Bioelectromagnetics 43:245–256 (2022)

Electromagnetic Fields Generated by the IteraCoil Device Differentiate Mesenchymal Stem Progenitor Cells Into the Osteogenic Lineage

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Rapid advances in mesenchymal stem progenitor cells (MSPCs) have rendered impetus into the area of cell therapy and regenerative medicine. The main promise of future stem cell therapies is their reliance on autologous stem cells derived from adipose tissue, which also includes treatments of bone fractures and degeneration. The effectiveness of different electric devices utilized to reprogram MSPCs toward osteogenic differentiation has provided varying degrees of effectiveness for clinical use. Adipose tissue-derived MSPCs were flow-cytometrically characterized and further differentiated into osteoblasts by culturing either in growth medium with pro-osteogenic supplements or without supplements with alternating electromagnetic field (EMF) generated by IteraCoil. IteraCoil is a multi-solenoid coil with a specific complex geometry that creates a 3D-EMF with desired parameters without directly applying electrodes to the cells and tissues. The flow-cytometric analysis of highly enriched (≥95%) adipose-derived MSPCs ($CD34^-$, $CD73^+$, $CD90^+$, and $CD105^+$) was utilized for the study. Osteoblasts and chondrocyte differentiations were then assessed by specific staining and quantified using ImageJ (National Institutes of Health). The osteoblastic differentiation of MSPCs cultured in regular medium and exposed to EMF at 0.05 and 1 kHz frequencies was compared with MSPCs cultured in a pro-osteogenic supplemented medium. In this study, we demonstrated that EMF from IteraCoil might have affected the signaling pathways that induce the osteogenic differentiation of human adipose-derived MSPCs in the absence of exogenous osteogenic factors. Therefore, EMF-generated osteogenic differentiation of reprogrammed adipose-derived autologous MSPCs may treat the loss of osteoblasts and osteoporosis and open new avenues for the development of regenerative cellular therapy. Bioelectromagnetics. 43:245–256, 2022. © 2022 Bioelectromagnetics Society.

Keywords: **mesenchymal stem progenitor cells (MSPCs); alternating electromagnetic field (EMF); alternating current (AC); osteogenic differentiation; chondrogenic differentiation**

INTRODUCTION

Osteoporosis and osteoporosis-related fractures are becoming the most prevalent degenerative bone diseases within the aging population. More than 75 million people suffer from osteoporosis in the United States, the European Union, and Japan [Bicer et al., 2021], and it is anticipated that by 2050 the number of patients affected by osteoporosis will gradually increase by one-third of the currently reported cases. Conventional therapies such as bisphosphonates, calcitonin, and estrogen-like drugs used to treat degenerative bone diseases are often associated with serious side effects including the development of esophageal cancer, ocular inflammation, severe

Grant sponsor: SkinAxis LLC.

Conflicts of interest: None.

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Received for review 14 November 2021; Revised 11 January 2-022; Accepted 20 March 2022

DOI:10.1002/bem.22401
Published online 07 April 2022 in Wiley Online Library (wileyonlinelibrary.com).

musculoskeletal pain, and osteonecrosis of the jaw [Kim and Mikos, 2020; Bicer et al., 2021]. The use of autologous mesenchymal stem progenitor cells (MSPCs) is a possible alternative therapeutic approach to recover osteoblast loss and treat osteoporosis [Jamal and de Guzman, 2017].

In the last 20 years, the use of MSPCs has been growing in different therapeutic areas and the use of autologous stem cells isolated from adipose tissue holds great promise for future stem cell therapies [Tsuchiya et al., 2019; Zhang et al., 2020]. Over 300 clinical trials worldwide have been registered for different diseases, and about a hundred new trials are added each year. The safety of MSCs was reported for both autologous and allogeneic MPSC therapies-systematic meta-analyses of side effects reported in MPSC clinical trials showed transient fever with no infusion toxicity, organ system complications, infection, death, or malignancies [Lalu et al., 2018].

Subcutaneous fat is a rich source of multipotent adipose-derived stem cells (ASC) or MSCs [Tang et al., 2008]. These cells share several properties with bone marrow-derived MSPCs including similar anti-inflammatory cytokine secretion profiles and their ability to differentiate into cell types that hold great potential for tissue engineering and clinical regenerative medicine [Boregowda et al., 2018; Holm et al., 2018]. MSC isolation from the stroma of adipose tissue is much easier and less invasive than stem cells derived from bone marrow [Oliva et al., 2019].

The characterization of MSC populations depends in part on the methods used to evaluate their differentiation potential [Phinney, 2007]. Clonal studies have shown that adherent populations of MSPCs are functionally heterogeneous and contain undifferentiated progenitors with varying capacities to differentiate into different cell types. In order to increase their therapeutic potency, MSPCs require priming and fine-tuning by utilizing various culture conditions supplemented with different growth factors [Boregowda et al., 2018]. A number of in vitro culture conditions have been exploited to induce differentiation of MSPCs into various terminally differentiated cells such as adipocytes, fibroblasts, endothelial cells, osteoblasts [Mussano et al., 2018], chondrocytes, cardiomyocytes, neural-like cells [Di Summa et al., 2018; Lo Furno et al., 2018; Petersen et al., 2018], hepatocytes [Liau et al., 2018], and pancreatic cells [Arzouni et al., 2017]. In multiple sclerosis, human MSPCs promote endogenous myelin repair by stimulating Th2 polarized immune response in vivo [Bai et al., 2009a,b]. The phenotypic signatures of MSPCs ($CD73^+$, $CD90^+$, and $CD105^+$) exhibit differential potential toward differentiation of various cell types including adipocytes, osteoblasts, and chondrocytes in the presence of suitable culture conditions [Boquest et al., 2005; Tsuchiya et al., 2019].

Numerous studies have demonstrated that the application of electric currents may be a useful tool for triggering desirable changes in living cells and tissues [Levin, 2014]. One such method is the effects of electrical current on tissue regeneration, stem cell differentiation, and induction of apoptosis in cancer cells [Levin et al., 2017; Saria and Kesari, 2016]. Reports indicate that both alternating current (AC) and direct current (DC) electric (electromagnetic) fields were equally capable of inducing osteogenic differentiation in MSPCs [Hu et al., 2019]. Different laboratories have utilized a great variety of parameters such as electric current signal shape (sinusoidal, quasi-rectangular, etc.), voltage, duty cycle, time interval, and treatment duration. However, the majority of studies have focused on demonstrating the effectiveness of AC at lower frequencies. It has been reported that electrical stimulation with 448 kHz promotes musculoskeletal lesion repair by activating the proliferation of stem cells present in the injured tissues [Sun et al., 2007; Hernandez-Bule et al., 2014]. By contrast, osteoblast differentiation and improved bone restoration have been obtained at a very low frequency of 15 Hz [Zeighami et al., 2019]. Furthermore, electric pulse current ACE at 1 Hz of AC resulted in sinusoidal electric field-induced cytoplasmic calcium level fluctuations that corroborated with enhanced osteogenic differentiation of stem and/or progenitor cells [McCullen et al., 2010]. However, the different frequencies of pulsed electromagnetic fields (EMFs) starting from 1 Hz to 448 kHz showed the highest efficiency at a range lower than 150 Hz. Another study specifically tested the whole range from 1 to 150 Hz for differentiation of osteoblasts from MSPCs and observed the effective peak at 50 Hz [Luo et al., 2012].

In these studies, bone markers and genes were found to be upregulated in the absence of pro-osteogenic factors in the culture medium when MSCs were exposed to electric currents, and in some other studies, the bone markers and genes were upregulated even long after the exposure was stopped [Eischen-Loges et al., 2018; Wu et al., 2018]. The widest spectrum of therapeutic effects on reprogramming of different stem cells has been documented by utilizing electric currents from accelerated wound healing at their potential of

differentiation into various cellular lineages required for improved bone healing, retinal disease improvement (retinitis pigmentosa), and human stem/progenitor cell differentiation [Ross et al., 2015; Wagner et al., 2017; Ashrafi et al., 2019].

After a wide spectrum of innovative research in EMF in a decade, it has become imperative to translate this rapidly developing technology into clinical regenerative practices [Ross et al., 2015; Wagner et al., 2017; Ashrafi et al., 2019]. Although MSCs have shown promising results in the preclinical and clinical studies, there are still unmet challenges regarding manipulation of target cellular/tissue area through easy access for utilizing electric impulses for a variety of reasons such as: (i) identifying the target cells' localization could be difficult without using cumbersome identification methods; (ii) direct application of electric current to the target tissue may cause undesirable chemical interaction (e.g., electrolysis) generated in the actual contact area of electroconductive material and the tissue under treatment; (iii) the physical access to target cells for applying electricity directly may prove to be difficult or even impossible to achieve the goals due to existence of highly differentiated cells/tissues surrounding the target cells that could be damaged by the electrode passing through them (e.g., brain tissue); (iv) complex, elaborate, and expensive techniques may be required for delivering electricity to cells and tissues (molecular scaffolds, networks, salt bridges, or other methods of delivering electric currents or fields to the cells) [Kim et al., 2009; McCullen et al., 2010; Hronik-Tupaj et al., 2011; Creecy et al., 2013; Cakmak et al., 2016; Zhang et al., 2016].

To deliver EMFs of desired parameters to MSPCs in the growth medium, we utilized a multi-solenoid coil (IteraCoil). A comparative analysis was performed between MSPCs exposed to electric fields of 0.05 and 1 kHz frequencies in a growth medium lacking growth supplements, and un-exposed MSPCs grown in the medium with or without pro-osteogenic supplements [Guasti et al., 2012].

MATERIALS AND METHODS

Isolation of MSPCs From Adipose Tissue

Freshly harvested human breast-derived adipose tissue was purchased from the National Disease Research Interchange (Philadelphia, PA). Adipose tissue was centrifuged at 20*g* for 6 min to obtain about 50 ml of concentrated tissue. Concentrated adipose tissue was mixed with 50 ml of digestion solution (560 U/ml-collagenase type I) in phosphate-buffered saline (PBS) and incubated for 1 h at 37 °C [Domenis et al., 2015; Meyer et al., 2015]. The digested tissue was centrifuged at 300*g* for 10 min. The upper fraction containing mature adipocytes was removed. The remaining lower stromal fraction was treated with red blood cell lysis buffer (Sigma, St. Louis, MO) for 10 min at room temperature. The suspension was filtered through a 100 μm nylon mesh (BD Biosciences, San Diego, CA) to remove cellular debris and centrifuged at 300*g* for 10 min. Cells were seeded at a concentration of $2.5 \times 10^3/cm^2$ in DMEM (Dulbecco's modified Eagle's medium)-high glucose medium supplemented with 10% FBS (fetal bovine serum; Invitrogen, Carlsbad, CA) with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml Fungizone) in a humidified atmosphere of 5% CO_2 at 37 °C.

Cells were cultured in T75 corning cell culture flasks with high glucose DMEM with 10% FBS for 10 days with replacement of medium to fresh medium every 2–3 days. The cells were 70-80% confluent on Day 14, then FACS analyses were performed. The cells used in all experiments were from the fourth passage.

Flow Cytometry

MSPCs (~1×10^6 cells) were labeled with 10 μl of either mouse anti-human CD44-fluorescein isothiocyanate (BD Biosciences), mouse anti-human CD90-, CD34-, CD45-, and CD14-phycoerythrin (PE) (Thy; BioLegend, Cambridge, UK), or mouse anti-human CD105-PE (SouthernBiotech, Birmingham, AL) in PBS, and 1% bovine serum albumin (BSA). After two washes in 1% BSA in PBS, cells were fixed in 4% PFA and analyzed with Beckman Coulter instrument (Beckman Coulter, Fullerton, CA), and histographs were created using FlowJo software (Tree Star, Ashland, OR).

Differentiation of MSPCs Into Osteoblasts By Using Pro-Osteogenic Medium

To differentiate MSPCs into osteoblasts, the growth medium of MSPCs (2×10^6 cells) was replaced with a pro-osteogenic differentiation medium. For in vitro mineralization assay, MPSC were maintained in pro-osteogenic differentiation medium for 3 weeks with replacement of medium to fresh medium every 2–3 days. The pro-osteogenic medium contained high glucose DMEM, supplemented with 10% FBS with 1 nM dexamethasone, 2 mM β-glycerol phosphate disodium salt, 50 μM 2 phospho-LA Ascorbic acid, and 1% penicillin/streptomycin. Cells were grown in humidified incubators at 37 °C with 5% CO_2. Osteogenic lineage differentiation was assessed by Alizarin Red staining,

and Alcian blue staining was used for chondrogenic differentiation [Guasti et al., 2012].

Differentiation of MSPCs Into Osteoblasts By Using IteraCoil

To differentiate MSPCs into osteoblasts by EMF, cells cultured in a growth medium without supplements were stimulated with EMF at 0.05 kHz and 1 kHz frequencies for 1 h twice per week for three consecutive weeks.

The selection of the investigated frequencies of 0.05 kHz (50 Hz), and 1 kHz (1000 Hz) was guided by data in previous literature. Specifically, the 50 Hz frequency for stimulation of the cells was chosen based on previous work from various research groups [Lim et al., 2013; Zeighami et al., 2019], which demonstrated that the best osteogenic induction occurred in the frequency range of 1–150 Hz with the peak effect at 50 Hz. However, they treated cells every day for 4 h a day. We were interested in finding out if less frequent electrical stimulation at 50 Hz (twice a week instead of every day, as they reported) could induce osteogenic differentiation of MSPCs by using the IteraCoil device and in culture conditions without pro-osteogenic supplements. This was not an unreasonable expectation, as there are reports of lesser time and frequency of exposure to electric fields (even a single exposure of MSPC at 15 Hz for 10 min) that successfully induced osteogenic differentiation of MSPC's [Parate et al., 2017]

We used stimulation of cells twice a week because it was stressful for cells to be trypsinized every day before electrical stimulation. We also employed a lesser time of electrical stimulation (1 h instead of 4 h as in some other studies) because the viability of cells was consistent at 1 h post-electrical stimulation, and with longer exposure time cell viability had gradually decreased (data not shown).

Osteogenic differentiation was assessed by Alizarin Red staining and chondrogenic differentiation with Alcian blue staining.

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Magnetic protection film (MuMetal Magnetic Shielding foil 0.012" × 8" × 12") used for the IteraCoil device was purchased from Magnetic Shield (Bensenville, IL).

IteraCoil Device

IteraCoil device was custom-manufactured by A. Tsaghikian, Ph.D. Its electromagnetic coil portion has the following parameters: the spool is 3D-printed using ABS plastic, has a cylindrical tube shape with a length of 80 mm, an inner diameter of 12 mm, and outer diameter of 14 mm. Enameled Copper wire (diameter—0.4 mm, length—15.6 m, total resistivity —2.12 Ω; Remington Industries, Johnsburg, IL) is tightly wound without gaps around the annealed iron wire (mild steel, diameter—1.0 mm, length—1600 mm; GoodFellowUSA.com)—secondary solenoid, which is then tightly wound without gaps around the spool—primary solenoid. The secondary solenoid, in which the core is made of iron wire with high magnetic permeability, was structured to create an alternating magnetic field with lines of force parallel to the long axis of the core. As the iron wire core was wound into the primary solenoid, it would create an alternating electric field within its core (the test tube) with lines of force parallel to its long axis. To register the electric fields in the test tube with media, the electrodes were connected to an oscilloscope (Hantek DSO5072P Digital Oscilloscope, 70 MHz Bandwidth, purchased from M&A Instruments, Arcadia, CA).

Statistical Analysis

Experiments were performed on cells from a single donor. For all experiments, four to six biological replicates were used with proper controls. Data are presented as means ± SEM. Quantitative analysis using ImageJ (National Institutes of Health, Bethesda, MD) to determine the percentage of the area presented in the photograph that contained Alizarin Red or Alcian Blue staining was performed. Statistical significance was evaluated by analysis of variance and Student's t test, and P-value < 0.05 was considered statistically significant.

RESULTS

Device Description and Testing

A particular embodiment of the IteraCoil device (Fig. 1A and B) was utilized for differentiation of adipose-derived MSPCs, having two separate IteraCoils on a platform with an attached ventilation system (Fig. 1B) to keep the temperature within the coils below 37 °C. The inner space within each IteraCoil was designed to fit one individual standard lab test tube (10 mm × 80 mm). Each individual coil was wrapped in a magnetic protection film to prevent electromagnetic interaction between the two coils, allowing two tests to run at the same time. The electronic portion of the device was well placed within the platform. It was designed to deliver a

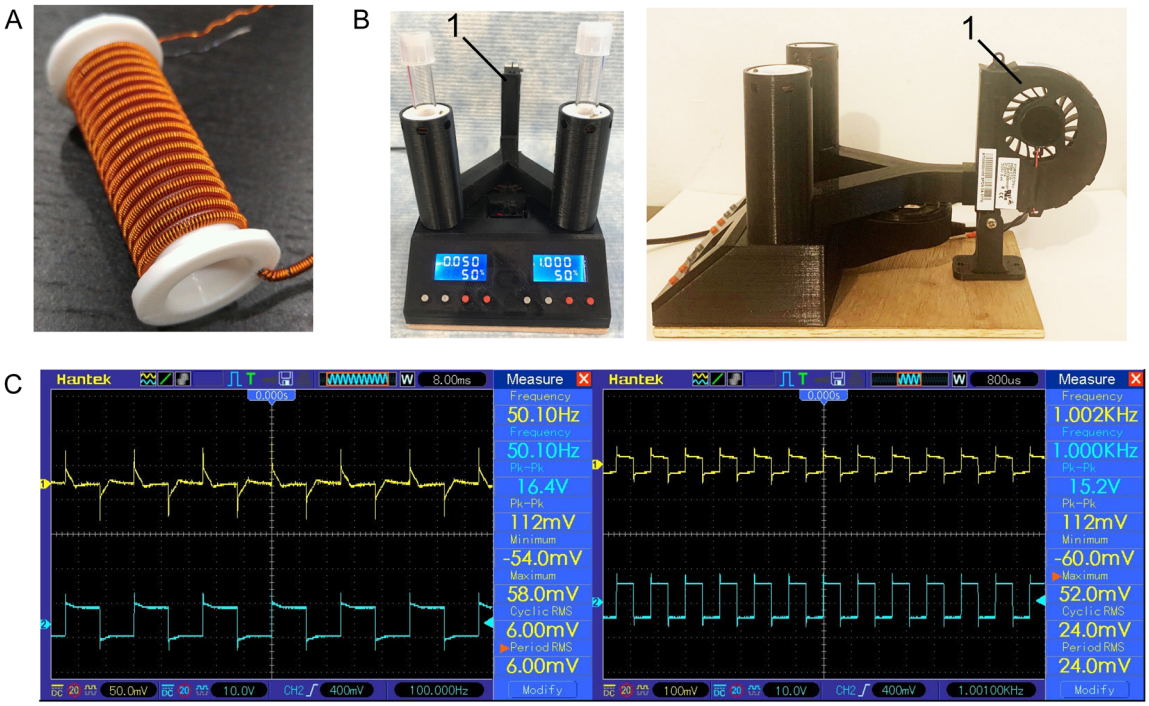

Fig. 1. IteraCoil device and electrical signals measured within the test tubs. (**A**) IteraCoil is a solenoid copper wire wrapped around an iron wire, which is then curved into another solenoid (**B**) Electrical Device and B1-cooling fan (**C**) Electric signals within the test tube are measured directly by an oscilloscope. The shapes of the input (blue) and output (yellow) curves in left and right panels, respectively.

quasi-rectangular alternating electric current of specific frequency and duty cycle to each IteraCoil. The frequency could be set on the dashboard between 1 and 10,000 Hz, and the duty cycle could be selected to be between 1 and 100%. Prior to carrying the experiments, the temperature of the inner surface of the wall of each coil's inner space was continuously monitored using a 1-wire programmable digital thermometer DS18B20 (Kynix, Shenzhen, China) for 2 h with the power block in ON position, and with following settings: Left Coil—duty cycle 50%, frequency 50 Hz, Right Coil—duty cycle 50%, frequency 1000 Hz. The temperatures fluctuated between 30.2 °C and 36.8 °C.

The particular embodiment of IteraCoil used in our experiments is a dual solenoid – it is a solenoid copper wire wrapped around an iron wire, which is then curved into another solenoid (Fig. 1A). When AC current is brought to the copper solenoid wire (Fig. 1A), an alternating magnetic field is created in the iron solenoid wire, which in turn creates an alternating electric field within its cylindrically shaped space. The selection of wire materials is based on the requirement to have a high electric conductivity in the first wire (copper), and high magnetic permeability in the second (iron). The quasi-rectangular shape of the AC current in copper wire translates into an electric field within the iron solenoid space that has a similar shape and frequency.

The use of the IteraCoil device avoided several undesired challenges and side effects such as electrochemical reactions at the point of contact of the cells/tissue with the electrode, or the necessity to create molecular scaffolds, networks, or salt bridges, or other methods of delivering electric charges or fields to the cells [Kim et al., 2009; McCullen et al., 2010; Hronik-Tupaj et al., 2011; Creecy et al., 2013; Cakmak et al., 2016; Zhang et al., 2016].

Before starting the experiment with MSPCs, the IteraCoil device was tested with 4 different cell culture media and buffers—DMEM, PBS, RPMI (Roswell Park Memorial Institute), and Normal Saline. A glass tube (same material and size as the lab test tubes were used in the actual experiment) individually containing each one of the media, with electrodes inserted on both ends, was placed in the coil space.

The electrodes were connected to an oscilloscope (Hantek DSO5072P Digital Oscilloscope, 70 MHz bandwidth; M&A Instruments) for registering

the electric fields in the test media (Fig. 1B). The input electric signals from the IteraCoil's electronic block were set on three different frequencies (50, 500, 1000 Hz) and duty cycle settings (20%, 50%, 80%) for testing within each culture medium/buffers and control (empty test tube) (Fig. 1B). For each run of testing, a quasi-rectangular signal of one setting (frequency, duty cycle) was applied to the coil, and the electric potential within the liquid was registered on the oscilloscope (Fig. 1C). The results demonstrated that the coil had consistently created within the media the electric potentials of the same frequency and duty cycle as the input, mostly of quasi-rectangular shape. The shapes of the input (blue) and output (yellow) curves are apparent in Figure 1C left and right panels. Preliminary studies have been conducted to assess the magnitudes of electric fields within the test tube. Metallic electrodes were inserted at both ends of the test tube; the distance between the electrodes was 80 mm. Four different media were used for testing: Normal Saline, PBS, DMEM, and RPMI. The AC power source was set at 3 different frequencies—50, 500, 1000 Hz, and in each case—at three different duty cycles—20%, 50%, 80%. All four media were tested with all three frequencies and all three duty cycle parameters (total of 36 different combinations); results are presented in Table 1. The Input Peak-Peak Voltage varied between 13.2 and 16.8 Volt. The Output Pk-Pk varied between 92 and 116 mV (1.15–1.45 mV/mm), while RMS varied between 8 and 20 mV (0.1–0.25 mV/mm) (Table 1).

When performing the actual experiments, two test tubes containing 2×10^6 MPCSs in PBS were inserted in each of the IteraCoil spaces. A quasi-rectangular AC current with a duty cycle of 50% for all testing, and a frequency of either 50 Hz (coil #1—left) or 1000 Hz (coil #2—right), was selected for each IteraCoil, and was applied as per protocol for 1 h each time twice a week for 3 consecutive weeks. The temperatures of the mediums within the coils were measured periodically every 10 min with the use of a laser thermometer BT980D (MachineSense, Baltimore, MD), and recorded prior to the start of the experiments, and they remained within a narrow range of 35.6– 36.8 °C with the help of a cooling fan (Fig. 1B1).

TABLE 1. Results of Testing Different Control Solutions in Electrical Device for Output Measurement of Electricity (mV/mm)

Frequency Hz (duty cycle %)	Empty test tube	PBS	DMEM	RPMI	Saline
50 (50%)	0	1.325	1.4	1.45	1.325
50 (20%)	0	1.35	1.425	1.3	1.275
50 (80%)	0	1.35	1.425	1.325	1.275
500 (50%)	0	1.4	1.35	1.3	1.45
500 (20%)	0	1.45	1.5	1.35	1.5
500 (80%)	0	1.45	1.5	1.4	1.5
1000 (50%)	0	1.3	1.4	1.3	1.25
1000 (20%)	0	1.3	1.3	1.3	1.3
1000 (80%)	0	1.25	1.35	1.3	1.25

DMEM = Dulbecco's modified Eagle's medium; PBS = phosphate-buffered saline; RPMI = Roswell Park Memorial Institute.

MSPCs' Characterization and Differentiation

Isolated MPSCs (as described in the Materials and Methods section by enzymatic digestion from normal adipose tissue) were expanded in DMEM plus high glucose and 10% FBS for 2 weeks. The expanded cells were phenotypically stained with MSPC surface markers such as anti-CD34, -CD73, -CD90, -CD105) antibodies. Flow cytometry analysis confirmed the enrichment of MSPCs ($CD73^+$, $CD90^+CD105^+$; Fig. 2, upper panel); and, the expression of these phenotypic markers remained unaltered following the cell freezing (Fig. 2, lower panel).

Previous reports were greatly focused on the usage of exogenous chemicals and biological compounds to induce osteogenic differentiation of MSPCs. The role of electrical stimulation has been demonstrated in the osteogenic differentiation of MSPCs [Wan et al., 2007; Cakmak et al., 2016]. Electrical signals of different frequencies (e.g., 15 and 1 Hz) were shown to facilitate bone restoration by stimulating osteoblasts [McCullen et al., 2010; Zeighami et al., 2019]**.** In contrast to our experiments, the electrical stimulation of MSPCs in these experiments was performed in the presence of an osteogenic medium [McCullen et al., 2010; Zeighami et al., 2019]**.**

Furthermore, Kammerer et al. [2020] demonstrated that electrical stimulation of MSPC with 1 kHz induced proliferation, but not differentiation. We also used 1 kHz electrical stimulation of MSPCs with the expectation of determining the proliferation of cells, but not differentiation. However, under these conditions, we observed osteogenic differentiation of the cells.

The MSPCs were subjected to EMF stimulation at high (1 kHz) and low (0.05 kHz) frequencies for 1 h twice a week for 3 consecutive weeks while culture was maintained with growth medium plus high glucose-containing DMEM supplemented with 10% FBS and 2 mM glutamine. The temperatures of the cell-containing mediums within the coils were measured periodically every 10 min with the use of a laser

thermometer BT980D during the experiments and again remained within the range of 35.6–36.8 °C.

Untreated MSPCs were grown in parallel either in the same medium or in the pro-osteogenic medium as a negative and/or positive control, respectively (Fig. 3). Alizarin Red staining demonstrated osteoblast formations when MSPCs were cultured in the pro-osteogenic medium but failed to differentiate into osteoblasts in the control medium (Fig. 4A and B). Similarly, MSPCs pulsed with 1 kHz and 0.05 kHz EMF showed increased calcium deposits (Fig. 4C and D). Alizarin Red staining of calcium deposits was clearly detectable in MSPCs following culture in osteogenic medium or EMF stimulation, but not in MSPCs grown in control medium.

In addition, quantitative analysis using ImageJ demonstrated that the percentage area that stained positively for Alizarin Red was significantly higher in osteogenic medium cultured cells (25.5%±4.8) and cells treated with ACE frequencies of 1 kHz (30.8% ± 8.03) and 0.05 kHz (30% ± 1.3), as compared to the cells grown in only growth medium referred to as control (5.6% ± 1.65) and stained 3 weeks after treatment (Fig. 4E).

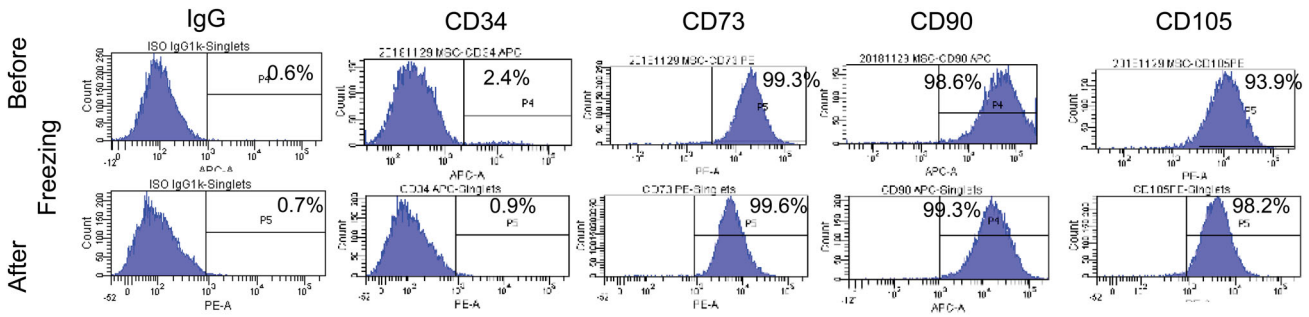

Fig. 2. Cell freezing does not affect the expression of MSPCs markers. The cytographs depict the flow cytometric analysis of the expression of surface markers, CD73, CD90, CD105, gated on the isotype control of MPSCs. Before freezing (upper panel) and after freezing (lower panel). MSPC = mesenchymal stem progenitor cell.

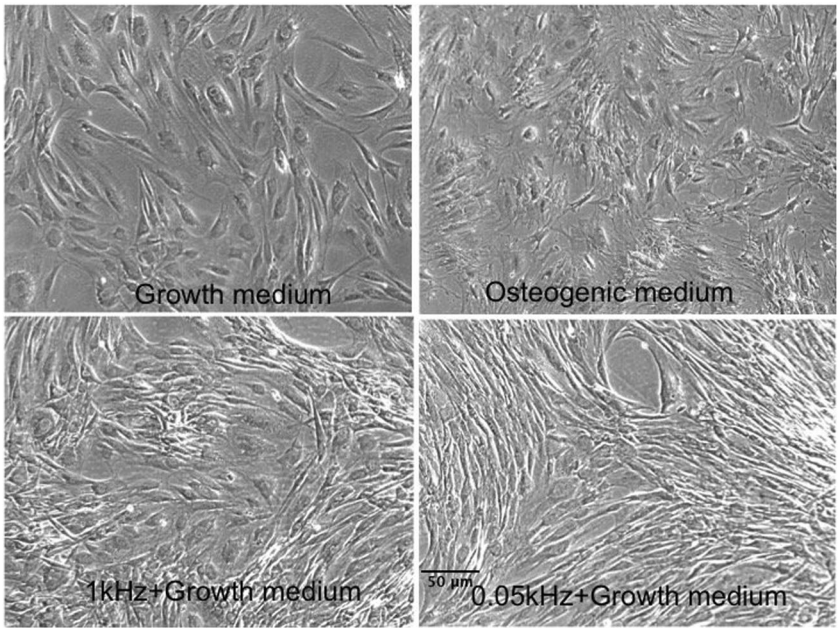

Fig. 3. Phase-contrast photomicrographs of MSPCs grown under different conditions. MPSCs were grown either in growth medium (GM) (Control) or osteogenic medium (upper panel) or cultured in the GM and subjected to electrical stimulation (lower panel) twice a week for three consecutive weeks (at 1 or 0.05 kHz) (magnification: ×200). MSPC = mesenchymal stem progenitor cell.

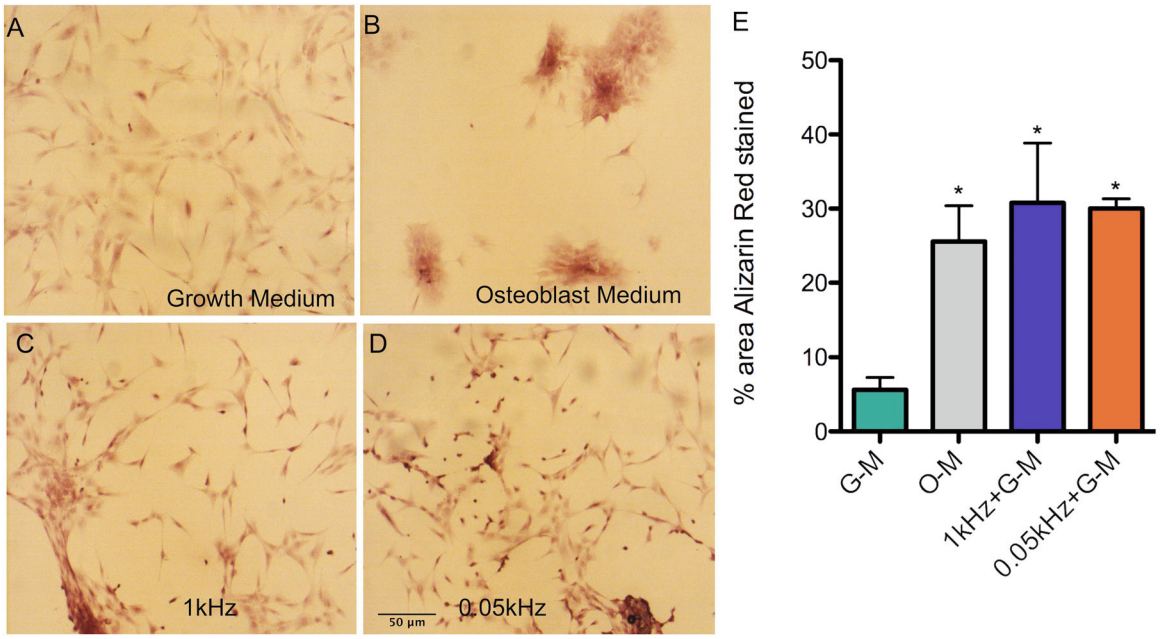

Fig. 4. Osteogenic medium or EMF treatment of MSPCs stimulates calcium deposits in the extracellular matrix of differentiated cells. MPSCs cultured in (**A**) growth medium (GM) or (**B**) osteogenic differentiation medium (ODM), or in growth medium (GM) and stimulated with (**C**) EMF 1 kHz or (**D**) 0.05 kHz for 1 h twice a week for 3 consecutive weeks, and then stained with Alizarin Red (**E**). The quantitative analysis using ImageJ revealed that the percentage area stained positively for Alizarin Red was significantly higher in treated cells than in growth medium cultured cells. One-way ANOVA: data are expressed as mean ± SD, $n = 6$, $^*P < 0.05$ vs. growth medium treated cells. ANOVA = analysis of variance; EMF = electromagnetic field; MSPC = mesenchymal stem progenitor cell.

Kwon et al. [2016] have demonstrated that the EMF triggers MSPC condensation and subsequently involves Ca^{2+}/ATP signaling cascades required for the process of chondrogenesis. A low-frequency electromagnetic stimulation on the differentiation of stem cells to osteoblasts appears to play role in chondrogenesis [Mayer-Wagner et al., 2011]. Therefore, we explored the possibility that osteogenic medium and different alternating electrical current (AEC) treatments of MSPCs could induce chondrogenesis. This was further validated by Alcian Blue staining of proteoglycan accumulation during chondrogenesis.

Positive staining for Alcian Blue was detected in MSPCs cultured in the osteogenic medium as well as cells cultured in growth medium and further stimulated by different frequencies of AEC (Fig. 5B–D). To quantitatively determine the percentage area that contained Alcian blue-stained cells by utilizing different treatments, ImageJ analyses were performed. The percentage of Alcian Blue positive cells was significantly higher (7.2%) in osteogenic medium, or when stimulated at 1 kHz (8.5%) or 0.05 kHz (9.7%), as compared to cells cultured in growth medium (1.8%; Fig. 5E).

Altogether, these data were consistent with osteogenesis and chondrogenesis induction in cells stimulated by AEC. Furthermore, Alizarin Red staining demonstrated a greater number of osteogenic induction of cells than chondriogenic cells stained with Alcian Blue (Figs. 4E and 5E). The comparative analysis of differences between the percentages of positive area for Alizarin Red or Alcian Blue stained cells grown in different conditions was not found statistically different from each other.

DISCUSSION

Bioelectrical signals can be potent regulators of cellular and tissue functions [Levin, 2021]. Several experiments conducted in the last decade illustrate how electrical signals can affect genetic networks and signaling pathways, and influence wound healing, embryonic stem cell differentiation, regenerative cell therapy, and cancer [Levin and Stevenson, 2012; Cai et al., 2017; Ashrafi et al., 2019].

Tissue-specific stem cells are regarded as the source for tissue repair and therefore considered as important players in regenerative medicine because of

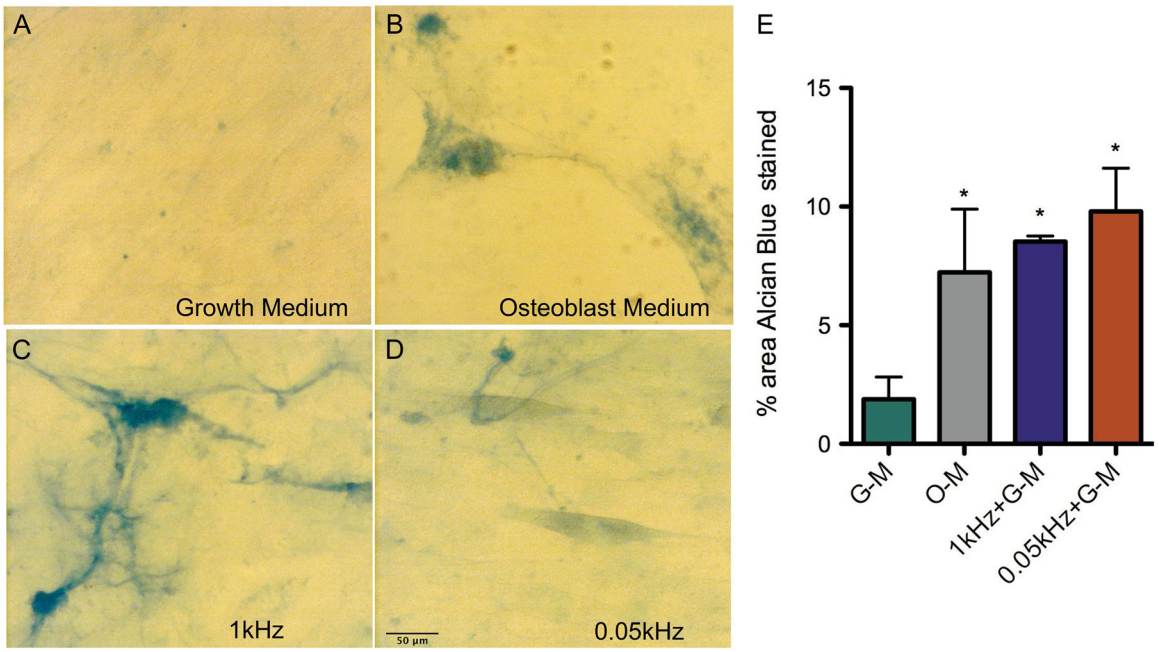

Fig. 5. Osteogenic medium or EMF treatment of MSPCs induces proteoglycan accumulation in the extracellular matrix of differentiated cells. MSPCs cultured in (**A**) growth medium (GM) or (**B**) osteogenic differentiation medium (ODM), or in GM and stimulated with (**C**) EMF 1 kHz or (**D**) 0.05 kHz for 1 h twice a week for 3 consecutive weeks and then stained with Alcian Blue (**E**). The quantitative analysis using ImageJ revealed that the percentage of area stained positively for Alcian Blue was significantly higher in treated cells than in growth medium cultured cells. One-way ANOVA: data are expressed as mean ± SD, $n = 6$, $^*P < 0.05$ vs. growth medium treated cells. ANOVA = analysis of variance; EMF = electromagnetic field; MSPC = mesenchymal stem progenitor cell.

their ability to differentiate into osteocytes, adipocytes, chondrocytes, muscle cells, and neurons [Kang et al., 2007; Takada et al., 2009; Guasti et al., 2012; Creecy et al., 2013; Hakim et al., 2019]. However, the molecular mechanisms underlying these differentiation processes are poorly understood to date. Stem cell differentiation and tissue regeneration are tightly regulated by several other elements including their microenvironment [Levin, 2021].

Particularly, bone regeneration depends on the action of extracellular factors, cellular microenvironment, and signaling pathways. Several key bioactive molecules and intracellular signaling pathways control the process of MSPCs' differentiation into osteoblasts such as TGF-β1 insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), BMP4 (bone morphogenetic protein 4), and nerve growth factor (NGF) [Chun et al., 2019; Dubus et al., 2019].

Endogenous electric fields, ion flows, and membrane potential gradients constitute a powerful system that underlies and in response affects the critical aspects of cell proliferation, apoptosis, and differentiation [Levin et al., 2017]. The response of stem cells to applied electric fields indicates bioelectricity as an important new key player in cell differentiation, tissue remodeling, and regenerative medicine [Whited and Levin, 2019]. Electrical stimulation can activate several intracellular signaling pathways and influence the intracellular microenvironment, which as a result notably affect cell migration, proliferation, and differentiation [Whited and Levin, 2019].

The natural voltage gradients exist not only in neuronal cells but also in all types of cells and support the regulation of cellular behavior and gene expression [Levin, 2013]. In particular, ion channels and ion pumps are localized to specific membrane regions of some cell types, rendering differences in ion flux through the apical and basal membranes of epithelial cells, which results in the generation of signals that make up part of the complex signaling network that leads to inter-and intra-cellular communications, and further triggers cellular differentiation [Goganau et al., 2018; Whited and Levin, 2019].

Recent studies on the cellular differentiation of mammalian osteoblasts have clearly demonstrated the importance of ion channels. It was reported that

osteoblasts and chondrocytes require the activity of the potassium channel $K_{ir}2.1$ for proper cellular differentiation [Pini et al., 2018]. In addition, alternative electrical currents or DCs are known regulators of the development and regeneration of many tissues [Levin and Stevenson, 2012]. Electrical fields are used to stimulate bone fracture healing by applying different electrical methods [Kim et al., 2009; Isaacson and Bloebaum, 2010; Hronik-Tupaj and Kaplan, 2012]. We used the AEC device that does not require direct cell contact with electrodes while it creates electric fields with lines of force parallel to one axis of symmetry, and demonstrated osteoblast differentiation from reprogrammed MSPCs with 1 and 0.05 kHz of AEC.

Consistent with other reports, we observed that treatment of normal human MSPCs with both lower (0.05 kHz) and higher (1 kHz) AEC frequencies induced osteogenic differentiation [Kim et al., 2009; Creecy et al., 2013; Zeighami et al., 2019]. In our protocol, MSPCs were subjected to AEC stimulation at higher (1 kHz) and lower (0.05 kHz) frequencies for 1 h twice a week for 3 consecutive weeks, which resulted in osteogenic differentiation and chondrogenesis. However, Parate et al. [2017] have demonstrated that a single electrical pulse with 15 Hz for 10 min was more effective in chondrogenic stimulation of MSPCs than repeated or long time intervals of exposure. We may consider additional experiments to determine whether single short-term EMF exposure with IteraCoil pulse on MSPCs with 1 kHz and/or 0.05 kHz could trigger osteogenic differentiation. Nevertheless, EMF treatment could be one supplementary approach for enhancing tissue regeneration by stimulating cells along with additional chemical mediators (cytokines, chemokines, and growth factors) to promote synergistic cellular responses [Ross et al., 2015].

In this study, we report new insights into the effects of electrical stimulation on osteoblasts and chondrocyte differentiation from MSPCs. To transfer this knowledge to clinical practice, an understanding of underlying mechanisms involved with AEC stimulation of bone healing is critical. Therefore, one of the futuristic goals should be to conduct relevant in vitro and in vivo studies for suitable optimization of the treatment process. Altogether, the potential of reprogrammed MSPCs to differentiate into different cell types has created ample interest in their usage for better treatment outcomes in clinical settings.

CONCLUSION

IteraCoil is a new electromagnetic device designed to deliver specific pulsed EMF to cells, tissues, and organs. IteraCoil can deliver pulsed EMFs to the cells without direct physical contact with the cells, eliminating such issues as electro-chemical interactions of electrodes with the cells and poor and inaccurate access to them, thereby eliminating the scarce cell/tissue targeting. MSPCs stimulated by IteraCoil at higher (1 kHz) and lower (0.05 kHz) frequencies for 1 h twice a week for 3 consecutive weeks in the growth medium without supplements triggered osteogenic and chondrogenic differentiation of cells.

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