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Original Paper

Far-Infrared Irradiation Inhibits Adipogenic Differentiation and Stimulates Osteogenic Differentiation of Human Tonsil-Derived Mesenchymal Stem Cells: Role of Protein Phosphatase 2B

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Key Words
Far-infrared irradiation • Tonsil-derived mesenchymal stem cells • Adipogenesis • Osteogenesis • Protein phosphatase 2B

Abstract
Background/Aims: Far-infrared (FIR) irradiation has been reported to exhibit various biological effects including improvement of cardiovascular function. However, its effect on the differentiation of stem cells has not been studied. Using tonsil-derived mesenchymal stem cells (TMSC), we examined whether and how FIR irradiation affects adipogenic or osteogenic differentiation. ***Methods:*** TMSC were exposed to FIR irradiation (3-25 μm wavelength) for various times (0, 30, or 60 min), and then adipogenic or osteogenic differentiation was induced for 14 days with its respective commercially available differentiation medium. At the end of the differentiation, the cells were stained using Oil red O or Alizarin red S solution, and the expression of differentiation-specific proteins was analyzed by western blotting. ***Results:*** FIR irradiation did not alter cell viability or the expression of MSC–specific surface antigens (CD14, CD34, CD45, CD73, CD90, and CD105) in TMSC. However, FIR irradiation significantly inhibited adipogenic differentiation of TMSC, as evidenced by decreased Oil red O staining as well as protein expression of peroxisome proliferator-activated receptor γ and fatty acid binding protein 4. In contrast, FIR irradiation induced osteogenic differentiation, as evidenced by increased Alizarin red S staining as well as protein expression of osteocalcin and alkaline

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phosphatase. Treatment with heat alone did not inhibit the adipogenic differentiation of TMSC, suggesting that the inhibitory effect on adipogenic differentiation was not due to heat induced by FIR irradiation. However, heat alone did stimulate osteogenic differentiation, but to a lesser extent than FIR irradiation. Furthermore, FIR irradiation increased intracellular Ca^{2+} levels and the activity of protein phosphatase 2B (PP2B) in TMSC. Treatment with cyclosporin A, a specific PP2B inhibitor, reversed the inhibitory effect of FIR irradiation on adipogenic differentiation of TMSC, but had no effect on osteogenic differentiation. ***Conclusion:*** Our data demonstrate that FIR irradiation inhibits adipogenic differentiation but enhances osteogenic differentiation of TMSC; the inhibitory effect on adipogenic differentiation is non-thermal and mediated at least in part by activation of Ca^{2+}-dependent PP2B.

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Introduction

Stem cells are generally defined as undifferentiated cells capable of self-renewal and differentiation into specialized cells, and can be classified based on their origin [1]. Stem cells are largely classified into two groups; embryonic stem cells derived from totipotent cells of the early mammalian embryo [2] or mesenchymal stem cells (MSC) derived from a variety of adult tissues, including bone marrow, adipose, and umbilical cord tissue [3-6]. MSC can mainly be differentiated into skeletal cells, such as osteocytes, chondrocytes, and adipocytes [7, 8]. We previously established tonsil-derived MSC (TMSC) from human palatine tonsils, and they have been showing great differentiation potentials into several cell types, including adipocytes, osteocytes, chondrocytes, Schwann cells, muscle cells, insulin-releasing cells, tenocytes, and parathyroid hormone-releasing cells [9-15]. In addition to their superior differentiation potential, TMSC are obtained by completely non-invasive tonsillectomy and proliferate faster (~1.5 times faster) than bone marrow-derived MSC (BM-MSC). Because of these advantages, TMSC have been proposed as a potential new MSC for clinical studies [16-18].

Far-infrared (FIR) is one of the three infrared regions, and is defined by the International Commission on Illumination as an invisible electromagnetic wave with a wavelength of 3-1000 μm [19]. FIR irradiation is known to contribute to a variety of biological effects including improved vascular function [20], which is attributable in part to upregulation of the vasodilator nitric oxide (NO) in endothelial cells (EC) [21]. Furthermore, FIR irradiation has also been reported to inhibit cancer cell proliferation through regulation of heat shock protein 70A [22]. Although a single recent paper described the effect of FIR irradiation on the basic properties of murine BM-MSC, such as proliferation and survival [23], the effect of FIR on differentiation has not been reported.

Protein phosphatase 2B (PP2B, also known as calcineurin), a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, is an important mediator of intracellular Ca^{2+} signaling in a number of cell types, and is inhibited by cyclosporin A (CsA) and FK506 [24, 25]. A previous study revealed that increased activity of Ca^{2+}-dependent PP2B in 3T3-L1 preadipocytes inhibits adipocyte differentiation by inhibiting the expression of peroxisome proliferator-activator receptor γ (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) [26]. Furthermore, PP2B is expressed in osteoblasts and regulates bone formation *in vitro* and *in vivo* [27]. However, little is known about the direct effect of PP2B on adipogenic or osteogenic differentiation in stem cells.

We previously reported that FIR irradiation increases NO production through intracellular Ca^{2+} mobilization in bovine aortic EC (BAEC) [21]. Based on these findings, we investigated whether FIR irradiation also increases intracellular Ca^{2+} levels in TMSC and its potential effects on Ca^{2+}-dependent PP2B activity, which in turn affects the differentiation potential of TMSC into adipocytes and osteocytes.

Materials and Methods

Isolation and culture of TMSC

TMSC were isolated as described previously [9]. Briefly, TMSC were isolated from the tonsillar tissues of patients (aged ≤ 10 years) undergoing tonsillectomy. Informed written consent was obtained from the legal guardians of the patients who participated in this study and the study protocol was approved by the Institutional Review Board (ECT-11-58-37) of Ewha Womans University Mokdong Hospital.

Isolated tonsil tissues were digested in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 210 U/mL collagenase type I (Invitrogen) and 10 mg/mL DNase (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 min at 37°C. After incubation, the digested tissues were filtered using a wire mesh and washed twice in Dulbecco's Modified Eagle Medium containing high glucose (4,500 mg/L) (DMEM-HG; Welgene Inc., Gyeongsan, Korea) and 20% fetal bovine serum (FBS; Invitrogen) and once in DMEM-HG with 10% FBS. Mononuclear cells were obtained by density gradient centrifugation using Ficoll-Paque (GE Healthcare, Little Chalfont, UK) and plated in a T-150 culture flask (Corning, Tewksbury, MA, USA) in DMEM-HG with 10% FBS. After incubation for 48 h, non-adherent cells were removed by washing and adherent cells were further incubated in the cell culture incubator at 37°C under 5% CO_2.

FIR irradiation

FIR irradiation was performed using an AC-driven constant-power lamp controller (Model No. S-O.T.H 9H, Saeik Medical Co., Ltd., Bucheon, Korea) with a ceramic infrared radiator (Model No. IOT/90-250, Elstein-Werk M. Steinmetz GmbH & Co. KG, Northeim, Germany), as described previously [28] with slight modifications. The emission wavelength of the FIR radiator ranged from 3 to 25 μm, with a peak at 7.5 μm. The FIR radiator was pre-warmed for 30 min at room temperature, and then TMSC, cultured in a 60-mm cell culture plate with 4 mL medium, were placed at a distance of 30 cm from the radiator and exposed for the indicated times (0, 30, or 60 min). TMSC were further incubated in the culture chamber at 37°C under 5% CO_2. In a separate experiment, TMSC were incubated at 38°C on a Chamlide heat incubator (Model No. CU-109, Live Cell Instrument, Seoul, Korea) for 30 min.

Cell viability and proliferation

Cell viability was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution. Cells were seeded onto 48-well plates and exposed to FIR irradiation for the indicated times. MTT solution was added to each well, and the culture plate was incubated for 2 h in a 37°C incubator. The medium was removed and the purple formazan product was dissolved using dimethyl sulfoxide. Dissolved solutions were transferred to a 96-well plate and the absorbance was measured at 540 nm with a microplate reader. For the measurement of proliferation, TMSC exposed to FIR irradiation were further incubated for 18, 24, or 48 h and subjected to MTT assay. Proliferation was expressed as the absorbance at indicated times relative to that at time 0.

Fluorescence-activated cell sorting (FACS) analysis

After exposure of TMSC to FIR irradiation for 0 or 30 min, cells were collected and stained with phycoerythrin (PE)-conjugated anti-human CD14, CD34, CD73, and fluorescein isothiocyanate (FITC)-conjugated anti-human CD45, CD90, CD105 antibodies. Stained cells were analyzed using a FACSCalibur system (BD Biosciences, San Diego, CA, USA). All antibodies were purchased from BD Biosciences.

Adipogenic or osteogenic differentiation and drug treatments

TMSC at a confluence of 80-90% were exposed to FIR irradiation for the indicated times and incubated in commercially available adipogenic or osteogenic differentiation medium (Cat. No. A10070-01 or A10072-01; Invitrogen) for up to 14 days. The culture medium was changed every 3 or 4 days. In some experiments, TMSC were pretreated with CsA (0, 0.25, 0.5, and 1 μM; Sigma-Aldrich) before exposure to FIR irradiation and then subjected to differentiation. After differentiation, cells were fixed with 4% formalin for 30 min and washed with phosphate-buffered saline. Adipogenic differentiated cells were stained with Oil Red O solution and osteogenic differentiated cells with Alizarin red S solution for 1 h at room temperature. The remaining excessive staining solution was removed, and stained cells were visualized under a microscope.

Western blot analysis

Cells were extracted using lysis buffer containing Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN, USA). The protein samples (equal quantities of 20 μg) were separated using SDS gel electrophoresis and transferred onto nitrocellulose membranes. The blots were probed with primary antibodies against transcriptional coactivator with PDZ-binding motif (TAZ), PPARγ, or fatty acid binding protein 4 (FABP4) (each at a 1:1000 dilution; Cell Signaling Technology, Boston, MA, USA); osteocalcin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); alkaline phosphatase (ALP; 1:1000; Abcam, Cambridge, UK); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2500 dilution; AbFrontier, Seoul, Korea) followed by the corresponding secondary antibodies, and the proteins were detected by enhanced chemiluminescence detection methods (Amersham, Buckinghamshire, UK).

Intracellular calcium measurement

The membrane-permeable calcium indicator dye Fluo-4 AM (Invitrogen) was used to measure intracellular Ca^{2+} levels as described previously [21]. Fluorescence images were obtained using a confocal microscope (LSM5 Pascall, Carl ZEISS, Oberkochen, Germany).

Protein phosphatase 2B (PP2B) activity assay

PP2B activity was measured using the PP2B cellular activity kit (Enzo Life Sciences, Farmingdale, NY, USA) with minor modification. Briefly, TMSC were exposed to FIR irradiation for 0 or 30 min and rinsed in ice-cold tris-buffered saline (20 mM Tris, pH 7.2, 150 mM NaCl). After washing, cells were lysed in lysis buffer with protease inhibitors and centrifuged at 16,000 g for 20 min. To remove free phosphate, the supernatant extracts were exposed to desalting column resin. The extracted supernatant (500 μg) was immunoprecipitated using anti-PP2B antibody (Cell Signaling Technology) and washed three times with lysis buffer. The immunoprecipitated samples were assessed using a PP2B cellular activity kit (Enzo Life Sciences) as described in the instruction manual. PP2B activity was quantified by measuring absorbance at 620 nm and normalized using the controls.

Statistical analysis

All results are presented as the means ± standard deviations (S.D.). Statistical significance was determined using Student's t-test. A value of $p<0.05$ was considered significant. All experiments were performed at least three times.

Results

FIR irradiation does not affect cell viability, expression of MSC-specific surface antigen markers, and proliferation of TMSC

To assess whether FIR irradiation affects the viability of TMSC, cells were exposed to FIR irradiation for 0, 30, or 60 min at room temperature. Results of the MTT assay showed that FIR irradiation did not affect cell viability, even at the maximum exposure time of 60 min (Fig. 1A). Next, we examined whether FIR irradiation alters the surface immunophenotypic character of TMSC. FACS analysis revealed that the surface markers tested in this study were not altered by FIR irradiation (Fig. 1B); TMSC in the absence or presence of FIR irradiation were negative for hematopoietic surface markers (CD14, CD34, and CD45) and displayed significant positive expression of MSC-specific surface markers (CD73, CD90, and CD105). Cell proliferation was also tested by further incubation of TMSC for 0, 18, 24, or 48 h after FIR irradiation. FIR irradiation affected unlikely TMSC growth compared with untreated controls (Fig. 1C). Together, these results suggest that FIR irradiation did not alter the viability, surface antigen expression, or proliferation of TMSC under our experimental conditions.

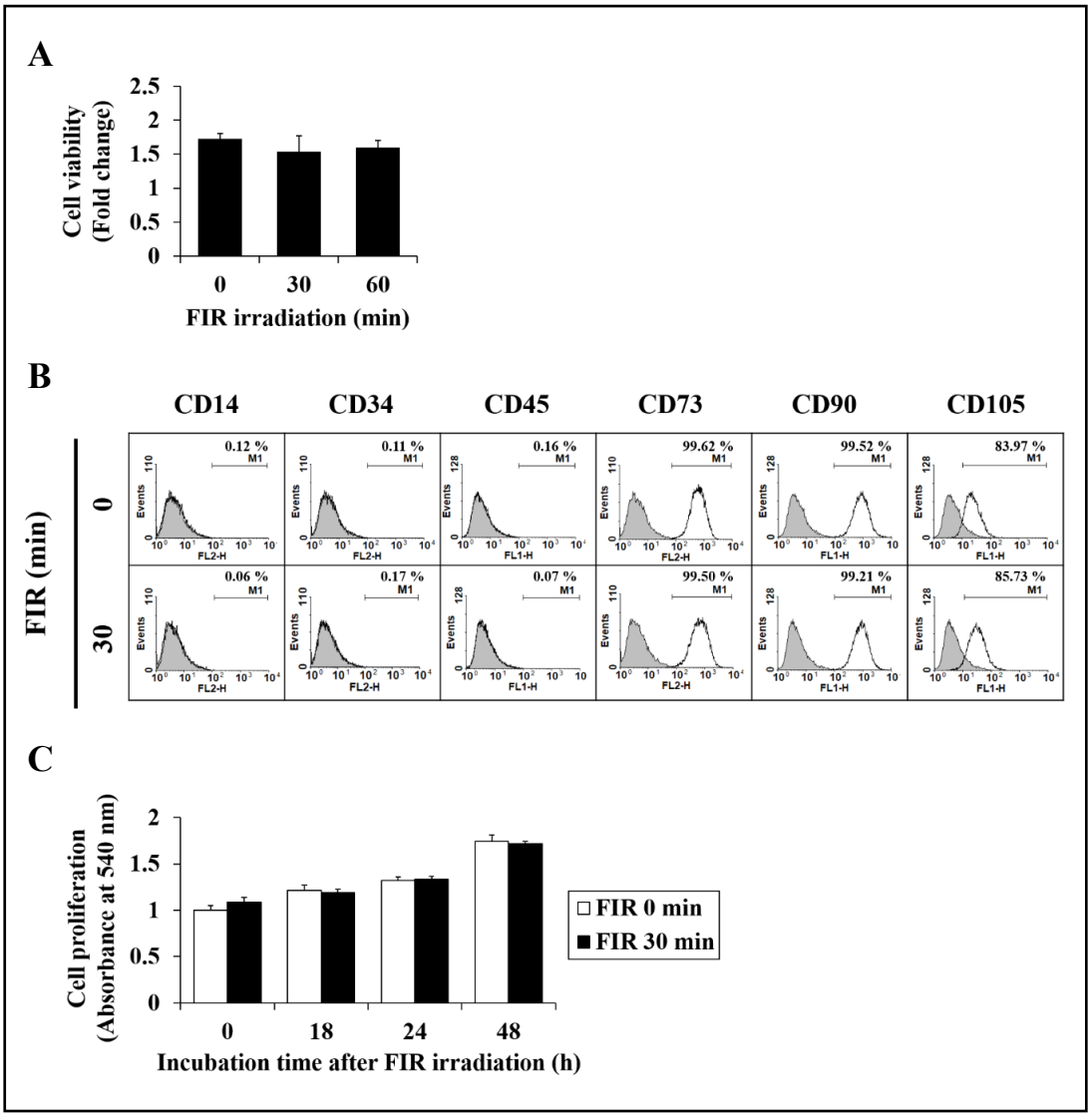

Fig. 1. FIR irradiation does not alter cell viability, expression of MSC-specific surface antigen markers, and proliferation of TMSC. TMSC were exposed to FIR irradiation at room temperature for 0, 30, and 60 min. (A) The viability of TMSC was measured by MTT assay. (B) The profiles of MSC-specific surface markers were analyzed by flow cytometry. (C) After FIR irradiation (30 min), the cells were further incubated for 0, 24, and 48 h in the cell culture incubator and cell growth was determined by MTT assay. The values of cell growth are expressed as means ± S.D (n=3).

Acute exposure to FIR irradiation inhibits adipogenic differentiation but promotes osteogenic differentiation of TMSC

To investigate whether FIR irradiation affects the differentiation of TMSC, cells were exposed to FIR irradiation for 30 and 60 min and then further differentiated using commercially available adipogenic or osteogenic differentiation medium for up to 14 days. As shown in Fig. 2A, lipid accumulation was significantly suppressed by FIR irradiation in a time-dependent manner. The expression levels of the adipocyte-specific markers PPARγ and FABP4 were also significantly downregulated (Fig. 2B). In contrast, as exposure time of FIR irradiation increased, mineralization was promoted and the protein expression of osteocyte-specific markers osteocalcin and ALP also significantly increased (Fig. 2C, D).

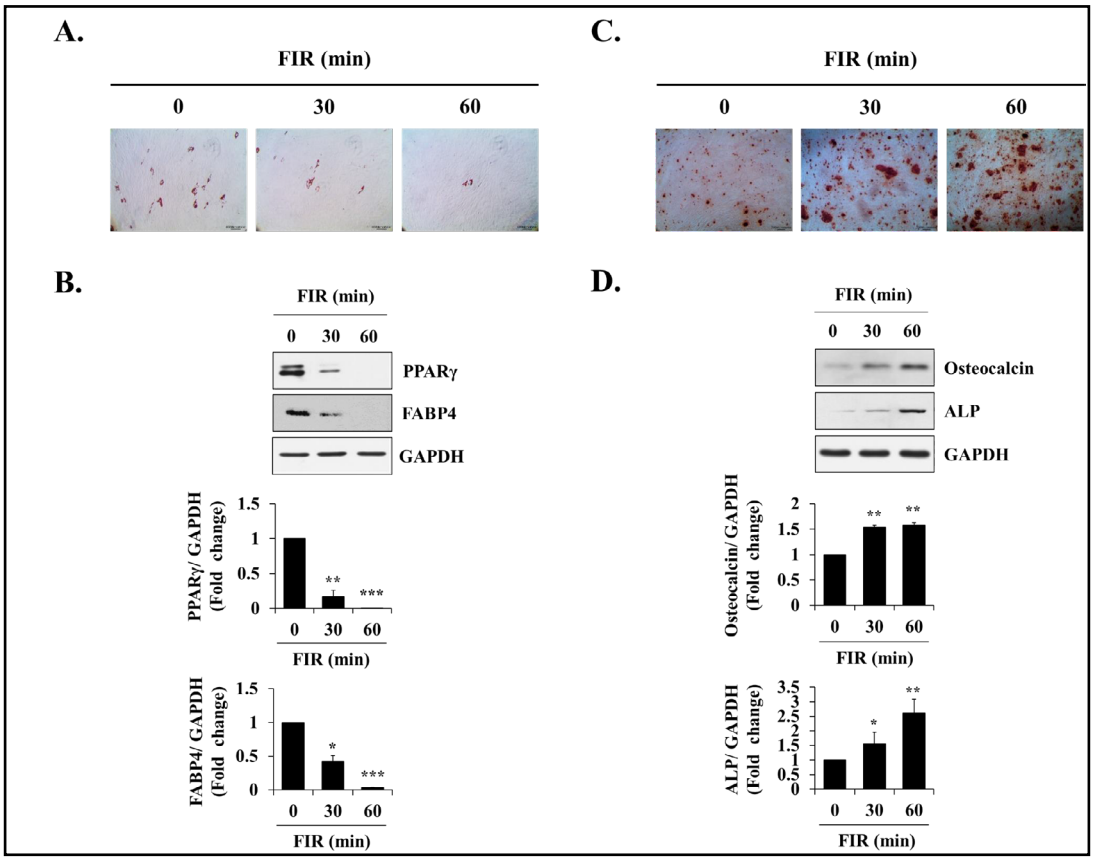

Fig. 2. Acute exposure to FIR irradiation inhibits adipogenic differentiation and promotes osteogenic differentiation of TMSC. TMSC were exposed to FIR irradiation for 0, 30, and 60 min and further incubated in commercially available adipogenic or osteogenic differentiation medium for up to 14 days. (A) The accumulation of lipid droplets was assessed using Oil red O staining. Scale bar = 100 μm. (B) Protein levels of PPARγ and FABP4 were measured by western blot analysis. GAPDH was used as a loading control for normalization. (C) Mineralization was assessed using Alizarin red S staining. Scale bar = 200 μm. (D) Protein levels of osteocalcin and ALP were measured by western blot analysis. GAPDH was used as a loading control for normalization. The results are representative of three independent experiments, and each bar represents the mean ± S.D. Differences were statistically significant at *p<0.05, **p<0.01, and ***p<0.001.

The inhibitory activity of FIR irradiation on adipogenic differentiation is not caused by thermal effects

Previously, we reported that FIR irradiation increases the temperature of the culture medium to 38 ± 1°C [28]. Based on this finding, we examined whether the heat alone induced by FIR irradiation regulates adipogenesis or osteogenesis of TMSC. Compared with untreated control cells, heat treatment (at 38°C) alone using the Chamlide heat incubator did not inhibit adipogenic differentiation of TMSC as evidenced by Oil red O staining (Fig. 3A) and the expression of PPARγ and FABP4 (Fig. 3B). Next, we tested the thermal effect of FIR irradiation on osteogenesis of TMSC. Although heat alone did stimulate osteogenic differentiation of TMSC, as evidenced by Alizarin red S staining, its effect was smaller than that with FIR irradiation (Fig. 3C). Furthermore, heat alone also increased osteocalcin expression but to a lesser extent than FIR irradiation (Fig. 3D). However, the protein expression of ALP was not significantly different between heat-treated and FIR-irradiated TMSC. Together, these results suggest that the inhibitory effect of FIR irradiation on adipogenic differentiation of TMSC is unlikely to be due to the thermal effect caused by FIR irradiation.

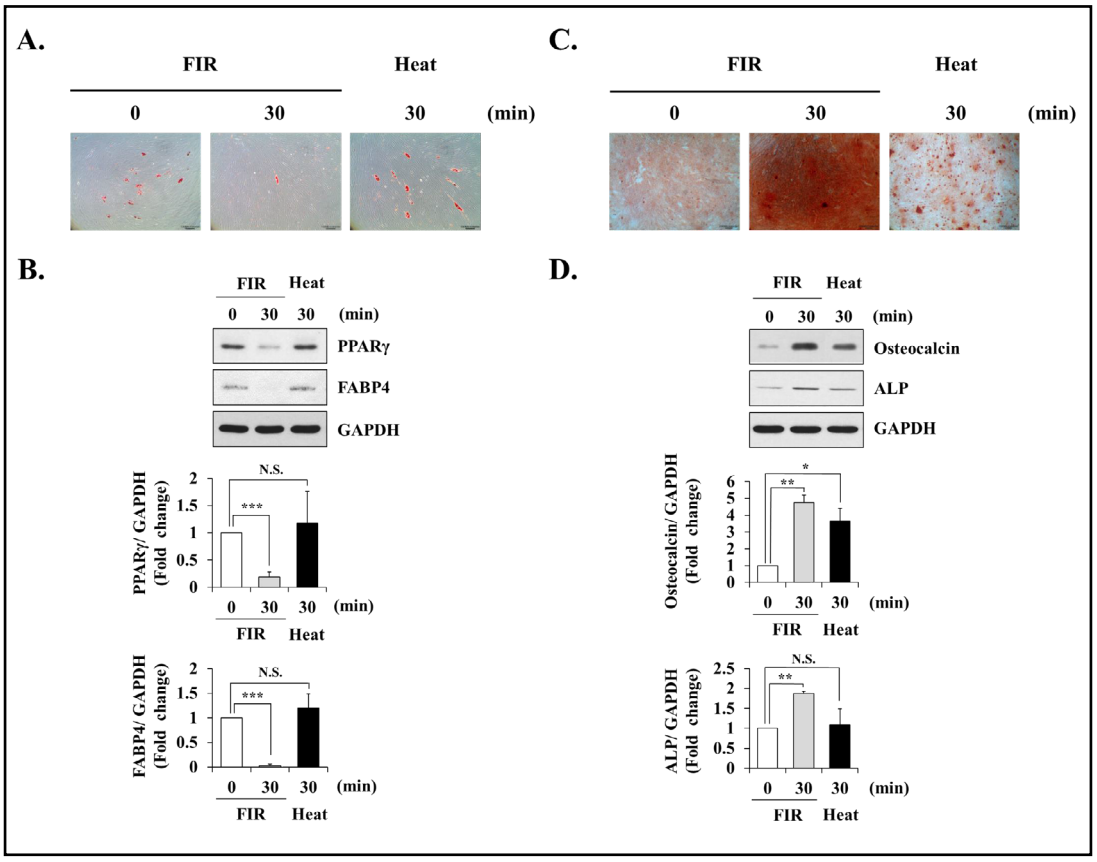

Fig. 3. Effects of thermal treatment on adipogenic or osteogenic differentiation of TMSC. TMSC were exposed to FIR irradiation or heat (38°C) using the Chamlide heat incubator for 30 min and further incubated in differentiation medium for up to 14 days. (A) Accumulation of lipid droplets, (B) protein levels of PPARγ, FABP4, and GAPDH, (C) mineralization, and (D) protein levels of osteocalcin, ALP, and GAPDH were measured as described in the legend of Fig. 2. The results are representative of three independent experiments, and each bar represents the mean ± S.D. Differences were statistically significant at *p<0.05, **p<0.01, and ***p<0.001. N.S., not significant.

Transcriptional coactivator with PDZ-binding motif (TAZ) is not involved in the adipogenic or osteogenic differentiation of TMSC under our experimental conditions

TAZ is a well-known transcriptional modulator that regulates adipogenesis and osteogenesis of MSC; it inhibits adipogenic differentiation and promotes osteogenic differentiation [29]. Based on this knowledge, we investigated whether TAZ regulates the FIR irradiation–induced decrease in adipogenesis and increase in osteogenesis of TMSC. If this is the case, we expected that FIR irradiation would increase TAZ protein expression. Under our conditions, however, FIR irradiation for 30 min decreased the protein level of TAZ (Fig. 4), suggesting no evidence for involvement of TAZ in the adipogenic or osteogenic differentiation of TMSC by FIR irradiation.

Cyclosporin A, a specific inhibitor of protein phosphatase 2B (PP2B) activity, reverses the effect of FIR irradiation on adipogenic differentiation but not osteogenic differentiation

Several studies have shown that PP2B, known as an intracellular Ca^{2+}-dependent phosphatase, exhibits a regulatory role in adipogenesis or osteogenesis [26, 27]. These reports, together with our previous study showing increased intracellular Ca^{2+} levels in FIR-irradiated BAEC [21], prompted us to examine whether PP2B is also involved in FIR irradiation–mediated effects on each type of differentiation in TMSC. As in BAEC, we found that FIR irradiation increased intracellular Ca^{2+} levels in TMSC (Fig. 5A). Furthermore, PP2B

activity was significantly elevated in FIR-irradiated TMSC (Fig. 5B). To examine whether PP2B is also involved in the effect of FIR irradiation on the differentiation of TMSC, we used a specific inhibitor of PP2B, CsA. As shown in Fig. 6A, treatment with CsA (up to 0.5 μM) clearly reversed the decrease in lipid accumulation induced by FIR irradiation. This reversal also occurred at a higher dose of CsA (1 μM), but without statistical significance. Similarly, the inhibitory effect of FIR irradiation on PPARγ and FABP4 expression was also significantly reversed when TMSC were treated with CsA at concentrations up to 0.5 μM (Fig. 6B) whereas no significant reversal occurred in TMSC treated with 1 μM CsA. Interestingly, none of the tested doses of CsA altered FIR irradiation-stimulated osteogenic differentiation with respect to mineralization (Fig. 6C) and protein expression of osteocalcin and ALP (Fig. 6D). These data suggest an important role for PP2B in regulating adipogenesis, but not osteogenesis, of TMSC induced by FIR irradiation.

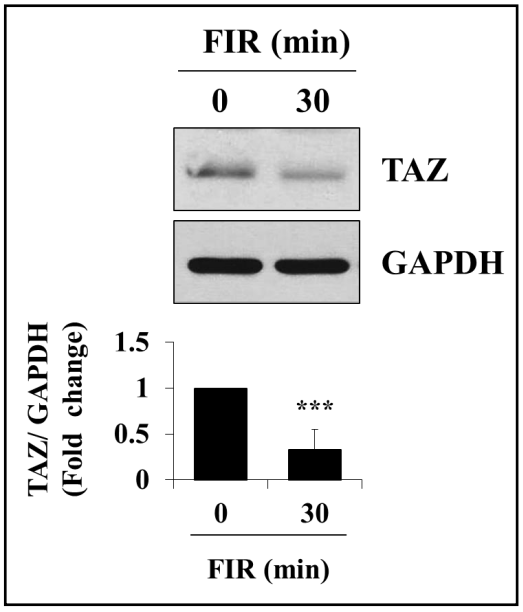

Fig. 4. Expression of TAZ as a modulator of MSC differentiation. TMSC were exposed to FIR irradiation for 30 min. Protein levels of TAZ and GAPDH were measured as described in the legend of Fig. 2. The results are representative of three independent experiments, and the bar represents the mean ± S.D. Difference was statistically significant at ***p<0.001.

Discussion

Previous studies have shown that FIR irradiation has therapeutic effects in various disease models, including models of cardiovascular disease and cancer [30, 31]. For the last decade, several types of cells, including EC and cancer cells [21, 22], have

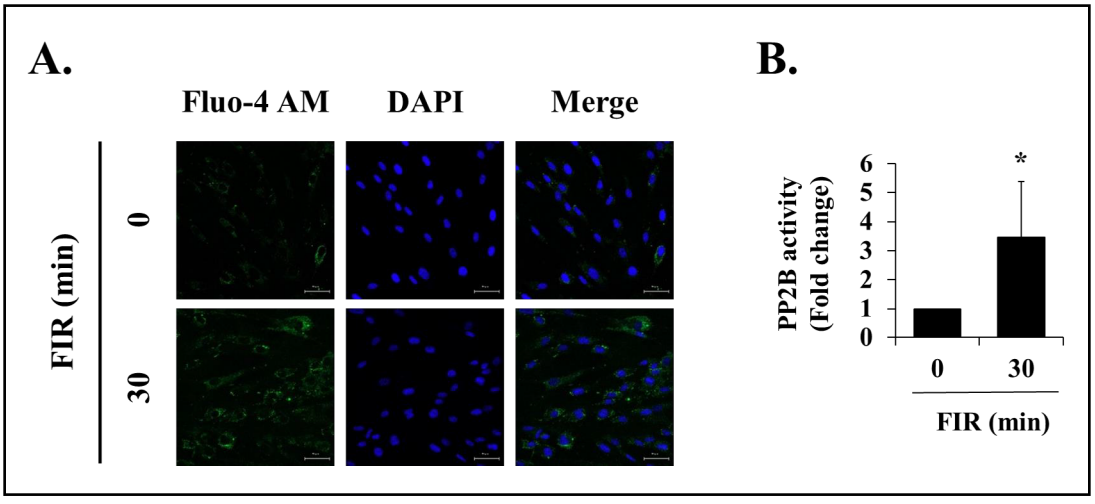

Fig. 5. FIR irradiation increases the activity of Ca^{2+}-mediated PP2B. (A) TMSC were acutely exposed to FIR irradiation for 30 min in media containing 1 μM Fluo-4 AM. Images of intracellular Ca^{2+} were visualized using a confocal microscope. Scale bar = 50 μm. (B) TMSC were exposed to FIR irradiation for 30 min. PP2B protein was immunoprecipitated using an anti-PP2B antibody and PP2B activity in the precipitates was measured using a PP2B cellular activity kit. Each bar represents the mean ± S.D. (n=3). Difference was statistically significant at *p<0.05.

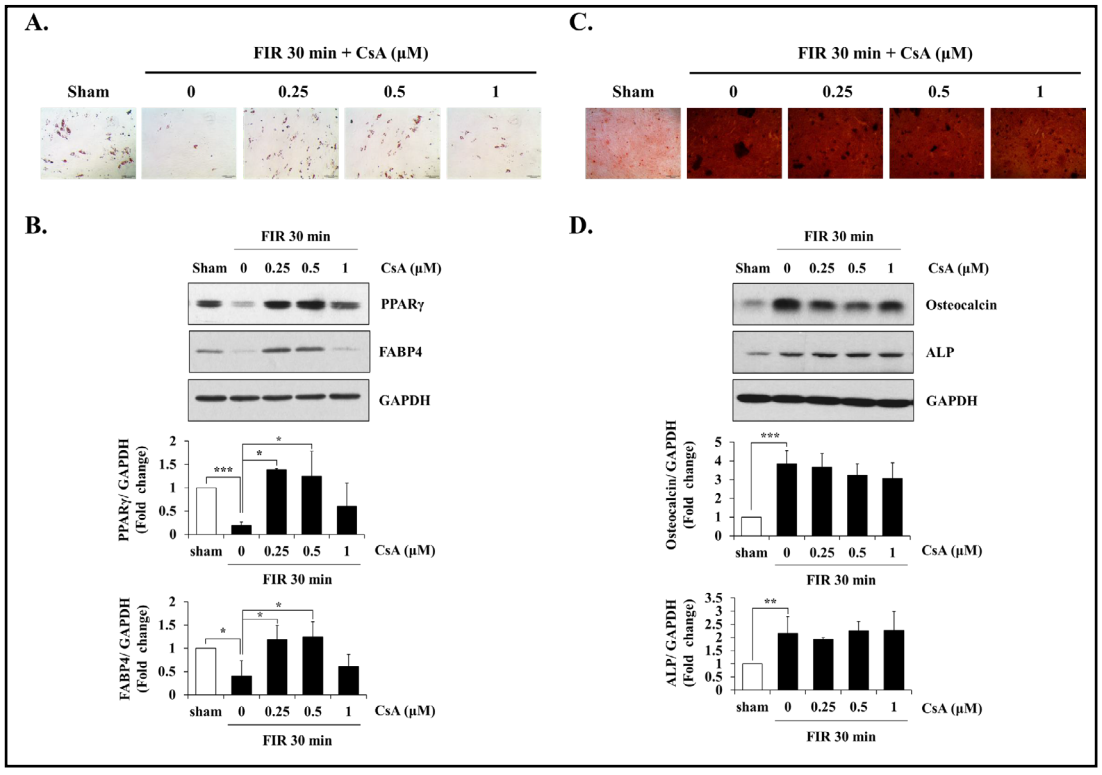

Fig. 6. Effects of activation of Ca^{2+}-mediated PP2B on adipogenic or osteogenic differentiation of TMSC. (A-B) After pretreatment with CsA (0, 0.25, 0.5, and 1 μM), TMSC were exposed to FIR irradiation for 30 min and further incubated in adipogenic differentiation medium for up to 14 days. Control cells without CsA pretreatment were used as Sham. (A) Accumulation of lipid droplets and (B) protein levels of PPARγ, FABP4, and GAPDH were measured as described in the legend of Fig. 2. (C-D) TMSC were pretreated with CsA before exposure to FIR irradiation, and further incubated in differentiation medium as described in the legend of Fig. 6A with the exception of using osteogenic medium instead of adipogenic medium. Control cells without CsA pretreatment were used as Sham. (C) Mineralization and (D) protein levels of osteocalcin, ALP, and GAPDH were measured as described in the legend of Fig. 2. The results are representative of three independent experiments, and each bar represents the mean ± S.D. Differences were statistically significant at *p<0.05, **p<0.01, and ***p<0.001.

been used to examine the molecular and cellular mechanisms underlying the therapeutic effects of FIR irradiation. Recent studies have identified stem cells as a potentially good source of cells for exploring mechanisms underlying the therapeutic effects of various bioactive drugs [32, 33]. Although one recent study showed that FIR irradiation slightly alters basic characteristics of murine BM-MSC, such as proliferation and survival [23], there is no study exploring the effects of FIR irradiation on differentiation of stem cells. Here, we demonstrate that FIR irradiation of TMSC significantly inhibits adipogenesis through a mechanism mediated by a Ca^{2+}-dependent PP2B signaling pathway. Furthermore, FIR irradiation also induces osteogenesis, but this does not appear to be related to a Ca^{2+}-dependent PP2B signaling pathway.

One of the most important findings in this study is that FIR irradiation inhibits adipogenic differentiation and promotes osteogenic differentiation of TMSC. There are accumulated data showing that several stimuli play a dual role in regulating adipogenic and osteogenic differentiation in MSC. For example, hypoxia was reported to inhibit adipogenesis and promote osteogenesis of BM-MSC in a hypoxia-inducible factor-1–dependent manner [34], which is consistent with our current data. Furthermore, microgravity alters both adipogenesis and osteogenesis in rat BM-MSC [35], and interestingly, these effects are dose dependent; longer exposure (10 days) to microgravity inhibits adipogenesis and promotes

osteogenesis whereas shorter exposure (3 days) shows opposite results. Similar to shorter exposure to microgravity, repeated exposure to mechanical vibration also promotes adipogenesis and inhibits osteogenesis of BM-MSC [36]. Based on our findings that a very short exposure time (only 30 min) is sufficient for inhibition of adipogenesis and promotion of osteogenesis by FIR irradiation, in contrast to the relatively long exposure times required for hypoxia and microgravity to regulate the differentiation of MSC, we suggest that FIR irradiation may provide a safer and more effective therapeutic treatment.

Unlike the previous study showing that heat stimulation alone significantly reduces the early adipogenesis of 3T3-L1 preadipocytes [37], we found that heat alone did not simulate the inhibitory effect of FIR irradiation on adipogenesis of TMSC, suggesting a heat-independent mechanism of FIR irradiation. At present, the reason for these inconsistent results has not been identified, but it may be attributable to the different conditions used: we used a 38°C heating system and TMSC whereas the previous study used a >43°C heating system and 3T3-L1 preadipocytes. Nonetheless, it is possible that the particular form of electromagnetic energy, but not the form of heat energy itself, produced by FIR plays a role in inhibiting adipogenesis of TMSC. In this regard, we also reported the heat-independent inhibitory effect of FIR irradiation on EC growth and angiogenesis [28]. However, our data showed partial heat dependence for FIR irradiation–stimulated osteogenic differentiation in TMSC, which reproduces previous data showing that heat stress at 38-40°C promotes osteogenesis of dental follicle stem cells [38].

Adipogenic and osteogenic differentiation of MSC are balanced by a transcriptional modulator, TAZ. TAZ is a coactivator of Runx2, which regulates osteocalcin expression to promote osteogenic differentiation, but simultaneously inhibits adipogenic differentiation by directly inhibiting PPARγ-dependent transcriptional events essential for adipogenesis [29]. Because our data showed that FIR irradiation inhibited adipogenic differentiation and stimulated osteogenic differentiation in TMSC, we predicted that TAZ might be involved in the FIR irradiation–induced TMSC differentiation into adipocytes and osteocytes. Previous reports showed that a reduced protein level of TAZ promotes adipogenic differentiation and inhibits osteogenic differentiation of MSC [39]. Surprisingly, however, FIR irradiation clearly decreased, rather than increased, the protein expression of TAZ. Based on this finding, we suggest that there is no evidence for involvement of TAZ in the dual effects of FIR irradiation on the adipogenic and osteogenic differentiation of TMSC; however, we cannot exclude involvement of some factor(s) other than TAZ in the observed effects of FIR irradiation in TMSC.

Ca^{2+} is an important mediator involved in various cellular processes, such as cell growth, apoptosis, and differentiation. In particular, intracellular and extracellular Ca^{2+} concentrations have been reported to affect adipogenic or osteogenic differentiation [40-42]. We previously reported that FIR irradiation increased intracellular Ca^{2+} levels in BAEC, mediating increased eNOS phosphorylation at serine 1977 and thus increasing NO production [21]. With respect to the increased levels of intracellular Ca^{2+}, the current data using TMSC reproduce our previous data in BAEC. Using CsA, we further demonstrate that Ca^{2+}-dependent PP2B activity mediates the regulatory effect of FIR irradiation on adipogenesis, but not osteogenesis, of TMSC. Consistent with our data, a previous study showed that CsA attenuates adipogenesis of 3T3-L1 preadipocytes induced by the Ca^{2+} ionophore ionomycin [26]. Furthermore, the authors of this previous study also reported that reversal of adipogenesis by CsA is mediated by the suppression of proadipogenic transcription factors (i.e., PPARγ and C/EBPα). Taking these findings together, it is clear that PP2B plays an important role in adipogenesis of several cells, including TMSC. Interestingly, our study showed that CsA (up to 0.5 μM) clearly reversed the inhibitory effect of FIR irradiation on adipogenesis. This reversal was also seen at a higher dose (1 μM) although it was not statistically significant. At present, we cannot explain why a higher dose of CsA attenuated reversal of the inhibitory effect on adipogenesis by FIR irradiation. A previous study reported that treatment with CsA (1 μg/mL) alone had no effect on adipogenic differentiation of 3T3-L1 preadipocytes [26], although another study reported that a higher dose (10 μg/mL) in itself inhibits the adipogenic differentiation of

3T3-L1 preadipocytes [43]. A concentration of 1 μg/mL CsA is equivalent to ~0.8 μM. Based on these previous studies, it is likely that under our experimental conditions CsA was an inhibitor of PP2B but not an inhibitor of adipogenic differentiation, which further validates our conclusion that the inhibitory effect of FIR irradiation on adipogenic differentiation is mediated by PP2B activation. Regarding the involvement of PP2B in osteogenic differentiation, it was reported that deficiency in PP2B catalytic subunit A exhibited osteoporosis *in vivo* and *in vitro* [27], suggesting a role for activation of PP2B in promoting osteogenic differentiation. However, from our findings, it seems unlikely that the stimulation of osteogenesis by FIR irradiation is mediated by PP2B activation. Identifying signaling molecules other than PP2B involving in FIR irradiation-induced osteogenic differentiation of TMSC is important and needs further investigations. Previously, we reported that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) plays an important role in promoting osteogenesis of human BM-MSC by the peptide with a novel collagen-binding motif derived from osteopontin [44]. Consistent with our previous study, a recent study also showed that a constant electric field induces osteogenic differentiation of rat BM-MSC on TiO_2 nanotubular layer via CaMKII [45]. With these findings, together with the previous data showing that FIR irradiation significantly activates CaMKII in BAEC [21], it seems likely that CaMKII is a potential candidate protein in stimulating osteogenic differentiation of TMSC. Further study is needed to clarify this issue.

Obesity is a medical condition of excessive fat accumulation in adipose tissues. Adipocytes, which are of mesodermal origin, are generally known to be derived from stem cells and are produced through a series of differentiation processes. Abnormal development of MSC into adipocytes leads to hyperplasia of adipocytes, which may cause obesity. Although the direct link between MSC and hyperplasia of adipocytes is not known, our study suggests that FIR irradiation can be used as a tool to understand its underlying mechanism and perhaps to treat obesity. In this regard, FIR sauna therapy was reported to significantly reduce body weight and body fat in obese patients [46]. There are also a few findings of *in vivo* beneficial effects of FIR irradiation on bone formation; for example, FIR irradiation was reported to promote bone-forming activity of osteoblasts and increase bone mineral density when natural FIR ceramics were implanted under the periosteum of rat skull [47]. Our current data, together with these previous findings, may extend the potential use of FIR therapy in clinical applications to treat obesity and repair bone defects.

Conclusion

We demonstrate that FIR irradiation inhibits adipogenic differentiation and promotes osteogenic differentiation of TMSC. Furthermore, the inhibition of adipogenic differentiation by FIR irradiation is mediated at least in part by Ca^{2+}-dependent PP2B activation, but the stimulation of osteogenesis is unlikely to be PP2B-dependent. Lastly, our findings may advance our understanding of the molecular and cellular mechanism by which FIR therapy decreases body fat in obese patients and repairs bone defects.

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Disclosure Statement

All authors have declared that there is no conflict of interests.

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