

Ultrasound stimulation of different dental stem cell populations: role of mitogen-activated protein kinase signaling

Gao, Qianhua; Walmsley, Anthony; Cooper, Paul; Scheven, Ben

DOI:

[10.1016/j.joen.2015.12.019](https://doi.org/10.1016/j.joen.2015.12.019)

License:

None: All rights reserved

Document Version

Peer reviewed version

Citation for published version (Harvard):

Gao, Q, Walmsley, A, Cooper, P & Scheven, B 2016, 'Ultrasound stimulation of different dental stem cell populations: role of mitogen-activated protein kinase signaling', *Journal of Endodontics*, vol. 42, no. 3, pp. 425-431. <https://doi.org/10.1016/j.joen.2015.12.019>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Eligibility for repository: Checked on 9/3/2016

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

**Ultrasound Stimulation of Different Dental Stem Cell Populations:
Role of MAPK signaling**

*Qianhua Gao, MS, A. Damien Walmsley, PhD, Paul R. Cooper, PhD and Ben A. Scheven,
PhD*

School of Dentistry, College of Medical and Dental Sciences, University of Birmingham,
Birmingham, UK

Correspondence:

Dr Ben Scheven
School of Dentistry, University of Birmingham
St. Chad's Queensway
Birmingham,
B4 6NN
UK

Tel: (44) 0121 466 5480
E-mail: b.a.scheven@bham.ac.uk

Running title: Ultrasound-stimulated MSC proliferation via MAPKs

Acknowledgments

This study was supported by the Li Siguang PhD Scholarship in conjunction with the University of Birmingham.

The authors deny any conflicts of interest related to this study.

Number of Figures: 4

Number of Tables: 1

Abstract

Introduction: Mesenchymal stem cells (MSCs) from dental tissues may respond to low-intensity pulsed ultrasound (LIPUS) treatment, potentially providing a therapeutic approach to promoting dental tissue regeneration. This work aimed to study compare LIPUS effects on the proliferation and MAPK signaling in MSCs from rodent dental pulp (DPSCs), compared with MSCs from periodontal ligament (PDLSCs) and bone marrow (BMSCs). **Methods:** Isolated MSCs were treated with 1 MHz LIPUS at an intensity of 250 or 750 mW/cm² for 5 or 20 min. Cell proliferation was evaluated by BrdU staining after 24h culture following single LIPUS treatment. Specific ELISAs were used to determine total and activated p38, ERK1/2, JNK MAPK signaling proteins up to 4h post-treatment. Selective MAPK inhibitors PD98059 (ERK1/2), SB203580 (p38) and SP600125 (JNK), were used to determine the role of activation of the particular MAPK pathways. **Results:** Proliferation of all MSC types was significantly increased following LIPUS treatment. LIPUS at 750 mW/cm² dose induced the greatest effects on DPSCs. BMSC proliferation was stimulated in equal measures by both intensities, whilst 250 mW/cm² LIPUS exposure exerted maximum effects on PDLSCs. ERK1/2 was activated immediately in DPSCs post-treatment. Concomitantly, DPSC proliferation was specifically modulated by ERK1/2 inhibition, whilst p38 and JNK inhibition exerted no effects. In BMSCs, JNK MAPK signaling was LIPUS-activated, and the increase in proliferation was blocked by specific inhibition of the JNK pathway. In PDLSCs, JNK MAPK signaling was activated immediately post-LIPUS, while p-p38 MAPK increased significantly in these cells 4h after exposure. Correspondingly, JNK and p38 inhibition modulated the LIPUS-stimulated PDLSC proliferation. **Conclusions:** LIPUS promoted MSC proliferation in an intensity and cell-specific dependent manner via activation of distinct MAPK pathways.

Key words: Ultrasound, LIPUS, DPSC, PDLSC, BMSC, cell proliferation, MAPK signaling, MSC, dental stem cells, dental tissue regeneration

Introduction

Ultrasound equipment has been used in clinical dentistry since the 1950s and its current applications range from periodontal scaling to endodontic root canal irrigation (1, 2). The acoustic energy from low-frequency kilohertz ultrasound (20 to 42 kHz) emitted by an oscillating tip of clinical dental scalers is utilized for surface cleaning by disrupting calculus, debris and bacteria on the external or internal surfaces of the tooth (2). Our previous work explored the molecular and biological effects of kHz ultrasound on dental cells demonstrating distinct changes in gene expression including expression of growth factors such as TGF β 1 and VEGF (3, 4). Ultrasound was also shown to promote the proliferation and differentiation of odontoblast-like cells (5), overall supporting the notion that low-intensity ultrasound has the therapeutic potential to promote endodontic tissue repair and dentine-pulp complex regeneration (6).

Unlike kHz ultrasound, low-intensity pulsed ultrasound (LIPUS) using a frequency in the low MHz range (1-3 MHz) is a more widely used approach as therapeutic application for tissue repair and regeneration, in particular for bone fracture healing (7-9). LIPUS delivers more focused, low-intensity acoustic pressure waves that produce small biomechanical interactions with the cells to elicit intracellular biological effects ultimately resulting in tissue repair and regeneration. Accumulating evidence indicates that LIPUS is effective to stimulate osteoblasts and promote bone formation (7). Moreover, research has suggested that stem cell-based tissue regeneration responds to LIPUS stimulation. LIPUS reportedly enhanced viability, proliferation and multilineage differentiation in a variety of postnatal mesenchymal stem cells (MSCs) including adipose-derived stem cells (ADSCs), bone marrow mesenchymal stem cells (BMSCs), periodontal ligament-derived stem cells (PDLSCs) and human umbilical cord-derived MSCs (10-14). Several studies have also suggested that LIPUS might be clinically beneficial in promoting dental tissue regeneration. Indeed, *in vitro* and *in vivo* studies indicated that exposure of dental tissues to LIPUS may promote dentinogenesis, accelerated periodontal tissue healing and dental implant osseointegration (15-17).

The mechanisms by which LIPUS stimulates cells and tissues has not yet been fully elucidated, but is in general attributed to its non-thermal biomechanical aspects. In particular, through acoustic microstreaming and physical radiation, LIPUS may affect the cell membrane

and cytoskeleton to trigger downstream signaling processes. MAPKs (ERK, JNK and p38 MAPK) have been demonstrated to play a role in mechanotransduction in various cell types, such as bone cells (18), periodontal ligament cells (19), and muscle cells (20). ERK and p38 MAPK pathways have been reported to control cell proliferation following mechanical stimulation (9, 21).

The use of MSCs, in particular dental pulp stem cells (DPSCs), BMSCs and PDLSCs, is increasingly being explored for application in dental tissue engineering strategies. Studies have suggested that MSCs from various tissue sources may show cell-specific responses under biomechanical stimuli via activation of different intracellular signaling pathways (22). In this study we investigated the effects of LIPUS on DPSC proliferation and the involvement of MAPKs in comparison with PDLSCs and BMSCs. We used primary cells isolated from rats which enabled us to establish and compare standardised and consistent cultures of MSC derived from the different tissue sources from the same donor animal (23). The findings of this study highlight the distinct differences between the responses of MSC from different tissues and underscore the potential of low-intensity ultrasound as therapeutic tool for dental tissue regeneration

Materials and Methods

Cell Cultures

Six-week-old male Wister Han rats (weight 120g) were used as tissue donors (Charles River Laboratories, UK). Dental pulp tissue was harvested from incisors and bone marrow from femora according to standard procedures as previously described (23). PDLSCs were isolated from periodontal ligament tissues dissected from rodent molars involving incubation in phosphate-buffered saline solution containing 0.25% trypsin and 1 mM EDTA (Gibco, UK) for 45 min at 37 °C in a rotary incubator (SI20H, Stuart Scientific, UK) and centrifugation at 1600g for 5 min (24). DPSC, PDLSC and BMSC were cultured in α -MEM/10 % FBS (Gibco, UK).

Characterization of Cell Cultures

Expression of CD29, CD90, vimentin (25), Nanog, Klf4, dentine matrix protein-1 (DMP1), osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP) was analyzed using semi-quantitative reverse transcription PCR (sqRT-PCR). RNA was extracted from cultures at passages 2 and 4 using the RNeasy minikit and reverse transcribed using an Omniscript RT kit according to the manufacturer's instructions (Qiagen, Manchester, UK). The house-keeping gene GAPDH was used to normalise gene intensity. Primers listed in Table 1.

To confirm differentiation potential, DPSC, PDLSC and BMSC were cultured in osteogenic medium [50 μ g/mL ascorbic acid (Sigma-Aldrich, UK), 10 mM β -glycerophosphate (Sigma-Aldrich, UK) and 10^{-9} M dexamethasone (Sigma-Aldrich, UK)] or adipogenic medium [0.5 mM 1-methyl-3-isobutylxantine (Sigma-Aldrich, UK), 60 μ M indomethacin (Sigma-Aldrich, UK) and 0.5 μ M hydrocortisone (Sigma-Aldrich, UK)]. After 3 weeks, the cultures were fixed and stained with alizarin red S (ARS) or oil red O (ORO) to demonstrate osteogenic and adipogenic differentiation, respectively, using described laboratory protocols (24).

LIPUS Stimulation

A DuoSon therapeutic ultrasound device (SRA Developments, Devon, UK) emitting pulsed ultrasound at a frequency of 1 MHz (pulsed at 63Hz repetition rate with pulse duration of 3.2ms) was used for ultrasound delivery. In brief, the MSC were seeded into the wells of a 6-well plate (Corning, UK) one day before ultrasound treatment (10,000 cell/well; 7ml medium/well). The following day, the 6-well culture plate was housed in a custom-built silicon anti-reflection

chamber on top of a 37 heat plate shaker and kept at 37 °C (5, 26). The ultrasonic transducer head was then carefully placed into the medium of a culture well to start ultrasound irradiation of the cultures. The DuoSon device, calibrated using a radiation force balance had two customized power settings to deliver 250 and 750 mW/cm². Both settings were used in all initial studies with two single treatment durations of 5 min and 20 min, respectively.

Cell Proliferation Analysis

Cell proliferation was evaluated by BrdU incorporation after 24h labeling using an immunocytochemical assay according to manufacturer's instructions (Roche Biosciences, No. 11299946001, Germany). Microscope images of the immunostained cultures were analysed using ImageJ software and a java program cell counter developed by Kurt De Vos (University of Sheffield, UK). Immunopositive and negative cell nuclei were marked manually by an observer. The percentage of BrdU positive cells (labelling index) as a measure for cell proliferation was calculated on the basis of the total cell number of five fields. Cell counts are presented as averages per time point from at least three independent experiments. In pathway inhibition experiments, cells were treated by the pharmacological MAPK inhibitors PD98059 (ERK1/2), SB203580 (p38) and SP600125 (JNK) (10 µM, TOCRIS, UK) 2h prior to ultrasound (27).

MAPK ELISA

ELISAs were used to determine total and phosphorylated p38, ERK1/2, JNK p38 MAP kinase using specific MAPK ELISA kits (Abcam, Cambridge, UK). Cytoplasmic protein extracts were collected following manufacturer's instructions at different time-points up to 4h after ultrasound treatment. Total protein concentration of each sample was determined by Bradford assay (28). The phosphorylated ratio of MAPK pathway proteins were calculated as p-MAPK/total MAPK.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and statistically analyzed using the student *t*-test and analysis of variance (ANOVA). *P*<0.05 was taken as statistically significant.

Results

Characterization of MSCs

sqRT-PCR analysis demonstrated the expression of CD29, CD90, vimentin, Nanog, Klf4, DMP1, OCN, OPN and BSP in all three MSC cultures (Fig. 1). CD29, CD90, and vimentin were regarded as MSC markers; Nanog and Klf4 as pluripotent markers; DMP1, OCN, OPN and BSP as hard-tissue, mineralization-related markers relevant to bone and dental tissues. In general, relatively lower expressions of these genes were observed in the stem cell cultures at passage 4 compared with passage 2 cultures. These data, in particular the significantly lower expression of the MSC marker CD90, led us to use passage 2 cultures for all ultrasound experiments.

After culture in osteogenic and adipogenic medium for three weeks, all MSC cultures displayed bi-differentiation potential (Fig. 2). PDLSCs seemed to show lower level of osteogenic and adipogenic differentiation when compared with DPSC and BMSC cultures as demonstrated by ARS and ORO staining, respectively, but this observation was not confirmed by quantitative analysis.

Effects of LIPUS Exposure on MSC Proliferation

LIPUS stimulated cell proliferation of all MSC types, mostly irrespective of the exposure time (Fig. 3). Proliferation of DPSC was significantly increased at both 250 and 750 mW/cm² intensities with the highest proliferation ratio resulting from the 750 mW/cm² treatment (Fig. 3A). BMSC proliferation was also significantly but equally increased in all ultrasound groups (Fig. 3B). Interestingly, PDLSC demonstrated a distinct response profile with proliferation significantly increased following exposure to the lower LIPUS power setting (250 mW/cm²), whereas at 750 mW/cm² no changes in DPSC proliferation were found (Fig. 3C). Moreover, the 5-min treatment group showed the relatively greatest stimulation in the PDLSC cultures.

MAPK Signaling in LIPUS Activated Proliferation in MSC

Specific ultrasound treatment parameters which promoted the highest level of cell proliferation for each cell type were selected for the subsequent analysis of the activation of MAPK signaling pathways. Thus, 5-min at 750 mW/cm² LIPUS was used for the DPSCs (see Fig. 3A). This treatment resulted in an immediate phosphorylation (i.e. activation) of ERK1/2 in DPSC, and following a transient decrease until 1h post-ultrasound treatment, an increase in ERK

phosphorylation which then remained relatively elevated up to 4h post-treatment (Fig. 4A). By adding PD98059, a selective ERK1/2 inhibitor 2h prior to ultrasound exposure, ultrasound-stimulated proliferation of DPSCs was abolished underscoring a key role of ERK1/2 activation in LIPUS-stimulated DPSC proliferation (Fig. 4B). However, addition of the JNK and p38 inhibitors SP600125 and SB203580 exerted no inhibitory effects on ultrasound-stimulated DPSC proliferation.

Ultrasound at an intensity of 750 mW/cm^2 was applied to BMSC for 5 min (see also Fig. 3); this led to a particular increase in phosphorylation of JNK MAPK (Fig. 4C). Interestingly, JNK was also activated immediately post-treatment, followed by a decrease until 1h after ultrasound, after which the JNK phosphorylation increased again remaining elevated up to 4h post-treatment, thus showing a similar pattern as was observed in LIPUS-induced ERK1/2 activation in DPSC cultures. The ultrasound-promoted proliferation was abolished in the specific JNK inhibition group confirming a critical role of JNK activation in BMSC stimulated proliferation. BMSC proliferation was not inhibited by PD98059 and SB203580, excluding an active role of ERK1/2 and p38 in LIPUS-stimulated BMSC proliferation (Fig. 4D).

For PDLSCs, 5 min of 250 mW/cm^2 LIPUS was applied based on results presented in Fig. 3. This ultrasound treatment immediately activated JNK MAPK and the phosphorylated JNK levels remained relatively high from 1h to 4h (Fig. 4E). Interestingly, phosphorylated p38 MAPK also significantly increased, albeit only 4h after ultrasound exposure (Fig. 4F). Both JNK inhibition by SP600125 and p38 inhibition by SB203580 in PDLSCs blocked the proliferation post-LIPUS treatment, suggesting essential involvement of p38 and JNK MAPK signaling in LIPUS-stimulated PDLSC proliferation (Fig. 4G).

Discussion

LIPUS is a safe and minimally non-invasive therapeutic tool that allows acoustic pulsed energy to be delivered to injured tissues facilitating bone repair and wounding healing (7). The relatively ease and inexpensive process of application may therefore provide an ideal therapy in the dental clinic for the regeneration of dental tissues (5, 6). Cell types involved in craniofacial and dental tissue homeostasis and repair including osteoblasts, periodontal ligament cells, odontoblasts, cementoblasts and gingival fibroblasts have been reported to be responsive to ultrasound exposure (5, 8, 29, 30). In stem cell-based tissue engineering, LIPUS may be used as an adjuvant therapy to accelerate regenerative processes by promoting stem cell expansion, differentiation and matrix production (8, 10, 12, 31).

LIPUS is considered to influence cells via a biomechanical mechanism entailing pressure waves and micro-steaming, but the precise mode of action is not well understood (8). Notably, mechanical forces and LIPUS may similarly affect cellular behavior activating common intracellular signaling pathways, such as the MAPK and Wnt pathways (21, 32, 33). Postnatal stem cells, in particular MSCs, isolated from a variety of tissues have been studied for tissue regeneration *in vitro* and *in vivo* (34). DPSCs, PDLSCs and BMSCs are mechanically sensitive MSCs and research indicated that mechanical loading, such as tensile stretch (35) and hydrostatic pressure (36) can affect matrix protein secretion, adhesion, proliferation and differentiation of these cells. This study focused on the effects of different LIPUS regimens on cell proliferation of three different MSC populations and also aimed to evaluate how LIPUS was able to bring about its cell type-dependent effects. Our study did not identify major phenotypic differences between the MSC derived from the two dental tissues (dental pulp, periodontal ligament) and bone marrow, a classical and most widely used MSC type. The RT-PCR analyses enabled us to screen and compare a range of MSC and pluripotent stem cell markers as well as markers representing hard-tissue biology, which would otherwise have been difficult to achieve for rat-derived cells. We recently confirmed the presence of CD29 and CD90 on the cell surface of rat DPSC and BMSC by flow cytometry, which corresponded with their genetic expression of these MSC markers (23). Our previous work also demonstrated particular differences in the differentiation potential and mineralisation patterns by different MSC types, which together with emerging evidence in the literature, strongly suggest that MSC from different niche sources may express

distinct biological features (23, 37, 38). The results from the present study did hint to subtle differences in the differentiation potential of PDLSC compared with DPSC and BMSC but further studies are needed to substantiate these observations. However, a remarkable finding from this study was that each of three different MSCs responded differently to LIPUS treatment which could be related to differences in the activation profiles of specific underlying MAPK signaling pathways.

Here we report that LIPUS increased proliferation of DPSCs, PDLSCs and BMSCs over a relatively short exposure time suggesting that LIPUS can rapidly activate and promote MSC expansion. Interestingly, the duration of a single LIPUS exposure (5 or 20 min) was not a major determinant in the level of increase in MSC proliferation. These observations may be explained by a well-established theory in biomechanics that mechanosensitive cells tend to become less sensitive to long-term mechanical loading suggesting that exposure times longer than 5 min did not significantly add to the biological effects (39). Clinically, a 20-min LIPUS treatment is widely used to accelerate and promote bone healing (9). Our findings suggesting that shorter duration of LIPUS may possibly achieve the same treatment efficacy warrants further (pre)-clinical research, including the study of the responsiveness of human-derived MSCs.

The present study showed that proliferation of DPSCs was particularly promoted at an intensity of 750 mW/cm^2 , whereas PDLSC proliferation was only increased at the lower intensity. This finding underscores the notion that postnatal stem cells derived from different tissues may present niche/site-specific biological characteristics, which may possibly explain why MSCs respond differently to a specific stimulus (37, 38). Here we speculate that DPSCs originated from the well-enclosed dental pulp may be less sensitive and responsive to mechanical intensity (needing a higher LIPUS power to respond) compared with PDLSCs which are derived from the mechanically dynamic periodontal ligament which is regularly and more directly subjected to various mechanical (e.g. masticatory) forces.

In this study the intracellular signal transduction via activation of MAPKs after ultrasound treatment was investigated by change in the phosphorylation level of the different MAPK signaling proteins. Adding specific MAPK inhibitors further confirmed the role of specific MAPK pathways in the dental stem cell proliferative response to ultrasound. In DPSC, ERK1/2

was shown to actively participate in control of ultrasound-induced proliferation, whilst in BMSC JNK appeared to be the main signaling pathway. Furthermore, our data suggested that both JNK and p38 may be both involved in stimulation of PDLSC proliferation. These particular MAPKs were activated immediately following LIPUS treatment, which reflect their established role and ability to respond in a very rapid manner to extracellular stimuli facilitating intracellular signaling leading to changes in gene expression and cell behavior (40). Remarkably, a common pattern seen in all the MSC types was that following the initial activation (phosphorylation) of the relevant MAPK proteins, a reduction occurred in the levels of phosphorylated MAPKs. This phenomenon likely reflected an overall de-phosphorylation phase as part of a physiological intracellular homeostatic mechanism involving alternate enzymatic phosphorylation and de-phosphorylation reactions to regulate cell function and mitosis (41). The fact that the single LIPUS treatment still resulted in a subsequent increase in MAPK activation underlines the potential long-reaching benefits of therapeutic ultrasound (5). It should be noted, however, that because of the limitations relating to the use of pharmacological pathway inhibitors, including non-specific off-target effects, cytotoxicity and transience of effect (42), further work such as the application of inhibitory siRNA will be needed to specify the role of each MAPK pathway in LIPUS stimulation.

In conclusion, our study underscores that LIPUS is an effective stimulus to promote proliferation of different MSC types. MSCs from different sources, in particular DPSC, PDLSC and BMSC, respond differently to different LIPUS treatment regimens with the ultrasound exposure duration less important than intensity. LIPUS-stimulated MSC proliferation was related to differential activation of distinct MAPK signaling pathways. These findings confirm the potential of low-intensity ultrasound as a non-invasive and safe therapeutic approach for dental and pulp tissue regeneration. Our study also highlighted intrinsic differences between MSCs from different sources including dental tissues, which might have implications for future stem cell-based dental tissue regeneration.

References

1. Zinner DD. Recent ultrasonic dental studies, including periodontia, without the use of an abrasive. *J Dent Res* 1955;34:748-49.
2. Walmsley AD, Laird WR, Lumley PJ. Ultrasound in dentistry. Part 2--Periodontology and endodontics. *J Dent* 1992;20:11-7.
3. Scheven BA, Man J, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ. VEGF and odontoblast-like cells: stimulation by low frequency ultrasound. *Arch Oral Biol* 2009;54:185-91.
4. Scheven BA, Millard JL, Cooper PR, Lea SC, Walmsley AD, Smith AJ. Short-term in vitro effects of low frequency ultrasound on odontoblast-like cells. *Ultrasound Med Biol*. 2007;33:1475-82
5. Man J, Shelton RM, Cooper PR, Scheven BA. Low-intensity low-frequency ultrasound promotes proliferation and differentiation of odontoblast-like cells. *J Endod* 2012;38:608-13.
6. Scheven BA, Shelton RM, Cooper PR, Walmsley AD, Smith AJ. Therapeutic ultrasound for dental tissue repair. *Med Hypotheses* 2009;73:591-3.
7. Claes L, Willie B. The enhancement of bone regeneration by ultrasound. *Prog Biophys Mol Biol* 2007;93:384-98
8. Romano CL, Romano D, Logoluso N. Low-intensity pulsed ultrasound for the treatment of bone delayed union or nonunion: a review. *Ultrasound Med Biol* 2009;35:529-36.
9. Padilla F, Puts R, Vico L, Raum K. Stimulation of bone repair with ultrasound: a review of the possible mechanic effects. *Ultrason* 2014;54:1125-45.
10. Cui JH, Park K, Park SR, Min BH. Effects of low-intensity ultrasound on chondrogenic differentiation of mesenchymal stem cells embedded in polyglycolic acid: an in vivo study. *Tissue Eng*. 2006;12:75-82.
11. Lee HJ, Choi BH, Min BH, Son YS, Park SR. Low-intensity ultrasound stimulation enhances chondrogenic differentiation in alginate culture of mesenchymal stem cells. *Artif Organs*. 2006;30:707-15.
12. Wang YK, Chen CS. Cell adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation. *J Cell Mol Med* 2013;17:823-32.
13. Hu B, Zhang Y, Zhou J, Li J, Deng F, Wang Z, Song J. Low-intensity pulsed ultrasound stimulation facilitates osteogenic differentiation of human periodontal ligament cells. *PLoS One*. 2014;9:e95168.
14. Marvel S, Okrasinski S, Bernacki SH, Lobo E, Dayton PA. The development and validation of a LIPUS system with preliminary observations of ultrasonic effects on human adult stem cells. *IEEE Trans Ultrason Ferroelec Freq Con* 2010;57:1977-84.
15. Ikai H, Tamura T, Watanabe T, Ito M, Sugaya A, Iwabuchi S, et al. Low-intensity pulsed ultrasound accelerates periodontal wound healing after flap surgery. *J Periodont Res* 2008;43:212-6.
16. Al-Daghreer S, Doschak M, Sloan AJ, Major PW, Heo G, Scurtescu C, Tsui YY, El-Bialy T. Long term effect of low intensity pulsed ultrasound on a human tooth slice organ culture. *Arch Oral Biol* 2012;57:760-8.

17. Wu G, Chen L, Zhu G, Wang Y. Low-intensity ultrasound accelerates mandibular implant bone integration in dogs with mandibular osteoradionecrosis. *J Surg Res.* 2013;182:55-61.
18. Liedert A, Kaspar D, Blakytyn R, Claes L, Ignatius A. Signal transduction pathways involved in mechanotransduction in bone cells. *Biochem Biophys Res Commun* 2006;349:1-5.
19. Kook SH, Lee JC. Tensile force inhibits the proliferation of human periodontal ligament fibroblasts through Ras-p38 MAPK up-regulation. *J Cell Physiol* 2012;227:1098-106.
20. Li C, Xu Q. Mechanical stress-initiated signal transduction in vascular smooth muscle cells in vitro and in vivo. *Cellular signalling.* 2007;19:881-91.
21. Zhou S, Schmelz A, Seufferlein T, Li Y, Zhao J, Bachem MG. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J Biol Chem* 2004;279:54463-9.
22. Wang YK, Chen CS. Cell adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation. *J Cell Molec Med* 2013;17:823-32.
23. Davies OG, Cooper PR, Shelton RM, Smith AJ, Scheven BA. A comparison of the in vitro mineralisation and dentinogenic potential of mesenchymal stem cells derived from adipose tissue, bone marrow and dental pulp. *J Bone Miner Metab* 2014; 33:371-82
24. Techawattanawisal W, Nakahama K, Komaki M, Abe M, Takagi Y, Morita I. Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. *Biochem Biophys Res Commun.* 2007;357:917-23.
25. Karaoz E, Aksoy A, Ayhan S, Sariboyaci AE, Kaymaz F, Kasap M. Characterization of mesenchymal stem cells from rat bone marrow: ultrastructural properties, differentiation potential and immunophenotypic markers. *Histochem Cell Biol* 2009;132:533-46.
26. Patel US, Ghorayeb SR, Yamashita Y, Atanda F, Walmsley AD, Scheven BA. Ultrasound field characterization and bioeffects in multiwell culture plates. *J Ther Ultrasound* 2015;3:8.
27. Motta EM, Calixto JB, Rae GA. Mechanical hyperalgesia induced by endothelin-1 in rats is mediated via phospholipase C, protein kinase C, and MAP kinases. *Exp Biol Med* 2006;231:1141-5
28. Carlsson N, Borde A, Wolfel S, Kerman B, Larsson A. Quantification of protein concentration by the Bradford method in the presence of pharmaceutical polymers. *Anal Biochem* 2011;411:116-21.
29. Inubushi T, Tanaka E, Rego EB, Kitagawa M, Kawazoe A, Ohta A, et al. Effects of ultrasound on the proliferation and differentiation of cementoblast lineage cells. *J Periodont* 2008;79:1984-90.
30. Shiraishi R, Masaki C, Toshinaga A, Okinaga T, Nishihara T, Yamanaka N, et al. The effects of low-intensity pulsed ultrasound exposure on gingival cells. *J Periodont* 2011;82:1498-503.
31. Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, et al. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. *Tissue Eng* 2004;10:921-9.

32. Mahoney CM, Morgan MR, Harrison A, Humphries MJ, Bass MD. Therapeutic ultrasound bypasses canonical syndecan-4 signaling to activate rac1. *J Biol Chem* 2009;284:8898-909.
33. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res*. 2009;88:792-806.
34. Han M-J, Seo Y-K, Yoon H-H, Song K-Y, Park J-K. Effect of mechanical tension on the human dental pulp cells. *Biotechnol Bioprocess Eng* 2008;13:410-7.
35. Perry MJ, Parry LK, Burton VJ, Gheduzzi S, Beresford JN, Humphrey VF, Skerry TM. Ultrasound mimics the effect of mechanical loading on bone formation in vivo on rat ulnae. *Med Eng Phys*. 2009;31:42-7.
36. Yu V, Damek-Poprawa M, Nicoll SB, Akintoye SO. Dynamic hydrostatic pressure promotes differentiation of human dental pulp stem cells. *Biochem Biophys Res Commun* 2009;386:661-5.
37. Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater*. 2010;20:121-33.
38. Ghaemi SR, Harding FJ, Delalat B, Gronthos S, Voelcker NH. Exploring the mesenchymal stem cell niche using high throughput screening. *Biomaterials* 2013;34:7601-15.
39. Schriefer JL, Warden SJ, Saxon LK, Robling AG, Turner CH. Cellular accommodation and the response of bone to mechanical loading. *J Biomech* 2005;38:1838-45.
40. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res*. 2002;12:9-18.
41. Bononi A, Agnoletto C, De Marchi E, Marchi S, Patergnani S, Bonora M, Giorgi C, Missiroli S, Poletti F, Rimessi A, Pinton P. Protein kinases and phosphatases in the control of cell fate. *Enzyme Res* 2011;329098.
42. Wauson EM, Guerra ML, Barylko B, Albanesi JP, Cobb MH. Off-target effects of MEK inhibitors. *Biochemistry*. 2013;52:5164-6.

Figure 1. DPSC, BMSC and PDLSC sqRT-PCR gene expression profiles at culture passages 2 and 4. All three MSC types expressed CD29, CD90, vimentin, Nanog, Klf4, DMP1, OCN, OPN, BSP. Values represent the mean \pm SD of three independent assays; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 2. Multilineage differentiation of DPSC, BMSC and PDLSC cultures. Controls were cultured in standard media without osteogenic or adipogenic supplements; the osteogenic and adipogenic groups were cultured with the osteogenic and adipogenic differentiation media for 3 weeks, respectively. Osteogenic differentiation and mineralisation was made evident by Alizarin Red S (ARS) staining. Oil red O (ORO) staining demonstrated lipid droplet-filled cells to signify adipogenic differentiation. Images were obtained using phase-contrast microscopy and are representative of three experiments. Scale bars represent 100 μm .

Figure 3. LIPUS effect on cell proliferation as determined by BrdU labelling 24h after ultrasound treatment. Four ultrasound parameters were tested: 5 min 250 mW/cm^2 , 20-min 250 mW/cm^2 , 5-min 750 mW/cm^2 and 20 min 750 mW/cm^2 . * $p < 0.05$ and ** $p < 0.01$ versus the untreated controls. Values represent mean \pm SD of three independent experiments.

Figure 4. Activation of MAPK pathways involved in ultrasound-stimulated MSC proliferation. Total and phosphorylated MAPK pathway protein (p-ERK1/2, p-JNK and p-p38) were determined by specific ELISAs. Phosphorylated ratio was calculated up to 4h after ultrasound application (A, C, E, F). To confirm the functional role of specific MAPK pathways, inhibitors PD98059 (PD, ERK1/2 inhibitor) SB203580 (SB, p38 inhibitor) and SP600125 (SP, JNK inhibitor) were used for the BrdU cell proliferation assays (B, D, G). LIPUS activated ERK1/2 pathway in DPSC up to 4h (A). Ultrasound-stimulated DPSC proliferation was inhibited by the ERK1/2 inhibitor PD98059 (B). LIPUS-activated JNK pathway in BMSC up to 4h (C). Ultrasound-stimulated BMSC proliferation was inhibited by the JNK inhibitor SP600125 (D). LIPUS activated JNK (E) and p38 (F) MAPK pathways in PDLSC. Ultrasound-induced PDLSC proliferation was inhibited by JNK inhibitor SP600125 and p38 inhibitor SB203580 (G). * $p < 0.05$ and ** $p < 0.01$ versus the untreated controls. # $p < 0.05$ and ## $p < 0.01$ versus the experimental groups. Values represent the mean \pm SD of three independent assays.