Ultrasound Effect on Neural Differentiation of Gingival Stem/Progenitor Cells

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Associate Editor Dan Elson oversaw the review of this article.

Abstract—Dental pulp loss due to caries or pulpitis can affect the longevity of teeth. Dental pulp tissue engineering necessitates the use of progenitor cells that has the potential to differentiate into neural, vascular and odontoblasts like cells. Previous reports have shown that human gingival progenitor cells (HGPCs) can be differentiated into different cell types; however neural differentiation of these cells, to the best of our knowledge, has not been reported. Low intensity pulsed ultrasound (LIPUS) has been reported to enhance cell differentiation. The aims of this study were (1) to explore the potential neural differentiation of HGPCs and (2) to investigate the effect of LIPUS on the differentiation of HGPCs when incubated under neuroinductive conditions. The HGPCs were isolated from human interdental papilla proximal to the premolar teeth that were extracted for orthodontic purpose. The HGPCs were induced to differentiate into neural lineage using a neuroinductive culture medium. HGPCs were divided into four groups; control group, neuro-induction (NI) group, ultrasound group (LIPUS), and a combined NI + LIPUS group. HGPCs were harvested for immunostaining and q-PCR after 1 day. Immunostaining for neuron specific antigens and q-PCR suggested that HGPCs can be differentiated into neural lineage and that selected neurodifferentiation markers can be enhanced by LIPUS.

Keywords—Human gingival progenitor cells, Low intensity pulsed ultrasound, Neural differentiation.

INTRODUCTION

Loss of dental pulp tissue due to pathology or trauma may lead to extraction of the affected tooth with multiple unwanted sequences such as bone resorption, movement of adjacent and opposing teeth, and/or the need for costly replacement of the missing tooth as well as reduced quality of life.[13,28] There has been recently an increasing interest in dental tissue engineering including the dental pulp.[10,13,14,24,25] Dental pulp tissue engineering requires the availability of odontoblast-like cells, nerve-like cells, and vascular cells/tissues. Previous attempts of dental pulp tissue engineering aimed at complete restoration of lost dental pulp to its original function by utilizing different types of adult progenitor cells.[13,14,24,25] Progenitor cells that have been previously utilized include dental pulp stem cells, bone marrow stem cells, dental follicle stem cells, and adipose tissue stem cells.[13,14,24,25] However, donor-site morbidity and/or non-availability (e.g., dental pulp stem cells in an adult for example) may be considered as unfavorable considerations for potential clinical application of this approach. Human gingival progenitor cells (HGPCs) are more accessible for harvest with minimal donor-site morbidity.[36] Previous studies have shown that HGPCs possess multipotent-progenitor cell characteristics as they express stem cell surface markers, can be differentiated into osteogenic lineage, and assume morphological changes similar to cementoblasts *in vitro*.[3,22,27,36] Moreover, the clinical application of HGPCs for therapeutic purposes to regenerate attached gingiva and interdental papilla has been reported.[19,20] The neurogenic differentiation of HGPCs is yet to be explored. Also, multiple previous studies have shown that low intensity pulsed ultrasound (LIPUS) enhances differentiation of mesenchymal cells into different types of specialized cells such as chondrogenic, osteogenic and cementoblasts and odontoblasts.[1,5,6,8,12,16,29] Furthermore, it has been reported that LIPUS has an anabolic effect on HGPCs and can enhance their differentiation into osteogenic lineage.[21]

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The aim of this study was to explore the potential neurogenic differentiation of HGPCs and to demonstrate the effect of LIPUS on their potential neurogenic differentiation. The hypothesis of this study is that HGPCs can be differentiated into neurogenic lineage and the application of LIPUS has a positive effect on their neurogenic differentiation.

MATERIALS AND METHODS

The study utilized HGPCs obtained from three human donors (age 14–16 years old) after extraction of their premolars (total $N = 12$ premolars) for orthodontic purpose in accordance with the approved health ethics board at the University of Alberta, Edmonton, Canada. Experiment below was run three times on the pooled cells collected from all donors. The HGPCs were harvested and isolated according to a previously published protocol.[3,22] In brief, the gingival explants were cut into small pieces, isolated on glass slides, placed in a culture plate, and incubated with a basic medium (DMEM, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. When the cells around gingival explants were confluent following 2–3 weeks of culture, they were transferred to 75-cm^2 tissue culture flasks using 0.08% trypsin/0.04% ethylenediaminetetraacetic acid. Identification of the multipotent potential of these cells was confirmed according to Dominici et al.[7] by flowcytometry for stem cell specific markers (positive for CD 90 and negative for CD 31, CD 34, and CD 45) (Fig. 1). Also, previous reports that showed that these cells can be differentiated into osteogenic lineage, and assume morphological changes similar to cementoblasts in vitro [3,22,27,36]

Neural induction was performed as previously described by Yaghoobi et al.[33] Briefly, neuroinductive medium (NIM) was added to the culture. The composition of NIM was: αMEM with 2% DMSO, 10 ng/mL bFGF, 100 μM butylated hydroxyanisole (Sigma), 10 μM Forskolin (Sigma), 25 mM KCl, 2 mM valproic acid and 5 μg/mL insulin. Cells from each donor were expanded and divided into four groups in seven 25-mL flasks. For each test, cells were used in triplicates. LIPUS was applied to each respective group using LIPUS device (SmileSonica Inc., Edmonton, Canada) with output pulse of 1.5 MHz that is repeated at 1 K Hs with spatial average—temporal average intensity of 30 mW/cm^2 for 10 min/day for 3 days under the tissue culture flasks using ultrasound gel and aseptic technique inside the incubator according to previously published protocol.[1,5,6,8,12,29] Group 1 was HGPCs with regular medium (negative control); group 2 was HGPCs + NIM; group 3 was HGPCs + LIPUS; and group 4 was HGPCs + NIM + LIPUS. The HGPCs at passage 2 were grown in 48-well plates at an initial seeding density of 2.5×10^3 cells/well. Cells were harvested for immunohistochemistry and quantitative polymerase chain reaction (qPCR) after 1 day of using either regular medium or NIM. Immunohistochemistry was performed as follows. The cells were fixed with 4% paraformaldehyde and stored in PBS at 4 °C before incubation with the primary antibody. Endogenous peroxidase was suppressed with 10% H_2O_2 for 20 min. The primary antibodies used were: (a) mouse monoclonal anti β-tubulin (1:250, Sigma); (b) mouse monoclonal anti-MAP2 (1:200, Sigma); (c) mouse monoclonal anti-neurofilament 200 (1:50, Sigma); and, (d) mouse monoclonal anti-synaptophysin (1:250, Sigma). For murine antibodies, 2° antibody = rabbit anti-mouse HRP conjugated secondary antibody (1:500, Dako, Glostrap Denmark). For nucleostemin, 1° antibody was goat polyclonal anti-nucleostemin (1:100, Abcam, Cambridge, UK) and 2° antibody was rabbit polyclonal antibody against goat HRP conjugated (1:500, Abcam, Cambridge, UK). Finally, for rabbit GFAP, 1° antibody was rabbit polyclonal anti-glial fibrilary acidic protein (GFAP) antibody (1:500, Chemicon) and 2° antibody was biotinylated goat anti-rabbit IgG (1:500, Vector, Burlingame, CA) and Avidine/HRP (1:500, Dako, Glostrap, Denmark) was used for incubation.

For qPCR, the total RNA from the cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and the absorbance were measured at 260 nm for RNA quantification. First-strand cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. In brief, 1.5 μg of total RNA from each sample was added to a mix of 2.0 μL 10× RT buffer, 0.8 μL 25× dNTP mix (100 mM), 2.0 μL 10× RT random primers, 1.0 μL MultiScribe™ reverse transcriptase, and 4.2 μL nuclease-free water. The final reaction mix
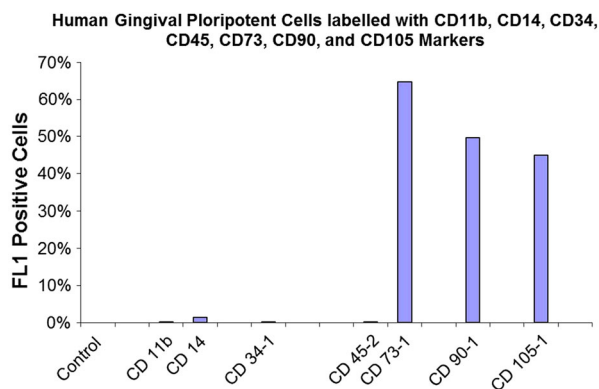

FIGURE 1. Flowcytometry of HGPCs.

was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated for 85 °C for 5 s, and finally cooled to 4 °C. Quantitative analysis of specific gene mRNA expression was performed by real time-PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems). The amount of 25 μL of the reaction mix contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 nM final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study were the housekeeping gene: GAPDH (forward) = CCTGCCA AGTATGATGACATCAA and GAPDH (reverse) = AGCCCAGGATGCCCTTTAGT and target genes: Neurofilament-M (NM-017029): Forward: GCACTA AGGAGTCCCTGGAAC; Reverse: GCCTCGACTT TGGTCTTCTG; Nucleostemin (NM-175580): Forward: TCCGAAGTCCAGCAAGTATTG; Reverse: AATGAGGCACCTGTCCACTC; Vimentin (NM-03 1140): Forward: AATTGCAGGAGCTGAATGAC; Reverse: AATGACTGCAGGGTGCTCTC.

The amplification mixture contains 1 μL RNA, 2 L of each primer and 12.5 L of the components of the SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA, USA) in a final volume of 25 L. Real-time PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems) with the following cycle conditions: 2 min at 50 °C, 15 min at 95 °C, 40 cycles of 15 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in each run. Normalized fluorescence was plotted against cycle number (amplification plot) and the threshold as suggested by the software was used to calculate Ct (cycle at threshold). Results of the real-time PCR were expressed as Ct, and the expression levels of specific genes were indicated by the number of cycles required to achieve the threshold level of amplification.

Statistical Analysis

All samples were performed in triplicate for each group and the data were analyzed with one-way analysis of variance (ANOVA) using SPSS version 16.0 software package (SPSS, Chicago, IL, USA). Intergroup differences were determined using the Boneferroni *post hoc* test, and statistical significance was defined by p-values <0.05.

RESULTS

To evaluate whether HGPCs have mesenchymal progenitor characteristics, flowcytometry was performed and the results are shown in Fig. 1. HGPCs were positive for CD 90 and negative for CD 31, CD 34, and CD 45 which confirmed their multipotent/progenitor characteristics.[7] The HGPCs cultured with regular medium with or without LIPUS showed spindle shaped cells (Figs. 2a and 2b). After 6 h of adding NIM to the HGPCs, morphological changes, e.g. cells became round with neurite-like processes extending from cell peripheries, were noted with or without LIPUS (Figs. 2c and 2d). Immunohistochemistry staining revealed no staining of the negative–negative control group for all neural cells specific antibodies (Fig. 3). On the other hand, HGPCs that were cultured in NIM showed increased staining and morphological changes after 96 h of culture with NIM with or without LIPUS application.

Quantitative PCR results showed that mRNA for neurofilament is significantly increased with NIM than the control group or LIPUS alone group (Fig. 4). Also, when LIPUS was applied in addition to NIM, mRNA for neurofilament was statistically increased than that of NIM alone ($p = 0.026$), LIPUS alone ($p = 0.01$) or control group ($p = 0.006$). Also, mRNA for neurofilament was statistically increased for NIM alone compared to negative control ($p = 0.008$). Similar pattern was noted for Vimentin mRNA as NIM + LIPUS showed significantly increased expression than the NIM alone ($p = 0.024$), LIPUS alone ($p = 0.048$) or control group ($p = 0.025$). Vimentin mRNA for NIM group was not significantly increased than the control group ($p = 0.14$) same as between NIM alone and LIPUS alone ($p = 0.58$). Nucleostemin mRNA was significantly decreased in NIM + LIPUS group than in control group ($p = 0.004$). Similar pattern of decrease was also noted for nucleostemin mRNA in LIPUS alone group than the control group ($P = 0.0025$).

DISCUSSION

Research in the field of dentofacial tissue engineering, especially dental pulp tissue engineering, has long been investigating different sources for stem cells. These sources include bone marrow stem cells, periodontal ligament stem cells, and dental pulp stem cells. However, these cells are difficult to obtain due to the invasive nature of their harvesting techniques.[2,4,9,11,34] The difficulty in obtaining these cells limits their potential for prospective clinical use in dental tissue engineering. Gingival progenitor cells, also known as gingival fibroblasts, can be differentiated into different cell linages. This study was oriented to explore the potential neural differentiation of HGPCs.
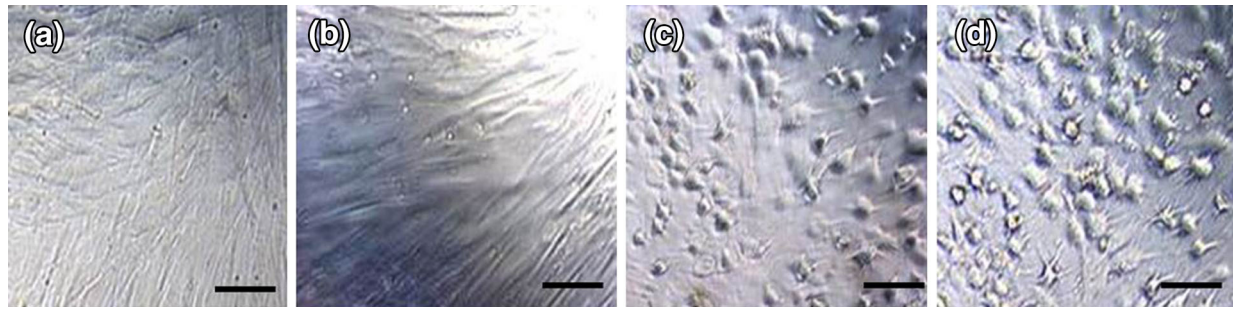
FIGURE 2. HGPCs, (a) control (Spindle shape); (b) LIPUS treated cells, (c) NIM only, and (d) NIM+LIPUS after 96 h in cell culture. Magnification is ×100. Note morphological changes as the cells have big round cell bodies and neurite-like processes are of HGPCs with NIM and with NIM+LIPUS. (Bar = 30 μm).
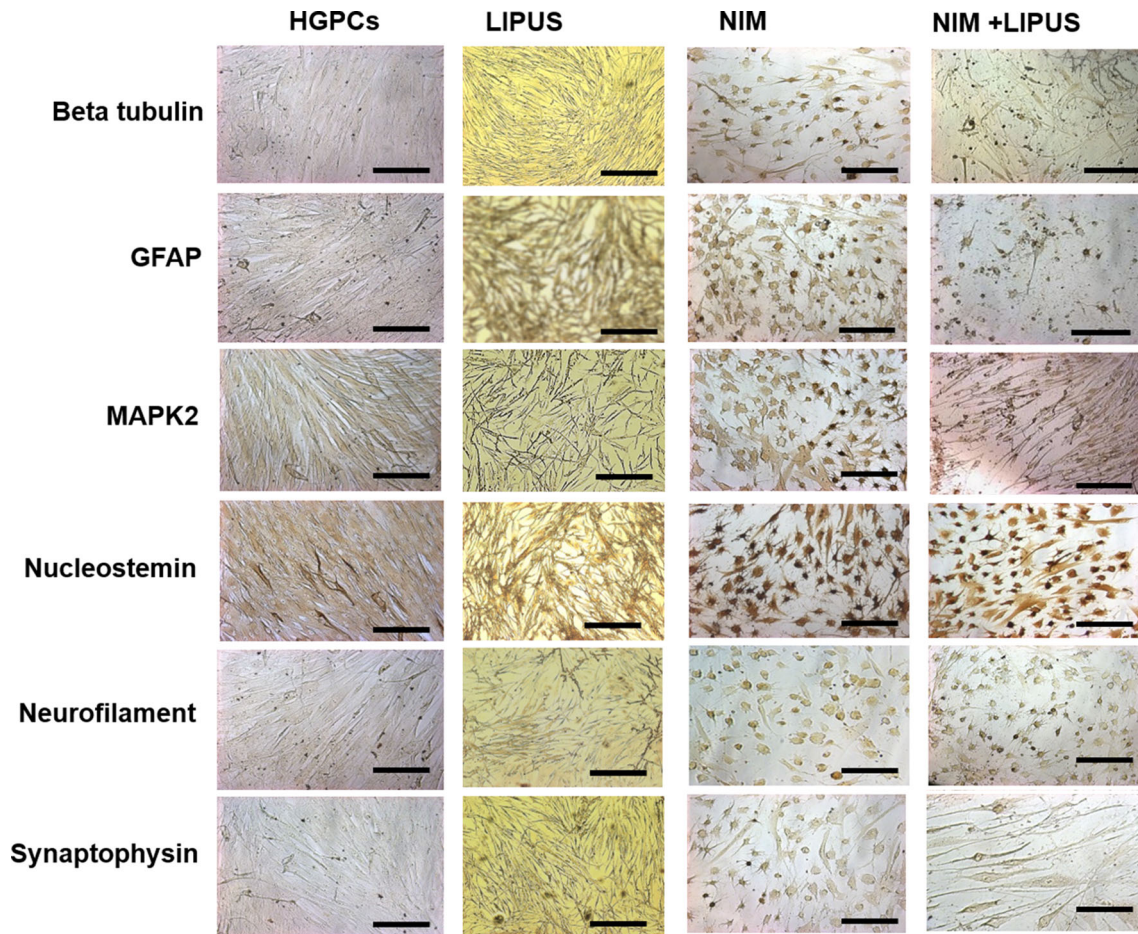

FIGURE 3. Immunohistochemistry of HGPCs stained with anti-Beta-tubulin; Glial Fibrillary; MAPK2; nucleostemin; neurofilament and synaptophysin antibodies in the negative control group; after LIPUS treatment; after NIM treatment and after NIM and LIPUS. It can be seen that control group show no staining to any of the antibodies used. However HGPCs show increased staining with most of the antibodies used. LIPUS alone slightly increased staining than the control group. Most of the HGPCs show deep staining after differentiation using NIM or NIM+LIPUS. (Bar = 100 μm).

Previous reports have shown that bone marrow stem cells can be differentiated into neuron-like cells.[33] In this study, we have utilized neural induction medium as reported by Yaghoobi et al.[33] The morphogenesis phenotype of the neuron-like cells was apparent in few hours following the addition of NIM (Fig. 2). This is in alignment with previous report about the neurogenic potential of bone marrow stem cells.[33] Cells showed large rounded cell bodies with neurite-like processes similar to cultured neuronal cells.[33] Neuron specific antibodies were used to identify the neurogenic differentiation of HGPCs.[33] Neuroinduced HGPCs
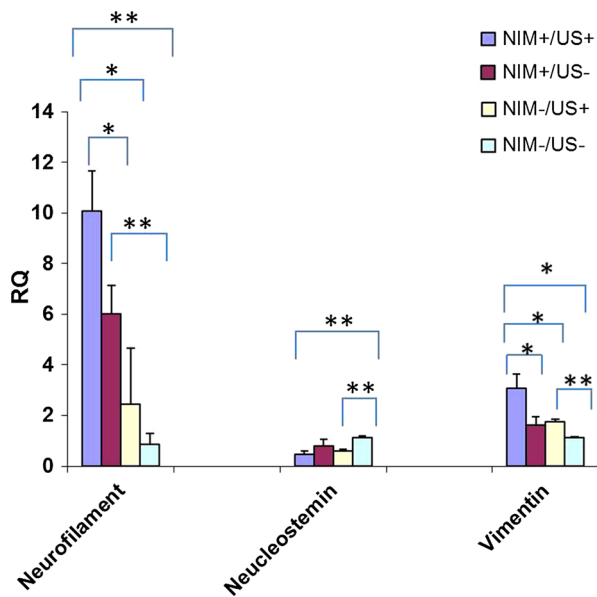
FIGURE 4. qPCR data showing significant up-regulation of Neurofilament (NF) gene by NIM and by LIPUS+NIM. Neucle-ostemin (NCT) is down-regulated by LIPUS and by LIPUS+NIM (non-significant). Vementin is significantly up-regulated by LIPUS+NIM than the control. $*p < 0.05$; $**p < 0.005$.

with or without LIPUS showed high reaction to these antibodies (Fig. 3).

Nucleostemin is expressed in stem cells and is then turned off upon differentiation.[32] Vimentin is selectively enriched in bone marrow stem cells.[15,30,31,33] The expression of HGPCs of Vimentin supports the hypothesis that HGPCs cells have stem cell characteristics. The decreased expression of nucleostemin by LIPUS as evaluated by qPCR in this study may support the hypothesis that these cells are differentiating, but not fully yet, into neural lineage and this differentiation is complemented by LIPUS. However, immunostaining of nucleostemin showed increased staining with NIM and LIPUS. This contradiction between qPCR and immunostaining could be due to the fact that qPCR deals with part of the nucleostemin protein, so NIM and LIPUS might have stimulatory effect on other parts of nucleostemin protein that is shown by immunostaining. This phenomenon warrants further investigation. In addition, it has been recently shown that there is more than one nucleostemin alleles that have different behaviors.[26] This contradiction between the qPCR and immunostaining might suggest that LIPUS might have stimulatory effect on one nucleostemin allele while has inhibitory effect on another allele. This observation also warrant further investigation. Also, HGPCs showed increased expression of neurofilament gene when induced to neural differentiation by NIM, which was complemented by LIPUS application more than control cells.

The qPCR results suggest that LIPUS has neural induction to HGPCs as there was statistically induced neurofilament and vimentin expression by LIPUS and this was supported by NIM. Also, there was no statistical difference between the NIM and LIPUS in vimentin expression which may indicate that LIPUS has similar neural induction as NIM. The enhanced neurofilament and vimentin expression by NIM suggest neural differentiation of HGPCs and this expression was enhanced by LIPUS. Also, the down regulation or nucleostemin by LIPUS support the hypothesis that LIPUS enhances neural differentiation of HGPCs. The exact mechanism of the mechano-transduction pathway involved in cellular responses to LIPUS remains unknown. Possible explanation that LIPUS stimulates neural differentiation is that LIPUS is a mechanical stimulation that stimulates intracellular signaling pathway. Previous studies have shown that LIPUS activate cell differentiation and integrins as well as the downstream signaling pathway in vitro.[23,37] Also, possible mechanism of LIPUS effect on neural differentiation of gingival stem cells is that LIPUS upregulates neurotrophin-3 (NT-3),[35] which is an important regulator of neural survival, development, function, and neuronal differentiation.[17,18] Future research may be directed to explore more details about the mechanisms by which LIPUS enhances stem cell neural differentiation.

CONCLUSION

To our best knowledge, this is the first report that HGPCs can be differentiated into neural lineage and this effect can be complemented by LIPUS application. This report suggests that HGPCs may be used in nerve tissue engineering including dental pulp as well as other craniofacial nerve tissue engineering. Also, our report supports that LIPUS can be used as a complementary technique in neural differentiation of stem cells.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

[1]Angle, S. R., K. Sena, D. R. Sumner, and A. S. Virdi. Osteogenic differentiation of rat bone marrow stromal cells by various intensities of low-intensity pulsed ultrasound. *Ultrasonics* 51(3):281–288, 2011.

[2]Bakopoulou, A., G. Leyhausen, J. Volk, A. Tsiftsoglou, P. Garefis, P. Koidis, and W. Geurtsen. Comparative analysis of *in vitro* osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch. Oral Biol.* 56(7):709–721, 2011.

[3]Carnes, D. L., C. L. Maeder, and D. T. Graves. Cells with osteoblastic phenotypes can be explanted from human gingiva and periodontal ligament. *J. Periodontol.* 68:701–707, 1997.

[4]Casagrande, L., M. M. Cordeiro, S. A. Nör, and J. E. Nör. Dental pulp stem cells in regenerative dentistry. *Odontology* 99(1):1–7, 2011; (Epub 2011 Jan 27).

[5]Dalla-Bona, D. A., E. Tanaka, T. Inubushi, H. Oka, A. Ohta, H. Okada, M. Miyauchi, and K. Tanne. Cementoblast response to low- and high-intensity ultrasound. *Arch. Oral Biol.* 53(4):318–323, 2008.

[6]Dalla-Bona, D. A., E. Tanaka, H. Oka, E. Yamano, N. Kawai, M. Miyauchi, T. Takata, and K. Tanne. Effects of ultrasound on cementoblast metabolism *in vitro*. *Ultrasound Med. Biol.* 32(6):943–948, 2006.

[7]Dominici, M., K. L. Blanc, I. Mueller, I. Slaper-Cortenbach, F. C. Marini, S. Krause, R. J. Deans, A. Keating, D. J. Prockop, and E. M. Horwitz. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4):315–317, 2006.

[8]El-Bialy, T., H. Uludag, N. Jomha, and S. Badylak. *In vivo* ultrasound assisted tissue engineered mandibular condyle: a pilot study in rabbits. *Tissue Eng. Part C* 16(6):1315–1323, 2010.

[9]Estrela, C., A. H. Alencar, G. T. Kitten, E. F. Vencio, and E. Gava. Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. *Braz. Dent. J.* 22(2):91–98, 2011.

[10]Ferreira, C. F., R. S. Magini, and P. T. Sharpe. Biological tooth replacement and repair. *J. Oral Rehabil.* 34(12):933–939, 2007.

[11]Huang, C. Y., D. Pelaez, J. Dominguez-Bendala, F. Garcia-Godoy, and H. S. Cheung. Plasticity of stem cells derived from adult periodontal ligament. *Regen. Med.* 4(6):809–821, 2009.

[12]Inubushi, T., E. Tanaka, E. B. Rego, M. Kitagawa, A. Kawazoe, A. Ohta, H. Okada, J. H. Koolstra, M. Miyauchi, T. Takata, and K. Tanne. Effects of ultrasound on the proliferation and differentiation of cementoblast lineage cells. *J. Periodontol.* 79(10):1984–1990, 2008.

[13]Iohara, K., K. Imabayashi, R. Ishizaka, A. Watanabe, J. Nabekura, M. Ito, K. Matsushita, H. Nakamura, and M. Nakashima. Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1. *Tissue Eng. Part A* 17(15–16):1911–1920, 2011.

[14]Ishizaka, R., K. Iohara, M. Murakami, O. Fukuta, and M. Nakashima. Regeneration of dental pulp following pulpectomy by fractionated stem/progenitor cells from bone marrow and adipose tissue. *Biomaterials* 33:2109e2118, 2012.

[15]Jia, L., M. F. Young, J. Powell, L. Yang, N. C. Ho, R. Hotchkiss, P. G. Robey, and A. F. Clair. Gene expression profile of human bone marrow stromal cells: high-throughput expressed sequence tag sequencing analysis. *Genomics* 79:7–17, 2002.

[16]Man, J., R. M. Shelton, P. R. Cooper, and B. A. Scheven. Low-intensity low-frequency ultrasound promotes proliferation and differentiation of odontoblast-like cells. *J. Endod.* 38(5):608–613, 2012.

[17]McAllister, A. K., L. C. Katz, and D. C. Lo. Neurotrophins and synaptic plasticity. *Annu. Rev. Neurosci.* 22:295–318, 1999.

[18]McAllister, A. K., D. C. Lo, and L. C. Katz. Neurotrophins regulate dendritic growth in developing visual cortex. *Neuron* 15:791–803, 1995.

[19]McGuire, M. K., and E. T. Scheyer. A randomized, double-blind, placebo-controlled study to determine the safety and efficacy of cultured and expanded autologous fibroblast injections for the treatment of interdental papillary insufficiency associated with the papilla priming procedure. *J. Periodontol.* 78(1):4–17, 2007.

[20]Mohammadi, M., M. A. Shokrgozar, and R. Mofid. Culture of human gingival progenitor cells on a biodegradable scaffold and evaluation of its effect on attached gingiva: a randomized, controlled pilot study. *J. Periodontol.* 78(10):1897–1903, 2007.

[21]Mostafa, N. Z., H. Uludag, D. N. Dederich, M. R. Doschak, and T. H. El-Bialy. Anabolic effects of low intensity pulsed ultrasound on gingival fibroblasts. *Arch. Oral Biol.* 54(8):7 43–7 48, 2009.

[22]Mostafa, N. Z., H. Uludağ, M. Varkey, D. N. Dederich, M. R. Doschak, and T. H. El-Bialy. *In vitro* osteogenic induction of human gingival fibroblasts for bone regeneration. *Open Dent. J.* 5:139–145, 2011; (Epub 2011 Aug 27).

[23]Nakamura, T., S. Fujihara, K. Yamamoto-Nagata, T. Katsura, T. Inubushi, and E. Tanaka. Low-intensity pulsed ultrasound reduces the inflammatory activity of synovitis. *Ann. Biomed. Eng.* 39(12):2964–2971, 2011.

[24]Nakashima, M., and A. Akamine. The application of tissue engineering to regeneration of pulp and dentin in endodontics. *J. Endod.* 31(10):711–718, 2005.

[25]Nakashima, M., and K. Iohara. Regeneration of dental pulp by stem cells. *Adv. Dent. Res.* 23(3):313–319, 2011.

[26]Nomura, J., M. Maruyama, M. Katano, H. Kato, J. Zhang, S. Masui, Y. Mizuno, Y. Okazaki, M. Nishimoto, and A. Okuda. Differential requirement for nucleostemin in embryonic stem cell and neural stem cell viability. *Stem Cells* 27(5):1066–1076, 2009.

[27]Pi, S. H., S. K. Lee, Y. S. Hwang, M. G. Choi, S. K. Lee, and E. C. Kim. Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival progenitor cells and periodontal ligament cells. *J. Periodontal Res.* 42:104–113, 2007.

[28]Salehrabi, R., and I. Rotstein. Endodontic treatment outcomes in a large patient population in the USA: an epidemiological study. *J. Endod.* 30:846–850, 2004.

[29]Scheven, B. A., J. L. Millard, P. R. Cooper, S. C. Lea, A. D. Walmsley, and A. J. Smith. Short-term *in vitro* effects of low frequency ultrasound on odontoblast-like cells. *Ultrasound Med. Biol.* 33(9):1475–1482, 2007.

[30]Silva, W., T. Dimas, Jr., A. Rodrigo, P. S. Rodrigo, L. C. S. Jorge, L. Z. Dalila, R. Anemari, and M. Zago. The profile of gene expression of human marrow mesenchymal stem cells. *Stem Cells* 21:661–669, 2003.

[31]Tsai, R. Y. L., and R. D. G. McKay. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. *Genes Dev.* 16:2991–3003, 2002.

[32]Wada, N., D. Menicanin, S. Shi, P. M. Bartold, and S. Gronthos. Immunomodulatory properties of human periodontal ligament stem cells. *J. Cell Physiol.* 219(3):667–676, 2009.

[33]Yaghoobi, M. M., S. J. Mowla, and T. Tiraihi. Nucleostemin, a coordinator of self-renewal, is expressed in rat marrow stromal cells and turns off after induction of neural differentiation. *Neurosci. Lett.* 390(2):81–86, 2005.

[34]Zhang, W., H. Abukawa, M. J. Troulis, L. B. Kaban, J. P. Vacanti, and P. C. Yelick. Tissue engineered hybrid tooth-bone constructs. *Methods* 47(2):122–128, 2009.

[35]Zhang, H., X. Lin, H. Wan, J. H. Li, and J. M. Li. Effect of low-intensity pulsed ultrasound on the expression of neurotrophin-3 and brain-derived neurotrophic factor in cultured Schwann cells. *Microsurgery* 29(6):479–485, 2009.

[36]Zhang, Q., S. Shi, Y. Liu, J. Uyanne, Y. Shi, S. Shi, and A. Le. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation—related tissue destruction in experimental colitis. *J. Immunol.* 183:7787–7798, 2009.

[37]Zhou, S., A. Schmelz, T. Seufferlein, Y. Li, J. Zhao, and M. G. Bachem. Molecular mechanisms of low intensity pulsed ultrasound in human skin fibroblasts. *J. Biol. Chem.* 279:54463–54469, 2004.