OSTEOGENIC DIFFERENTIATION OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS ON BONE-DERIVED SCAFFOLDS: EFFECT OF MICROVIBRATION AND ROLE OF ERK1/2 ACTIVATION

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Abstract

Although *in vivo* studies have shown that low-magnitude, high-frequency (LMHF) vibration (LM: < 1 ×g; HF: 20-90 Hz) exhibits anabolic effects on skeletal homeostasis, the underlying cellular/molecular regulation involved in bone adaptation to LMHF vibration is little known. In this report, we tested the effects of microvibration (magnitude: 0.3 ×g, frequency: 40 Hz, amplitude: ±50 μm, 30 min/12 h) on proliferation and osteodifferentiation of bone marrow-derived mesenchymal stromal cells (BMSCs) seeded on human bone-derived scaffolds. The scaffolds were prepared by partial demineralisation and deproteinisation. BMSCs were allowed to attach to the scaffolds for 3 days. Morphological study showed that spindle-shaped BMSCs almost completely covered the surface of bone-derived scaffold and these cells expressed higher ALP activity than those cultured on plates. After microvibration treatment, BMSC proliferation was decreased on day 7 and 10; however, numbers of genes and proteins expressed during osteogenesis, including Cbfa1, ALP, collagen I and osteocalcin were greatly increased. ERK1/2 activation was involved in microvibration-induced BMSC osteogenesis. Taken together, this study suggests that bone-derived scaffolds have good biocompatibility and show osteoinductive properties. By increasing the osteogenic lineage commitment of BMSCs and enhancing osteogenic gene expressions, microvibration promotes BMSC differentiation and increase bone formation of BMSCs seeded on bone-derived scaffolds. Moreover, ERK1/2 pathway plays an important role in microvibration-induced osteogenesis in BMSC cellular scaffolds.

Keywords: Bone-derived scaffold, bone marrow-derived mesenchymal stromal cells, microvibration, osteogenesis, ERK1/2.

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Introduction

Current consensus for bone tissue engineering includes three essential elements, i.e., biomaterial scaffold, osteogenic cell lineage and bone inducing factors (e.g., mechanical stimulus, Ashammakhi and Ferretti, 2003; Khan *et al*., 2005; Mistry and Mikos, 2005). Scaffold materials should provide the support for cell attachment and have osteoinductive property (Langer and Vacanti, 1993; Ashammakhi and Ferretti, 2003). Due to the limited supply and donor-site morbidity of autogenous bone grafts, different physical structures and insufficient osteoinductive ability of synthetic materials (Ashammakhi and Ferretti, 2003; Silber *et al*., 2003), scaffolds derived from different individuals (allografts) and species (xenografts) provide a promising resource and approach to address the significant drawbacks of existing scaffolds, because these scaffolds have similar structures to autogenous bone (Salkeld *et al*., 2001; Simion *et al*., 2004). Additionally, with the proper chemical and physical process on these bone materials, including demineralisation and deproteinisation (Tadjoedin *et al*., 2003; Xu *et al*., 2003), we can minimise immune rejection of bone-derived scaffolds (Deijkers *et al*., 1997; Norman-Taylor and Villar, 1997), but preserve their osteoinductive abilities and maintain their physical structures.

However, the simple loading of osteogenic cell sources (e.g., BMSCs) to bone-derived scaffolds has largely been limited by the challenge of lacking stimulus to promote bone formation. Since bone is a dynamic tissue and constantly remoulding to adapt mechanical loading (Burger and Klein-Nulend, 1999; Huiskes *et al*., 2000; Frost, 2003). Numerous studies have confirmed that a variety of mechanical stimuli, including fluid flow, hydrostatic pressure, mechanical stretching and vibration, influence bone remoulding through the coupling of osteoblastic and osteoclastic activities (Weyts *et al*., 2002; Huang *et al*., 2009; Hess *et al*., 2010; Lau *et al*., 2010). The vibration with proper combination of frequency and magnitude can induce bone anabolic response (Turner *et al*., 1995; Rubin *et al*., 2002; Tanaka *et al*., 2003; Rubin *et al*., 2004; Rubin *et al*., 2007). Low-magnitude (LM: < 1×g, g = 9.81 m/s^2), high-frequency (HF: 20-90 Hz) vibrations have been demonstrated by studies that such kinds of mechanical stimuli exhibit anabolic roles on bone homeostasis in animals (Rubin *et al*., 2002; Garman *et al*., 2007; Rubin *et al*., 2007), postmenopausal osteoporotic women (Rubin *et al*., 2004), and children with musculoskeletal diseases such as cerebral palsy

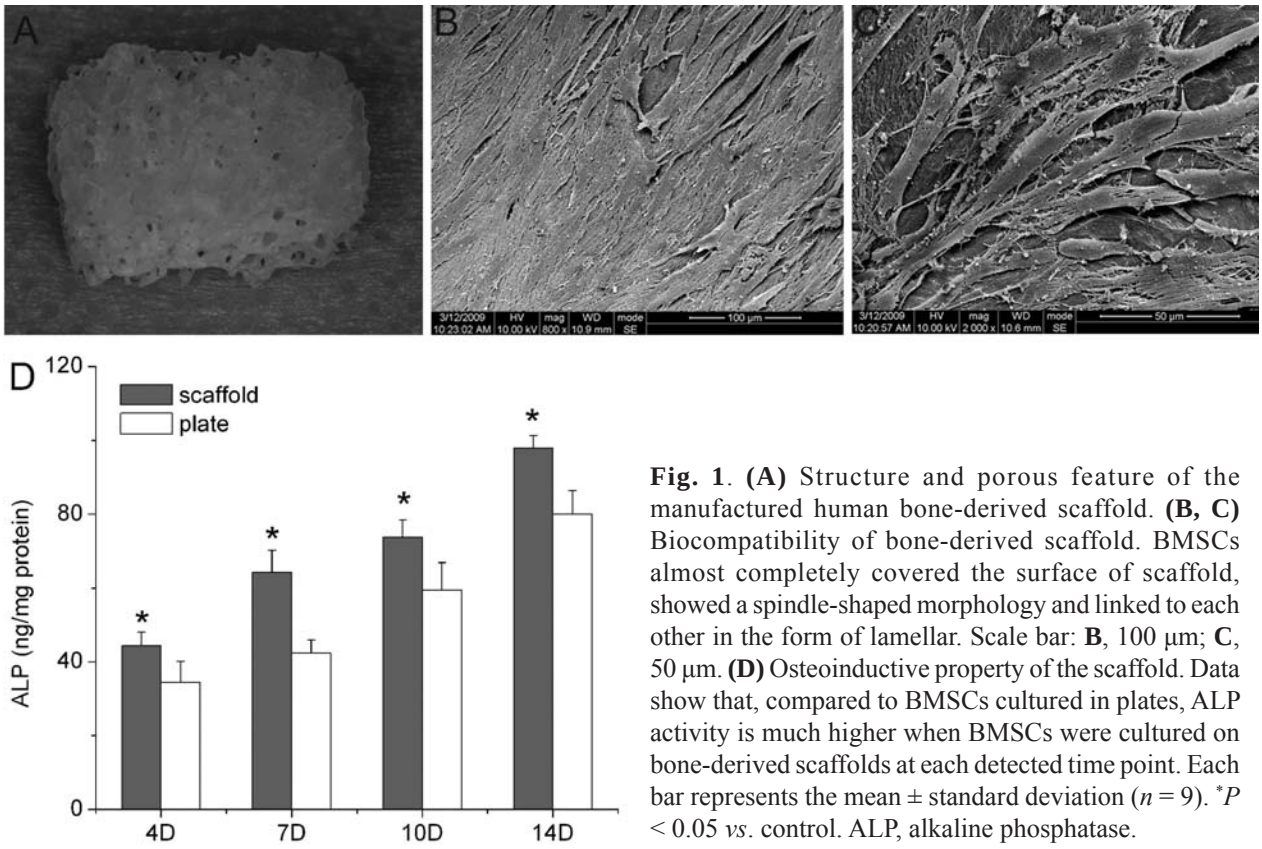

Fig. 1. **(A)** Structure and porous feature of the manufactured human bone-derived scaffold. **(B, C)** Biocompatibility of bone-derived scaffold. BMSCs almost completely covered the surface of scaffold, showed a spindle-shaped morphology and linked to each other in the form of lamellar. Scale bar: **B**, 100 μm; **C**, 50 μm. **(D)** Osteoinductive property of the scaffold. Data show that, compared to BMSCs cultured in plates, ALP activity is much higher when BMSCs were cultured on bone-derived scaffolds at each detected time point. Each bar represents the mean ± standard deviation ($n = 9$). $^{*}P < 0.05$ *vs*. control. ALP, alkaline phosphatase.

(Ward *et al*., 2004). However, the underlying mechanism of the anabolic and anti-resorptive role of LMHF vibration on bone remains unknown. Lau *et al*. (2010) recently reported that osteocytes are the sensing cells to LMHF vibration and produce soluble factors that suppress osteoclast formation. Additionally, Patel *et al*. (2009) observed that LMHF vibration is capable of stimulating osteoblast differentiation. However, whether LMHF vibration exhibits any effect on BMSC differentiation, the progenitors of osteocytes and osteoblasts, is currently unknown. We thus put forward a hypothesis that LMHF vibration may be able to regulate the osteodifferentiation of BMSCs. The postulation is supported by the study of Dumas *et al*. (2010), which showed the indirect evidence that extracellular matrix produced by osteoblasts under LMHF vibration is favourable to BMSC osteodifferentiation.

The differentiation of BMSCs into osteoblasts is primarily controlled by Cbfa1/Runx2 (Ducy *et al*., 1997). Cbfa1/Runx2 phosphorylation and activation is mediated by ERK1/2 (Xiao *et al*., 2000; Xiao *et al*., 2002). The activation of ERK1/2 has been demonstrated by studies to be involved in BMSC and osteoblast differentiation in response to various mechanical stimuli, including shock wave, hydrostatic pressure, fluid flow and cyclic strain (Wang *et al*., 2002; Weyts *et al*., 2002; Simmons *et al*., 2003; Kim *et al*., 2007). Moreover, ERK1/2 activation is also associated with collagen synthesis, bone specific protein production and calcium deposition (Lai *et al*., 2001; Wang *et al*., 2002; Weyts *et al*., 2002; Simmons *et al*., 2003; Kim *et al*., 2007). This may suggest that ERK1/2 is an essential pathway in the mechanotransduction process. We therefore hypothesized that ERK1/2 activation may also play an essential role in LMHF vibration mediated-BMSC osteodifferentiation.

Here, it is relevant to note that the *in vitro* data presented in two studies by Lau *et al*. (2010) and Patel *et al*. (2009) to provide an explanation for the anabolic and anti-resorptive role of LMHF vibration observed *in vivo*, are obtained from bone cells in two dimensional (2D) cultures. However, investigating the role of LMHF vibration on osteogenic cells in 3D cultures might mimic *in vivo* conditions. Therefore, we subjected BMSCs seeded on human bone-derived scaffolds (3D) to LMHF vibration at a magnitude of 0.3 ×g and a frequency of 40 Hz. To test our hypotheses, we examined the cell proliferation, osteogenic markers, and whether ERK1/2 activation is involved in osteogenesis process after LMHF vibration treatment. Since the amplitude of this kind of LMHF vibration is ± 50 μm, we defined it as microvibration.

Materials and Methods

Animals

Sprague-Dawley rats (8-10 weeks) were purchased from the Laboratorial Animal Center, Huaxi Medical Centre, Sichuan University (Chengdu, China) and received care according to the Guide for the Care and Use of Laboratory Animals issued by the US National Institute of Health (NIH Publication NO. 85-23). The experimental procedures on rats were approved by the Care and Use of Experiment Animals Committee of Huaxi Medical Centre, Sichuan University, Chengdu, China.

Isolation and culture of rat BMSCs

BMSCs were isolated and cultured as previously described (Zhou *et al*., 2010). Briefly, rats were anaesthetised with Nembutal (intramuscular: 25 mg kg^{-1}; Sigma-Aldrich, St. Louis, MO, USA) and killed by heart puncture, the tibias and femurs were then dissected. BMSCs were harvested by flushing out the bone marrow with 1 % low glucose Dulbecco's Modified Eagle Medium (L-DMEM; Gibco BRL, Gaithersburg, MD, USA) in a syringe. The cells were resuspended and placed in the culture medium containing 10 % foetal bovine serum (FBS; Gibco BRL), 100 units mL^{-1} penicillin (Sigma-Aldrich) and 100 μg mL^{-1} streptomycin (Sigma-Aldrich), and grown in a humidified atmosphere of 5 % CO_2 at 37 °C. The culture medium was replenished every 2 d until the cells were 80-90 % confluent; then cells were detached with 0.25 % trypsin (Gibco BRL) and subcultured. The passages 2-3 of BMSCs were used in all experiments.

Preparation of human bone-derived scaffold

Frozen tibias and femurs of human cadavers were obtained from the Bone Bank, Sichuan Province Tissue Bank, with the approval of the ethics committee, Chengdu, Sichuan, China. The bones were cut into blocks with a size of 18×10×8 mm, which consisted of both cancellous and cortical bone tissue. The blocks were washed with physiological saline, and processed a series of physical and chemical treatments (Tadjoedin *et al*., 2003; Xu *et al*., 2003; Mauney *et al*., 2005), including partial deproteinisation, partial demineralisation and lyophilisation. Briefly, bone blocks were sequentially immersed in 10 % hydrogen peroxide (Sigma-Aldrich) for 1 d at 38 °C, 0.6 N hydrochloric acid (Sigma-Aldrich) for 4 h at room temperature, chloroform/methanol (1:1; Sigma-Aldrich) for 1 h at room temperature, 0.25 % trypsin for 12 h at 4 °C and finally 0.5 % SDS (Sigma-Aldrich) for 6 h at room temperature, followed by lyophilisation and sterilisation using ^{60}Co γ-ray irradiation (20-25×10^3 Gy; Beijing Dede Chuangye Technology Company Limited, Beijing, China). The processed scaffolds were then trimmed to a size of 13.4×8×5 mm, without cortical structures (Fig. 1A) and tightly placed in 24-well plates. The pore size of the processed scaffolds is 90-700 μm and the interval porosity is 88 %.

Culture of BMSCs on bone-derived scaffold

The bone-derived scaffolds were soaked in DMEM for 1 d prior to cell seeding. The excessive volume of medium in the scaffolds was removed using sterile cotton balls and 100 μL BMSCs suspension (1×10^7 cells mL^{-1}) was slowly dripped onto scaffolds to avoid overflow. These scaffolds seeded with BMSCs were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 3 h, after which the additional culture medium was added to fully cover the scaffolds. To make sure cells can attach to scaffolds, the composites needed to be further incubated in the humidified atmosphere of 5 % CO_2 at 37 °C for 3 d. BMSC cellular scaffolds were then prepared for scanning electron microscopy (SEM) or microvibration experiments.

Fig. 2. View of GJX-5 vibration sensor and fixture.

GJX-5 vibration sensor

The panel of GJX-5 vibration sensor (Beijing Sending Technology, Beijing, China; Fig. 2) has a platform for the fixture to be mounted on (Fig. 2). The 24-well plates cultured with BMSCs cellular scaffolds can be placed tightly in the fixture. Once the fixture was in parallel with the ground, BMSC cultures received mechanical stimuli (magnitude: 0.3 ×g, frequency: 40 Hz, amplitude: ± 50 μm) for 30 min every 12 h for various time periods as indicated in each experiment.

Microvibration culture *vs*. static culture *vs*. plastic culture

Cells seeded on scaffolds or in plastic plates (2×10^4 cells/well) were then randomly divided into microvibration culture and static culture groups, both of which were cultured in a humidified atmosphere of 5 % CO_2 at 37 °C with a change of osteogenic medium (90 % DMEM, 10 % FBS, 10^{-6} M dexamethasone, 10^{-2} M β-glycerol phosphate and 50 μg/mL ascorbic acid; Sigma) prior to application of mechanical stimulus. After microvibration treatment, all experiments, mRNA and protein collection were performed immediately.

DNA content assay

The total DNA content in BMSC cellular scaffolds cultured in different environments was assayed by using a Picogreen DNA Quantification kit on day 0, 1, 3, 7, 10 (Molecular Probes, Eugene, OR, USA). DNA was extracted from each scaffold in aliquots of enzymatic cocktail containing 0.1 % collagenase A (Roche, Penzberg, Germany) and 0.1 trypsin at 37 °C for 2 h, with vortex every 30 min and followed by three cycles of freeze and thaw. The measurement was conducted following the manufacturer's instructions.

Thymidine incorporation assay

The proliferation of BMSCs in the scaffolds under different culture conditions were examined on day 0, 1, 3, 7, and 10. BMSC cellular scaffolds were incubated at 37 °C for

5 h with addition of 1 μCi of [^{3}H]-thymidine (Shanghai Institute of Nuclear Research, Shanghai, China). After washing with PBS three times, aliquots of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 1 % Triton X-100 and 0.1 % SDS; Sigma-Aldrich) were used to dissolve cells in the scaffolds. The lysate solutions were added onto glass-fibre filter paper and dried at 37 °C. The paper was then immersed in 5 mL scintillation solution (5 mg mL^{-1} 2, 5-diphenyl oxazole and 0.3 g mL^{-1} 1, 4-bis (5-phenyl-2-oxazolyl) benzene dissolved in dimethyl benzene; Sigma-Aldrich) at dark room for 2 h. [^{3}H]-thymidine incorporation was analysed by using a liquid scintillation analyser (Beckman Coulter, Brea, CA, USA). The results are shown as count per minute (CPM; Zhou *et al*., 2010).

Quantitative real-time RT-PCR

Total mRNA was extracted from BMSC cellular scaffolds by adding 1 mL of RNAiso plus (TAKARA, Dalian, China) on day 1, 4, 7, 10, 14, 18, 22, and 26. 1 μg mRNA was then subjected to cDNA synthesis in a 20 μL reaction volume containing 0.5 μL PrimeScript™ RT Enzyme Mix I, 0.5 μL oligo dT Primer, 2.0 μL PrimeScript™ Buffer and 0.5 μL Random 6 mers. The procedures of SYBY Green PCR assay on cDNA samples using iCycler iQ™ Multicolor real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) included initial denaturation at 95 °C for 10 s, 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 45 s, in a 25 μL reaction volume containing 2 μL cDNA, 1 μL forward primer, 1 μL reverse primer, 12.5 μL SYBR® Premix Ex Taq™, and 8.5 μL sterile water. Fluorescence data was analysed by using Optical system software version 3.1 (Bio-Rad) to get CT values. The CT values were calculated in relation to GAPDH CT values by the $2^{-\Delta\Delta CT}$ method (Zhou *et al*., 2010).

Western blots

The Cbfa1/Runx2 contents in BMSCs cellular scaffolds under different culture conditions were measured on day 4, ERK1/2, phospho-ERK1/2 and tubulin on 30 min, day 1, 3, 7. Scaffolds were ground into small pieces with mortar and pestle, and proteins were harvested with lysis solution (50 mM Tris-HCl, 150 mM NaCl, 1 % Triton X-100, 0.25 % Na-deoxycholate, 1 mM activated Na_3VO_4, 1 mM EDTA, 1 mM NaF, 1 mM DTT, 1 mM phenylmethylsulphonylfluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin, pH 7.4; Sigma-Aldrich). After centrifugation of cell lysates at 14,000 g for 15 min at 4 °C, supernatant protein samples were harvested and total protein concentration was determined using BCA protein assay (Pierce, Rockford, IL, USA). The samples were separated on 10 % SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Roche Diagnostics). These membranes were then immunoblotted with primary antibody at 4 °C for 12 h. After incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA, USA) at room temperature for 1 h, Cbfa1/Runx2, tubulin, ERK1/2 and phospho-ERK1/2 (Santa Cruz; Neven *et al*., 2010; Li *et al*., 2010) were visualised using enhanced chemiluminescence reagents (Pierce). The immunoblots were quantified with Scion Image Beta 4.0.2 software (Scion Corporation, Frederick, MD, USA).

Alkaline phosphatase (ALP) activity assessed by spectrophotometry

The intracellular ALP activities under different culture conditions were compared on days 4, 7, 10, 14. Cell lysates were obtained as described above. Alkaline phosphatase (ALP) activity was analysed by adding aliquots of lysate solution in a 100 μL reaction volume containing 50 mM p-nitrophenylphosphate (Sigma-Aldrich), 1 mM $MgCl_2$ (Sigma-Aldrich), and 50 mM glycine (Sigma-Aldrich), at 37 °C for 30 min. The optical density of catalysate was detected at 405 nm using SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA; Zhou *et al*., 2010).

Quantitation of osteocalcin (OC) by ^{125}I radioimmunoassay

After being cultured in two different conditions for 4, 7, 10 and 14 d, the OC content in BMSC cellular scaffolds were assayed as previously described (Gundberg *et al*., 1998). Briefly, the assay is based on the competition of radioactively labelled OC (Beijing Puer Weiye Biotechnology Company, Beijing, China) and an identical non labelled OC for binding to a specific antibody. In the reaction system, the amount of labelled OC bound to the antibody is conversely proportional to the amount of unlabelled OC. The assays consisted of a known concentration of standard OC or unknown concentrations of OC samples, 2×10^4 CPM of ^{125}I OC and 100 μL antiserum in assay buffer, according to the manufacturer's instructions. The reaction systems were then incubated at 25 °C for 20 h with constant shaking before further detection.

ERK1/2 inhibition study

To assess the effects of ERK1/2 inhibition on BMSC osteogenesis, 10 μM U0126 (Promega, Madison, WI, USA), an inhibitor of phospho-ERK1/2 (Goueli *et al*., 1999; Simmons *et al*., 2003; Kim *et al*., 2007), was added to the serum-free cell culture media with the purpose of reducing basal ERK1/2 activity 1 h prior to application of microvibration. BMSC cellular scaffolds were then placed into osteogenic media with or without U0126 and exposed to microvibration for 14 d. The inhibitor was dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich). The untreated cells were pre-incubated with same amount of 0.04 % DMSO alone. ALP was harvested on day 14 and assayed as described above.

Statistical analysis

The data were presented as mean ±standard deviation and statistically analysed using paired Student's *t*-test with SPSS soft ware, version 17.0 (SPSS Inc., Chicago, IL, USA). Statistically significant values were defined as $P < 0.05$. Thymidine incorporation assay, DNA content assay, quantitative real-time PCR, Western blot and ERK1/2 inhibition study were repeated from three independent experiments. All other assays were carried out in triplicate with three independent experiments.

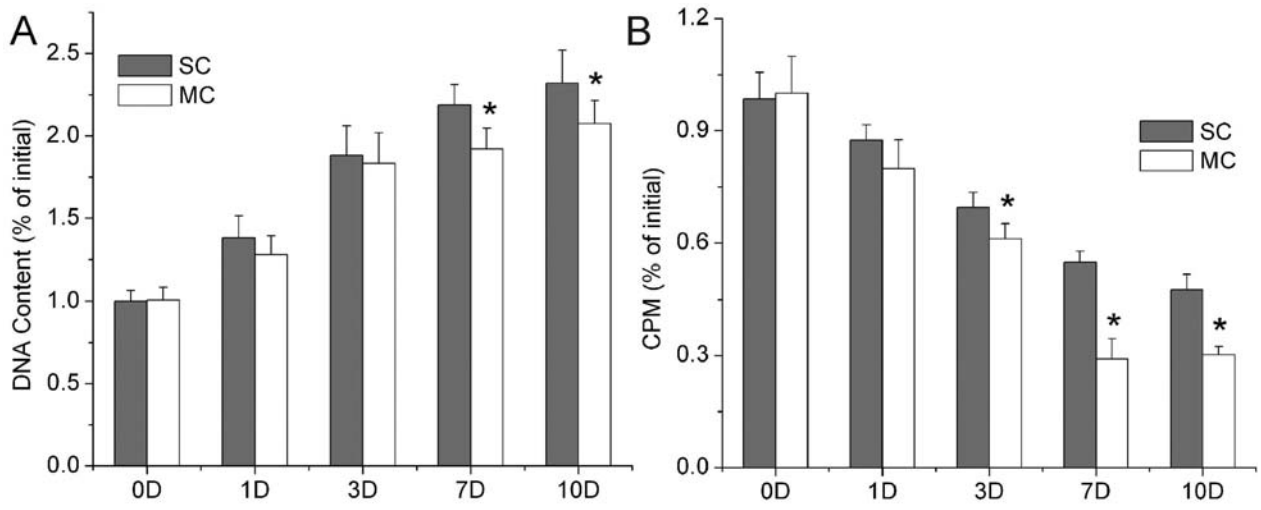

Fig. 3. Effect of microvibration on BMSC proliferation. **(A)** DNA content: DNA contents in both groups were increased progressively. However, with microvibration treatment, it is lower on day 7 and 10. **(B)** Thymidine incorporation: BMSC's ability to incorporate thymidine was decreased as culture progress. The inhibitory effect of microvibration on cell's ability to incorporate thymidine was observed on day 3, 7 and 10. Bars represent the mean ± standard deviation (n = 3); *P < 0.05. CPM, count per minute. SC, static culture. MC, microvibration culture.

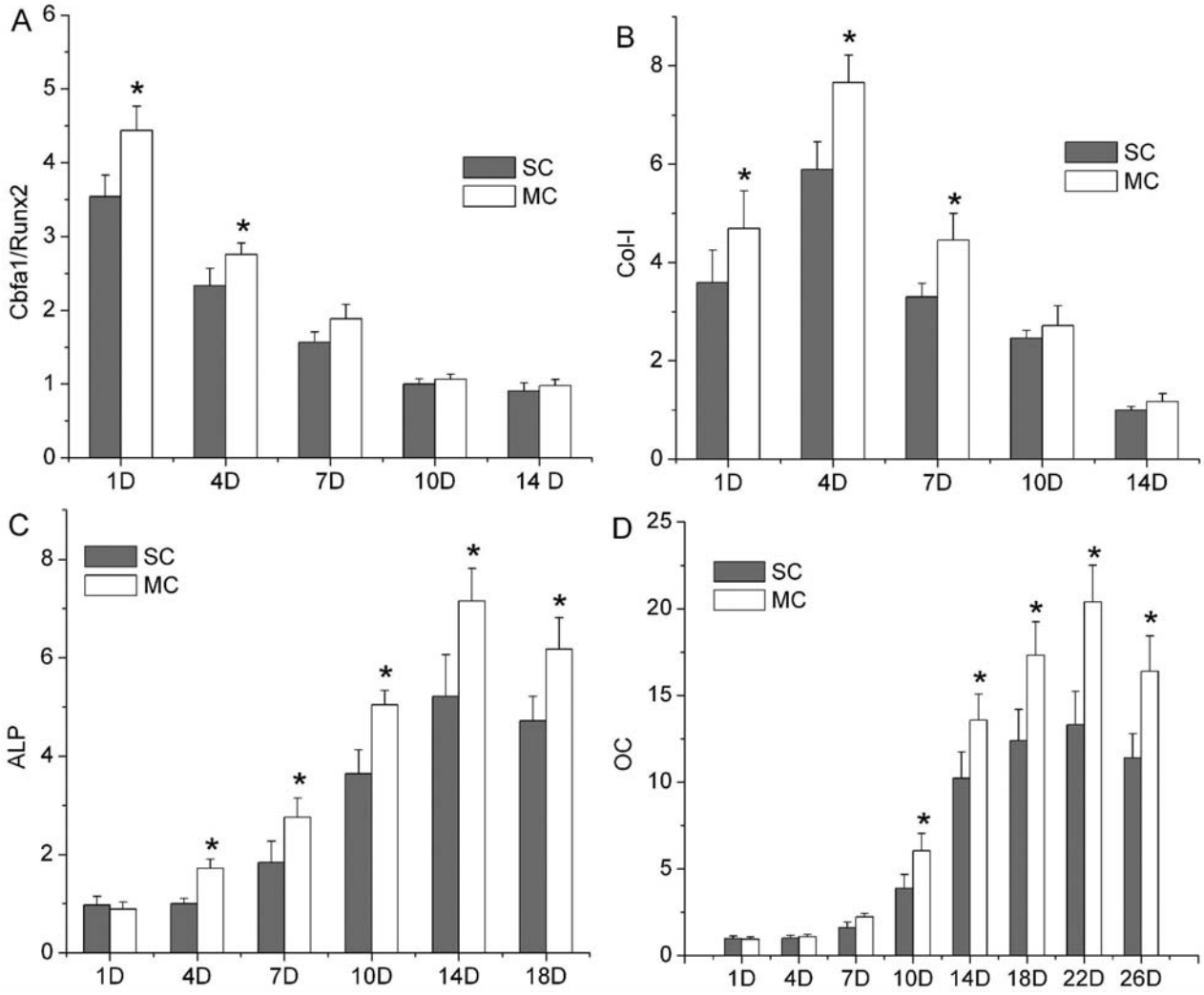

Fig. 4. Effect of microvibration on osteogenic gene expressions in BMSC cellular scaffolds. Cbfa1/Runx2, Col-I, ALP and OC mRNA expressions were assayed on day 1, 4, 7, 10, 14, 18, 22 and 26. Data show that microvibration greatly upregulated these mRNA levels at different stages of osteogenesis. Each bar represents the mean ± standard deviation (n = 3); *P < 0.05. SC, static culture. MC, microvibration culture. Col-I, collagen I. ALP, alkaline phosphatase. OC, osteocalcin.

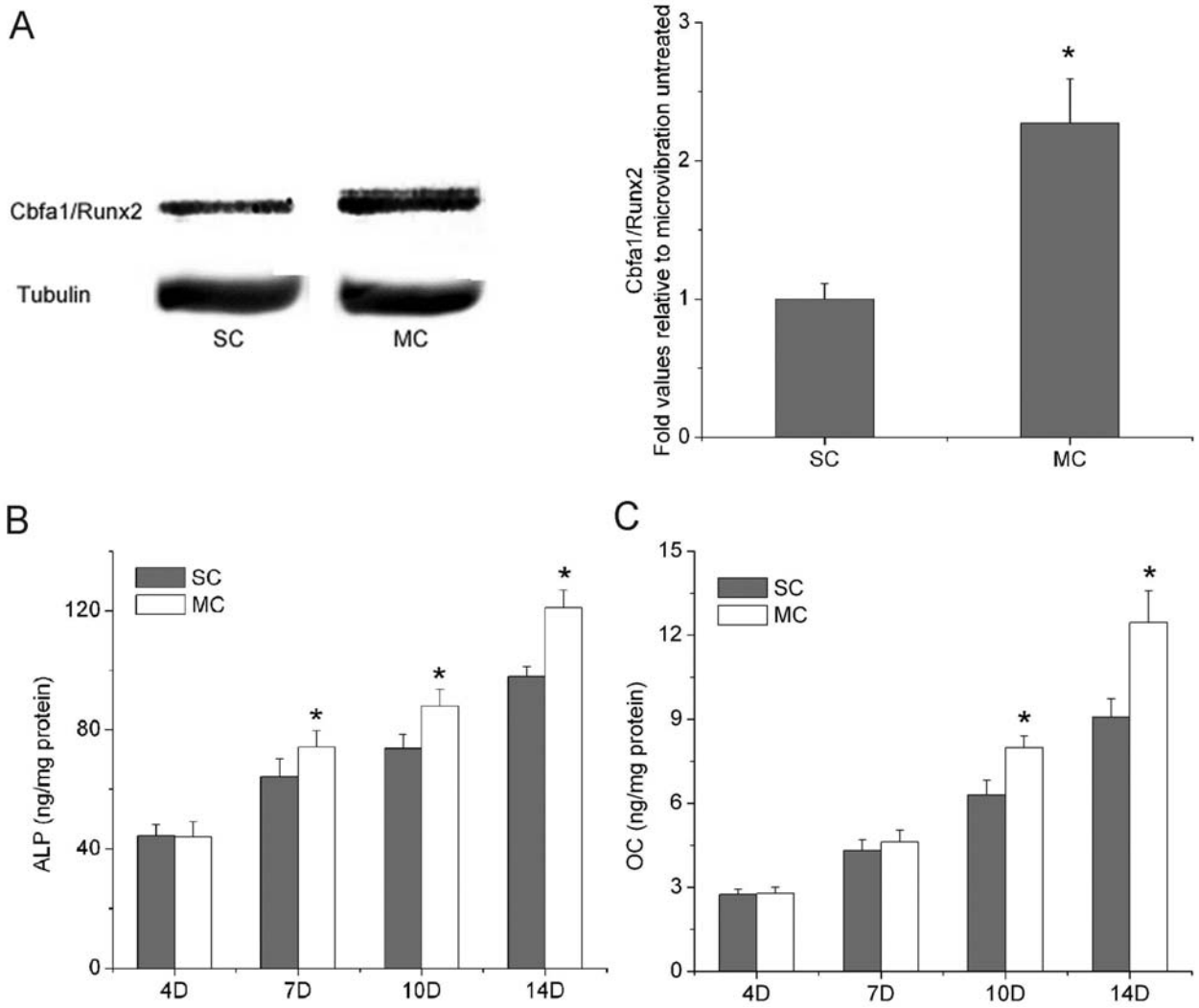

Fig. 5. Effect of microvibration on bone specific proteins. **(A)** Microvibration significantly increased Cbfa1/Runx2 expression on day 4. Each bar represents the mean ± standard deviation ($n = 3$); $^{*}P < 0.05$. **(B)** ALP activity was upregulated under microvibration culture on day 7, 10 and 14. Each bar represents the mean ± standard deviation ($n = 9$); $^{*}P < 0.05$. **(C)** OC level was enhanced by microvibration on day 10 and 14. Each bar represents the mean ± standard deviation ($n = 9$); $^{*}P < 0.05$. SC, static culture. MC, microvibration culture. ALP, alkaline phosphatase. OC, osteocalcin.

Results

Biocompatibility and osteoinductive property of bone-derived scaffold

As observed under SEM, BMSCs almost completely covered the surface of human bone-derived scaffold and showed a spindle-shaped morphology. These cells linked to each other and presented in the form of lamellae (Fig. 1B, C). The observation suggested that bone-derived scaffolds could provide support for BMSCs to attach, implying that scaffolds had good biocompatibility property. To further determine whether the processed scaffolds promoted osteogenesis, BMSCs were cultured in osteogenic medium and ALP activity was assayed on day 4, 7, 10 and 14. In comparison to BMSCs cultured in plates, ALP activity was significantly increased in BMSC cellular scaffolds, suggesting that scaffolds produced as described above had osteoinductive properties (Fig. 1D; $P < 0.05$).

Decrease in BMSC proliferation in bone-derived scaffolds by microvibration

The impact of microvibration on BMSC proliferation was assayed by DNA content in each scaffold. As shown in Fig. 3A, total DNA contents in both groups were gradually increased and appeared to reach the maximal levels around day 7. However, the DNA content in microvibration-treated group was lower on day 7 and 10, when compared to control group ($P < 0.05$).To further confirm that microvibration had adverse effect on BMSC proliferation, the ability of BMSCs to incorporate thymidine was estimated on day 0, 1, 3, 7 and 10. As shown in Fig. 3B, the thymidine incorporations in both groups were gradually decreased in a time-dependent manner. However, the ability for cells to incorporate thymidine in microvibration-treated group was significantly inhibited on day 3, 7 and 10, compared to the control group ($P < 0.05$).

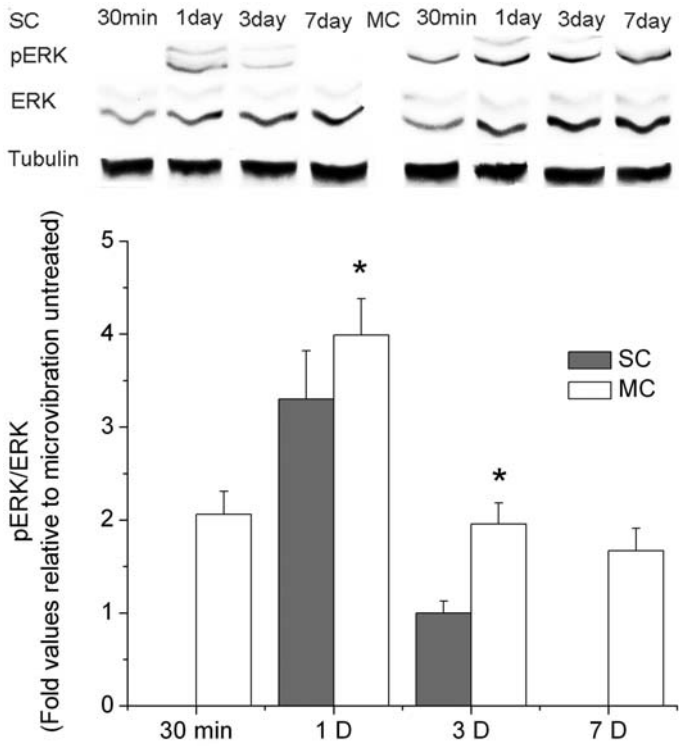

Fig. 6. pERK1/2 was upregulated and sustained over time in the presence of microvibration. Data show that microvibration induced ERK1/2 activation at 30 min and the phosphorylation of ERK1/2 was sustained more time when compared to microvibration-untreated group. Bars represent the mean ± standard deviation ($n = 3$); *$P < 0.05$. SC, static culture. MC, microvibration culture.

Changes in mRNA expression of BMSCs in bone-derived scaffolds by microvibration

To investigate whether microvibration affected BMSC osteogenic differentiation, genes associated with osteogenesis, including Cbfa1/Runx2, collagen I (Col-I), ALP and OC, were measured by real-time RT-PCR on day 1, 4, 7, 10, 14, 18, 22 and 26. Microvibration-stimulated Cbfa1/Runx2 mRNA expression was apparent on the 1st day (Fig. 4A; $P < 0.05$). Although the expression of Cbfa1/Runx2 showed no obvious difference on day 7, 10 or 14 (Fig. 4A; $P > 0.05$), the elevated level of Cbfa1/Runx2 mRNA lasted for 3 days (Fig. 4A; $P < 0.05$). Similarly, with microvibration treatment, the mRNA expression of Col-I showed a marked accumulation on day 1, 4 and 7 (Fig. 4B; $P < 0.05$); ALP mRNA expression was also significantly increased after microvibration exposure for 4, 7, 10 and 14 d (Fig. 4C; $P < 0.05$). The exposure of BMSC cellular scaffolds to microvibration induced an increase of OC mRNA expression on day 10, 14, 18, 22 and 26 (Fig. 4D; $P < 0.05$), although no visible difference of OC mRNA expression was observed on day 1, 4 or 7 (Fig. 4D; $P > 0.05$).

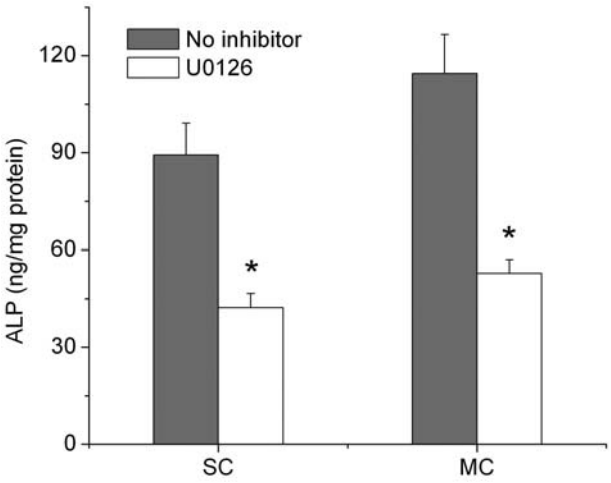

Fig. 7. ERK1/2 pathway was involved both in scaffold- and microvibration-stimulated osteogenesis. Data show that ALP activity was significantly inhibited when BMSCs were treated with U0126 inhibitor regardless of microvibration treatment. Bars represent the mean ± standard deviation ($n = 3$); *$P < 0.05$. SC, static culture. MC, microvibration culture. ALP, alkaline phosphatase.

Increased expression of bone specific proteins in BMSC cellular scaffolds by microvibration

The favourable effects of microvibration on osteogenesis were further testified by the increased expressions of bone specific proteins. As shown in Fig. 5A, western blot revealed that Cbfa1/Runx2 was greatly enhanced under microvibration culture ($P < 0.05$). Moreover, microvibration also resulted in an increase of ALP activity with a time course similar to that observed in ALP mRNA expression from day 1 to day 14 (Fig. 5B; $P < 0.05$). The pattern of OC protein up-regulation also paralleled that of an increase in OC mRNA expression from day 1 to day 14 (Fig. 5C; $P < 0.05$).

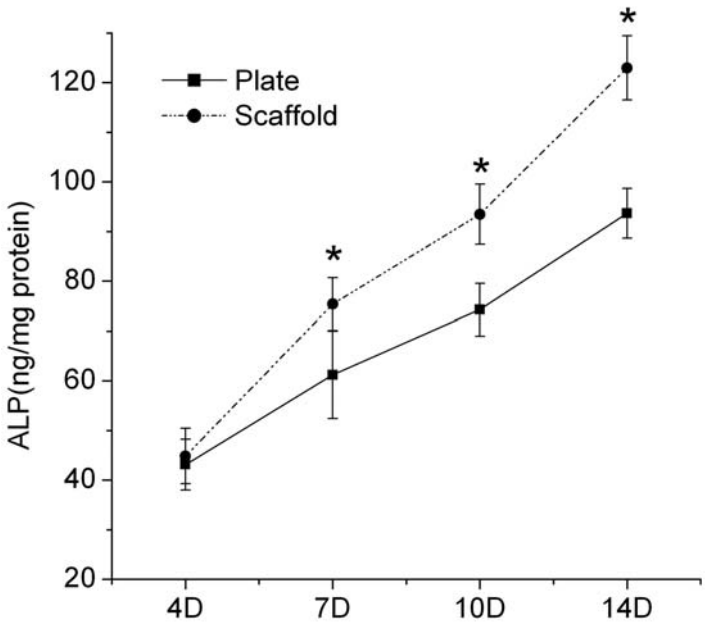

Fig. 8. ALP activity was increased when the cells were cultured in 3D. The data show that, compared to BMSCs cultured in plates with microvibration treatment, the ALP activity is much higher when BMSCs were seeded on bone-derived scaffolds and simultaneously treated with microvibration. Bars represent the mean ± standard deviation (n = 9); *P < 0.05. ALP, alkaline phosphatase.

Involvement of ERK1/2 activation in microvibration-increased osteogenesis

Since many studies have demonstrated that mechanical stimuli-induced ERK1/2 activation is involved in various osteogenic responses (Lai *et al*., 2001; Ziros *et al*., 2002), western blot analysis was performed to determine whether ERK1/2 pathway was responsible for the increased osteogenesis stimulated by microvibration. As shown in Fig. 6, microvibration stimulus induced the phosphorylation of ERK1/2 at 30 min, but no phosphorylated ERK1/2 was detected in the static culture. The activation of ERK1/2 was increased or sustained over time in the presence of microvibration (P < 0.05), although ERK1/2 phosphorylation was observed in static culture on day 1 and 3. To further confirm the involvement of ERK1/2 in the microvibration-induced osteogenesis, 10 μM U0126, an inhibitor of phospho-ERK1/2, was added to BMSC cellular scaffolds and ALP activity was measured on day 14. As shown in Fig. 7, ALP activity was significantly inhibited in the treated groups (P < 0.05), implying that ERK1/2 pathway plays an important role both in scaffold- and microvibration-induced osteogenesis.

Discussion

In this work, we examined the potential cellular and molecular regulation by which microvibration induced BMSC osteodifferentiation in 3D culturing environment. We have demonstrated that BMSCs seeded on modified bone-derived scaffolds responded to microvibration at both transcript and protein levels during osteogenesis, and EKR1/2 activation was responsible for the microvibration-induced osteogenesis.

Recently, the most commonly employed approaches used for manufacturing allograft bones included demineralisation and deproteinisation (Tadjoedin *et al*., 2003; Xu *et al*., 2003; Mauney *et al*., 2005). Studies have demonstrated that properly controlling the balance of demineralisation and deproteinisation can preserve or improve the osteoinductive properties of bone scaffolds (Ashammakhi and Ferretti, 2003; Mistry and Mikos, 2005). The reason for these optimised scaffolds may be that the process can expose both soluble and insoluble osteogenic factors from the calcified matrix, including bone morphogenetic proteins and other non-collagenous proteins (Rosenthal *et al*., 1999; Colnot *et al*., 2005). To obtain partially or fully demineralised scaffolds, cancellous bone needs to be immersed in hydrochloric acid for 15 min and 12 h, respectively (Mauney *et al*., 2005). The partially demineralised bone-derived scaffold processed in our study is obtained by hydrochloric acid treatment for 4 h. Therefore, the osteogenic factors may be exposed more within the partially demineralised scaffolds even after partial deproteinisation and thus mainly contribute to the osteoinductive property. This property was demonstrated by ALP activity, which is much higher in BMSC cellular scaffolds compared to those cultured in plastic plates. In addition, the porous structures in scaffolds can easily support the localization, adhesion and growth of xenogenic cells (rat BMSCs), implying that the produced scaffolds show good biocompatibility.

Microvibration caused a reduction of BMSC proliferation in our study. Oh *et al*. (2010) also demonstrated that vibration was capable of inhibiting 3T3-L1 cell proliferation after exposure to vibration for 2 or 3 d. However, Patel *et al*. (2009) found no effect of LMHF vibration on 2T3 cell proliferation. The inconsistency for

the effects of LMHF vibration on cell proliferation may be due to different cell types, culturing conditions and vibration protocols. Interestingly, BMSC proliferation in our study was gradually decreased and seemed to cease on day 7, regardless of microvibration treatment. Since the concomitant increase in cell differentiation is generally accompanied by a parallel reduction in cell growth (Franceschi, 1999), the arrest of cell growth indicates the stimulation of BMSC osteogenic differentiation in both two groups (see below).

The osteogenic differentiation of the cells is usually divided into three discrete stages: commitment to osteogenic lineage, matrix synthesis, and matrix mineralisation (Beck, 2003). Cbfa1/Runx2 is an essential transcriptional activator for osteogenic lineage commitment (Ducy *et al*., 1997). It has been demonstrated that Cbfa1/Runx2 knockout could result in the impairment of bone formation (Komori *et al*., 1997). The enhancement of Cbfa1/Runx2 expression induced by microvibration (frequency: 40 Hz) on day 1 and 4 suggests that microvibration can affect osteogenesis at early stage by increasing the commitment of BMSCs to osteogenic lineage. On the other hand, Oh *et al*. (2010) reported that vibration at 20 Hz and 30 Hz enhances cell commitment to adipogenic lineage as demonstrated by increased adipogenic markers, but vibration at 40 Hz is unable to induce cell adipogenic differentiation. Taken together, these data may imply the existence of a frequency-dependent effect of vibration on determining the cell commitment to different lineages. The hypothesis is further supported by *in vivo* LMHF vibration reports, where vibration at 90 Hz is more anabolic than vibration at 45 Hz in ovariectomised rats (Judex *et al*., 2007). Moreover, in contrast to the role of low frequency (20-30 Hz) vibration in inducing cell adipogenic differentiation (Oh *et al*., 2010), vibration at high frequency (90 Hz) in *in vivo* animal studies favours cell osteogenic differentiation but inhibits adipogenic differentiation (Rubin *et al*., 2007; Luu *et al*., 2009). Since the concomitant increase in cell differentiation is generally accompanied by a parallel reduction in cell proliferation (Franceschi, 1999), the increased commitment of BMSCs to osteogenic lineage in microvibration culture, as demonstrated by increased Cbfa1/Runx2 expression, can partially explain the inhibitory effect of microvibration on BMSC proliferation (Franceschi, 1999). The increased matrix synthesis and maturation by microvibration was shown by elevated expressions of the middle (Col-I, ALP) and late (OC) markers, as well as their proteins.

The osteogenic cells usually follow similar paths to differentiation and exhibit a similar pattern and time frame of gene expression. Cbfa1/Runx2 is usually highly expressed at early stage (commitment to osteogenic lineage), Col-I and ALP at the middle stage (matrix synthesis) and OC at the late stage (matrix mineralisation) during osteogenesis (Franceschi, 1999; Beck, 2003). Based on the results of PCR analysis, the peak levels of Cbfa1/Runx2 expression in BMSCs without microvibration exposure is observed at day 1, Col-I at day 4, ALP at day 14, and OC at day 22. The gene expression presented in BMSC cellular scaffolds under static culture exhibits the pattern similar to the one occurring in osteogenic differentiation (Franceschi, 1999), implying that scaffold is favourable to osteogenic differentiation and further confirming the osteoinductive property of scaffold. Interestingly, microvibration can enhance the peak levels of these genes and intensify the pattern occurred in osteogenic differentiation. The observation could thus prove that microvibration is beneficial for osteogenesis.

3D culture has been demonstrated to promote cell osteogenic differentiation and mineralisation (Kinoshita *et al*., 1999; Rattner *et al*., 2000). To test the advantage of 3D culture on osteogenic differentiation in response to LMHF vibration, ALP activity was measured on day 14. As shown in Fig. 8, compared to BMSCs cultured in plates under microvibration, ALP activity is elevated when BMSCs were cultured on bone-derived scaffolds and simultaneously treated with microvibration.

In response to mechanical stimuli, ERK1/2 activation has been demonstrated in many studies to play an essential role in cell osteogenic differentiation (Wang *et al*., 2002; Weyts *et al*., 2002; Simmons *et al*., 2003; Kim *et al*., 2007). Since microvibration is capable of increasing Cbfa1/Runx2 expression, of which the phosphorylation and activation is regulated by ERK1/2 (Xiao *et al*., 2000; Xiao *et al*., 2002), we postulated that ERK1/2 activation may be also involved in microvibration-mediated BMSC osteodifferentiation. The novel finding that microvibration-induced BMSC osteodifferentiation on bone-derived scaffolds is *via* ERK1/2 signalling further confirmed the essential role of ERK1/2 pathway in cell osteogenic differentiation during the mechanotransduction process. In our study, blockade of ERK1/2 phosphorylation with U0126 downregulated ALP activity in BMSC cellular scaffolds without microvibration exposure, implying that ERK1/2 pathway also plays a role in the scaffold osteoinductive property. The hypothesis is supported by previous studies that the increased osteogenic differentiation by cell-matrix interactions also involves ERK1/2 activation (Xiao *et al*., 2000; Xiao *et al*., 2002). Taken together, these data indicates that ERK1/2 pathway may be not only an essential signal for mechanical stimuli, but also a common mediator for non-mechanical signal.

Osteoblasts and adipocytes are two major lineages differentiating from MSCs and the relationship is reciprocal (Jaiswal *et al*., 2000; David *et al*., 2007). The commitment of MSCs into osteogenic or adipogenic lineage is primarily regulated by activation or inhibition of ERK1/2, respectively (Jaiswal *et al*., 2000). Although there is no evidence supporting the involvement of ERK1/2 activation in determining the commitment of mechanical stimuli-induced cell differentiation, mechanical stimuli such as cyclic stretching have been shown to favour osteodifferentiation but inhibit the induction of adipogenesis (Tanabe *et al*., 2004; David *et al*., 2007). Moreover, mechanical stretching also exhibits the ability to activate ERK1/2 to promote cell osteogenic differentiation (Huang *et al*., 2009). These results may suggest that the reciprocal roles of mechanical stimuli on mediating cell osteogenic and adipogenic differentiation may be *via* a differently regulating ERK1/2 pathway. Recent animal studies have proved that LMHF vibration is able to conduct the lineage commitment of BMSCs by biasing cell fate in

favour of osteogenesis over adipogenesis (Rubin *et al*., 2007; Luu *et al*., 2009). However, whether microvibration-activated ERK1/2 signalling is involved in biasing the concomitant cell differentiation remains unknown. Further investigation may elucidate the exact role of microvibration induced-ERK1/2 activation on BMSC differentiation.

It has been proved that the addition of osteogenic cells into the scaffold before *in vivo* implantation could significantly promote bone formation and osteointegration due to osteogenic proteins secreted by these loaded cells (Cancedda *et al*., 2003; Mauney *et al*., 2004; Mauney *et al*., 2005; Zhu *et al*., 2006). MSCs are able to produce bone tissues and enhance the osteogenic ability of demineralised bone materials (Cancedda *et al*., 2003; Mauney *et al*., 2004; Mauney *et al*., 2005; Zhu *et al*., 2006). *In vitro*, we have demonstrated that osteogenic markers are greatly increased by microvibration stimuli. However, in response to microvibration, whether these markers can be highly expressed in BMSC cellular scaffolds after *in vivo* implantation is unknown. The transmissibility of whole-body vibration has been elucidated to have anabolic effects on skeletal homeostasis (Rubin *et al*., 2002; Rubin *et al*., 2007). There is ongoing effort in using BMSC cellular scaffolds to repair bone defects in animals to understand whether whole body microvibration shows any positive effect on the healing process of bone defects after *in vivo* implantation.

In conclusion, our study provided a first glimpse at how BMSCs seeded on bone-derived scaffolds respond to microvibration. BMSC differentiation appears to be mediated by microvibration. At the transcript and protein levels, microvibration drives gene and protein changes that favour osteogenesis. In addition, the activation of ERK1/2 plays an important role in microvibration-induced osteogenesis.

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Discussion with Reviewers

Reviewer I: The extracellular matrix of bones is made out of proteins and a mineral phase. What is left when the human bone is demineralised and deproteinised like in this study?

Authors: The bone extracellular matrix has two main components (Sommerfeldt and Rubin, 2001, additional reference). Of the matrix, 65-70 % is the mineral part consisting of hydroxyapatite. The remainder is the organic part consisting of proteins such as collagen I, osteocalcin, osteopontin, osteonectin, fibronectin, biglycan, and bone sialoprotein. A series of physical and chemical procedures was conducted to process scaffolds in our study, including partial demineralisation by 0.6N hydrochloric acid, partial deproteinisation by 1:1 chloroform/methanol and lyophilisation and sterilisation by 60 Co γ-ray irradiation. The partial demineralisation can expose both soluble and insoluble osteogenic factors from calcified matrix, including bone morphogenetic proteins and other non-collagenous proteins (Rosenthal *et al*., 1999; Colnot *et al*., 2005, text references). Therefore, what is left in the scaffolds is demineralised hydroxyapatite and some proteins released from decalcifying matrix even after partial deproteinisation.

Reviewer I: If microvibrations improve osteoblast differentiation, would we improve the system by applying a constant treatment instead of a 30 min treatment every 12 h?

Authors: It has been recently been shown by many *in vivo* studies that LMHF vibration and other kinds of mechanical stimuli exhibit favourable influence on bone homeostasis (Rubin *et al*., 2002; Rubin *et al*., 2004; Ward *et al*., 2004; Garman *et al*., 2007; Rubin *et al*., 2007, text references; Rubin *et al*., 2001; Maddalozzo *et al*., 2008; de Oliveira *et al*., 2010, additional references). Although the anabolic role of LMHF vibration on bone is achieved by different magnitudes and frequencies of vibration produced with different devices, the time for vibration imposed on these objects is temporal and intermittent. Additionally, in *in vitro* studies, LMHF vibration has been demonstrated to promote cell osteogenic differentiation and inhibit osteoclast activity when cells were exposed to vibrations for 10-60 min/d (Patel *et al*., 2009; Lau *et al*., 2010, text references). In our preliminary study, ALP activity was significantly decreased when BMSCs seeded on scaffolds were treated with vibration for a whole day. Bone is a complicated biological system and the process of bone formation and bone resorption needs mechanical stimuli (Burger and Klein-Nulend, 1999; Huiskes *et al*., 2000; Frost, 2003, text references). However, under physiological conditions, bone experiences temporal and intermittent – but not constant mechanical stimuli. Therefore, our vibration protocol is appropriate for mimicking physiological conditions.

Reviewer II: Why did the authors choose to combine rat BMSCs with human bone-derived scaffolds?
Authors: The aim of our study is firstly to demonstrate that *in vitro* microvibration exhibits a favourable influence on osteogenic differentiation of BMSCs seeded on bone-derived scaffolds, and then to understand whether microvibration shows any positive influence on the healing process of bone defects after *in vivo* implantation by using BMSC cellular scaffolds to repair bone defects in an animal study. Rat is a convenient model to create a bone defect (Kikuchi *et al*., 2008, additional reference). Moreover, the diameter of rat femur is 4.24 ±0.08 mm (Beall *et al*., 1984, additional reference). Considering the size of scaffold presented in our study, it may be easy for us to remould the shape of scaffolds and then repair the bone defects after creating bone defects in rat femur. In addition, given that using the xenogenic BMSCs cellular scaffolds may cause immune reaction after *in vivo* implantation in rats, we thus decided to choose to combine rat BMSCs with human bone-derived scaffolds. As shown in our study, rat BMSCs attached well in the porous scaffolds and experienced osteogenic differentiation. However, whether the processed scaffolds in our study can be further used in the clinic remains unknown. In our opinion, an animal study is the first step to understand how these BMSC cellular scaffolds function *in vivo*.

Reviewer II: Can you imagine clinical applications of this special method? How would such a setting look like?
Authors: The sensor presented in our study may be not suitable for clinical use. Therefore, significant changes to the device need to be carried out if clinical application is required. For example, to the best of our imagination, when patients receive an *in vivo* implantation in their limbs, we can put a platform under the surgical site. The surface shape of platform must mimic the outline of limbs so patients will feel comfortable when their limbs are placed on the platform. Once limbs are fixed on the platform, we adjust the parameters of microvibration produced by sensor and the signalling can be transferred to the platform by a transmitter (e.g., a cable). Thus, patients can receive local body microvibration to accelerate the healing process. However, when patients receive more than one *in vivo* implant in different body parts, whole body microvibration will be much better, and placing the sensor under the patient's bed will be a good method. Anyway, modification on the device to suit clinical needs may require knowledge from different specialities, including medical science, mechanics, and so on. In our opinion, animal experiments should be the first step to test whether microvibration exhibits any positive effects on the healing process of bone defects after *in vivo* implantation.

Reviewer II: Are there any possible restrictions to the types of stem cells used for this special approach? Could one make use of cells other than BMSCs?
Authors: The scaffolds derived from bone may mainly be used in bone tissue engineering. As shown in our study, the bone-derived scaffolds show osteoinductive property. So any type of stem cells that is capable of differentiating into osteogenic lineage and is able to attach on the surface of scaffolds should be useful for this cell-scaffold composite. Among various stem cells, MSC, primarily originating from mesenchymal tissues, can be differentiated into a variety of cell types, including osteoblasts and chondrocytes (Pittenger *et al*., 1999, additional reference). Therefore, MSC derived from other non-bone marrow tissues, such as umbilical cord blood, adipose tissue, muscle or the dental pulp of deciduous baby teeth (Pittenger *et al*., 1999; Minguell *et al*., 2001; Huang *et al*., 2009, additional references) could be proposed as a promising cell resource for this cell-scaffold composite.

Reviewer III: If the aim of the study is to evaluate the role of body microvibration on osteogenesis involved *in vivo* scaffold implantation, is it possible to assimilate the LMHF applied on the scaffold and the body microvibrations in terms of magnitude and frequency?
Authors: The microvibration regimen was determined based on existing literatures and our preliminary studies. The optimal parameters of vibration in our study are different from those *in vitro* and *in vivo* studies (Rubin *et al*., 2002; Rubin *et al*., 2004; Garman *et al*., 2007; Lau *et al*., 2010; Patel *et al*., 2009, text references; Rubin *et al*., 2001; Maddalozzo *et al*.; 2008, de Oliveira *et al*., 2010, additional reference). The disparity may due to the various factors, including different devices to produce vibration, different culturing environment and cell types for *in vitro* study, different subjects for *in vivo* study, and different conditions between *in vivo* and *in vitro* study. The purpose of our next study is to understand whether microvibration shows any positive influence on the healing process of bone defects after *in vivo* implantation by using BMSC cellular scaffolds to repair bone defects in animals. We plan to use rats as animals. The rats receiving *in vivo* implantation will be placed in a cage that can be tightly placed in a fixture. By fastening the fixture into the platform of the sensor, the rats can receive body microvibration at about the magnitude (0.3 g) and frequency (40 Hz) described in our *in vitro* study.

Reviewer IV: How can you explain that the DNA content increases (Fig. 3) whereas the incorporation of thymidine decreases?
Authors: Thymidine is a labelled DNA precursor. Once a cell divides, it will be incorporated into the cell's DNA. Therefore, the level of radioactive signal depends on the proliferation rate. The higher the proliferation rate, the more cells can be harvested thus the more radioactive DNA and accordingly the higher signal. Based on the principle of thymidine incorporation, to get higher signal, cells should firstly be viable and secondly be proliferative. The scaffolds and microvibration presented in our study show ability to promote osteogenic differentiation, thus the proliferative cells will decrease as the osteogenesis process goes on. Since these differentiated cells can't undergo cell division, the radioactive signal (CPM) will become weaker and weaker as osteogenesis goes on. As for DNA content, it reflects cell number. The more the number of cells, regardless of whether cells are differentiated or

remain viable, the higher DNA content will be achieved. Moreover, although more and more cells experience osteogenic differentiation in our study, there are still some cells can proliferate and DNA content will thus increase.

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