Electromagnetic fields enhance chondrogenesis of human adipose-derived stem cells in a chondrogenic microenvironment in vitro

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Electromagnetic fields enhance chondrogenesis of human adipose-derived stem cells in a chondrogenic microenvironment in vitro. J Appl Physiol 114: 647–655, 2013. First published December 13, 2012; doi:10.1152/japplphysiol.01216.2012.—We tested the hypothesis that electromagnetic field (EMF) stimulation enhances chondrogenesis in human adipose-derived stem cells (ADSCs) in a chondrogenic microenvironment. A two-dimensional hyaluronan (HA)-coated well (2D-HA) and a three-dimensional pellet culture system (3D-pellet) were used as chondrogenic microenvironments. The ADSCs were cultured in 2D-HA or 3D-pellet, and then treated with clinical-use pulse electromagnetic field (PEMF) or the innovative single-pulse electromagnetic field (SPEMF) stimulation. The cytotoxicity, cell viability, and chondrogenic and osteogenic differentiations were analyzed after PEMF or SPEMF treatment. The modules of PEMF and SPEMF stimulations used in this study did not cause cytotoxicity or alter cell viability in ADSCs. Both PEMF and SPEMF increased the chondrogenic gene expression (SOX-9, collagen type II, and aggrecan) of ADSCs cultured in 2D-HA and 3D-pellet. The expressions of bone matrix genes (osteocalcin and collagen type I) of ADSCs were not changed after SPEMF treatment in 2D-HA and 3D-pellet; however, they were enhanced by PEMF treatment. Both PEMF and SPEMF increased the cartilaginous matrix (sulfated glycosaminoglycan) deposition of ADSCs. However, PEMF treatment also increased mineralization of ADSCs, but SPEMF treatment did not. Both PEMF and SPEMF enhanced chondrogenic differentiation of ADSCs cultured in a chondrogenic microenvironment. SPEMF treatment enhanced ADSC chondrogenesis, but not osteogenesis, when the cells were cultured in a chondrogenic microenvironment. However, PEMF enhanced both osteogenesis and chondrogenesis under the same conditions. Thus the combination of a chondrogenic microenvironment with SPEMF stimulation can promote chondrogenic differentiation of ADSCs and may be applicable to articular cartilage tissue engineering.

INJURY TO ARTICULAR CARTILAGE caused by traumatic and age-related degeneration are major health problems worldwide (24). The difficulty of a cartilage defect repairing itself is well known. The conventional surgical methods used to repair cartilage defects may not lead to hyaline cartilage regeneration (15). For this reason, attempts have been made to use chondrocytes or undifferentiated mesenchymal stem cells (MSCs) that are expanded in vitro in functional hyaline cartilage engineering to treat the defects. Due to the poor proliferative capacity and dedifferentiation of chondrocytes during in vitro expansion, MSCs are becoming an important cell source for articular cartilage tissue engineering (4, 14, 23, 36). In attempting to produce the functional hyaline cartilage from MSC-based tissue engineering, it is important to direct committed MSCs into the chondrogenic lineage and promote the subsequent cartilaginous matrix formation, mainly collagen type II and glycosaminoglycans (GAGs) (17). Chondrogenic induction factors like proteins or chemical compounds have been used to induce MSC differentiation into chondrogenic lineage (13). However, the drawbacks of using proteins or chemicals were reported to include protein denaturation, the carrying of pathogens, or the causing of undesired side effects (13).

Other than using proteins or chemicals, physical stimulation was suggested to induce MSC differentiation into desired lineages (13). Electromagnetic field (EMFs) stimulation systems like pulse electromagnetic field (PEMF) stimulation have been approved by the Food and Drug Administration (FDA) for the treatment of bone fractures, especially in nonunions (1, 11, 12). A report also indicated that PEMF stimulation is effective in promoting the osteogenic differentiation of bone marrow-derived MSCs (16).

The microenvironment that regulates stem cell differentiation can be cell-matrix adhesions or cell-cell interactions (6). An appropriate microenvironment is helpful for maintaining MSC survival, commitment, and differentiation (6, 35). Our previous report also showed that a hyaluronan-enriched microenvironment can both initiate and promote the chondrogenic differentiation of human adipose-derived stem cells (ADSCs) (35). In this study, we further hypothesized that under chondrogenic induction, PEMF and single-pulse electromagnetic...
field (SPEMF) stimulation may promote chondrogenic differentiation and cartilaginous matrix formation of MSCs, and that combined treatment with chondrogenic inductive factor and EMFs may be used for articular cartilage tissue engineering. To test the hypothesis, we evaluated the effects of PEMF and SPEMF stimulation on the mRNA expression of chondrogenic marker genes and the cartilaginous matrix formation of ADSCs cultured in two-dimensional hyaluronic (HA)-coated wells (2D-HA) and a three-dimensional pellet culture system (3D-pellet).

MATERIALS AND METHODS

Materials. Sodium hyaluronate, grade FCH-200 (mol mass = 2,000–2,100 kDa), was obtained from Kibun Food Chemicals (Tokyo, Japan). Dulbecco’s modified Eagle medium (DMEM) and antibiotics were purchased from Gibco BRL (Gaithersburg, MD). The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO).

Isolation and culture of human ADSCs. After obtaining informed consent from all the patients and approval from the hospital ethics committee (KMU-IRB-970267), leftover subcutaneous adipose tissue was acquired from six male patients aged 19–40 yr undergoing orthopedic surgery. The ADSCs were isolated from human subcutaneous adipose tissue following the previously described method (8, 35). The isolated ADSCs were cultured and expanded at 37°C under the neumous adipose tissue following the previously described method (8, 35). The isolated ADSCs were cultured and expanded at 37°C under 5% CO2 in K-NAC medium containing keratinocyte-SFM (Gibco BRL, Rockville, MD) supplemented with the EGF-BPE (Gibco BRL, Rockville, MD), N-acetyl-L-cysteine, L-ascorbic acid 2-phosphate sesquimagnesium salt (Sigma, St. Louis, MO), and 5% FBS (8, 35). In the test of real-time PCR, Alcian blue staining and dimethylmethylene blue (DMMB) assay, individual ADSCs from six different patients were used. In the test of lactate dehydrogenase (LDH) leakage, MTT, alizarin red staining, and histological analysis, individual ADSCs from four different patients were used. All experiments were performed by using individual ADSCs from a single patient each time and repeated at least three times. Data merged from all the examinations were analyzed for statistical significance. Analyses were performed by the merge of all tests.

EMF stimulation systems. Two different types of EMF stimulation were used in this study. The first was PEMF (Physio-Stim) stimulation. The PEMF-treated ADSCs were treated with PEMF modules with the following characteristics: maximum magnetic flux density of 2 mT, frequency of 15 Hz, magnetic field of 20 Gauss, pulsed period of 67.1 ms (Fig. 1A). The second was the innovative single-pulse electromagnetic field (SPEMF) composed of a single pulse repeated in adjustable times and magnetic fields. The pulsed period was 5 ms in sine wave per stimulation. The magnetic field was produced by every single pulse and the magnitude of the magnetic field was adjustable, in ranges from 0.6 T up to 1 T. The SPEMF-treated ADSCs were treated with SPEMF modules with the following characteristics: a pulsed period was produced by every single pulse and the magnitude of the magnetic field was 5 ms in sine wave per stimulation. Every pulse needed 5 s to store energy for the next pulse; the magnetic flux density was 1 T (104 Gauss) (Fig. 1B). The daily stimulation was <3 min. In brief, the stimulation module of PEMF was pulsed with a period of 5 ms repeated in 15 Hz, with a magnetic magnitude of 20 Gauss, 8 h daily, and the module of SPEMF had a magnitude of 1 T/pulse, and a pulsed period of 5 ms for 30 times, about 3 min daily. In our preliminary study, we found PEMF is most effective in chondrogenic induction 8 h daily, rather than 2 and 4 h daily, by mRNA expressions (data not shown). We also found 60 pulses in SPMEF were not more effective in chondrogenic induction than 30 pulses in SPMEF by GAG formation in 2D-HA (data not shown). Therefore, we chose 8 h daily for PEMF and 30 pulses for SPEMF in this study.

Lactate dehydrogenase (LDH) leakage analysis. The lactate dehydrogenase (LDH) leakage from ADSCs after EMF stimulation was measured using a cytotoxicity detection kit (Roche, Germany) (5, 26). The ADSCs were seeded into 24-well plates at 5 × 104 cells per well with a standard medium. Twenty-four hours after cell seeding, the cells were treated with PEMF stimulation at durations of 2, 4, or 8 h per day with or with SPEMF stimulation at durations of 10, 30, or 60 pulses per day. At every indicated time interval after PEMF or SPEMF treatment, the supernatants and cell layers of the cultures were collected for assay. The supernatants were removed and cell layers were lysed with 1% Triton X-100 and moved into 96-well plates. Briefly, 100 μl of catalyst solution was added to each assay well for 20 min. Absorbance was measured with an ELISA reader with a 490-nm filter. LDH leakage from ADSCs was calculated using the following formula: LDH leakage = supernatant/(supernatant + cell lysate).

Cell viability analysis. The effect of different modules of EMF stimulation, specifically PEMF and SPEMF, on the cell viability of ADSCs was tested using the MTT method (19). The ADSCs were seeded into 96-well plates at a density of 2 × 103 cells/well in standard medium. Twenty-four hours after cell seeding, the cells were treated with PEMF stimulation at durations of 2, 4, or 8 h per day or with SPEMF stimulation at durations of 10, 30, or 60 pulses per day. At every indicated time interval after PEMF or SPEMF treatment, 10 μl MTT solution was added to each well (at a final concentration of 5 mg/ml) and incubated at 37°C for 4 h. Cell viability was determined by measuring the cellular reduction of MTT to the crystalline formazan product which was dissolved in 100 μl dimethyl sulfoxide (DMSO), and the absorbance at 570 nm was measured.

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Cell culture on 2D HA-coated wells (2D-HA). HA-coated wells were used to develop 2D microenvironment-induced chondrogenesis. ADSC cultures were divided into four groups. 1) In the control (Ctrl) group, the ADSCs were cultured in 24-well culture plates without HA coating or EMF treatment. 2) In the HA group, the ADSCs were cultured in 2D HA-coated wells, but were not subsequently treated with EMF stimulation. 3) In the HA + PEMF group, the ADSCs were first cultured in 2D HA-coated wells, and then treated with PEMF stimulation for 8 h per day. 4) In the HA + SPEMF group, the ADSCs were cultured in 2D HA-coated wells, and then treated with SPEMF stimulation for 30 pulses per day. For the preparation of 2D HA-coated wells, purified HA dissolved in PBS was coated on 24-well plates (0.5 mg/cm²) for 48 h at 37°C, followed by two washes with PBS (35). The ADSCs were seeded at a density of 1 × 10⁶ cells per 500 μl of a standard medium. Twenty-four hours after seeding on HA-coated wells, cells were treated with PEMF or SPEMF stimulations. The standard medium in the plates was changed every 2 days. At every indicated time interval after EMF stimulation, cells were collected for further experimental analysis.

Cell culture in the 3D pellet culture system (3D-pellet). The 3D-pellet culture system (3D-pellet) was also used to develop 3D microenvironment-induced chondrogenesis. ADSC cultures were divided into four groups. 1) In the Ctrl group, the ADSCs were pellet-cultured in a 15-ml polypropylene conical tube, but not subsequently treated with EMF stimulations. 2) In the PEMF group, the ADSCs were cultured in a tube using pellet culture, and then they were treated with PEMF stimulation for 8 h per day. 3) In the SPEMF group, the ADSCs were cultured in a tube using pellet culture, and then they were treated with PEMF stimulation for 30 pulses per day. 4) In the chondroinduction (CI) group, first, the ADSCs were cultured in a tube using pellet culture, and then they were treated with PEMF stimulation for 8 h per day. 3) In the SPEMF group, the ADSCs were cultured in a tube using pellet culture, and then they were treated with PEMF stimulation for 30 pulses per day. 4) In the chondroinduction (CI) group, first, the ADSCs were cultured in a tube using pellet culture, and then they were treated with CI medium. For preparation of the pellet culture, aliquots of 1 × 10⁶ cells in 1 ml of standard medium were spun down and a 3D cell pellet was developed (2). For the PEMF and SPEMF groups, cell pellets were cultured in the presence of standard medium, and then treated with PEMF or SPEMF. For the CI group, cell pellets were cultured in the presence of CI medium: standard medium supplemented with 1% nonessential amino acid, 6.25 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 10 ng/ml transforming growth factor (TGF)-β1 (Sigma-Aldrich), and 50 μmol/l L-ascorbate-2-phosphate (20). The medium was changed every 2 days. At the indicated time intervals, cell pellets were collected for further experimental analyses.

RNA isolation and real-time polymerase chain reaction (real-time PCR). Total RNAs were extracted from cells using TRIzol (Gibco BRL, Rockville, MD), following the manufacturer’s instructions. Briefly, 0.5–1 μg of total RNA per 20 μl of reaction volume were reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed and monitored using the iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and the quantitative real-time PCR detection system (Bio-Rad Laboratories). cDNA samples (2 μl for a total volume of 25 μl per reaction) were analyzed for genes of interest and for the reference gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Fold changes (x-fold) in gene expression levels were calculated by the 2^-ΔΔCt method (21). Four readings of each experimental sample were performed for each gene of interest, and experiments were repeated at least three times. The primer sequences used are shown in Table 1.

Alcian blue staining for 2D culture. Cell cultures were fixed with 3.7% formaldehyde in PBS for 30 min, washed once with PBS, and rinsed with distilled water (dH2O); then they were processed for Alcian blue staining. Specimens were incubated with 0.05% Alcian blue solution overnight. Excess stain was removed by washing in PBS, rinsing with 5% acetic acid to remove nonspecific staining, and washing with PBS again.

Histological analysis and dimethylmethylene blue (DMMB) assay for 3D-pellets. To assess cell morphology and the presence of cartilage-specific matrix proteins, cells cultured in 3D-pellet culture were fixed overnight using 4% paraformaldehyde in PBS and transferred to 70% ethanol until processing. Constructs were embedded in paraffin, and cut into 5-μm sections. For histological analysis, sections were stained with Alcian blue (Fluka, Buchs, Switzerland) for the presence of cartilage sulfated GAG (sGAG) depositions. The sections were counterstained with nuclear Fast Red to identify cellular nuclei, which is a reflection of the cell number. At indicated time intervals, DNA content and the sGAG accumulation by cells were quantified spectrophotometrically using 33258 Hoechst dye and DMMB, respectively (7, 34). Total sGAG content was quantified using Blyscan Glycosaminoglycan Assay Kit (Biocolor, Newtonabbey, Northern Ireland, UK) according to the manufacturer’s instructions. The standard curve for DMMB assay was generated using an aqueous solution of chondroitin sulfate C (Sigma-Aldrich), with concentrations ranging from 0 to 25 μg/l.

Alizarin red staining and quantification. Calcium deposition was determined by staining with Alizarin red solution. Cell cultures were fixed with 3.7% formaldehyde in PBS for 30 min and then processed for Alizarin red staining. Specimens were incubated with 2% Alizarin red solution (Junsei Chemical, Tokyo) for 10 min. After that, cells were washed four times with dH2O. The image acquisition was carried out with a digital camera. For quantitative analysis after the image acquisition, 10% acetic acid was added to each well. Cells were scraped and transferred to a 1.5 ml microcentrifuge tube and centrifuged for 15 min at 12,000 g. The quantity of Alizarin was measured in a spectrophotometer at 405 nm.

Statistical analysis. Each experiment was repeated at least three times, and data (expressed as means ± SE) from a representative

<table>
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<th>Human Gene</th>
<th>PCR Primer Sequences (Forward and Reverse)</th>
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<td>Reverse: 5'-TGC TGC AGG GAA GTT TTG-3'</td>
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<td>Reverse: 5'-GTC GAA TGC AGA GGT TCT-3'</td>
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<tr>
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<td>Cycling conditions</td>
<td>Denature:95°C for 30 s, 95°C for 4 min, followed by 35 cycles of 95°C for 10 s, 58.4–61.5°C (see Anninging Temperature column) for 15 s and 72°C for 15 s</td>
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experiment are shown. Statistical significance was evaluated by Kruskal-Wallis one-way analysis of variance (ANOVA). A $P < 0.05$ was considered significant.

RESULTS

Effect of different modules of PEMF and SPEMF stimulation on cytotoxicity of ADSCs. The results showed that neither PEMF nor SPEMF stimulations have a cytotoxic effect on ADSCs, compared with the non-EMF-treated control. The results from LDH leakage of ADSCs after PEMF stimulation for a period ranging from 2 to 8 h per day for 3 and 5 days showed no significant difference from the control group (Fig. 2A). The LDH leakage of ADSCs after SPEMF stimulation amounting to 10 to 60 pulses per day for 3 and 5 days also did not show any significant difference from the control group (Fig. 2B).

Effects of different modules of PEMF or SPEMF stimulation on cell viability of ADSCs. The MTT analysis results showed that different modules of EMF stimulation did not affect cell viability after 3 and 5 days of treatment (Fig. 2). There were no significant differences among the control, PEMF-treated, and SPEMF-treated groups after 3 and 5 days of treatment (Fig. 2, C and D).

Effect of PEMF and SPEMF stimulation on mRNA expressions of chondrogenic and osteogenic marker genes, and sGAG deposition of ADSCs cultured in a 2D chondrogenic microenvironment. The mRNA expression of chondrogenic and osteogenic marker genes in ADSCs cultured in a 2D-HA microenvironment after PEMF (8 h per day) and SPEMF (30 pulses per day) stimulation were tested to determine the effect of EMF stimulation. The results indicated that both PEMF and SPEMF stimulation increased the chondrogenic marker genes of ADSCs in 2D-HA. PEMF stimulation also upregulated the osteogenic marker genes of ADSCs under 2D-HA, but SPEMF did not. Upregulation of mRNA expression in chondrogenic marker genes was found in the HA group from day 1 to day 7 (See Supplemental Table 1, available with the online version of this article) (Fig. 3, A–C). With the combination of PEMF or SPEMF stimulation, the mRNA expression of chondrogenic marker genes in ADSCs was further upregulated. More enhanced chondrogenic marker gene expressions were found in the HA + PEMF and HA + SPEMF groups than in the HA group (Supplemental Table 1) (Fig. 3, A–C). There were more of the chondrogenic marker gene expressions of ADSCs in the HA + SPEMF than in the HA + PEMF groups on day 1 and day 3 (Fig. 3, A–C) (Supplemental Table 1). The effects of PEMF and SPEMF stimulation on the osteogenic marker gene expressions (osteocalcin and collagen type I) of ADSCs were tested in each culture and calculated as described in MATERIALS AND METHODS. Cultures were treated with PEMF stimulation 2–8 h/day for 3 and 5 days (A and C); or treated with SPEMF at 10–60 pulses/day for 3 or 5 days (B and D). Each bar represents means ± SE of 4 replicate cultures.

Results from Alcian blue staining showed obvious deposition of sGAG in the HA + PEMF and HA + SPEMF groups after 10 days of culture (Fig. 3F). Compared with the control group, the quantity of sGAG deposition in the HA + PEMF, and HA + SPEMF groups was significantly increased at day 10 of culture ($P < 0.001$) (Fig. 3G). More pronounced sGAG deposition was found in the HA + SPEMF ($P < 0.05$) groups than in the HA group (Fig. 3G). These results indicate that in a 2D-HA microenvironment, both PEMF and SPEMF stimulation promotes chondrogenic marker gene expression as well as sGAG deposition of ADSCs.

Effect of PEMF and SPEMF stimulation on mRNA expression of chondrogenic and osteogenic marker genes, and sGAG deposition of ADSCs cultured in a 3D-pellet microenvironment. The mRNA expression of chondrogenic and osteogenic marker genes on ADSCs cultured in a 3D-pellet chondrogenic microenvironment after PEMF and SPEMF stimulation was tested to determine the effect of EMF stimulation. The results indicated that PEMF and SPEMF stimulation both increased the chondrogenic marker genes of ADSCs in 3D-pellet. Com-
pared with the control group, enhanced expressions of chondrogenic marker genes were found in the PEMF and SPEMF groups from day 1 to day 7 (see Supplemental Table 2, available with the online version of this article) (Fig. 4, A–C). In addition, compared with the CI group, the PEMF and SPEMF groups showed no significant difference in chondrogenic gene expression on ADSCs from day 1 to day 7 (see Supplemental Table 2, available with the online version of this article) (Fig. 4, A–C). The mRNA expression of osteogenic marker genes, including osteocalcin and collagen type I, by ADSCs was also tested. Compared with the control group, the mRNA expression of osteocalcin and collagen type I by ADSCs was increased in the PEMF group (Fig. 4, D and E), but not in the SPEMF group. Moreover, the mRNA expression of collagen type I in ADSCs was decreased in the SPEMF group compared with the PEMF group (Supplemental Table 2) (Fig. 4, D and E).

For GAG synthesis, ADSCs were harvested after 10 days of PEMF, SPEMF, or CI stimulation (Fig. 4F). In the DNA content, only CI enhanced ADSCs proliferation, but neither PEMF nor SPEMF did (Fig. 4G). Compared with the control group, the sGAG produced by ADSCs was significantly higher in the PEMF, SPEMF, and CI groups, but there was no significant difference in the levels produced (Fig. 4H).
Effect of PEMF and SPEMF stimulation on calcium deposition of ADSCs cultured under 2D-HA. The calcium deposition of ADSCs cultured in a 2D-HA chondrogenic microenvironment after PEMF and SPEMF stimulation was tested. The results from Alizarin red staining on day 10 showed increased calcium deposition in the HA + PEMF group, but not in the HA + SPEMF group. Compared with the control group, the quantity of calcium deposition in the HA + PEMF group was significantly increased on day 10 of culture ($P = 0.001$), but that of the HA + SPEMF group was not (Fig. 5B). These results indicate that in a chondrogenic microenvironment, PEMF stimulation promotes more calcium deposition of ADSCs, but SPEMF does not.

DISCUSSION

Regeneration of articular hyaline cartilage, which has a limited ability to self-repair, is a major challenge for orthopedic research (15). Most recent studies have focused on the development of new techniques, such as implantation of cells and tissue-engineered constructs to improve cartilage repair (18). Among these, cartilage regeneration through stem cell-based tissue engineering may provide more advantages for current cutting-edge research (3, 10, 18). Compared with bone marrow mesenchymal stem cells (BMSCs), ADSCs may be a more potent cell source for cartilage regeneration because of their abundant cell numbers and easily accessible MSCs (3, 10). However, the most important issue is to find a better bioactive factor to enhance the chondrogenesis of ADSCs. Recent literature suggests that physical-chemical interaction enhances the tissue-specific differentiation of stem cells. We have recently reported that the HA-modified PLGA scaffold provides a better microenvironment and enhances chondrogenesis in ADSCs (35). To build upon our previous finding, we herein explored the effect of physical stimuli, EMFs, on the chondrogenesis of ADSCs in a chondrogenic microenvironment.

Although numerous studies have examined the effects of EMF on the proliferation and chondroprotection of chondrocytes (9, 32, 33), the effects of EMF on the chondrogenesis of stem cells, especially ADSCs, have not been reported. In this study, we investigated the effects of PEMF (Physio-Stim) and our newly developed SPEMF on the chondrogenesis of ADSCs. The EMFs generated a pulse EMF with the Physio-Stim (Orthofix, McKinney, TX), which is FDA-approved for the treatment of nonunion or delayed union bone fractures (Fig. 1A). At the present time, no EMF apparatus is approved for the treatment of arthritis or cartilage regeneration. We found no adverse toxicity and no altered cell viability in ADSCs treated with 2–8 h of PEMF or 10–60 pulses of SPEMF daily.

We recently reported that an HA-enriched microenvironment induced chondrogenesis in ADSCs (35). Therefore, in this study, 2D-HA was used as a chondrogenic microenvironment to examine whether PEMF or SPEMF stimulation can further enhance the chondrogenesis of ADSCs in an HA microenvironment. The PEMF- and SPEMF-treated ADSCs showed more mRNA expression of chondrogenic marker genes and higher sGAG synthesis than those without EMF treatment in 2D-HA. These results indicated that in an appropriate chondrogenic microenvironment, physical stimuli like PEMF and SPEMF stimulations can also be used as a tool for enhancing chondrogenic differentiation of ADSCs, without adding any protein or chemical induction factors. The results were further confirmed in 3D-pellet cultures without chondrogenic induction factors in the media. Our results indicate that EMFs are as effective as a chondrogenic medium in inducing ADSC chondrogenesis. EMFs may be alternative methods to

![Image](http://jap.physiology.org/)

Fig. 5. Calcium deposition of ADSCs stimulated by PEMF or SPEMF and cultured under 2D-HA. Alizarin red-stained image of ADSCs cultured in the Ctrl, HA, HA + PEMF, and HA + SPEMF groups after 10 days. (A). Quantification of calcium depositions from ADSCs cultured in the Ctrl, HA, HA + PEMF, and HA + SPEMF groups for 10 days ($B$). Values are means ± SE; $n = 4$. **$P < 0.01$ vs. Ctrl group. $\#P < 0.01$ vs. HA + PEMF group.

Fig. 4. Effect of PEMF and SPEMF stimulation on chondrogenic and osteogenic mRNA expression, and sGAG deposition of ADSCs cultured under 3D-pellet. PEMF, SPEMF, and chondroinduction medium (CIM) stimulation of chondrogenic and osteogenic gene expressions of ADSCs from day 1 to day 7 cultured in 3D-pellet. Real-time PCR showed SOX-9 (A), collagen type II (B), aggrecan (C), osteocalcin (D), and collagen type I (E). Values are means ± SE; $n = 6$. sGAG deposition of ADSCs stimulated by PEMF or SPEMF cultured in 3D-pellet. Alcian blue-stained image of ADSCs in the Ctrl, PEMF, SPEMF and CIM groups after 10 days of culture (F). Quantification of sGAG content from ADSCs cultured in the Ctrl, PEMF, SPEMF, and CIM groups after 10 days of culture (H). Values are means ± SE; $n = 6$. *$P < 0.05$, **$P < 0.01$ vs. Ctrl group. $SP < 0.05$, $SSP < 0.01$ vs. PEMF group. $\#P < 0.05$, $\#\#P < 0.01$ vs. SPEMF group. See Cell culture in the 3D-pellet culture system for description of groups.

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induce ADSCs toward chondrogenic differentiation without a chondrogenic medium.

Previous reports have emphasized that PEMF stimulation leads to osteogenic differentiation in MSCs (22). In this study, PEMF also significantly increased osteocalcin mRNA expression in ADSCs cultured in 3D-pellet and 2D-HA. On the other hand, SPEMF did not induce, and even suppressed bone matrix gene expression and mineralization in ADSCs cultured in both 3D-pellet and 2D-HA-coated dishes. These results suggest that SPEMF may be more appropriate than PEMF for use in enhancing the chondrogenesis of ADSCs.

Current PEMF treatment requires 8 h daily, which is very time-consuming, but effective SPEMF treatment requires only 3 min a day. Although SPEMF requires a higher-magnitude magnetic field of 0.8 T, this magnitude is less than that of the clinical use of MRI (3 T). We also found no toxic effect on the ADSCs in this study. There may be concern that a high magnetic field can cause damage to the human body; however, reports indicated that static EMF from 0.3 T up to 1 T for more than 8 h/day did not have deleterious effects (25, 27–29). Even though the World Health Organization is concerned about the in vivo and in vitro effects of high EMF of more than 1 T, there is no definite evidence that high EMF will lead to damage in vivo and in vitro (30, 31). Schiffer et al. (30) reported that no detectable side effects of magnetic fields (static magnetic fields ranging from 1.5 to 7.05 T and exposure periods ranging from 1 to 24 h) were observed in cell cycle progression in HL60 cells and EA2 cells. The formula for our innovative SPEMF is below the intensity and time of the above-mentioned for inducing chondrogenesis of ADSCs. Therefore, the safety of this innovative SPEMF may be reliable. The daily treatment duration for SPEMF is much shorter than that for PEMF, indicating SPEMF may be more beneficial for clinical application. To our knowledge, this is the first study to compare the effect of PEMF and SPEMF on chondrogenesis of ADSCs in a CI microenvironment. The findings of this study not only provide new insight for using EMFs in the chondrogenic differentiation of human ADSCs, but also offer a possible adjunctive tool for enhancing the chondrogenic differentiation of ADSCs in tissue engineering.

In conclusion, we found that both PEMF and SPEMF treatment can enhance the chondrogenesis of human ADSCs cultured in a CI microenvironment, whether 2D-HA or 3D-pellet. Furthermore, the gene expression profile and extracellular matrix formation were evidence that SPEMF treatment leads to more ADSC commitment to chondrogenic lineage, but not osteogenic lineage, than PEMF treatment. Of even more importance, there is no obvious expression of bone matrix and fibrous cartilage marker genes, such as osteocalcin and type I collagen, in SPEMF treatment, but PEMF treatment causes osteocalcin expression and enhances matrix calcium deposition. The daily treatment time required for this innovative SPEMF to enhance the chondrogenesis of ADSCs is much less than that for PEMF. Thus the combination of a CI microenvironment with SPEMF treatment for <5 min daily can promote the chondrogenic differentiation of ADSCs, which may be applied in tissue engineering for articular cartilage. The molecular mechanisms of the effects of SPEMF on ADSCs require further investigation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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