Ultrasound in Med. & Biol., Vol. 41, No. 7, pp. 1842–1852, 2015
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0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2015.03.017

● *Original Contribution*

# OSTEOBLASTOGENESIS OF MESENCHYMAL STEM CELLS IN 3-D CULTURE ENHANCED BY LOW-INTENSITY PULSED ULTRASOUND THROUGH SOLUBLE RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND

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(*Received* 5 *September* 2014; *revised* 12 *February* 2015; *in final form* 11 *March* 2015)

**Abstract—This study was performed to investigate osteoblastogenesis of human mesenchymal stem cells (hMSCs) cultured in 3-D scaffolds stimulated with low-intensity pulsed ultrasound and to identify the underlying mechanism mediated by soluble receptor activator of nuclear factor kappa B ligand (sRANKL) secreted by hMSCs. The results indicate that the mRNA levels of *core-binding factor subunit alpha subunit 1 (CBFA1)*, *osterix (OSX)*, *alkaline phosphatase (ALP)*, *osteocalcin* and *osteoprotegerin (OPG)* and sRANKL production of hMSCs stimulated by ultrasound were significantly increased compared with the levels without ultrasound stimulation. Attenuating the sRANKL activity of ultrasound-treated hMSCs significantly reduced the mRNA expression of *CBFA1*, *OSX*, *ALP* and *OPG*. Adding sRANKL in hMSC culture significantly increased the mRNA expression of *CBFA1*, *OSX* and *OPG*. Together, the results suggest that osteoblastogenesis of hMSCs enhanced by ultrasound stimulation is mediated by endogenous sRANKL. (E-mail: li@ortho.wisc.edu)** **© 2015 World Federation for Ultrasound in Medicine & Biology.**

***Key Words:*** **Low-intensity pulsed ultrasound, Osteoblastogenesis, Receptor activator of nuclear factor kappa B ligand, Mesenchymal stem cell.**

## INTRODUCTION

Ultrasound stimulation has been extensively used to enhance bone fracture healing (Malizos et al. 2006; Rubin et al. 2001; Siska et al. 2008) and osteoblast differentiation (Suzuki et al. 2009; Takayama et al. 2007). Ultrasound stimulation, specifically low-intensity pulsed ultrasound, has also been approved by the U.S. Food and Drug Administration (21 CFR1050 2013) for clinical treatment of pathologic defects and traumatic fractures of bone. Such acoustic energy is transmitted to a biological tissue through an impedance-matching medium, inducing micromassage and microstream without local temperature alteration. Although the non-thermal effects of ultrasound stimulation are considered the essential mediators of cell and tissue response, the intracellular and extracellular regulators induced by ultrasound remain unclear.

Healthy bone constantly undergoes tissue remodeling to maintain homeostasis between bone formation and resorption (Rodan 1998) mediated by osteoblasts and osteoclasts, respectively. Ultrasound stimulation plays a critical role in modulation of bone homeostasis. Previous studies have found that ultrasound stimulation is capable of upregulating the expression of osteogenic markers and increasing production of bone matrix proteins in mesenchymal stem cells (MSCs) and osteoblasts. Low-intensity pulsed ultrasound is capable of increasing transient expression of osteoblast-related markers, such as *osteonectin* and *osteopontin*, in rat bone marrow-derived stromal cells (Sena et al. 2005). Other studies have found that low-intensity ultrasound stimulation is able to upregulate the expression of osteoblast-related markers, including *alkaline phosphatase (ALP)*, *core-binding factor subunit alpha 1* (*CBFA1*), *osterix* (*Osx*), *msh homeobox homologue 2*, *distal-less homeobox 5*, *bone sialoprotein (Bsp)* and *bone morphogenetic protein*

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*2 (Bmp2)*, and increase ALP activity and mineralization in osteoblastic lineage cells (Angle et al. 2011; Chen et al. 2014; Harle et al. 2001; Suzuki et al. 2009; Unsworth et al. 2007). Though interesting, these findings are limited to studies using 2-D culture. Given that cell behavior differs between 2-D and 3-D culture, and 3-D culture is more physiologically relevant than 2-D culture, we were interested in studying the osteogenic capacity of human MSCs (hMSCs) stimulated with ultrasound in 3-D culture.

Receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) are dominant mediators regulating bone formation and resorption. Specifically, RANKL can bind to membrane-anchored RANK of osteoclastic progenitors to initiate osteoclastogenesis (Takahashi et al. 1999; Wada et al. 2006). In addition, RANKL is involved in the regulation of bone formation (Atkins et al. 2003), and OPG is a decoy receptor capable of preventing RANKL from binding to RANK to induce osteoclastogenesis (Simonet et al. 1997; Khosla 2001). Recently, our group reported that OPG is able to enhance osteogenesis of hMSCs (Palumbo and Li 2013). When these results are considered together, RANKL and OPG appear to play a critical role in the regulation of hMSC osteogenesis. However, it is unclear whether ultrasound stimulation can regulate RANKL and/or OPG to modulate hMSC osteogenesis. A previous study found that ultrasound stimulation upregulates *OPG* and downregulates *RANKL* mRNA and protein expression in human osteoblast-like cells (Maddi et al. 2006). Therefore, we aimed to investigate the role of RANKL and OPG in the regulation of hMSC osteoblastogenesis during ultrasound stimulation.

In this study, we hypothesized that ultrasound stimulation regulates osteoblastogenesis of hMSCs through the expression of RANKL and/or OPG. To test the hypothesis, we evaluated the effect of ultrasound stimulation on osteoblastogenesis of hMSCs seeded in 3-D scaffolds composed of $\beta$-tricalcium phosphate ($\beta$-TCP) and poly(L-lactic acid) (PLA) by analyzing the mRNA expression of osteoblast-related markers and protein production of CBFA1 and osteocalcin (OC). The roles of soluble RANKL (sRANKL) and OPG in the regulation of hMSC osteoblastogenesis during ultrasound stimulation were also investigated.

## METHODS

### *Cell culture*

Human bone marrow was isolated from femoral heads of two patients, a 49-y-old man and a 53-y-old woman, undergoing total hip replacement surgery that had been approved by the institutional review board (IRB) of the University of Wisconsin—Madison. Approved by the IRB, informed consent from patients was waived in accordance with federal regulations for human tissue obtained as surgical waste for biomedical research. In brief, whole bone marrow was curetted from the interior compartment of a femoral neck and head, reconstituted in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA), separated from bone debris using an 18-gauge needle, centrifuged at 2,000 rpm for 5 min and reconstituted in Hanks' balanced salt solution (HBSS; HyClone, Logan, UT, USA). Mononucleated cells were retrieved from the reconstituted cell solution using the Ficoll (GE Healthcare, Pittsburgh, PA, USA) density gradient approach, which consists of centrifugation at 500*g* for 30 min, two washes with HBSS, resuspension in culture medium composed of 1 g/L D-glucose DMEM (DMEM-LG; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics (10,000 IU/mL penicillin, 10,000 $\mu$g/mL streptomycin and 25 $\mu$g/mL amphotericin) and plating in tissue culture flasks (Corning, Corning, NY, USA). Culture medium was replaced with fresh medium the next day to remove non-adherent cells. The culture was maintained at 37°C in a humidified incubator with 5% $CO_2$ (Thermo Scientific, Asheville, NC, USA). Cells were passaged using 0.05% trypsin–EDTA (Gibco) after reaching 80% density confluence and replated at a seeding density of $2 \times 10^4$ cells/cm$^2$. Cells isolated from different patients were individually cultured and used separately in replicates of each assay. Specifically, each analysis of the study was conducted with one donor's cells and then repeated with the other donor's cells to confirm the reproducibility of experimental data. The results of each assay presented in this report are the representative results of repeated assays using cells of either donor.

The 3-D scaffolds composed of 80% (w/w) $\beta$-TCP and 20% (w/w) PLA were obtained from Kensey Nash (Exton, PA, USA) through research collaboration. The scaffold is a circular disk (10 mm in diameter and 4 mm in thickness) with a porosity of 82%. A total of $10^6$ hMSCs at passage 4 in a 50-$\mu$L cell solution were seeded on each side of the scaffold. To ensure that cells were securely attached, seeded scaffolds were placed in a cell culture incubator for 2 h before basal medium was added to the culture. Within 24 h, the cell-laden scaffolds were induced with osteogenic medium containing 10% FBS basal medium supplemented with 10 mM $\beta$-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 50 $\mu$g/mL L-ascorbic acid (Sigma-Aldrich) and $10^{-7}$ M dexamethasone (Sigma-Aldrich).

### *Ultrasound stimulation*

To stimulate the hMSC-laden scaffolds with ultrasound, a transducer (Metron/Patterson, Bolingbrook, IL,

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