Runx2 and Osterix Gene Expression in Human Bone Marrow Stromal Cells Are Mediated by Far-Infrared Radiation

Ming-Tzu Tsai*, Yung-Sheng Lin, Wan-Chi Chen, Chien-Hua Ho, Heng-Li Huang, and Jui-Ting Hsu

***Abstract*—Runx2 and Osterix are the key transcription factors initiating and regulating the early osteogenesis and late mineralization of bone. To investigate whether the expression of these osteogenic genes in human bone marrow stromal cells (hBMSCs) was regulated by physical stimulation, far infrared (FIR) was applied to this present work. hBMSCs were cocultured and physically separated with the their adjacent cell, human osteoblasts (hOBs) in a transwell system. Both cells were placed onto 200-mg FIR ceramic powders packed into a plastic bag for uniform FIR irradiation. 0-, 30-, 60-, and 120-min treatment of FIR irradiation were performed inside an incubator. Each group was fully wrapped by foils to prevent FIR interruption. The electrophoretic RT-PCR data showed that hBMSCs exhibited higher gene expression of Runx2, Osterix and collagen type I under 120-min/day FIR treatment at day 14. Histochemical staining results also revealed that FIR-treated hBMSCs expressed more ALP and calcium deposits after 28-day exposure. It suggested that appropriate physical stimulus may be an alternative tool for the control of osteogenic differentiation in hBMSCs in vitro.**

***Keywords*—bone marrow stromal cells, far-infrared irradiation, Osterix, Runx2**

I. Introduction

BONE marrow stromal cells (BMSCs), which can differentiate into osteoblasts (OBs) by appropriate induction, have become one of the most important cells for bone tissue engineering [1,2]. Due to the mulipotent differentiation capability of BMSCs, it is valuable to develop the technique of controlling, maintaining and promoting the osteogenesis of BMSCs.

Runx2 is essential for osteoblast differentiation and skeletal development during the early stages of embryogenesis [3]. The role of Runx2 on regulating osteogenic differentiation is revealed by inhibiting the formation of mineralized nodules with Runx2 antisense oligonucleotides, while forced expression of Runx2 in nonosseous mesenchymal cells induces expression of osteoblast phenotypic genes [4,5]. The role of Runx2 on regulating bone phenotypic genes was demonstrated that Runx2 can upregulate the expression of OCN, but also interacts with other genes, such as Osterix (Osx) [6,7]. Subsequent studies revealed that Runx2 can also downregulate various bone-related genes, including the collagen type I (Col I) [8] and bone sialoprotein. Col I is the major organic component of bone extracellular matrix produced by osteoblasts [9]. OCN is the bone-specific gene exhibit the osteoblastic phenotype. OCN is also the late bone mark during the osteogenic differentiation and mineralization [10]. Osx is a novel zinc finger–containing transcription factor that is essential for osteoblast differentiation and bone formation [11,12]. In *osx*-null mutant mice, neither endochondral nor intramembranous bone formation occurs, and osteoblast differentiation is arrested [12].

Far-infrared (FIR) radiation is an invisible and short electromagnetic waves ranges from 8–14 μm. FIR therapy has been used to treat vascular-related disorders for many years. Although the mechanism on human is still unknown, most data show that FIR irradiation can increase skin microcirculation by elevating the blood flow and nitric-oxide synthesis [13-15]. It demonstrates that FIR promotes intracellular event by exerting some biological effects. Prior studies also exhibit that FIR radiation enhances the proliferation of fibroblasts and increases the immunity by strengthening leukocytes may have a promotion on wound healing [13,16-18]. The biological effects of FIR on skin microcirculation by interacting with intracellular factors may occur in bone marrow during osteogenic differentiation.

In this present work, we used FIR ceramic powders as a physical stimulus applied to hBMSCs cocultured with hOBs, to explore the osteogenic gene expression of Runx2, Osx, Col I, and OCN. The electrophoretic RT-PCR data showed that hBMSCs exhibited higher gene expression of Runx2, Osx, Col I under 120-min/day FIR treatment at day 14. Histochemical staining results also revealed that FIR-treated hBMSCs expressed more ALP and calcium deposits after 28-day exposure. It suggested that appropriate physical stimulus may be an alternative tool for the control of osteogenic differentiation in hBMSCs in vitro.

Manuscript received March 7, 2011; revised April 7, 2011.

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II. MATERIALS AND METHODS

A. Isolation and Cultivation of hBMSCs

hBMSCs were isolated from the bone marrow aspirate obtained from four donors (43–78 years old) undergoing total hip replacement (IRB approval, Taichung Veterans General Hospital, Taiwan), as previously described by Caterson et al. Briefly, adherent cells derived from marrow stroma were maintained as monolayer cultures and expanded in basal medium containing Dulbecco's modified Eagle's medium (DMEM), 10% selected lots of fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells at passage 3–4 were cultured in 12-well culture plate at initial seeding cell densities of 5,000 cells/cm^2. To induce osteogenic differentiation of hBMSCs, cells were cultured in an osteogenic medium supplemented with 100 nM dexamethasone, 50 µg/ml L-ascorbic acid, and 10 mM β-glycerophosphate. Half of the conditioned medium was replaced with fresh medium every 2–3 days. After one day for cell attachment, hBMSCs were cocultured with hOBs pre-seeded onto inserts in a transwell system without cell-cell contact. Cells were placed onto 200-g ceramic powders and exposed to FIR radiation for 21 days.

B. Isolation and Cultivation of hOBs

hOBs were derived and isolated from trabecular bones of bone chips obtained from the same patients undergoing the surgery. These bone chips were washed by aseptic saline for several times, and the trabecular bones were scraped and collected in calcium-free Dulbecco's modified Eagle's medium/F12K medium. Bone chips were cut and limited to 3–5 mm in length, and reacted with collagenase P to remove the extracellular matrix. Bone chips were cultured and expanded in DMEM medium with 10% FBS, 2 mM L-glutamine, 50 ug/mL ascorbate, and 50 ug/mL penicillin-streptomycin for 10–15 days. After hOBs migrated from chips and reached to the confluence, 110 mM $CaCl_2$ was added into the medium for cell survival.

C. Preparation of FIR Ceramics

The ceramic powder consisted of micro-sized particles produced from several ingredients, mainly mineral oxides. The average emissivity of the ceramic powder was 0.98 at wavelengths of 8−14 µm, which was proven by tests run by the industrial technology research institute, and this represented an extremely high ratio of FIR ray intensity. Equal amounts of 200 g of FIR powder (FIR-treated groups) were enclosed in each plastic bag for equal FIR radiation exposure.

D. Experimental Design

hBMSCs and hOB at passages 3–5 were cocultured in a transwell system (Fig. 1). hBMSCs and hOB were seeded onto 12-well culture plate and insert with 0.3 µm pore size at 5,000 and 10,000 cells/cm^2. After cell attachment for one day, these cells were cocultured in basal medium. Culture plates and inserts were placed onto the top of FIR ceramic powder bag for uniform FIR irradiation. Cells were divided into four groups: control (non teatment), 30-min group (FIR treatment for 30 min/day), 60-min group (FIR treatment for 60 min/day), and 120-min group (FIR treatment for 120 min/day). Cells were cultured for 14−28 days. 200-g FIR ceramic powders were packed into each plastic bag, and the outside of bags was asepticized by 70% ethanol. Both FIR-treated and non-treated cells were fully rapped and covered by foils to prevent the interruption of FIR radiation (Fig. 1).

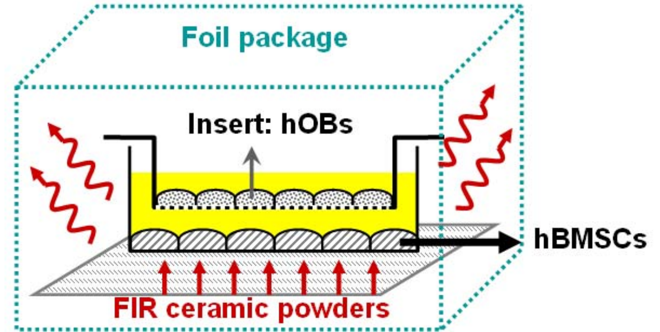

Fig. 1. Schematic of the FIR-exposure system. hBMSCs and hOBs were separately cultured onto the bottom and insert of 12-well culture plate. Cells were exposed to uniform FIR irradiation for 0-, 30-, 60-, and 120-min/day.

E. Total RNA Isolation and RT-PCR Reaction

The mRNA expression of *Runx2* (a critical transcriptional factor that regulates skeletogenesis) [19], osterix (Osx; an essential transcription factor that initiates mineralization) [12], type I collagen (Col I; a major organic component that exists in bone extracellular matrix) [20], osteocalcin (OCN; a late and specific marker of bone formation) [10], and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; as an internal control for RNA loading) in control and FIR-treated cocultures were determined at each time point by reverse transcriptase– polymerase chain reaction (RT-PCR) assay. Total RNA was extracted and collected by using TriZol reagent and the first-strand cDNA was synthesized by using the SuperScript First-Strand Synthesis System followed by the amplification of cDNA product using Platinum Taq DNA polymerase. The sense and antisense sequences of the primers used for semiquantitative RT-PCR reactions are listed in Table 1. The reaction was performed under the following conditions: incubation at 94°C for 2 min; denaturation at 94°C for 45 s, annealing at 51°C (Runx2), 58°C (Osx), 60°C Col I and OCN), and 52°C (GAPDH) for 30 s, and polymerization at 72°C for 60 s for 30–35 cycles; followed by a final extension at 72°C for 5 min. The expression of amplified products was evaluated using electrophoresis with 2% agarose gel and ethidium bromide staining. All electrophoresis images were analyzed quantitatively using NIH Image J software, and normalized to their respective GAPDH values.

F. Histochemical Staining

The matrix mineralization of hBMSCs in cocultures with or without FIR treatment was detected by histochemical staining at day 28. Matrix mineralization was visualized by staining. Osteogenic differentiation was evaluated on the basis of ALP production histochemically detected in cultures fixed with 2% paraformaldehyde in methanol using the Leukocyte Alkaline Phosphatase Kit according to the manufacturer's protocol. Matrix mineralization was visualized by staining cultures fixed in 60% isopropanol with 2% alizarin red solution for 5 min at room temperature.

TABLE I
PRIMERS SEQUENCES FOR RT-PCR ANALYSIS

Target Gene	Annealing Temp (C)	Sequence
Runx2	51	Forward: CCG CAC GAC AAC CGC ACC AT Reverse: CGC TCC GGC CCA CAA ATC TC
Osx	58	Forward: TAG TGG TTT GGG GTT TGT TTT ACC GC Reverse: AAC CAA CTC ACT CTT ATT CCC TAA GT
Col I	60	Forward: GGA CAC AAT GGA TTG CAA GGC CGC Reverse: TAA CCA CTG CTC CAC TCT GGA TGG
OCN	60	Forward: ATG AGG ACC CTC TCT CTG CTC CGT TA Reverse: ATG AGG ACC CTC TCT CTG CTC AGC GG
GAPDH	52	Forward: GGA CAC AAT GGA TTG CAA GG Reverse: TAA CCA CTG CTC CAC TCT GG

G. *Statistical Analysis*

All assays were repeated, with a minimum of n=3 per group. Data are expressed as mean and SD values. Statistical significance was determined using one-way ANOVA to compare means between groups, with a p value of less than 0.05 being considered significant.

III. RESULTS

This present work showed that a relatively higher increase without significance in Rux2 gene expression in hBMSCs treated with FIR radiation compared to the control from day 3 to 7 by electrophoretic RT-PCR analysis (Fig. 2). A significant increase in Runx2 exhibited in 60-min group with FIR treatment at day 14 (Fig. 2). All FIR-treated groups showed similar and higher Osx gene expression compared to the control at day 3 (Fig. 3). hBMSCs showed much higher Osx gene expression after FIR treatment for 60- or 120-min/day at day14 (Fig. 3). 120-min FIR-treated group was remarkable on the higher expression of osteogenic genes (Runx2, Osx, and Col I) at day 14 (Fig. 2–4). OCN gene expression did not show obvious difference between each group (Fig. 5). Due to Osx in hBMSCs was induced by FIR radiation, 14-day culture period may not be enough to enhance OCN gene expression in FIR-groups. A longer culture period of 3–4 weeks may be necessary to examine OCN gene expression in all groups.

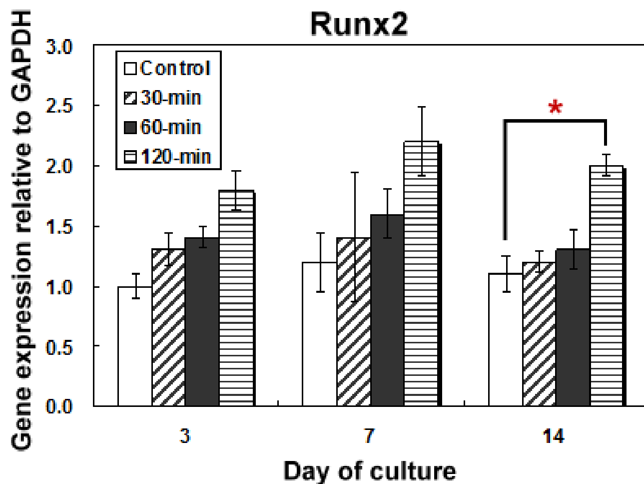

Fig. 2. Electrophoretic RT-PCR analysis of Runx2 gene expression from hBMSCs cocultured with hOBs and exposed to FIR irradiation. Data presented are from two independent experiments, with triplicate samples (12-well culture plate) per experiment (N=3 total). *, p < 0.05.

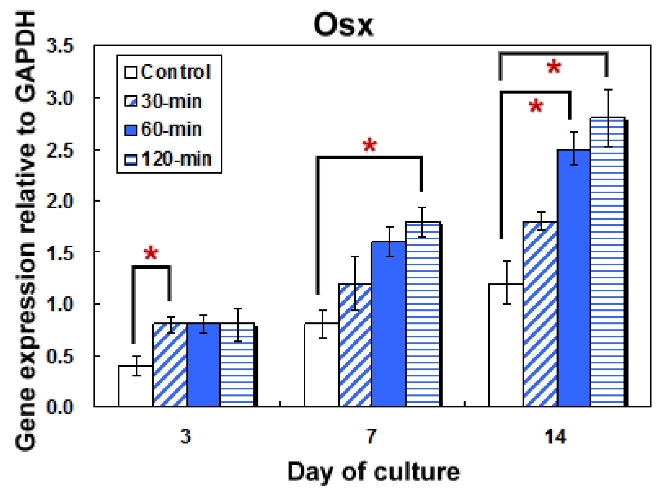

Fig. 3. Electrophoretic RT-PCR analysis of Osx gene expression from hBMSCs cocultured with hOBs and exposed to FIR radiation. Data presented are from two independent experiments, with triplicate samples (12-well culture plate) per experiment (N=3 total). *, p < 0.05.

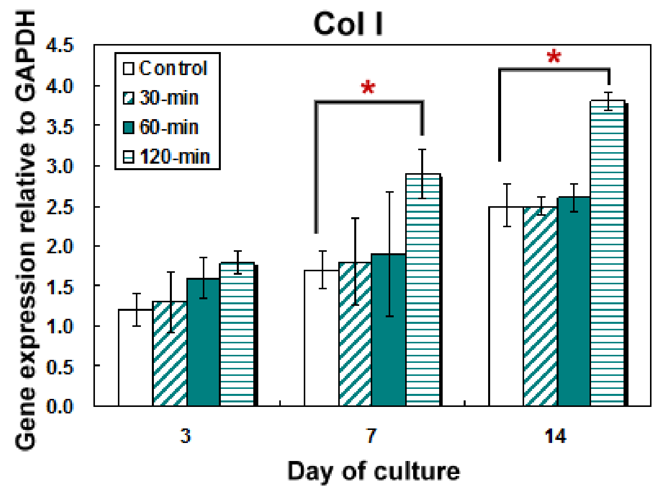

Fig. 4. Electrophoretic RT-PCR analysis of Col I gene expression from hBMSCs cocultured with hOBs and exposed to FIR irradiation. Data presented are from two independent experiments, with triplicate samples (12-well culture plate) per experiment (N=3 total). *, p < 0.05.

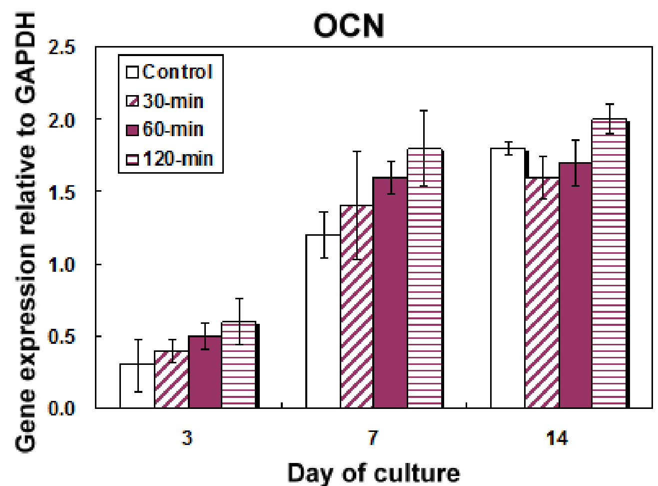

Fig. 5. Electrophoretic RT-PCR analysis of OCN gene expression from hBMSCs cocultured with hOBs and exposed to FIR irradiation. Data presented are from two independent experiments, with triplicate samples (12-well culture plate) per experiment (N=3 total). *, p < 0.05.

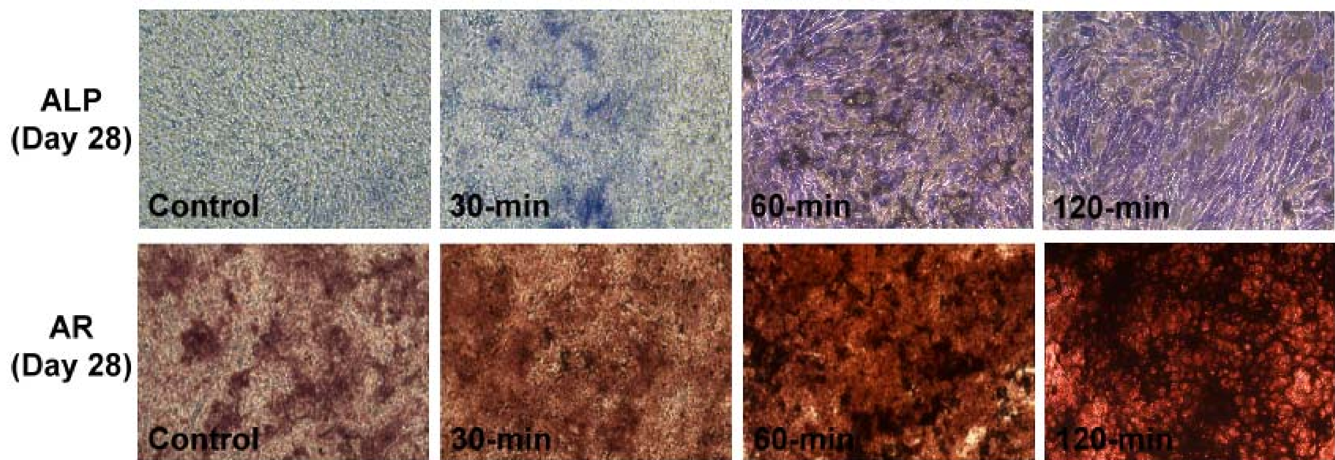

Fig. 6. The osteogenic differentiation and mineralization of hBMSCs were estimated by ALP and alizarin red S staining at day 28. calcium deposit accumulated over time and reached to the highest level at the end of culture period.

Compared to the control, all FIR-treated groups showed obvious ALP expression and calcium deposit in hBMSCs at the end of culture during the osteogenic differentiation and mineralization by ALP and alizarin red S staining (Fig. 6). Osteogenic differentiation was induced by 30-min FIR irradiation, and reached to the highest by 120-min FIR irradiation for 28 days (Fig. 6).

IV. Discussion

The therapeutic effects and safety of FIR on skin wound healing have been proved from numerous studies in patients and animals for many years. The purpose of this study was to investigate the possible biological effects of FIR on the osteogenic–specific genes expression of hBMSCs cocultured with hOBs on the ceramic powders emitting FIR irradiation. To evaluate the expression of Runx2, Osx, Col I, and OCN genes, hBMSCs were exposed to FIR-emitted ceramic powders for different time period.

The runt family transcription factor Runx2 plays a crucial role and regulates the skeletogenesis and bone formation during the early embryogenesis [3,5,21]. Increase in the gene expression and cellular protein levels of Runx2 have been reported in early embryos, murine and human osteoblasts, and BMSCs [19,22]. Our data showed that Runx2 had a remarkable higher level of expression in 120-min FIR group at day 14 compared to others (Fig. 2). Also, Osx and Col I both revealed significant increase (Fig. 3–4). Runx2 is also identified as a key regulator of osteoblast-specific genes expression, and various studies have revealed that Runx2 may commit cells to osteogenesis though mediating other osteogenic-target genes, such as Osx, Col I, and OCN [23,24].

Osx, a specific osteogenic transcription factor, which is identified as a late bone marker required for the differentiation of preosteoblasts into fully functioning osteoblasts [7,11,25]. Increase in Osx gene expression was found in FIR-treated groups throughout the culture period (Fig. 2). All FIR-treated groups showed higher expression in Osx at day 3 compared to the control (Fig. 3). Although group with 120-min FIR irradiation exhibited the highest ALP activity and calcium accumulation at day 28, the control and other FIR-treated groups showed positive calcium deposition eventually at the end stages of culture (Fig. 6). OCN levels were greatly elevated in all groups during the culture period, but no significant difference between each group (Fig. 5). OCN is another osteogenic-specific gene especially identified as a late bone marker in osteoblasts [10].

Studies demonstrate that Osx is one of the downstream genes of Runx2 [7,11,25,26]. Previous data show the level of OCN gene expression is elevated by forced expression of Osx [27,28]. However, the high level of Osx, did not enhance osteogenic differentiation, and did not up-regulate collagen I expression in primary human fetal mesenchymal stem cells [9]. Ectopic expression of Osx in fibroblasts suppressed the expression of collagen type I, but enhances the proliferation of fibroblasts and increased the expression of osteopontin during osteogenic differentiation [29]. Mice with Osx-deficiency do not form endochondral or intramembranous bones due to the lack of osteoblasts [12]. From these above, it seems that Runx2 and Osx may control different stages of differentiation in osteoblasts. Interestingly, Osx is not expressed in Runx2-null mice but Runx2 is expressed normally in Osx-deficient mice [12]. It suggested that Osx may act as a downstream factor of Runx2 [12].

Col I is the most abundant protein exists in the bone extracellular matrix with high level of expression in osteoblasts [9]. The expression of Col I gene exhibits at all stages during the bone development [20,30]. Col I expression in all FIR-treated groups accumulated over time, especially the 120-min group (Fig. 3). It believes that Runx2 is one of the positive regulators of these osteogenic-specific genes described above.

Although the biological effects of FIR on BMSCs remain poorly understood, its application on skin wound healing has been well known and performed for decades [13,16-18]. The speed of full-thickness skin wound healing in rats is significantly more rapid with FIR irradiation. No change is found in skin blood flow and skin temperature during FIR irradiation. Histological findings also reveal greater collagen regeneration and infiltration of fibroblasts in wounds in the FIR-treated groups [16,17]. Our data showed a time-dependent manner of FIR radiation on increasing the levels of osteogenic-specific genes in hBMSCs during osteogenic differentiation. It suggested that FIR may control hBMSC osteogenesis though mediating Runx2 and its

downstream target genes.

V. Conclusion

Osteogenic-specific gene expression and calcium accumulation in FIR-treated hBMSCs cocultured with osteoblasts were greatly elevated especially for the exposure time of 120 min/day. It indicated that an appropriate physical stimulus may be an alternative tool for the control of osteogenic differentiation in hBMSCs in vitro, and may become an effective therapy for bone disorders.

Acknowledgment

The authors thank Taichung Veterans General Hospital, Taiwan for providing human specimen for this work.

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