Vibrational stimulation induces osteoblast differentiation and the upregulation of osteogenic gene expression in vitro

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Received: 2 December 2015 / Accepted: 24 August 2016 / Published online: 17 September 2016
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Abstract Vibrational stimulation is an accepted non-invasive method used to improve bone remodeling. However, the underlying mechanisms of this phenomenon remain unclear. In this study, we developed a new vibration-loading system to apply vibrational stimulation to cells based on a previously reported in vivo study. We hypothesized that osteoblasts respond to vibrational strain by expressing osteogenic marker genes, such as alkaline-phosphatase (ALP), Runx2, and Osterix. To test our hypothesis, we developed a vibration-loading system to apply a precise vibrational force to an osteoblast culture on a silicone membrane. The system regulated frequency and acceleration of the vibration, and strain on the silicone membrane culture surface was measured using the strain gauge method. After vibrational stimulation, cellular gene expression was analyzed using real-time polymerase chain reaction. We obtained clear strain signals from the culture surface at vibrational ranges of 1.0–10 m/s² acceleration and frequencies of 30, 60, and 90 Hz, respectively. The strain increased in a linear fashion, depending on the acceleration magnitude. Vibrational stimulation also significantly upregulated expression of the osteogenic

marker genes Runx2, Osterix, type I collagen, and ALP. In conclusion, we developed a new vibration-loading system that can precisely regulate frequency and acceleration, and we established the presence of dynamic cellular strain on a culture surface. Our findings suggest that vibrational stimulation may directly induce osteoblast differentiation.

Keywords Vibration · Strain · Strain gauge · Osteogenic gene expression · Osteoblast differentiation

Introduction

Low-magnitude, high-frequency (LMHF) vibrations applied to the whole body (whole-body vibration, WBV) have beneficial effects on bone (Ozcivici et al. 2010; Edwards and Reilly 2015; Beck 2015). WBV has been shown to increase trabecular bone density of the proximal femurs in sheep (Rubin et al. 2001), increase the rate of bone formation and trabecular bone density in ovariectomized rats (Judex et al. 2007), facilitate callus formation in a rat fracture model (Leung et al. 2009), and accelerate peri-implant bone healing (Ogawa et al. 2014). Local LMHF vibrational stimulation can also impart beneficial effects on bone. It is reported that orthodontic tooth movement is accelerated by local resonant vibration (Nishimura et al. 2008). However, the optimal

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parameters necessary to maximize the beneficial effects of vibration on bone, such as acceleration and frequency, remain unknown.

To determine the optimal parameters for vibrational stimulation, it is necessary to identify the cells that respond to vibration. Additionally, *in vitro* experiments are important to understand the role that each cell type plays. Finally, vibration will need to be precisely controlled to understand the parameters that mimic the actual environment when vibrational stimulation has been applied.

Osteoblasts appear to be sensitive to vibration *in vitro*. However, it is difficult to determine the optimal vibration parameters for osteoblasts, as prior studies applied different frequency ranges. The vibration technique used in these studies was also different. Some vibrated the culture plates perpendicularly (Hou et al. 2011), while others applied vibration horizontally (Bacabac et al. 2006; Rosenberg et al. 2002; Tjandrawinata et al. 1997). The manner by which vibrational strain was applied to cells on a plastic substrate is different. A resonant frequency was used to maximize vibrational displacement of the plastic culture plates (Dumas et al. 2010). The amplitude of the vibrational strain is limited by the mechanical properties of the plastic substrate. Therefore, collagen gels are used as scaffolds and cells are vibrated using a mixed broad frequency vibration noise (Tanaka et al. 2003). The osteoblasts are sensitive to vibration, although it remains unclear what the optimal parameters are.

Based on challenges highlighted by prior studies, we developed a novel *in vitro* vibration-loading system that precisely regulates acceleration and frequency and creates a known minute strain on silicone membranes. Osteoblasts were cultured on silicone membranes coated with type I collagen, which mimics the stiffness of an *in vivo* environment when vibrational stimulation is applied.

The aim of this study was to investigate the effects of vibrational stimulation on osteoblasts and to identify the optimal parameters for osteoblast response.

Materials and methods

Cell culture

Mouse osteoblast-like MC3T3-E1 cells were kindly provided by Dr. H. Kodama, Ohu University,

Koriyama, Japan. Cells were cultured in α -modified minimal essential medium (α -MEM; Wako, Osaka, Japan) supplemented with 10 % (v/v) fetal bovine serum (BioWest, Nuaille, France), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37 °C in a 5 % CO₂ environment.

Vibration-loading system

The system for delivering the vibrational stimulation comprises a vibration controller (RC-1120, IMV Corp., Osaka, Japan), a power amplifier (PET-0A, IMV Corp.), a charge amplifier (5011B, Kistler Instrumente AG, Winterthur, Switzerland), and a vibrator (PET-01, IMV Corp.). A force sensor (Kistler Instrumente AG) and an accelerometer (Endevco Corp., San Juan Capistrano, CA, USA) were built into the vibrator (Fig. 1). Flexible-bottomed (type I collagen-coated silicon membrane, 4.9 cm²/well) culture plates (Flexcell International Corp., Hillsborough, NC, USA) were positioned on the acrylic stage (Fig. 1c) with a hole that was 12 mm in diameter. A 780 g/125 cm² acrylic plate was placed on top of the culture plates to prevent vertical displacement. The center of the underside of the silicone membrane was stimulated perpendicularly by a vibrator in contact with the membrane, allowing for the control of the magnitude of the peak acceleration and the frequency of the sine wave. Table 1 shows the calculated peak displacement of the vibrator at 1.0, 5.0 and 10 m/s² of peak acceleration and frequencies of 30, 60 and 90 Hz.

Vibrational stimulation

Cells were seeded on flexible-bottomed (type I collagen-coated silicon membrane, 4.9 cm²/well) culture plates (Flexcell International Corp., Hillsborough, NC, USA) at a density of 2×10^5 cells/cm² in 1 ml of medium, and incubated at 37 °C in a 5 % CO₂ environment. After 48 h, the medium in each well was replaced with 1 ml of fresh medium. The cells were then subjected to vibrational stimulation for 10 min at room temperature in a 5 % CO₂ environment. We confirmed that there were no standing waves on the fluid surface in the range of the accelerations and frequencies used in this study. To avoid variance because of environmental factors, additional cells prepared as controls were seeded into other wells, cultured and exposed to the same conditions but not stimulated.

Fig. 1 **a** Photograph of the in vitro vibration-loading system. **b** Schema of the strain measurement system. **c** Application of vibrational stimulation to osteoblastic MC3T3-E1 cells. (Color figure online)

Measurement of strain

To apply a precise vibrational force to the silicone membrane, we measured the strain exerted on the silicone membrane using two types of strain gauges: triple-rosette strain gauges and single-element strain gauges.

To clarify the direction and magnitude of the strain placed on the silicone membrane, a triple-rosette strain

gauge (KFG-1-120-D17-11, Kyowa, Tokyo, Japan) (Fig. 2a) was placed on the silicone membrane 7.5 mm from the center of the membrane using an adhesive (CC-33A Strain Gauge Cement, Kyowa, Tokyo, Japan) (Fig. 2b). One of the three different axis elements were attached along a line that passed through the center of the silicone membrane. Strain signals from the three elements included in the triple-rosette strain gauge were simultaneously recorded by a

Table 1 Peak displacement of the vibration rod (μm)

Frequency	Peak acceleration (m/s^2)		
	1.0	5.0	10
30 Hz	28	141	281
60 Hz	7	35	70
90 Hz	3	16	31

data logger (NR-ST04, Keyence, Osaka, Japan) at $200\ \mu\text{s}$ of sampling time for a total of 100 ms. The signals were digitized at a 16-bit resolution. Data were analyzed using WaveLogger NR-H7 W (Keyence, Osaka, Japan) analysis software. Representative strain data at a 10-m/s^2 peak acceleration and a 60-Hz frequency are shown in Fig. 2c. The maximum strain was calculated from the peak-to-peak strain (Fig. 2g) for a randomly chosen 0.1-s period using the fast Fourier transform algorithm (FFT). Detected maximum strain was defined as ε_a , ε_c and ε_b on channels 1, 2 and 3, respectively. The angle θ , defined as the major principal strain direction between the center line of the silicone membrane and channel 3 of the triple-rosette strain gauge, was calculated using the following equation: $\theta = \frac{1}{2} \tan^{-1} \frac{2\varepsilon_c - \varepsilon_a - \varepsilon_b}{\varepsilon_a - \varepsilon_b} - 45^\circ$. The major principal strain $\varepsilon_{\max}$ and minor principal strain $\varepsilon_{\min}$ were calculated as follows: $\varepsilon_{\max} = \frac{1}{2}[(\varepsilon_a + \varepsilon_b) + \sqrt{2\{(\varepsilon_a - \varepsilon_c)^2 + (\varepsilon_c - \varepsilon_b)^2\}}]$ and $\varepsilon_{\min} = \frac{1}{2}[(\varepsilon_a + \varepsilon_b) - \sqrt{2\{(\varepsilon_a - \varepsilon_c)^2 + (\varepsilon_c - \varepsilon_b)^2\}}]$.

To test for site-dependent differences in the silicone membrane, three single-element strain gauges (KFG-03-120-C1-11, Kyowa, Tokyo, Japan) (Fig. 2d) were positioned at 0 mm (channel A), 2.5 mm (channel B) and 7.5 mm (channel C) from the center of the silicone membrane (Fig. 2e). The gauges were positioned along a straight line that passed through the center of the silicone membrane. Figure 2f shows representative detected strain data at a peak acceleration of 10 m/s^2 and a frequency of 60 Hz. Strain ($\Delta L/L$) was defined as the ratio of changes in length (ΔL) to the original length of the membrane (L).

Real-time PCR

Total RNA extraction from the MC3T3-E1 cells was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions 24 h following vibrational stimulation. After the measurement of the RNA concentration of each sample using the Nanodrop2000 (Thermo Scientific, Waltham, MA, USA), 500 ng of total RNA was subjected to first-strand cDNA synthesis using Prime-Script RT Master Mix (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. A volume of $2\ \mu\text{l}$ of cDNA, equivalent to 20 ng of total RNA, was used as a template in the subsequent real-time polymerase chain reaction (PCR) analysis using SYBR Premix Ex Taq (TaKaRa Bio) according to the manufacturer's instructions. To evaluate osteoblast differentiation we investigated the gene expression of Runx2, osterix, type I collagen, osteocalcin, osteopontin, ALP, and bone sialoprotein (BSP) as osteogenic markers, using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as a housekeeping gene. Primer sequences are listed in Table 2.

Cell viability assay

An alamarBlue[®] assay was performed to quantify cell viability. A total of 0.1 ml of alamarBlue[®] solution (BioSource, Camarillo, CA, USA) was added to each well 20 h after vibrational stimulation. Cells were incubated at 37°C in 5 % CO_2 for 4 h. The absorbance of each well was measured at 570- and 595-nm wavelengths using a spectrophotometer (Bio-Rad, Hercules, CA, USA).

Statistical analysis

A one-way ANOVA followed by Dunnett's post hoc test was performed for cellular response results at 1.0, 2.5, 5.0, and 10 m/s^2 at 60 Hz ($n = 1$) (Fig. 5). A Student's t test was performed for cell viability assay results and cellular response results at 1.0 and 5.0 m/s^2 at 60 Hz ($n = 3$), and 5.0 m/s^2 at 30 Hz (Figs. 6, 7). A p value <0.05 was considered statistically significant. All statistical analyses were performed using JMP Pro software (version 11, SAS Institute Inc., Cary, NC, USA).

Triple rosette strain gauge

Single element strain gauge

Calculation of maximum strain

Fig. 2 Strain measurement. Triple rosette strain gauge. **a** Photograph of the triple-rosette strain gauge (KFG-1-120-D17-11, Kyowa, Tokyo, Japan). **b** The triple-rosette strain gauge placed on the silicone membrane at a point 7.5 mm from the center of the silicone membrane. **c** Example of the detected strain data from the triple-rosette strain gauge for 50 ms at a 10-m/s^2 peak acceleration and a frequency of 60-Hz. The red, green and black lines indicate the strain signal from channels 1, 2 and 3 of the triple-rosette strain gauge, respectively. Single element strain gauge. **d** Photograph of the single-element strain gauge (KFG-

03-120-C1-11, Kyowa, Tokyo, Japan). **e** Three single-element strain gauges placed on the silicone membrane at 0 (channel A), 2.5 (channel B), and 7.5 mm (channel C) from the center of the silicone membrane. **f** Example of the detected strain data from the three single-element strain gauges for 50 ms at a 10-m/s^2 peak acceleration and a frequency of 60-Hz. The blue, red and green lines indicate the strain signal from channels A, B and C of the single-element strain gauges, respectively. Calculation of maximum strain. **g** Method to calculate the maximum strain using the peak-to-peak strain. (Color figure online)

Table 2 Primer sequences used in this study for real-time reverse transcription (RT)-PCR

Gene	Forward primer	Reverse primer	Accession number
Gapdh	5'-AGG TCG GTG TGA ACG GAT TTG-3'	5'-TGT AGA CCA TGT AGT TGA GGT CA-3'	NM_008084
Runx2	5'-AAC GAT CTG AGA TTT GTG GGC-3'	5'-CCT GCG TGG GAT TTC TTG GTT-3'	NM_001271630
Osterix	5'-ATG GCG TCC TCT CTG CTT G-3'	5'-TGA AAG GTC AGC GTA TGG CTT-3'	NM_130458
Collagen-1	5'-GGG CAA GAC AGT CAT CGA AT-3'	5'-GGT GGA GGG AGT TTA CAC GA-3'	NM_007742
Osteocalcin	5'-CCG CCT ACA AAC GCA TCT AT-3'	5'-ACT TGC AGG GCA GAG AGA GA-3'	NM_031368
Osteopontin	5'-TCT GAT GAG ACC GTC ACT GC-3'	5'-CCT CAG TCC ATA AGC CAA GC-3'	AF515708
ALP	5'-CGG GAC TGG TAC TCG GAT AA-3'	5'-ATT CCA CGT CGG TTC TGT TC-3'	AF285233
BSP	5'-AGG GAG GCA GTG ACT CTT CA-3'	5'-CCC GAG AGT GTG GAA AGT GT-3'	NM_008318

Results

Strain on the culture surface of a type I collagen-coated silicone membrane

The direction and magnitude of the strain applied to the silicone membrane was measured using a triple-rosette strain gauge. Table 3 shows the maximum strains ε_a , ε_b and ε_c detected on channels 1, 2 and 3, the angle of the major principal strain θ , the major principal strain $\varepsilon_{\max}$ and the minor principal strain $\varepsilon_{\min}$. The average θ was $0.2 \pm 4.7^\circ$ ($n = 6$, mean $\pm$ SD). The major principal strain is directed along a line passing through the center of the silicone membrane. The average $\varepsilon_{\max}$, the maximum radial strain, and $\varepsilon_{\min}$, the minimum circumferential strain, were 8.8 ± 2.6 and -7.2 ± 1.8 at 60 Hz, 10 m/s², respectively.

To analyze the distribution of the maximum strain on the silicone membrane in greater detail, smaller single-element strain gauges were used instead of the triple-rosette strain gauges. The waveforms obtained are shown in Fig. 3a, b, c, d, e, f, g, h, i. The strain

profile in Fig. 3j shows no vibrational input, and represents environmental noise. The noise level was $\pm 0.8 \mu$ strain. In Fig. 3b, c, d, e and 3g, h, clear sinusoidal strain signals were detected at each position. The phase of strain at channel C was the opposite of that observed on channels A and B. At 1.0 m/s² and 60 Hz, sinusoidal strain signals were not detected on channel C because the signals were weaker than the external noise (Fig. 3f). At 1.0 m/s² and 90 Hz, no sinusoidal strain was detected on any channel (Fig. 3i). At 10 m/s² and 30 Hz, oscillated signals but not sinusoidal signals were detected (Fig. 3a).

Figure 4 shows the peak acceleration dependence at maximum strain when the silicone membrane was vibrated at 1.0–10 m/s² and 30, 60 or 90 Hz. The maximum strain increased proportionally with the magnitude of the peak acceleration. Table 4 lists the maximum strain values obtained at 1.0, 5.0 and 10 m/s². At a peak acceleration of 7.1–10 m/s² and a frequency of 30 Hz, the maximum strain could not be calculated using peak-to-peak strain because sinusoidal signals were not detected.

Table 3 Strain measurement by triple rosette strain gauge

Triple rosette strain gauge Lot.	ε_a (μ strain)	ε_b (μ strain)	ε_c (μ strain)	θ ($^\circ$)	$\varepsilon_{\max}$ (μ strain)	$\varepsilon_{\min}$ (μ strain)
No. 1	1.4 ± 0.2	11.4 ± 0.7	0.8 ± 0.4	2.3 ± 1.4	11.7 ± 1.0	-10.8 ± 1.6
No. 2	0.9 ± 0.5	7.6 ± 5.6	1.1 ± 0.4	-1.6 ± 2.4	8.7 ± 5.4	-6.0 ± 6.1
No. 3	0.1 ± 0.0	5.8 ± 1.3	0.3 ± 0.3	1.5 ± 1.6	6.8 ± 1.2	-5.8 ± 2.2
No. 4	1.2 ± 0.3	12.7 ± 5.6	5.6 ± 1.9	-7.5 ± 4.0	12.8 ± 4.7	-7.6 ± 5.7
No. 5	1.9 ± 0.4	6.5 ± 3.4	1.3 ± 0.9	8.0 ± 4.7	7.3 ± 3.5	-5.6 ± 3.6
No. 6	1.4 ± 0.8	4.8 ± 1.8	1.3 ± 0.9	-1.6 ± 3.4	5.6 ± 1.5	-7.2 ± 1.8
Average $\pm$ SD	1.2 ± 0.6	8.1 ± 2.9	1.7 ± 1.8	0.2 ± 4.7	8.8 ± 2.6	-7.2 ± 1.8

ε_a , ε_b , and ε_c : detected strain from the triple-rosette strain gauge at channels 1, 3, and 2, respectively. θ : angle of major principal strain, $\varepsilon_{\max}$: major principal strain, $\varepsilon_{\min}$: and minor principal strain. Strain experiments were repeated 5 times for 6 lots. at a 10-m/s² peak acceleration and 60-Hz frequency. Mean $\pm$ SD

Fig. 3 Dynamic strain waveforms obtained from three strain gauges showing a range of frequencies and accelerations. Strain measurements on the silicone membrane coated with type I collagen were performed using three strain gauges. Strain profiles were observed at 30 Hz and accelerations of 10, 5.0, and 1.0 m/s² (a–c). Strain profiles observed at 60 Hz and

accelerations of 10, 5.0 and 1.0 m/s² (d–f). Strain profiles observed at 90 Hz and accelerations of 10, 5.0 and 1.0 m/s² (g–i), respectively. **j** To evaluate the background noise level a strain profile was measured without external stimulation. Blue, red and green lines show the data on strain gauge channels A, B and C, respectively. (Color figure online)

Gene expression of osteoblast-specific transcriptional factors and osteogenic markers

To survey the acceleration needed to induce osteogenic gene expression in osteoblasts, we investigated the effects of peak acceleration on osteogenic gene expression. Peak accelerations were set at 1.0, 2.5, 5.0 or 10 m/s² with a fixed frequency of 60 Hz. Runx2, osteocalcin and ALP expression were markedly upregulated at an acceleration of 5.0 m/s² (Fig. 5a, d, f). We therefore selected 5.0 m/s²

as the most effective acceleration for subsequent experiments.

Cell responses were measured at 5.0 m/s² and 60 Hz to confirm statistical significance. The expressions of Runx2, osterix, type I collagen and ALP (1.4-, 1.3-, 1.4- and 1.4-fold respectively, Fig. 6a, b, c, f; V2) were significantly upregulated by vibration compared with controls ($p < 0.05$). When the cells were stimulated at 1.0 m/s² and 60 Hz, no significant differences were detected between the control and vibration groups (Fig. 6; V1).

Fig. 4 Peak acceleration dependence of the maximum strain. Channels A, B and C indicate the position of strain gauges A, B and C, respectively (Fig. 2e). The results obtained are presented as mean $\pm$ standard errors. Peak acceleration dependencies of

the strain were observed at frequencies of 30 (a), 60 (b) and 90 Hz (c). At 30 Hz and 7.1–10 m/s^2 , maximum strain could not be calculated by measuring the peak-to-peak strain (Fig. 3a). (Color figure online)

Table 4 Maximum strain observed on three single element strain gauges

Frequency (Hz)	Peak acceleration (m/s^2)	Maximum strain (μ strain)		
		Channel A	Channel B	Channel C
30	1.0	9.0	14.4	4.0
	5.0	40.2	68.4	19.2
	10	— ^a	— ^a	— ^a
60	1.0	1.4	2.2	— ^b
	5.0	10.9	17.4	5.0
	10	22.0	35.6	9.8
90	1.0	— ^b	1.5	— ^b
	5.0	4.8	7.7	2.2
	10	9.4	15.3	4.1

Three single element strain gauges were placed on the silicone membrane at 0 (channel A), 2.5 (channel B), and 7.5 mm (channel C) from the center of the silicone membrane

^a Maximum strain was not calculated by peak-to-peak strain because sinusoidal signals were not detected

^b Strain was not detected because the signal was less than external noise

To investigate whether a 5.0 m/s^2 peak acceleration induced osteoblast differentiation at different frequencies, we also examined the effects of vibration at 30 Hz. Runx2, osterix and ALP expression increased significantly by 1.4-, 1.9- and 1.3-fold, respectively, compared with controls ($p < 0.05$) (Fig. 6a, b, f; V3).

Cell viability assay

The viability of the cells stimulated by vibration at 60 Hz and 5.0 m/s^2 , 60 Hz and 1.0 m/s^2 and 30 Hz and 5.0 m/s^2 was analyzed. No significant difference

was observed between the control group and the vibration group (Fig. 7).

Discussion

In the present study, we developed and evaluated a new in vitro vibration-loading system. We first analyzed the vibration-induced dynamic strain on the cell culture surface of a silicone membrane coated with type I collagen. The direction of the major principal strain was along a line passing through the

Fig. 5 Survey of the peak acceleration dependencies of osteogenic gene expression **a** Runx2, **b** Osterix, **c** Type I collagen (Collagen I), **d** Osteocalcin, **e** Osteopontin, **f** Bone alkaline phosphatase (ALP) and **g** Bone sialoprotein (BSP). All measurements were standardized with glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Peak acceleration was set at 1.0, 2.5, 5.0 and 10 m/s². The frequency was fixed at 60 Hz, and the duration of the stimulation was 10 min. Unstimulated cells set up on the vibrational system under the same conditions represented a control. Values represent the results of triplicate measurements (mean $\pm$ standard error). Statistical analysis for one sample at each point ($n = 1$) was performed using a one way-ANOVA followed by Dunnett's test. A p value < 0.05 was considered statistically significant: * $p < 0.05$; ** $p < 0.01$

Fig. 6 Comparisons of relative osteogenic gene expression levels between the control group (C) ($n = 3$) and the vibration group ($n = 3$). Cells were stimulated by vibration at 60 Hz and 1.0 m/s² (V1), 60 Hz and 5.0 m/s² (V2), or 30 Hz and 5.0 m/s² (V3), respectively. Duration of vibrational stimulation was 10 min. Control cells were prepared for each vibrational condition experiments; unstimulated cells on the vibrational system under the same conditions represented the control group. Data are presented as mean $\pm$ standard deviation. Standard deviations for the controls under each vibration condition were 0.08, 0.09, 0.06, 0.07, 0.02, 0.15, and 0.07 (for V1), 0.04, 0.07, 0.12, 0.15, 0.10, 0.26, and 0.09 (for V2), and 0.10, 0.09, 0.13, 0.08, 0.07, 0.21, and 0.08 (for V3) for gene expression of Runx2 (**a**), Osterix (**b**), Col1 (**c**), OCN (**d**), OPN (**e**), ALP (**f**), and BSP (**g**), respectively. Statistical analyses were performed using the Student's t test. A p value < 0.05 versus control was considered to be statistically significant: * $p < 0.05$; ** $p < 0.01$

Fig. 7 AlamarBlue[®] assay to examine cell viability. MC3T3-E1 cell viability in the control (C) and vibration groups. Cells were stimulated by vibration at 60 Hz and 1.0 m/s² (V1) (a), 60 Hz and 5.0 m/s² (V2) (b), or 30 Hz and 5.0 m/s² (V3) (c), respectively. Duration of vibrational stimulation was 10 min. Data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using the Student's *t* test. A *p* value <0.05 was considered statistically significant

center of the silicone membrane. Triple-rosette strain gauge measurements demonstrated that the major and minor principal strains were similar in absolute value (8.8 and -7.2μ strain, respectively, at 60 Hz, 10 m/s²) at a point 7.5 mm from the membrane center.

We used a silicone membrane as a cell culture substrate, instead of plastic. In our model, silicone has several advantages compared with plastic cell culture plates. These advantages include: (1) a flexible

silicone membrane to accurately apply a broad range of 1–100 μ strain on the culture surface, while approximately 15–40 μ strain was induced by resonant frequency on the plastic culture plates (Dumas et al. 2010); (2) the phase of the dynamic strain-induced sinusoidal wave was position-dependent, which suggests that the vibration wave was conducted through the entire membrane; (3) in our in vitro vibration system, the fluid shear stress induced by the vibration was not considerably large. We confirmed that there was no standing wave on the fluid surface within the range of accelerations and frequencies used in this study. Finite element methods revealed that the range of fluid shear stress placed upon the cell layer was 0.04–0.94 Pa (Uzer et al. 2013) when the plate was horizontally vibrated (LMHF; 30–100 Hz, 0.98–9.81 m/s²). Because the vertical displacement of the vibrator at 60 Hz and 5 m/s² was 35 μ m, this was too small to induce a horizontal flow. Therefore, it appears that the vibration-induced fluid shear stress acting on the cell layer in this study was less than that measured in Uzer's report (Uzer et al. 2012, 2013); (4) the maximum strain increased proportionally with peak acceleration (Fig. 4). The peak displacement of the vibrator was proportional to the peak acceleration (Table 1; $m\omega^2 = ma$, where *m*, *r*, ω , and *a* are the mass, the displacement, the angular frequency, and the acceleration of the vibrator, respectively). Thus, the maximum strain was proportional to the peak displacement of the vibrator, suggesting that maximum strain was precisely controlled within the elastic region of the silicone membrane.

Conversely, as a disadvantage, the magnitude of dynamic strain changed throughout the silicone membrane (Figs. 3, 4). The maximum strain distribution on the silicone membrane was not homogeneously induced. Gilbert et al. (1994) compared the strain distribution between thick (the same membrane used in this study) and thin silicone membranes with the Flexercell Strain Unit (Flexcell International Corp., Hillsborough, NC, USA) using finite element methods. Based on their findings, it may be advantageous in future studies to use thin silicone membranes to create a relatively uniform strain.

To improve the clinical utility of vibration, it is necessary to understand the exact molecular interactions and cascades caused by vibration, and to identify the optimal parameters necessary for inducing an

osteoblastic response as simply as possible. This study, therefore, focused on the direct effects of vibration on osteoblasts.

We investigated the effects of vibrational stimulation on MC3T3-E1 cell viability. There were no significant differences between control and vibration groups, indicating that vibrational stimulation caused no cytotoxic effects or cell death (Fig. 7). This allows us to conclude that the gene expression data obtained in this study came only from the osteoblast response to vibration.

Early-stages of osteoblast differentiation were observed when we applied a single vibrational stimulation for 10 min based on mRNA levels from a single cell type, which was measured 24 h after stimulation. Alterations to repetition time, duration, and time course of experiment will be important for future studies.

In this study, we carefully selected the osteoblast differentiation markers. The osteoblastic differentiation process could be divided into several stages, including proliferation, extracellular matrix deposition, matrix maturation, and mineralization (Stein and Lian 1993). To investigate osteoblast differentiation, expression levels of distinct differentiation marker genes are used, such as alkaline phosphatase (ALP), type I collagen (Col1), bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OC). ALP is considered an early marker, and OC is considered a late marker for osteoblast differentiation (Stein and Lian 1993). Transcriptional regulators impact osteoblast lineage specific differentiation. For instance, Runx2 is the earliest transcription factor essential for osteoblast differentiation (Komori 2006), and Osterix is a factor downstream of Runx2 that is necessary for differentiation of pre-osteoblasts into mature osteoblasts (Nakashima et al. 2002).

Therefore, because sequential osteoblast differentiation can be identified by stage-specific osteogenic marker gene expression, we speculated that vibration mechanisms induce osteoblast differentiation as follows. Initially vibration upregulates expression of the osteoblast-specific master gene, Runx2, which leads to increased Osterix expression as the cells differentiate into osteoblast precursors. Runx2 and Osterix transcription factors may regulate subsequent extracellular matrix gene transcription, such as ALP, Col1, BSP, OPN, and OC (Harada et al. 1999; Jang et al. 2012; Javed et al. 2001; Welch et al. 1998). Immature osteoblasts then differentiate into mature osteoblasts

through matrix production and mineralization (Eriksen 2010). Increased Runx2 activation and Osterix expression have been reported after application of cyclic force (Kanno et al. 2007). Also, ALP activation increased following 60-Hz vibration (Rosenberg et al. 2002). However, it should be noted that the more complex in vivo tissue structures might compound or modulate the presently studied phenomena; the balance between osteoblast and osteoclast activity is important for net bone remodeling. Further studies focused on other cells stimulated by the in vitro-vibration-loading system used in this study might help to illustrate a more detailed cellular mechanism.

A vibrational acceleration of 5.0 m/s² and a frequency of 60 Hz were found to be the most effective conditions for osteogenesis in our in vitro study. At these settings, Runx2, Osterix, and ALP upregulation took place after only 10 min of vibrational stimulation, suggesting that this exposure time may be sufficient to promote effective bone formation. The clinical implications of short periods of vibration to induce bone healing might be valuable, because a shorter effective duration of vibrational stimulation minimizes patient discomfort while increasing therapeutic applications. Vibration may facilitate a shorter retention period in orthodontic treatment, stronger osseointegration of dental implants, and faster fracture healing.

The signal transduction pathway affected by vibrational stimulation should be discussed. A vibrational force that is perpendicularly induced at peak acceleration results in increased pro-osteogenic gene expression, even though smaller maximum strains <20 μ strain were observed at 60 Hz and at 1.0 or 5.0 m/s², respectively. This is in contrast with prior work describing enhanced osteoblast differentiation with 0.1 Hz of cyclic stretch (Zeng et al. 2015). They found that a 10 % elongation (or a $1 \times 10^5 \mu$ strain) was necessary to upregulate Runx2 gene expression. A vertically applied vibrational force might cause osteoblast differentiation through a mechanism other than stretch. We propose that differences in nucleus and cytoplasm motion are important for cells to sense vibration (Bacabac et al. 2006; Uzer et al. 2012). The vibration parameters in an in vivo system, as well as the intra- or extracellular signaling pathways involved in the cellular reaction to vibration, remain unclear, but future studies will focus on these mechanisms.

In conclusion, we developed a new vibration-loading system that can precisely regulate frequency

and acceleration of the vibration applied to an *in vitro* model. We also measured the magnitude of dynamic strain applied to a culture surface, demonstrating that vibrational stimulation directly increased expression of genes encoding osteoblast-specific transcriptional factors and other osteogenic markers. Unexpectedly, the effective parameter to up-regulate expression of osteogenic marker genes might be acceleration rather than strain, because the detected vibration strain was small enough ($20\ \mu$ strain at 60 Hz, $5\ \text{m/s}^2$) to be neglected compared with the previous reported strain (1000 – $10000\ \mu$ strain) when stretched. These results provide valuable insights to help clarify signal transduction mechanisms for future studies. Our findings suggest that vibrational stimulation may directly induce osteoblast differentiation through acceleration-controlled cell motion, even though strains $<20\ \mu$ were observed at 60 Hz and $5.0\ \text{m/s}^2$.

Acknowledgments This work was supported by JSPS KAKENHI Grant Number 24592951, 26462963, and 13J05121. We are grateful to Prof. M. Wakamori, K. Igarashi, O. Suzuki, and Associate Prof. T. Ogawa at Tohoku University for reviewing this manuscript.

Compliance with ethical standards

Conflicts of interest The authors report no conflicts of interest.

References

- Bacabac RG, Smit TH, Van Loon JJ, Doulabi BZ, Helder M, Klein-Nulend J (2006) Bone cell responses to high-frequency vibration stress: Does the nucleus oscillate within the cytoplasm? *FASEB J* 20:858–864
- Beck BR (2015) Vibration therapy to prevent bone loss and falls: mechanisms and efficacy. *Curr Osteoporos Rep* 13:381–389
- Dumas V, Ducharme B, Perrier A, Fourier C, Guignandon A, Thomas M, Peyroche S, Guyomar D, Vico L, Rattner A (2010) Extracellular matrix produced by osteoblasts cultured under low-magnitude, high-frequency stimulation is favorable to osteogenic differentiation of mesenchymal stem cells. *Calcif Tissue Int* 87:351–364
- Edwards JH, Reilly GC (2015) Vibration stimuli and the differentiation of musculoskeletal progenitor cells: review of results *in vitro* and *in vivo*. *World J Stem Cells* 7:568–582
- Eriksen EF (2010) Cellular mechanisms of bone remodeling. *Rev Endocr Metab Disord* 11:219–227
- Gilbert JA, Weinhold PS, Banes AJ, Link GW, Jones GL (1994) Strain profiles for circular cell culture plates containing flexible surfaces employed to mechanically deform cells *in vitro*. *J Biomech* 27:1169–1177
- Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, Yamaguchi A, Komori T, Nakatsuka M (1999) Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem* 274:6972–6978
- Hou WW, Zhu ZL, Zhou Y, Zhang CX, Yu HY (2011) Involvement of Wnt activation in the micromechanical vibration-enhanced osteogenic response of osteoblasts. *J Orthop Sci* 16:598–605
- Jang WG, Kim EJ, Kim DK, Ryoo HM, Lee KB, Kim SH, Choi HS, Koh JT (2012) BMP2 protein regulates osteocalcin expression via Runx2-mediated Atf6 gene transcription. *J Biol Chem* 287:905–915
- Javed A, Barnes GL, Jasanya BO, Stein JL, Gerstenfeld L, Lian JB, Stein GS (2001) Runt homology domain transcription factors (Runx, Cbfa, and AML) mediate repression of the bone sialoprotein promoter: evidence for promoter context-dependent activity of Cbfa proteins. *Mol Cell Biol* 21:2891–2905
- Judex S, Lei X, Han D, Rubin C (2007) Low-magnitude mechanical signals that stimulate bone formation in the ovariectomized rat are dependent on the applied frequency but not on the strain magnitude. *J Biomech* 40:1333–1339
- Kanno T, Takahashi T, Tsujisawa T, Ariyoshi W, Nishihara T (2007) Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* 101:1266–1277
- Komori T (2006) Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* 99:1233–1239
- Leung KS, Shi HF, Cheung WH, Qin L, Ng WK, Tam KF, Tang N (2009) Low-magnitude high-frequency vibration accelerates callus formation, mineralization, and fracture healing in rats. *J Orthop Res* 27:458–465
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrughe B (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108:17–29
- Nishimura M, Chiba M, Ohashi T, Sato M, Shimizu Y, Igarashi K, Mitani H (2008) Periodontal tissue activation by vibration: intermittent stimulation by resonance vibration accelerates experimental tooth movement in rats. *Am J Orthod Dentofac Orthop* 133:572–583
- Ogawa T, Vandamme K, Zhang X, Naert I, Possemiers T, Chaudhari A, Sasaki K, Duyck J (2014) Stimulation of titanium implant osseointegration through high-frequency vibration loading is enhanced when applied at high acceleration. *Calcif Tissue Int* 95:467–475
- Ozicivici E, Luu YK, Adler B, Qin YX, Rubin J, Judex S, Rubin CT (2010) Mechanical signals as anabolic agents in bone. *Nat Rev Rheumatol* 6:50–59
- Rosenberg N, Levy M, Francis M (2002) Experimental model for stimulation of cultured human osteoblast-like cells by high frequency vibration. *Cytotechnology* 39:125–130
- Rubin C, Turner AS, Bain S, Mallinckrodt C, McLeod K (2001) Anabolism, low mechanical signals strengthen long bones. *Nature* 412:603–604
- Stein GS, Lian JB (1993) Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype. *Endocr Rev* 14:424–442

- Tanaka SM, Li J, Duncan RL, Yokota H, Burr DB, Turner CH (2003) Effects of broad frequency vibration on cultured osteoblasts. *J Biomech* 36:73–80
- Tjandrawinata RR, Vincent VL, Hughes-Fulford M (1997) Vibrational force alters mRNA expression in osteoblasts. *FASEB J* 11:493–497
- Uzer G, Manske SL, Chan ME, Chiang F-P, Rubin CT, Frame MD, Judex S (2012) Separating fluid shear stress from acceleration during vibration in vitro: identification of mechanical signals modulating the cellular response. *Cell Mol Bioeng* 5:266–276
- Uzer G, Pongkitwitoon S, Chan ME, Judex S (2013) Vibration induced osteogenic commitment of mesenchymal stem cells is enhanced by cytoskeletal remodeling but not fluid shear. *J Biomech* 46:2296–2302
- Welch RD, Jones AL, Bucholz RW, Reinert CM, Tjia JS, Pierce WA, Wozney JM, Li XJ (1998) Effect of recombinant human bone morphogenetic protein-2 on fracture healing in a goat tibial fracture model. *J Bone Miner Res* 13:1483–1490
- Zeng Z, Yin X, Zhang X, Jing D, Feng X (2015) Cyclic stretch enhances bone morphogenetic protein-2-induced osteoblastic differentiation through the inhibition of Hey1. *Int J Mol Med* 36:1273–1281