitro investigation.

A putative mechanosensor for detecting LMHF vibration is the osteocyte. Our laboratory found that osteocytes under LMHF vibration release soluble factors that inhibit osteoclast formation.[20] Furthermore, osteocytes communicate with other cell types, such as MSCs,[33] through gap junctions[34] and soluble factors,[33] and such communication is mechanically regulated.[26,33,35] Future studies are needed to investigate communication between osteocytes and MSCs and to elucidate the role of osteocytes in orchestrating MSC differentiation under vibration stimulation.

Our experimental set-up aimed to investigate whether the anabolic effect of LMHF vibration observed in vivo is a direct consequence of MSCs sensing and responding to the vibration signal. However, our system may not be replicating the in vivo environment as it lacks other cell types. Also, a vibration stimulus applied externally to the whole-body may translate to a different signal at the cellular level. However, the precise mechanical consequences of vibratory loading in vivo are currently unknown. Thus, as a first step, our in vitro system allows us to apply a controlled vibration signal that is isolated from possible confounding cellular responses of other cell types and any secondary mechanical stimuli. Furthermore, although most in vivo studies typically employed a loading duration of $<$20 min, we chose a 1 h stimulation for two reasons. First, in preliminary tests, osteocyte-like MLO-Y4 cells did not respond to brief periods of LMHF vibration. Second, many in vitro studies testing the effects of other types of mechanical stimulation on bone cells usually employed periods $\geq$1 h,[26,36] and showed that cellular response increased with increasing load duration from 0.5–2 h.[36] Thus, to strike a balance between existing in vitro and in vivo mechanical loading regimes, which would allow us to compare our results with existing literature, we employed a 1 h duration.

In conclusion, under our experimental conditions, we did not detect any OS effect of vibration on rMSCs, but instead observed decreased mRNA level of Osx and inhibited matrix calcification, suggesting that other factors contribute to the anabolic effect of vibration on bone. We speculate that the OS effect of LMHF is elicited through an alternative mechanism, where rMSCs may be activated by secondary mechanical stimuli induced by vibration or indirectly activated via communication with osteocytes that are more mechanosensitive to LMHF vibration. Our results provide a better understanding of the cellular mechanism that underlies bone's adaptive response to vibration. Such information is valuable in identifying the OS components of the physical signal and facilitates the translation of the physical treatment of osteoporosis to clinical settings.

## ACKNOWLEDGMENTS

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