

Low-magnitude, high-frequency vibration promotes the adhesion and the osteogenic differentiation of bone marrow-derived mesenchymal stem cells cultured on a hydroxyapatite-coated surface: The direct role of Wnt/ β -catenin signaling pathway activation

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Abstract. The positive effect of low-magnitude, high-frequency (LMHF) vibration on implant osseointegration has been demonstrated; however, the underlying cellular and molecular mechanisms remain unknown. The aim of this study was to explore the effect of LMHF vibration on the adhesion and the osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) cultured on hydroxyapatite (HA)-coated surfaces in an *in vitro* model as well as to elucidate the molecular mechanism responsible for the effects of LMHF vibration on osteogenesis. LMHF vibration resulted in the increased expression of fibronectin, which was measured by immunostaining and RT-qPCR. Stimulation of BMSCs by LMHF vibration resulted in the rearrangement of the actin cytoskeleton with more prominent F-actin. Moreover, the expression of β 1 integrin, vinculin and paxillin was notably increased following LMHF stimulation. Scanning electron microscope observations revealed that there were higher cell numbers

and more extracellular matrix attached to the HA-coated surface in the LMHF group. Alkaline phosphatase activity as well as the expression of osteogenic-specific genes, namely Runx2, osterix, collagen I and osteocalcin, were significantly elevated in the LMHF group. In addition, the protein expression of Wnt10B, β -catenin, Runx2 and osterix was increased following exposure to LMHF vibration. Taken together, the findings of this study indicate that LMHF vibration promotes the adhesion and the osteogenic differentiation of BMSCs on HA-coated surfaces *in vitro*, and LMHF vibration may directly induce osteogenesis by activating the Wnt/ β -catenin signaling pathway. These data suggest that LMHF vibration enhances the osseointegration of bone to a HA-coated implant, and provide a scientific foundation for improving bone-implant osseointegration through the application of LMHF vibration.

Introduction

An increasing number of patients requiring surgical implantation are expected to attend orthopedic clinics. As a consequence of age or systemic diseases, the risk of implant loosening is also increasing, becoming an intractable problem. Thus, increasing efforts are focusing on the development of methods or approaches to prevent implant loosening with particular focus on enhancing bone-implant osseointegration. Various approaches have been investigated to promote implant osseointegration, including modification of the implant surface and implant material (1,2), as well as the administration of medicines (3,4) and local growth factors (5). Unfortunately, these approaches are associated with several drawbacks including unstable implants, high costs and an adverse side effect profile (6,7). Thus, clinically, it is imperative to develop

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approaches that are effective, without side effects and that promote osseointegration between the host bone and the implant.

It has long been recognized that bone is capable of adapting its mass and microstructure in response to mechanical stimuli (8,9). Some researchers have focused on biophysical stimulation methods to treat osteoporosis (10). The non-invasive and non-pharmacological intervention of low-magnitude (LM, $<1 \times g$, $g=9.81 \text{ m/sec}^2$), high-frequency (HF, 20-90 Hz) (LMHF) vibrations has gained interest as studies have shown that such a mechanical signal may promote bone formation, prevent bone loss and stimulate bone healing (11-15). Additionally, the effect of LMHF vibration on bone-implant osseointegration has been confirmed (16-18). Particularly, our previous study demonstrated that LMHF enhanced bone-implant osseointegration in ovariectomized rats, which was confirmed by histomorphometrical and biomechanical analysis (19). However, the underlying mechanism through which LMHF vibration induces bone-implant osseointegration at the cellular and molecular level remains largely unknown.

Previously, several studies have determined the effect of the chemical composition of the implants and their surface properties on osteoblast behavior (20-22). However, these studies were performed under static conditions and did not take into account the presence of mechanical stimuli at the cell-implant interface and their effects on the osteoblastic phenotype. It is well known that the efficacy of orthopedic implants requires the creation of a direct structural and functional connection between the material and the bone. Adhesion is a factor involved in the first phase of cell-material interactions and the quality of adhesion determines the ability of cells to proliferate and differentiate in contact with the implant. Several *in vitro* studies have determined the effects of mechanical strain on the adhesion of osteoblastic cells (23,24). On the other hand, several studies have reported that the exposure of osteoblastic cells or bone marrow-derived mesenchymal stem cells (BMSCs) to LMHF vibration may stimulate osteoblast differentiation (25-27). However, whether LMHF vibration exerts an effect on the adhesion and the differentiation of BMSCs cultured on the surface of an implant currently remains unclear. Thus, we hypothesized that LMHF vibration enhances the adhesion and the osteogenic differentiation of BMSCs cultured on an implant surface *in vitro*, leading to the increased expression of adhesion molecules and osteoblastic markers, as well as enhancing extracellular matrix (ECM) synthesis.

Thus far, the molecular mechanism responsible for the effects of mechanical stimuli on bone formation has yet to be addressed. Several studies have demonstrated that Wnt/ β -catenin signaling is a normal physiological response to mechanical stimuli (28-30). Canonical Wnt signaling has been found to encourage mesenchymal progenitor cells to differentiate into osteoblasts (31). Hou *et al* (30) investigated the mechanobiological mechanisms of vibration-enhanced osteogenic responses in osteoblasts, and found that the protein expression of Wnt10B and osteoprotegerin (OPG) increased, whereas the levels of sclerostin and receptor activator of nuclear factor κ B ligand (RANKL) were reduced. These findings suggest that Wnt signaling is an essential pathway in the mechanotransduction process. Thus, we hypothesized that

Wnt/ β -catenin signaling may also play an important role in the LMHF vibration-mediated osteogenic differentiation of BMSCs.

In this study, we established an *in vitro* model of hydroxyapatite (HA)-coated substrates. We aimed to explore the cellular and molecular mechanisms responsible for the effects of LMHF vibration on the adhesion and the osteogenic differentiation of BMSCs cultured on HA-coated surfaces, and to examine whether Wnt/ β -catenin signaling is involved in osteogenesis following exposure to LMHF vibration.

Materials and methods

In vitro material preparation. Titanium substrates (circular substrates 34 or 14.75 mm in diameter and 1 mm in thickness) were made by the Engineering Research Center in Biomaterials of Sichuan University (Chengdu, China) and plasma sprayed with HA (100 μm in thickness) using a Metco MN Plasma System and an AR-2000 Thermal Spray Robot (Metco, Westbury, NY, USA).

Isolation and culture of BMSCs. Twenty male Sprague-Dawley (SD) rats (4-6 weeks old) were obtained from the Experimental Animal Center of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). The current study was approved by the Animal Care Committee of Sun Yat-sen University (approval no. [2014]52). The BMSCs were isolated from SD rats as previously described (26). Briefly, femora and tibiae were harvested from euthanized rats (excess 10% chloral hydrate by intraperitoneal injection), cut at each bone end, and then flushed with 5 ml Dulbecco's modified Eagle's medium (DMEM; Gibco, Beijing, China) culture medium twice using a syringe. The cells were resuspended following centrifugation (1,000 $\times g$, 5 min) and placed in the culture medium, and cultured in an 5% CO_2 incubator at 37°C. The culture medium was replaced every 2 days. The cells were subcultured to 80-90% confluence. Passage 3 (P3) BMSCs were used for all experiments (26).

LMHF vibration in vitro. HA-coated titanium substrates (Φ 34 or 14.75 mm) were placed into 6-well plates, 24-well plates and 35 mm cultured dishes after high temperature sterilization. The BMSCs were seeded on the HA-coated surface in 6-well plates/35 mm cultured dishes (5×10^4 cells/cm²) and 24-well plates (5×10^3 cells/cm²) and randomly divided into two groups: i) osteogenic medium (control group) and ii) osteogenic medium and LMHF vibration (LMHF group). In the control group, the medium was replaced with osteogenic medium (medium containing 10 nM dexamethasone, 10 mM β -glycerophosphate and 50 $\mu\text{g/ml}$ ascorbic acid) (Sigma, St. Louis, MO, USA) after 24 h. The osteogenic medium was replaced every 2 days for 14 days.

For the LMHF group, the plates cultured with BMSCs were mounted on the platform of a GJX-5 vibration sensor (Beijing Sendig Technology, Beijing, China) and subjected to mechanical stimuli (magnitude, 0.3 g; frequency, 40 Hz) for 30 min every 24 h starting from day 0. The vibration parameter (40 Hz, 0.3 g) was similar to that of previously published methods (26,32,33). After 30 min of vibration, the cells in the LMHF group received fresh osteogenic medium.

Table I. Primers used for RT-qPCR.

Gene	Primer sequences	Product size (bp)
Fibronectin	F: 5'-CGGTGGCTGTCAGTCAAAG-3' R: 5'-AAACCTCGGCTTCCTCCATAA-3'	130
β 1 integrin	F: 5'-TGAATGTGAATGCCAAAGCGA-3' R: 5'-CAATGTCTACCAACACGCC-3'	128
Vinculin	F: 5'-TCTGACCTCCTGCTTACCTTT-3' R: 5'-CAACTCCTGCTGTCTCTCATC-3'	188
Paxillin	F: 5'-GCCAGCAGCAGACCAGAA-3' R: 5'-TAGTGGGAGGAGGAGGAAGG-3'	171
Runx2	F: 5'-CCACCTCTGACTTCTGCCTC-3' R: 5'-GGGATGAAATGCCTGGGAAC-3'	111
Osterix	F: 5'-CAGTAATCTTCGTGCCAGACC-3' R: 5'-GGCTTCTTTGTGCCTCCTTT-3'	180
Collagen I	F: 5'-GTGTTCAAGGTGGCAAAGGTG-3' R: 5'-CAGGACCAGGGAGACCGAA-3'	141
Osteocalcin	F: 5'-GCGGAACCTATGCCTGCT-3' R: 5'-GTGGTGTGGTGATTTGGATGG-3'	118
β -actin	F: 5'-GGAAATCGTGCGTGACATTA-3' R: 5'-AGGAAGGAAGGCTGGAAGAG-3'	183

The culture medium in all groups was replaced every 48 h. All groups were cultured for 14 days (34).

Immunofluorescence staining. After 1, 3 and 5 days of administration, immunostaining was performed. The cells were then washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min at room temperature, and washed again with PBS. They were then immersed in 0.1% Triton X-100 for 4 min and washed in PBS. The cells were blocked with 1% bovine serum albumin (BSA) and incubated with anti-fibronectin (FN; mouse; 1:100; F7387) and anti-F-actin (1:100; 94072) primary antibodies (both from Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight followed by incubation with FITC-conjugated secondary antibody (anti-mouse; 1:2,000; A-21202; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h at room temperature. The cells were rinsed with PBS, and the nuclei were stained with 4',6-diamino-2-phenylindole hydrochloride (DAPI) and incubated for 30 min at room temperature. Imaging experiments were conducted using a fluorescence microscope (Leica DMI3000 B) and a laser scanning confocal microscope (ZEISS LSM710) (both from Leica Microsystems, Wetzlar, Germany).

Scanning electron microscope (SEM) observations. The cells in all groups were evaluated using an SEM to examine cell morphology and attachment. The BMSCs of all groups were seeded in 35-mm culture dishes (containing HA-coated titanium substrates) at a density of 5×10^4 cells/cm² in osteogenic medium for 14 days. The medium was removed and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer

overnight at 4°C. After fixation, the samples were dehydrated at room temperature in increasing concentrations of ethanol (30, 50, 70, 95 and 100%), and then point-dried with CO₂ (35). The specimens were mounted on aluminum stubs and sputter-coated with gold. Finally, the samples were observed under an SEM.

Assay of alkaline phosphatase (ALP) activity. To evaluate ALP activity, the BMSCs were seeded on the HA-coated surface in 6-well plates at a density of 5×10^4 cells/cm² and osteogenesis was induced in different groups. ALP activity was then measured using a SensoLyte p-nitrophenyl phosphate (pNPP) ALP assay kit (AnaSpec, Fremont, CA, USA) according to the manufacturer's instructions on days 3, 7, 10 and 14. Briefly, the cells were lysed with lysis buffer, which was provided in the kit. After three rapid freeze-thaw cycles, the proteins were extracted. The supernatant was collected after the cell lysate was centrifuged for 15 min (10,000 x g, 4°C) and subsequently combined with pNPP. ALP activity was detected at 405 nm using a microplate reader (Sunrise; Tecan, Männedorf, Switzerland). Values were normalized to total protein content, which was measured using a spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cultured cells in different groups using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was then used for cDNA synthesis with a SYBR-PrimeScript RT-PCR kit (Takara, Dalian, China). Each cDNA samples was analysed in triplicate in a 10 μ l reaction volume containing 1 μ l cDNA, 0.3 μ l forward primer, 0.3 μ l reverse primer, 5 μ l SYBR[®] Premix Ex Taq[™], and 3.4 μ l diethylpyrocarbonate (DEPC) water. Fluorescence data was analyzed using a CFX96[™] Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: initial denaturation, 95°C for 5min (1 cycle); denaturation, 94°C for 30 sec; annealing, 60°C for 10 sec; elongation, 72°C for 20 sec; and final extension step, 72°C for 10 min (for 40 cycles). Gene expression levels were calculated in relation to the β -actin CT value by the 2^{- $\Delta\Delta$ CT} method. Data are normalized to the control group. The primer sequences of FN, β 1 integrin, vinculin, paxillin, Runt-related transcription factor 2 (Runx2), osterix (Osx), collagen I (Col-I), osteocalcin (OCN) and β -actin are presented in Table I.

LMHF vibration and Dkk-1 administration. In order to examine whether Wnt/ β -catenin signaling is involved in osteogenesis after LMHF vibration treatment, the BMSCs were seeded in 6-well plates at a density of 5×10^4 cells/cm² and then randomly divided into three groups: i) osteogenic medium (control group), ii) osteogenic medium and LMHF vibration (LMHF group) and iii) osteogenic medium and LMHF vibration with 100ng/ml dickkopf-1 (Dkk-1; Sigma-Aldrich), a WNT signaling pathway inhibitor (Dkk-1 group). The culture medium in all groups was replaced every 48 h. All groups were cultured for 14 days (34).

Western blot analysis. The samples for western blot analysis were collected on day 4. To obtain whole-cell protein extracts, the cells were washed twice with ice-cold PBS and lysed

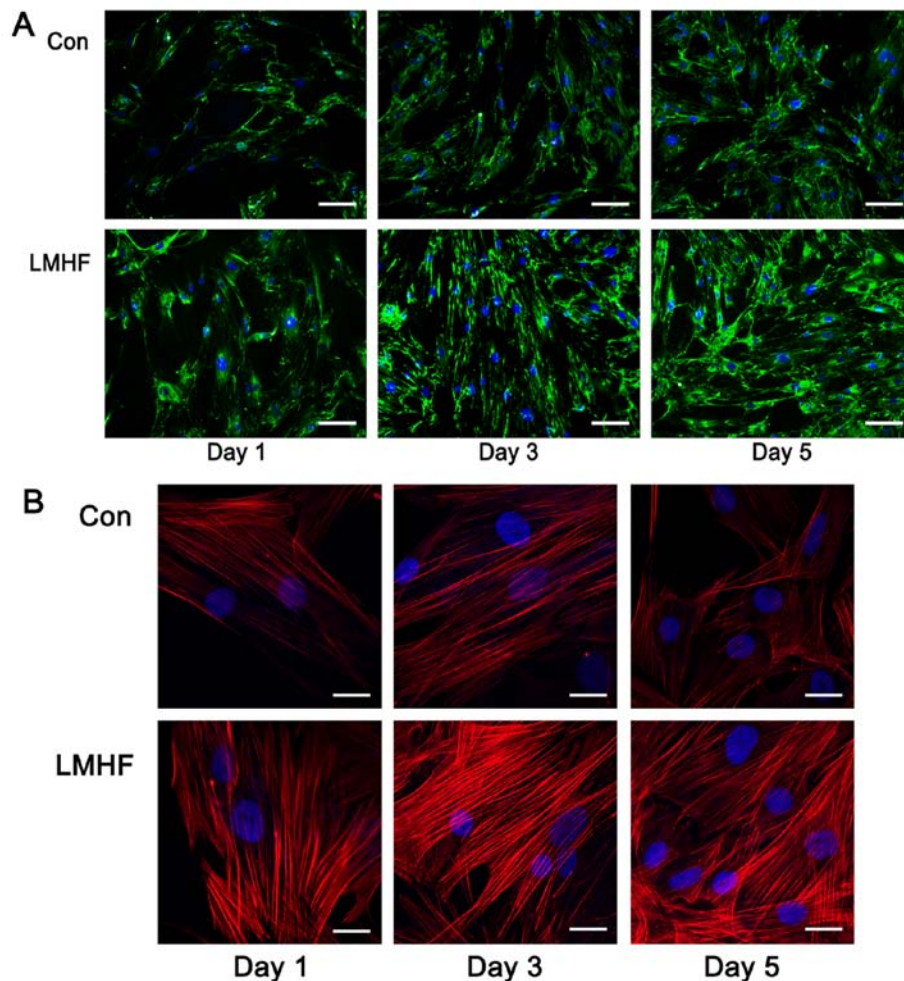


Figure 1. Effects of low-magnitude, high-frequency (LMHF) vibration on fibronectin (FN) organization and the reorganization of the actin cytoskeleton on days 1, 3 and 5 in bone marrow-derived mesenchymal stem cells (BMSCs). (A) Control and LMHF cultures were performed in parallel. FN was visualized by immunofluorescence staining. (B) F-actin immunostaining of the control group or the LMHF group after 1, 3 and 5 days of culture. Scale bars, 100 μ m. Con, control.

in 80 μ l Mammalian Protein Extraction reagent (Thermo Fisher Scientific, Inc.). The supernatant protein samples were harvested after centrifugation for 15 min (12,000 \times g, 4°C) and boiled for 5 min. Equal volumes of the samples were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and subsequently transferred to a PVDF membrane. The membrane was blocked for 1 h with 5% nonfat dry milk and then incubated overnight at 4°C with the following primary antibodies: Wnt10b (ab106522; Abcam, Cambridge, CA, USA); β -catenin (2968) and Runx2 (12556) (both from Cell Signaling Technology, Danvers, MA, USA); Osx (ab22552) and GAPDH (ab8245) (both from Abcam). Following incubation with secondary antibody (Cell Signaling Technology), the immunoreactive proteins were visualized using a chemiluminescence kit (Millipore, Billerica, MA, USA). Gray analysis was performed using Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis. All data are presented as the means \pm standard deviation. For the control and the LMHF cultures, comparisons were performed using the two-tailed Mann-Whitney test, and statistical analysis to compare the results among groups was conducted using one-way analysis of

variance (ANOVA), and the least significant difference (LSD) post hoc test was applied for multiple comparisons using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Immunofluorescence staining of matrix organization and cytoskeleton. The effect of LMHF vibration on FN matrix organisation was investigated on days 1, 3 and 5. As shown in Fig. 1, increasing fluorescence intensity and increasing numbers of FN fibres were observed after 1, 3 and 5 days of LMHF vibration (Fig. 1A), confirming the increased FN production quantified by RT-qPCR. Actin rearrangement is an important event in cell attachment. Cytoskeletal organization was assessed by F-actin labeling following LMHF stimulation (1, 3 and 5 days). Actin filaments were clearly visible after staining with the F-actin antibody (Fig. 1B). The stimulation of BMSCs with LMHF vibration resulted in the rearrangement of the actin cytoskeleton with more prominent F-actin. The majority of the actin filaments were situated parallel to the main direction of the cell, and the fluorescence intensity was significantly stronger than that in the control group.

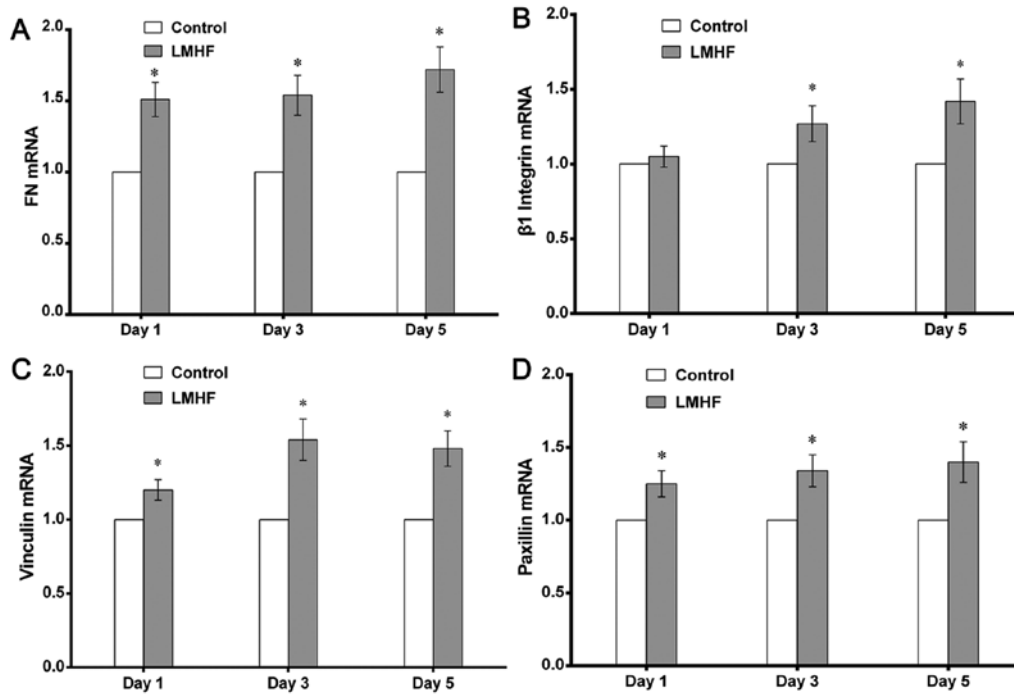


Figure 2. Effect of low-magnitude, high-frequency (LMHF) vibration on the gene expression of cell adhesion molecules. The mRNA expression of (A) fibronectin (FN), (B) β 1 integrin, (C) vinculin and (D) paxillin was assayed on days 1, 3, and 5. Data showed that LMHF vibration greatly upregulated these mRNA levels at different time-points. Data are expressed as the means \pm standard deviation (n=5). *P<0.05 compared with the control.

Analysis of the gene expression of cell adhesion molecules by RT-qPCR. To determine the effect of LMHF vibration on cell-biomaterial interactions, the mRNA expression of FN, β 1 integrin, vinculin and paxillin, which are involved in the adhesion of cells to the substrates, was measured by RT-qPCR on days 1, 3 and 5. The mRNA expression of FN was greatly enhanced in LMHF-vibrated cultures (P<0.05) (Fig. 2A); β 1 integrin mRNA expression was also significantly increased after LMHF vibration on days 3 and 5 (P<0.05) (Fig. 2B). Similarly, LMHF vibration also resulted in an increase in the mRNA expression of vinculin and paxillin on days 1, 3 and 5 (P<0.05) (Fig. 2C and D).

SEM observation of cell morphology. SEM images of the HA-coated surface without any cells are shown in Fig. 3a and b. Fourteen days after culture, the cells in the two groups adhered tightly to the HA-coated surface and SEM images of the cells are presented in Fig. 3A and B. The cells were connected with each other by filopodia and established their ECM on the HA surface, which suggests that the cells are capable of commendably integrating with the HA-coated substrate. Most strikingly, an increased number of cells and more plentiful ECM are present on the HA-coated surface in the LMHF group compared with that in the control group (Fig. 3A and B).

Assessment of ALP activity. The BMSCs were cultured in osteogenic medium in different treatment groups for measuring ALP activity on days 3, 7, 10 and 14 (Fig. 4). ALP activity gradually increased in a time-dependent manner in all groups. In comparison with the control group, the level of ALP activity of the LMHF group was significantly increased at different time-points (P<0.05).

Analysis of osteogenic-specific gene expression by RT-qPCR. To examine whether LMHF vibration affected the osteogenic differentiation of BMSCs, the expression of genes associated with osteogenesis, including Runx2, Osx, Col-I and OCN, was evaluated using RT-qPCR on days 3, 7, 10 and 14. LMHF vibration upregulated the mRNA expression of Runx2 and Osx on days 3, 7, 10 and 14 (P<0.05), and the expression of Runx2 and Osx was apparent on day 3 (P<0.05) (Fig. 5A and B). Similarly, with LMHF vibration treatment, the mRNA expression of Col-I showed a marked increase on days 3, 7, 10 and 14 (P<0.05) (Fig. 5C). The mRNA expression of OCN was also significantly increased following exposure to LMHF vibration on days 10 and 14 (P<0.05) (Fig. 5D), although no visible difference in the mRNA expression of OCN was observed on days 3 or 7 (P>0.05) (Fig. 5D).

Involvement of Wnt/ β -catenin signaling in LMHF vibration-induced osteogenesis. Western blot analysis and gray analysis showed that Wnt10b and β -catenin expression in the LMHF group was significantly increased compared with the control group (P<0.05) (Fig. 6A). Additionally, following the inhibition of Wnt/ β -catenin signaling by Dkk-1, the protein expression of Wnt10b and β -catenin was reduced when compared with that in the LMHF group (P<0.05), although there was no statistically significant difference compared with the control group (P>0.05) (Fig. 6A). Similar results were seen with regard to the protein expression of Runx2 and Osx (Fig. 6B). Runx2 and Osx protein levels were significantly increased in the LMHF group, when compared with those in the control group (P<0.05). Similarly, after the administration of Dkk-1, a significant decrease in Runx2

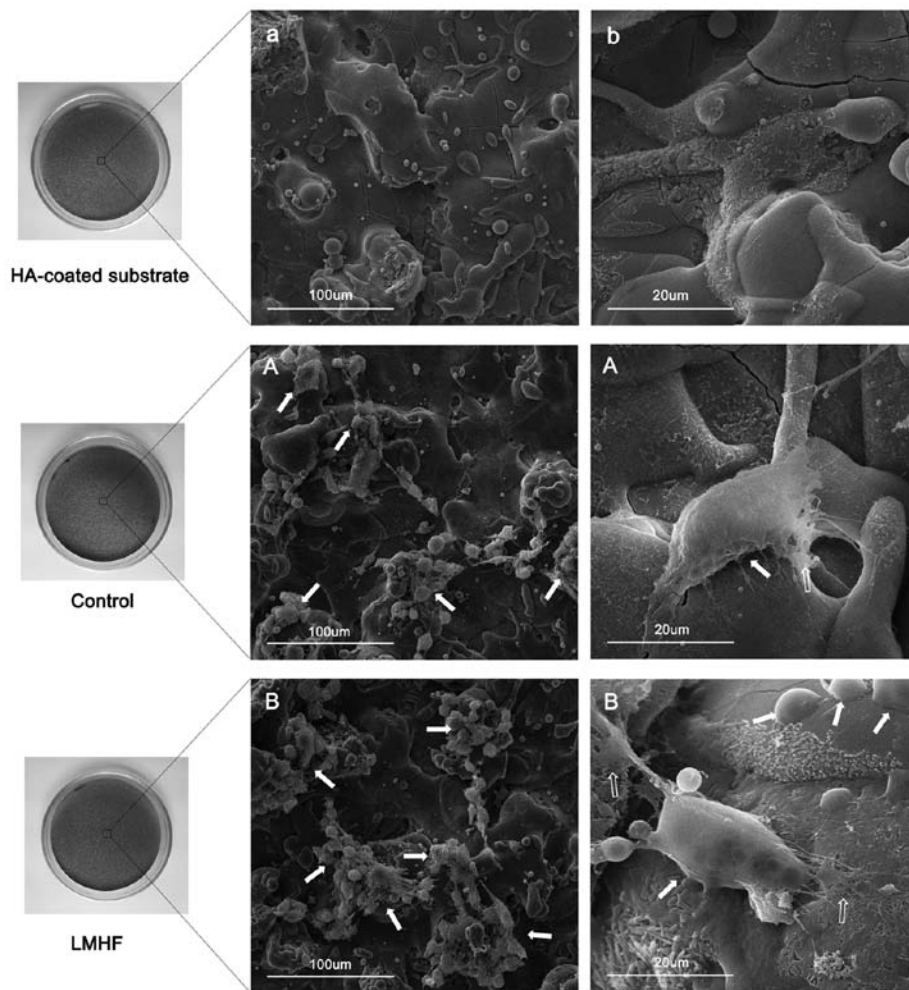


Figure 3. Scanning electron microscope (SEM) micrographs of cell growth and attachment on the HA-coated surface after 14 days. Cell adhesion to the HA-coated surface in the low-magnitude, high-frequency (LMHF) group was significantly increased compared with that in the control group. (a and b) HA-coated surface, (A) control group, (B) LMHF group. Solid arrows indicate cells and open arrows indicate extracellular matrix.

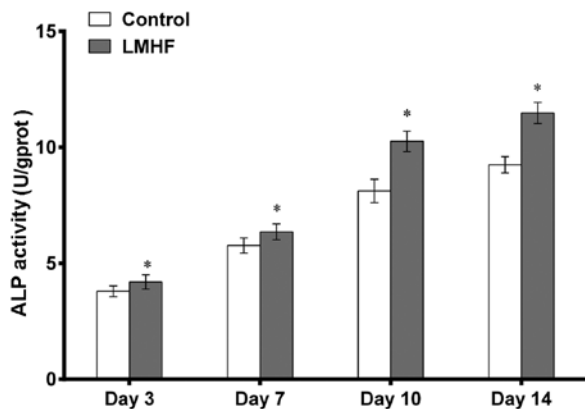


Figure 4. Measurement of alkaline phosphatase (ALP) activity (U/gprot) in the control and the low-magnitude, high-frequency (LMHF) groups on days 3, 7, 10 and 14. The LMHF group exhibited significantly improved ALP activity compared with that of the control group. Data are expressed as the means \pm standard deviation (n=5). *P<0.05 compared with the control.

and Osx protein expression was observed when compared with that in the LMHF group (P<0.05), and there was no significant difference between the control and Dkk-1 groups (P>0.05).

Discussion

A number of *in vivo* studies have proved that LMHF vibration is beneficial for osseointegration at the bone-implant interface (17-19). Previously, we established a rat model of osteoporosis and inserted implants into the proximal tibiae, and then treated the rats with LMHF vibration (0.3 g, 30-35 Hz). Our results indicated that LMHF vibration significantly increased bone-to-implant contact (%) and the peri-implant bone fraction, as well as biomechanical parameters, such as maximum push-out force and interfacial shear strength (19). As a continuation of our previous *in vivo* study, the current *in vitro* study aimed to examine the cellular and molecular mechanisms responsible for the effects of LMHF vibration on bone-implant osseointegration. Consistent with our hypothesis, the findings demonstrated that LMHF vibration enhanced the adhesion and the osteogenic differentiation of the BMSCs cultured on the HA-coated surface, and Wnt/ β -catenin was responsible for the LMHF vibration-induced osteogenesis.

In the present experiment, we used HA-coated titanium substrates. HA is the major mineral component of human bone tissue and has been widely used as a coating of load-bearing metallic implants due to the osteoconductivity and

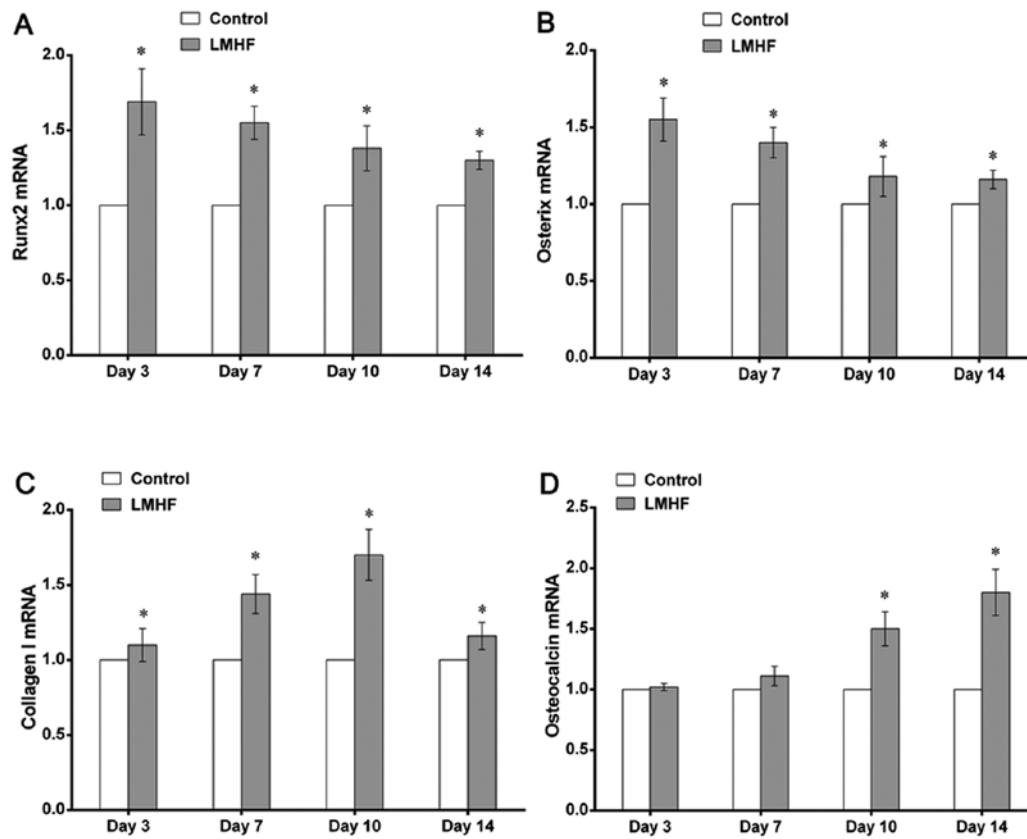


Figure 5. RT-qPCR analysis of the mRNA expression of (A) Runx2, (B) osterix, (C) collagen I and (D) osteocalcin in the control and the low-magnitude, high-frequency (LMHF) groups on days 3, 7, 10 and 14. Data showed that the mRNA levels were greatly elevated in the LMHF group. Data are expressed as the means \pm standard deviation (n=5). *P<0.05 compared with the control.

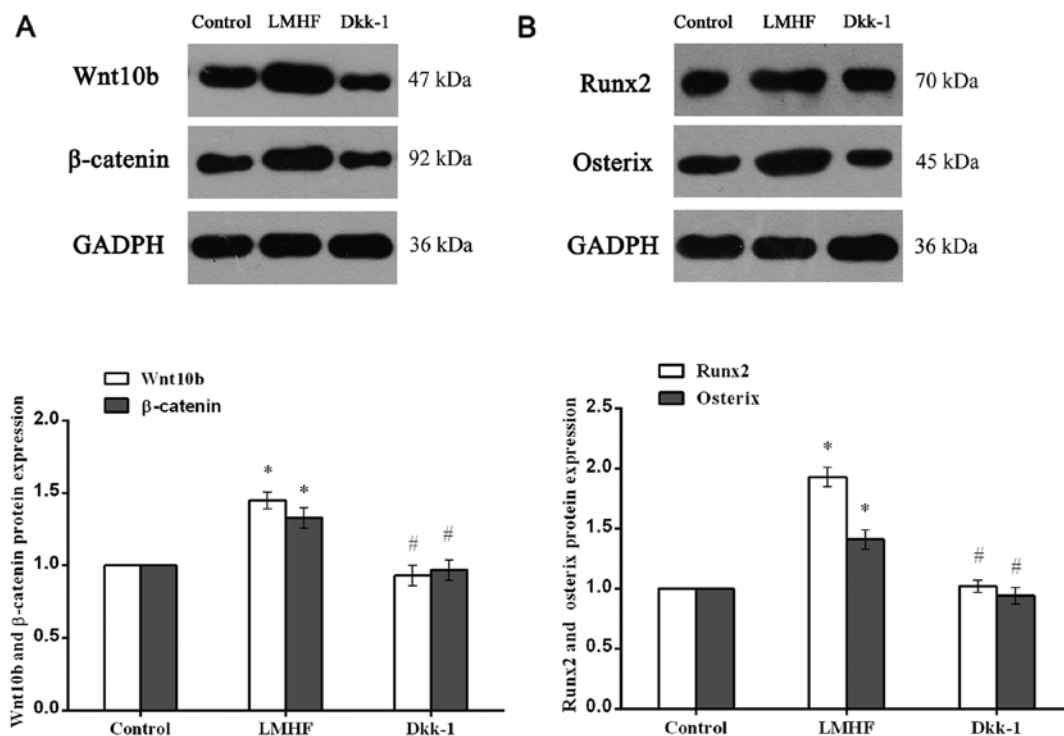


Figure 6. Effect of Wnt/ β -catenin signaling pathway on low-magnitude, high-frequency (LMHF) vibration-induced osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) evaluated using western blot analysis and gray analysis. The protein levels of (A) Wnt10b and β -catenin, as well as (B) Runx2 and osterix under different culture conditions were measured on day 4. The protein expression of Wnt10b, β -catenin, Runx2 and osterix in the LMHF group was significantly increased compared with that in the control group. The addition of the WNT signaling pathway inhibitor dickkopf-1 (Dkk-1) to LMHF vibration-induced osteogenic differentiation of BMSCs resulted in decreased protein expression of Wnt10b, β -catenin, Runx2 and osterix. Data are expressed as the means \pm standard deviation (n=5). *P<0.05 compared with the control; #P<0.05 compared with the LMHF group.

chemical similarity of HA to bone (35,36). The HA coating has been found to increase bone-implant contact and new bone formation around implant surfaces (37). Previous studies have demonstrated that the HA coating exhibits good *in vitro* cell adhesion and cytocompatibility (20,35-37). Thus, HA-coated substrates were used in this *in vitro* study to provide insights into the adhesion and the osteogenic differentiation of BMSCs in response to LMHF vibration, and this is a novel contribution of the present study.

In the present study, the LMHF vibration regime (0.3 g, 40 Hz) used has been previously demonstrated to exert an osteogenic effect (26,32,33). Zhou *et al* (26) demonstrated that microvibration (magnitude, 0.3 g; frequency, 40 Hz) has a stimulatory effect on the osteodifferentiation of BMSCs. Zhang *et al* (32) cultured periodontal ligament stem cells (PDLSCs) and treated them with vibration (magnitude, 0.3 g; frequency, 10-180 Hz), and the results suggested that the osteogenic markers were significantly increased by LMHF vibration at frequencies of 40 and 50 Hz. Additionally, in a study by Kim *et al* (33), human mesenchymal stromal cells (hMSCs) were subjected to vibration stimuli (magnitude, 0.1-0.6 g; frequency, 10-40 Hz), and they found that daily exposure to vibration increased the proliferation and the osteogenic differentiation of hMSCs, with the highest efficiency occurring at a peak magnitude of 0.3 g and a frequency of 30 to 40 Hz. Thus, based on the findings of these studies, a magnitude of 0.3 g and a frequency of 40 Hz was selected for use in this study.

The ability of BMSCs to adhere to the implant surface and their differentiation on the implant surface are important components of successful osseointegration (38,39). In order to analyze cell adhesion to the implant surface, we assessed the matrix organization (FN) and the cytoskeleton rearrangement (F-actin), as well as the gene expression of FN, $\beta 1$ integrin, vinculin and paxillin, which are involved in the adhesion of cells to substrates. FN is a major protein of the ECM and plays a central role in regulating cell adhesion (23). This study indicated that LMHF vibration significantly increased the expression of FN. This upregulation of FN by mechanical stress has also been reported in other studies (23,40). Mechanical strain is known to induce changes in cytoskeletal organization, and actin filaments are crucial for cell adhesion (41). The stimulation of BMSCs by LMHF vibration resulted in rearrangement of the actin cytoskeleton with more prominent F-actin, and the fluorescence intensity was significantly stronger. A similar actin fiber pattern has been observed in myoblasts following exposure to cyclic strain (42). To the best of our knowledge, various adhesion molecules are involved in the adhesion of cells to implants, such as $\beta 1$ integrin, vinculin and paxillin (43-45). The results showed that LMHF stimulation notably increased the mRNA expression of $\beta 1$ integrin, vinculin and paxillin. Dumas *et al* (40) suggested that LMHF stimulation increased the expression of vinculin in mesenchymal stem cells (MSCs). Carvalho *et al* (46) showed that human osteosarcoma cells treated with mechanical stimulation exhibited significantly increased mRNA expression of $\beta 1$ integrin. Furthermore, SEM observation was performed to evaluate cell morphology on the HA-coated surface. SEM images revealed that the cells were connected to each other by filopodia and were tightly adhered to the HA-coated surface, whereas there were higher cell numbers and more ECM

attached to the HA-coated surface in the LMHF group. The SEM images illustrated that LMHF vibration significantly promoted the integration of cells with the HA-coated surface. Taken together, these results demonstrate that LMHF vibration may promote cell adhesion to the HA-coated surface. This finding was unique in that it is the first to describe the effects of LMHF vibration on the cell-implant interaction *in vitro*, to the best of our knowledge.

The osteogenic differentiation of cells is usually divided into three discrete stages: commitment to osteogenic lineage, matrix synthesis, and matrix mineralization. Runx2 and Osx are usually highly expressed at the early stage (commitment to osteogenic lineage) (47,48), Col-I and ALP at the middle stage (matrix synthesis) (32,48), and OCN at the late stage (matrix mineralization) of osteogenesis (49). Based on the results of the ALP activity assay and RT-qPCR, we observed that ALP activity and the expression of Runx2, Osx, Col-I and OCN were increased by LMHF vibration. Taken together, these results regarding the osteogenic-specific markers indicated that LMHF vibration promotes the osteogenic differentiation of BMSCs cultured on HA-coated surfaces *in vitro*. Zhou *et al* (26) investigated the effect of LMHF vibration on the osteogenic differentiation of BMSCs seeded on human bone-derived scaffolds. They found that LMHF vibration promoted BMSC differentiation by upregulating the mRNA and protein expression of osteogenic markers including Runx2, ALP, Col-I and OCN. Zhang *et al* (32) cultured PDLSCs under conditions of LMHF vibration. The results showed that LMHF vibration increased the levels of ALP, Col-I, Runx2, Osx and OCN, which demonstrated that LMHF mechanical vibration promotes the osteogenic differentiation of PDLSCs. Additionally, Prè *et al* (27) treated BMSCs with mechanical vibration, and the results showed that the expression of ALP and Runx2 was significantly increased after mechanical vibration treatment. Even though a marked enhancement of osteogenic differentiation in different cell types was observed in these studies, none of them used HA-coated material to observe the effect of LMHF vibration on the osteogenic differentiation of BMSCs. Our experiment covered the limitations of the studies mentioned above. In addition, the results suggested that LMHF vibration is beneficial for the osteogenic differentiation of BMSCs cultured on HA-coated surfaces *in vitro*.

Although LMHF vibration has been demonstrated to promote the osteogenic differentiation of BMSCs cultured on implant surfaces, the molecular mechanism responsible for the effects of LMHF vibration on the osteogenic differentiation of BMSCs remains elusive. Several studies have suggested that cells convert the mechanical stimulus into a biochemical signal, thus playing a role in regulating osteogenic differentiation (29,30,50). Canonical Wnt signaling promotes MSCs to differentiate into osteoblasts. In osteoblasts, the Wnt pathway also promotes proliferation and mineralization whereas it blocks apoptosis and osteoclastogenesis by increasing the OPG/RANKL ratio (31). In the present study, we analyzed the protein expression of Wnt10b, β -catenin, Runx2 and Osx to probe the direct effect of LMHF vibration on the osteogenic differentiation of BMSCs through the activation of the Wnt/ β -catenin signaling pathway. We found that LMHF vibration significantly increased the expression of Wnt10b, β -catenin, Runx2 and Osx; however, after the administration

of Dkk-1, these proteins were downregulated markedly. These results suggest that LMHF vibration may directly promote the osteogenic differentiation of BMSCs through the Wnt/ β -catenin signaling pathway.

Previously, Robinson *et al* (28) demonstrated that Wnt/ β -catenin signaling is a normal physiological response to mechanical loading and that the activation of the Wnt/ β -catenin pathway enhances the sensitivity of osteoblasts to mechanical loading. Hou *et al* (30) investigated the mechanobiological mechanisms of vibration-enhanced osteogenic responses in MC3T3-E1 cells and they demonstrated that Wnt signaling was involved in mechanotransduction at LM vibrations and the RANKL/OPG ratio and levels of sclerostin were also significantly decreased. It has been found that RANKL/OPG is the major signaling axis that controls osteoclast formation, activation and survival, and furthermore, sclerostin was shown to modulate the activity of osteoblastic cells by reducing ALP activity, type I collagen synthesis, and mineralization (51). Thus, all of these studies have suggested that the Wnt/ β -catenin signaling pathway is activated by mechanical vibration in osteoblastic cells, and that mechanical vibration played a role in promoting osteogenesis indirectly by activating the Wnt/ β -catenin pathway. However, in the present study, we have demonstrated that LMHF vibration may directly promote the osteogenic differentiation of BMSCs by activating the Wnt/ β -catenin signaling pathway.

Our *in vitro* results demonstrated the positive effects of LMHF vibration on the adhesion and on the osteogenic differentiation of BMSCs cultured on HA-coated surfaces. Moreover, the effect of LMHF vibration on the osteogenic differentiation of BMSCs by activating the Wnt/ β -catenin signaling pathway was also proved. However, it remains unknown whether the activation of Wnt/ β -catenin signaling by LMHF vibration is involved in the regulation of bone-implant osseointegration. Further investigations may elucidate the in-depth role of LMHF vibration-induced activation of Wnt/ β -catenin on the osteogenic differentiation of BMSCs.

In conclusion, LMHF vibration promotes the adhesion and the osteogenic differentiation of BMSCs on HA-coated surfaces *in vitro*. LMHF vibration may directly induce osteogenesis by activating the Wnt/ β -catenin signaling pathway. These data provide a scientific foundation for improving bone-implant osseointegration through the application of LMHF vibration.

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