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Stimulation of Chondrogenic Differentiation of Adult Human Bone Marrow-Derived Stromal Cells by a Moderate-Strength Static Magnetic Field

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Tissue-engineering strategies for the treatment of osteoarthritis would benefit from the ability to induce chondrogenesis in precursor cells. One such cell source is bone marrow-derived stromal cells (BMSCs). Here, we examined the effects of moderate-strength static magnetic fields (SMFs) on chondrogenic differentiation in human BMSCs *in vitro*. Cells were cultured in pellet form and exposed to several strengths of SMFs for various durations. mRNA transcript levels of the early chondrogenic transcription factor *SOX9* and the late marker genes *ACAN* and *COL2A1* were determined by reverse transcription–polymerase chain reaction, and production of the cartilage-specific macromolecules sGAG, collage type 2 (Col2), and proteoglycans was determined both biochemically and histologically. The role of the transforming growth factor (TGF)-β signaling pathway was also examined. Results showed that a 0.4 T magnetic field applied for 14 days elicited a strong chondrogenic differentiation response in cultured BMSCs, so long as TGF-β3 was also present, that is, a synergistic response of a SMF and TGF-β3 on BMSC chondrogenic differentiation was observed. Further, SMF alone caused TGF-β secretion in culture, and the effects of SMF could be abrogated by the TGF-β receptor blocker SB-431542. These data show that moderate-strength magnetic fields can induce chondrogenesis in BMSCs through a TGF-β-dependent pathway. This finding has potentially important applications in cartilage tissue-engineering strategies.

Introduction

Osteoarthritis (OA) is a common joint disease.[1,2] Symptoms include joint pain, stiffness, tenderness, impaired mobility, and local inflammations of variable severity.[2,3] OA affects 9.6% of men and 18% of women aged >60 years, making it a major cause of disability in elderly people and causing significant utilization of healthcare resources.[4,5] The incidence of OA and the associated socioeconomic burden is predicted to increase due to greater obesity and an aging population.[4,6] The central hallmarks of OA are degradation of articular cartilage, remodeling of subchondral bone, and formation of osteophytes.[7,8] The primary changes observed in cartilage degeneration include progressive loss of proteoglycans and mineralization of the extracellular matrix (ECM).[1,7,9] These events are poorly characterized and result from an intricate interplay of a wide range of signaling events.[1,9–11]

Current treatments for OA aim at reducing pain and consequent immobility, and therapeutic strategies vary depending on the degree of severity.[3,4] Nonpharmacological management, which includes regular exercise, weight loss, and lifestyle changes, is considered for patients in early stages of OA.[4] Analgesics, for example, nonsteroidal anti-inflammatory drugs and intra-articular knee injections of corticosteroid and hyaluronic acid, are provided in more advanced cases.[4] When these treatments are insufficient, surgical interventions, for example, arthroscopic debridement, microfracture, mosaicplasty, and autologous chondrocyte implantation, are the primary therapeutic options that are used to regenerate damaged cartilage.[4,9,12,13] However, current therapeutic strategies are unable to counteract the development of OA and achieve cartilage regeneration.[12]

In recent years, mesenchymal stromal cells (MSCs) from bone marrow, synovium, adipose tissue, and periosteum[14–16] with chondrogenic potential have been considered as components of a treatment strategy for cartilage defects. However, existing cell therapy approaches have not been reported to provide a long-term clinical benefit.[17] This may,

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in part, be due to the intra-articular inflammation that is observed in OA, which may limit terminal chondrogenic differentiation to regenerate cartilage tissue. Thus, tissue-engineering strategies involving *in vitro* stimulation (e.g., biochemical and biophysical) to induce cell differentiation, followed by implantation of terminally differentiated cells to repair damaged tissues, have recently gained attention.

Biophysical forces such as electromagnetic fields (EMFs) and static magnetic field (SMF) have attracted interest for their effects on bone remodeling[18,19] and wound healing,[20–22] and have been used as adjuvant treatments for cartilage regeneration.[23–31] For example, EMFs apparently stimulated chondrocyte proliferation and proteoglycan synthesis in bovine and human cartilage cells.[23,24,27] In human chondrocytes, pulsed EMFs stimulated cell proliferation.[28,30,31] A moderate-intensity (40 mT) SMF led to a histological improvement in ECM of cartilage in rabbits.[26] Further, 72 h of exposure to a 0.6 T SMF also caused significant stimulation of human chondrocyte cell growth *in vitro*.[5,31] These reports suggest that an SMF might be useful in stimulating chondrogenic differentiation of adult human precursor cells; however, more detailed studies are lacking. Here, we examine the effect of SMFs on adult human bone marrow-derived stromal cell (BMSC) chondrogenic differentiation *in vitro*, including examining a possible molecular mechanism involved in SMF-mediated chondrogenic differentiation of BMSCs.

Materials and Methods

Reagents and culture media compositions

The present study used commercially available BD mosaic serum-free growth media (GM; BD biosciences) to scale up BMSCs. Chondrogenic media (CM), containing DMEM-high glucose, 40 μg/mL L-proline, 50 μg/mL ascorbate-2-phosphate, 100 nM dexamethasone, insulin, transferrin, selenium, and 1% penicillin/streptomycin (p/s) (all from Sigma), were used in combination with 10 ng/mL TGFβ3 (Peprotech) as selective culture media to promote BMSC chondrogenic differentiation.

Cell culture

Adult primary human BMSCs from three healthy male donors (aged 30–45 years, purchased at passage 1 from Lonza) were used in the present study to examine the effects of SMF on chondrogenic differentiation *in vitro*. BMSCs were cultured in GM supplemented with 1% p/s at 37°C in a humidified atmosphere of 5% CO_2 in air, as previously described.[32,33] Cells were used between passages 2 and 5.

Effects of SMF on BMSC chondrogenic differentiation

Effects of various SMF intensities (0.1–0.6 T) on sGAG production of BMSCs. Previous studies have suggested that moderate-strength SMFs (0.1–1 T) may influence the chondrogenic differentiation of chondrocytes.[23–26,31] We, thus, examined the effects of 0.1, 0.2, 0.4, and 0.6 T SMF on terminal chondrogenic differentiation of BMSCs cultured as pellets, as previously described.[32] Briefly, cells were scaled up in GM, trypsinized, and cultured (0.5×10^6 cells/pellet in 15 mL conical polypropylene Falcon tubes with caps loosened to enable gas exchange [Nunc]) in GM overnight to obtain cell pellets. Culture media were then replaced by fresh GM or CM + TGF-β3, as previously described.[32] Pellets were then cultured for 3 weeks in the presence or absence of 20 mm diameter neodymium (NdFeB) magnets (E-Magnets). Magnets were of field strengths 0.1, 0.2, 0.4, or 0.6 T and were fixed beneath the Falcon tubes at a distance of 3 mm from the cell pellets. After 3 weeks, terminal chondrogenic differentiation was evaluated by quantifying the sulphated glycosaminoglycan (sGAG) content in pellets using the commercially available Blyscan kit (Biocolor). Total DNA content was quantified using the PicoGreen® double-stranded DNA assay (Invitrogen). All sGAG amounts were normalized by the DNA content. sGAG and DNA quantification assays were carried out as per the manufacturer's instructions.

The experiments cited earlier showed that the optimal SMF intensity was 0.4 T. A time course study was then performed in which BMSCs were exposed to 0.4 T SMF for 1, 2, or 3 weeks. At the end of each time period, cell pellets were harvested and sGAG levels were measured as described earlier.

Histological and immunocytochemical evidence of BMSC chondrogenic differentiation. BMSC pellets were cultured in GM, CM + TGF-β3, or CM + TGF-β3 + 0.4 T SMF, as described earlier. After 3 weeks, Alcian blue staining for proteoglycans and glycosaminoglycans was carried out to assess terminal chondrogenic differentiation. Briefly, cell pellets were fixed in 4% paraformaldehyde at 4°C for 24 h, dehydrated in an ascending series of ethanols (40%, 70%, 90%, and 100% ethanol; 20 min/step), and embedded in paraffin, as previously described.[32] Three micrometer sections were cut and stained with 1% Alcian blue (pH 2.5; Sigma) for 5 min to enable proteoglycans and glycosaminoglycans to be visualized. Nuclei were counterstained using Harris hematoxylin.

Paraffin sections were also immunolabeled for collage type 2 (Col2), as previously described.[32,33] Briefly, tissue in sections was treated with 1 mg/mL pepsin (in 10 mM Tris-HCl, pH 2.0; Sigma) for 15 min at room temperature (RT) to retrieve antigen sites and then permeabilized using 0.1% Triton X (Sigma) for 15 min at RT, followed by 1 h incubation at RT with a blocking solution containing 10% normal goat serum (NGS; Life Technologies) in phosphate-buffered saline (PBS). Sections were then incubated for 2 h at RT with primary mouse monoclonal anti-Col2 (Abcam) diluted at 1:100 in PBS containing 1% NGS. Incubation was then carried out with goat anti-mouse Alexa Fluor secondary antibody (Life Technologies) diluted at 1:200 in PBS containing 1% NGS for 1 h at RT. Col2-positive cells were visualized by their green fluorescent cytoplasmic and ECM staining. Nuclei were counterstained using Hoechst dye (Life Technologies).

Gene expression evidence of BMSC chondrogenic differentiation. Total RNA was extracted from replicate pellet cultures (cultured in GM, CM + TGF-β3, or CM + TGF-β3 + SMF) for conventional reverse transcription–polymerase chain reaction (RT-PCR) analysis of the early chondrogenic transcription factor SOX9 (measured after 1 week of culture) and the late marker genes COL2A1 and ACAN (measured after 2 weeks of culture), as previously described.[32] Since mRNA transcripts are expressed much

earlier than the corresponding proteins, the *COL2A1* gene was measured at week 2, and immunostaining of Col2 protein was carried out at week 3 (as described earlier). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene, was used as an endogenous control to ensure the quality and the quantity of amplified cDNA and to enable comparisons between control and test conditions. The sequences of the forward and reverse primers (from Sigma) were as follows: *SOX9* forward: ATCTGAAGAA GGAGAGCGAG, reverse: TCAGAAGTCTCCAGAGCTTG; *COL2A1* forward: TTTCCCAGGTCAAGATGGTC, reverse: CTTCAGCACCTGTC CACCA; *ACAN* forward: TGAGGA GGGCTGGAACAAGTACC, reverse: GGAGGTGGTAATT GCAGGGAACA; *GAPDH* forward: CCACCCATGGCAAA TTCCCATGGCA, reverse: CTGGACGGCAGGTCAGGTCC ACC. SOX9, COL2A1, and ACAN primers were used at annealing temperatures of 58°C, 58°C, and 54°C, respectively, for 35, 35, and 30 cycles, respectively, and *GAPDH* primers were used at an annealing temperature of 55°C for 25 cycles. To obtain a semi-quantitative estimate of the relative level of mRNA transcript in cells, the cDNA intensity of the band corresponding to each PCR product was measured by densitometry using ImageJ (NIH software) and normalized to that of the *GAPDH* internal control.

Effects of SMF on TGF-β3 pathway during chondrogenic differentiation of BMSCs

To determine whether SMF and TGF-β3 act synergistically on BMSC chondrogenic differentiation, we examined the effect of a 0.4 T SMF on sGAG production by BMSCs cultured in media with or without exogenous TGF-β3. Cells were cultured in pellet form for 3 weeks under one of the following conditions: GM, GM + SMF, CM, CM + SMF, CM + TGF-β3, or CM + TGF-β3 + SMF. At the end of the experiment, the sGAG content was quantified as described earlier.

Further, to examine the effects of a 0.4 T SMF on TGF-β3 production by BMSCs cultured in the absence of exogenous TGF-β3, we measured total TGF-β3 (latent + active) by ELISA, as previously described.[34,35] Briefly, BMSCs were cultured in pellet form in GM, CM, or CM + SMF and supernatant from cells exposed to SMF at $t = 0$ was collected at $t = 6$, 24, and 48 h. TGF-β3 levels were measured using the DuoSet® ELISA kit (R & D) following the manufacturer's instructions. In order to activate the latent form of TGF-β3, cell supernatants were acidified in 1 N HCl for 15 min followed by a neutralization step (incubation with 1N NaOH), as previously described.[34] The present study determined total TGF-β3 levels (latent + active form).

To determine whether SMF-induced chondrogenesis involves a TGF-β-dependent pathway, the effect of SB-431542, a TGF-β3 antagonist,[36] was determined using BMSCs. Briefly, BMSC pellets were cultured in GM, GM + SB-431542, CM, CM + SB-431542, CM + SMF, CM + SMF + SB-431542, CM + TGF-β3, CM + TGF-β3 + SB-431542, CM + TGF-β3 + SMF, and CM + TGF-β3 + SMF + SB-431542. After 2 weeks, sGAG levels were quantified as described earlier.

Effects of various lengths of SMF exposure time on BMSC chondrogenic differentiation

In order to evaluate the duration of SMF exposure required for terminal chondrogenic differentiation, BMSCs were cultured in GM, CM + TGF-β3, and CM + TGF-β3 + 0.4 T SMF. For test conditions, the SMF was applied at time zero and removed after 4, 7, 10, or 14 days. Cells were then further cultured so that the total culture duration for all samples was 21 days. Pellets were then harvested, and sGAG and DNA contents were quantified, as described earlier.

Statistical analysis

The sGAG quantification and semi-quantitative PCR data are presented as the mean ± standard error (based on a total of three measurements from three patients [one measurement/patient]) after normalization of data to readouts from control conditions, defined as 1.0, unless otherwise stated. Multi-way ANOVA was carried out along with Bonferroni corrections for multiple comparisons (SPSS 18.0 software).

Results

Effects of increasing intensities of SMF (0.1–0.6 T) on BMSC sGAG production

SMFs stimulated the production of sGAG by BMSCs, a key indicator of terminal chondrogenic differentiation *in vitro* (Fig. 1). This effect was ''dose-dependent'': specifically, 0.4 and 0.6 T SMF increased sGAG levels by 590% and 507%, respectively, compared with control conditions of cell culture in GM ($p < 0.05$). Results showed maximum/optimal response at 0.4 T. These data demonstrate that SMFs promote terminal chondrogenic differentiation of BMSCs *in vitro*. Since 0.4 T SMF had the most pronounced effect on BMSC chondrogenic differentiation, this field strength was used for experiments reported next.

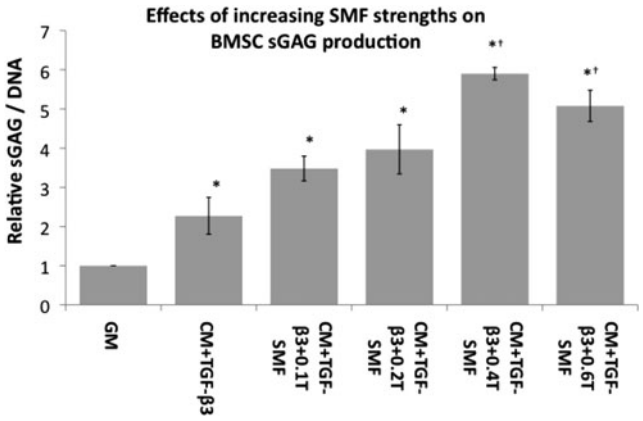

FIG. 1. Effects of increasing SMF intensity on sGAG amounts in human BMSC cultures. BMSCs obtained from three healthy donors were cultured in pellets in GM, CM + TGF-β3, or CM + TGF-β3 + SMF (0.1, 0.2, 0.4, or 0.6 T) for 3 weeks. The amounts of sGAG in pellets were quantified and divided by DNA content in order to examine the extent of terminal chondrogenic differentiation of BMSCs under the culture conditions mentioned earlier. The plotted quantities are the relative sGAG/DNA ratios normalized to values from cells grown in GM, defined as 1.0. *$p < 0.05$ compared with GM; †$p < 0.05$ compared with CM + TGF-β3. BMSCs, bone marrow-derived stromal cells; CM, chondrogenic media; GM, growth media; sGAG, sulphated glycosaminoglycan; SMF, static magnetic field; TGF, transforming growth factor.

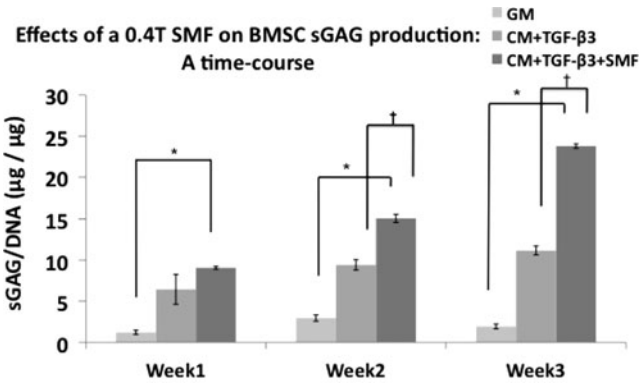

FIG. 2. Effects of a 0.4 T SMF on sGAG amounts in BMSC cultures after 1, 2, or 3 weeks. BMSCs were cultured in pellet form in GM, CM + TGF-β3, or CM + TGF-β3 + 0.4 T SMF for approximately 3 weeks. The amounts of sGAG in pellets after weeks 1, 2, and 3 were quantified and divided by DNA content. The plotted quantities are the sGAG/DNA (µg/µg) ratio. $^{*}p < 0.05$ compared with GM; $^{\dagger}p < 0.05$ compared with CM + TGF-β3.

A time course study showed that 0.4 T SMF had no marked stimulatory effect on BMSC sGAG production after 1 week of culture (Fig. 2). However, after 2 weeks, a significant increase ($p < 0.05$) in sGAG levels compared with CM + TGF-β3 was observed, an effect that was more pronounced at 3 weeks ($p < 0.05$).

Histological sections (Fig. 3) showed strong Alcian blue staining of the ECM in pellets cultured in the presence of 0.4 T SMF for 3 weeks, as compared with weak staining in control cultures grown in GM and CM + TGF-β3. Similarly, strong Col2 staining in the ECM of BMSC pellets was observed when cells were cultured in CM + TGF-β3 + 0.4 T SMF, compared with relatively low levels of Col2 staining observed in cultures grown with CM + TGF-β3 and little, if any, staining observed in cultures from nonselective growth conditions. These data are consistent with 0.4 T SMF stimulating terminal chondrogenic differentiation of BMSCs as measured by sGAG synthesis.

Effects of SMF on the expression of chondrogenic marker genes in BMSCs

RT-PCR studies (Fig. 4) show that BMSCs cultured in CM + TGF-β3 and CM + TGF-β3 + SMF expressed higher levels of the early chondrogenic transcription factor *SOX9* (1.1- and 3.2-fold higher, respectively; $p < 0.05$ for a comparison with cultures in GM) and the late genes *ACAN* (2.8- and 8.3-fold higher, respectively; $p < 0.05$) and *COL2A1* (3.0- and 9.5-fold higher, respectively; $p < 0.05$), compared with pellet cultures in GM. Notably, cultures in CM + TGF-β3 + SMF had significantly higher mRNA levels of *SOX9* (2.0-fold), *ACAN* (2.4-fold), and *COL2A1* (2.6-fold) transcripts, compared with cells cultured in CM + TGF-β3 ($p < 0.05$).

Effects of SMF on TGF-β3 pathway during chondrogenic differentiation of BMSCs

A 0.4 T SMF stimulated BMSC sGAG production even in the absence of the potent chondrogenic growth factor TGF-β3 (Fig. 5). BMSCs cultured in CM (without TGF-β3) in the presence of SMF exhibited elevated sGAG levels compared with cells cultured without SMF (167% increase; $p < 0.05$). Moreover, the effects of TGF-β3 and SMF were synergistic, with cells cultured with both stimuli present showing a 206% increase in sGAG levels, compared with TGF-β3 alone ($p < 0.05$) (Fig. 5). Notably, no significant change in sGAG levels was observed when BMSCs were cultured in nonselective growth condition with SMF (GM + SMF) compared with GM. Thus, these data suggest a possible synergistic effect of TGF-β3 and SMF on chondrogenic differentiation of BMSCs.

In view of these results, we examined the effects of SMF on TGF-β3 production by BMSCs grown in otherwise TGF-β3-free conditions. TGF-β3 ELISA results showed that SMF markedly stimulated BMSC TGF-β3 production. More specifically, BMSCs cultured in the presence of 0.4 T SMF

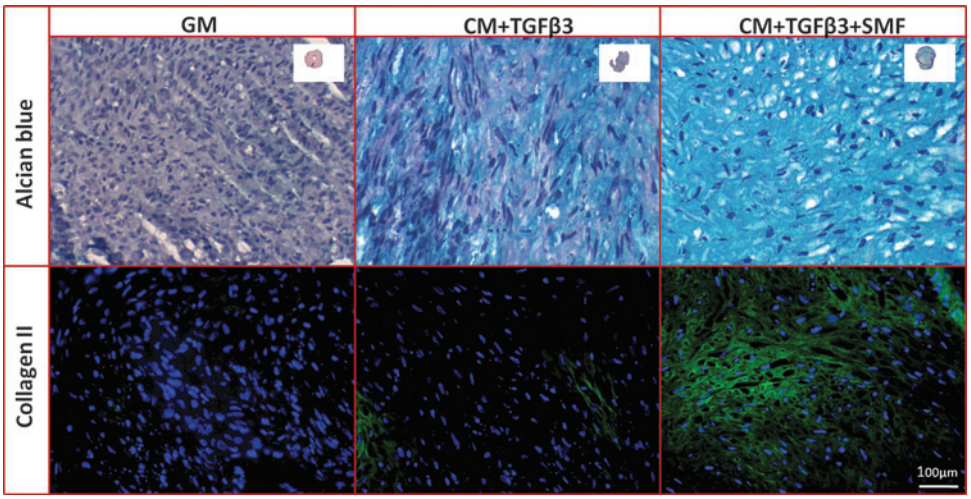

FIG. 3. Histological and immunocytochemical assessment of the effects of a 0.4 T SMF on chondrogenic differentiation. Alcian blue and Col2 immunostaining of paraffin sections of BMSC pellets maintained for 3 weeks in GM, CM + TGF-β3, or CM + TGF-β3 + 0.4 T SMF was carried out. Sections stained with Alcian blue were counter-stained with Harris Hematoxylin (purple nuclei). Sections stained with Col2 were counter-stained with Hoechst dye (blue nuclei). Inset shows cross-section of entire pellets. Col2, collage type 2.

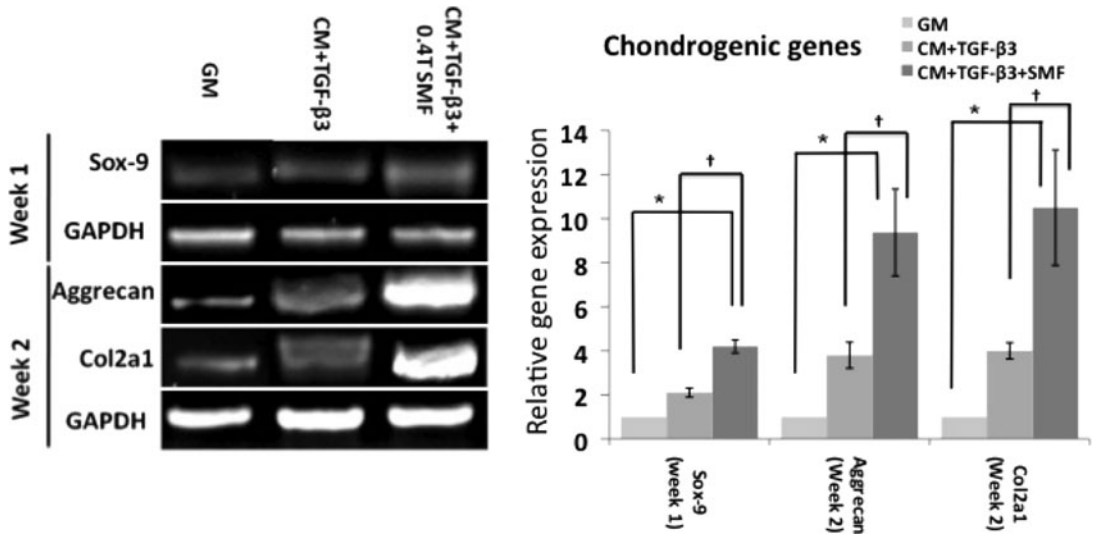

FIG. 4. Effect of a 0.4 T SMF on chondrogenic gene message levels in BMSCs. (1) A representative RT-PCR gel showing the expression of the early chondrogenic gene *SOX9* and the late genes *ACAN* and *COL2A1* from BMSCs cultured for approximately 2 weeks in GM, CM + TGF-β3, or CM + TGF-β3 + 0.4 T SMF. (2) PCR product band intensity, relative to *GAPDH*, from cells cultured in the presence of CM + TGF-β3 and CM + TGF-β3 + SMF. The plotted values have been normalized to levels from cells cultures in GM, defined as 1.0. The values are the means ± SE of triplicate measurements, as described in the text. $^{*}p < 0.05$ compared with GM; $^{\dagger}p < 0.05$ compared with CM + TGF-β3. *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription–polymerase chain reaction.

(CM + SMF) for 6, 24, and 48 h showed significantly higher levels of total TGF-β3 (4335, 4681, and 4864 pg/mL, respectively; $p < 0.05$) in culture media, compared with cells cultured in control conditions of GM (1499, 868, and 1619 pg/mL, respectively) and CM (2816, 3154, and 3111 pg/mL, respectively) (Fig. 6).

We also examined the functional consequences of a TGF-β pathway inhibitor, SB-431542, on SMF-mediated BMSC chondrogenic differentiation (Fig. 7). Notably, SB-431542 completely ablated SMF-induced BMSC sGAG production. Specifically, when BMSCs were cultured in CM + SMF with added SB-431542 (Fig. 7, bar F), there was a marked 56% reduction ($p < 0.05$) in sGAG levels, compared with cells cultured in CM + SMF (bar E). Similarly, for cells cultured in CM + TGF-β3 (bar G), the addition of SB-431542 (bar H) led to a 54% reduction in sGAG levels ($p < 0.05$). These results were consistent with observations of cells cultured with

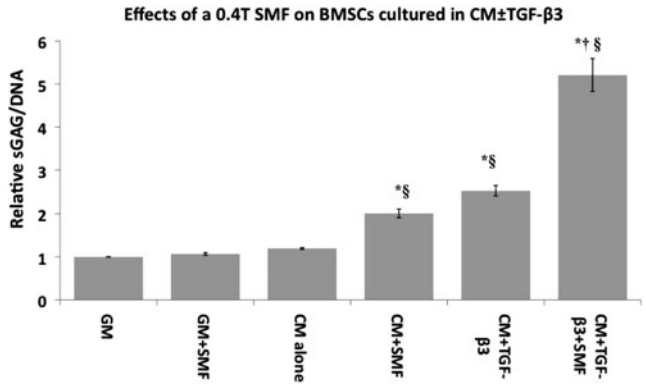

FIG. 5. Assessment of the SMF-mediated chondrogenic differentiation of BMSCs in the presence and absence of TGF-β3. BMSCs were cultured in pellet form in GM, CM, CM + SMF, CM + TGF-β3, or CM + TGF-β3 + 0.4 T SMF for 3 weeks. The amounts of sGAG in pellets after 3 weeks were quantified and divided by DNA content. The plotted quantities are the sGAG/DNA ratios, normalized to values for cells cultured in GM, defined as 1.0. $^{*}p < 0.05$ compared with GM; $^{\S}p < 0.05$ compared with CM; $^{\dagger}p < 0.05$ compared with CM + TGF-β3.

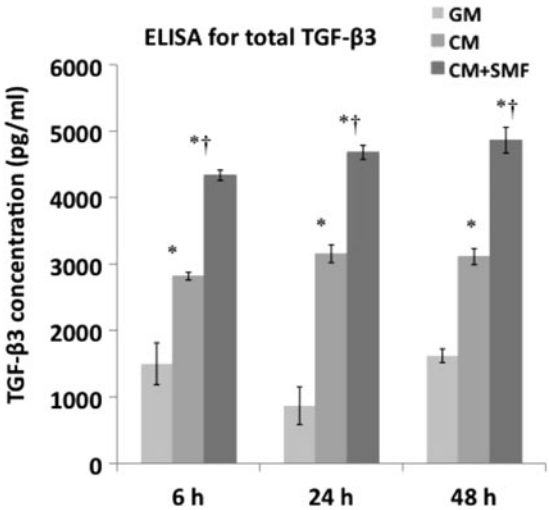

FIG. 6. Quantitative assessment of TGF-β3 secretion by BMSCs in the presence of a 0.4 T SMF. TGF-β3 levels in the supernatant from BMSCs cultured in GM, CM, or CM + SMF were examined using ELISA for TGF-β3. Supernatants were acidified (see text) to activate any TGF-β3 in latent form. The plotted quantities are TGF-β3 levels in culture media harvested from cells minus TGF-β3 levels in culture media without cells (background readings). $^{*}p < 0.05$ compared with GM; $^{\dagger}p < 0.05$ compared with CM.

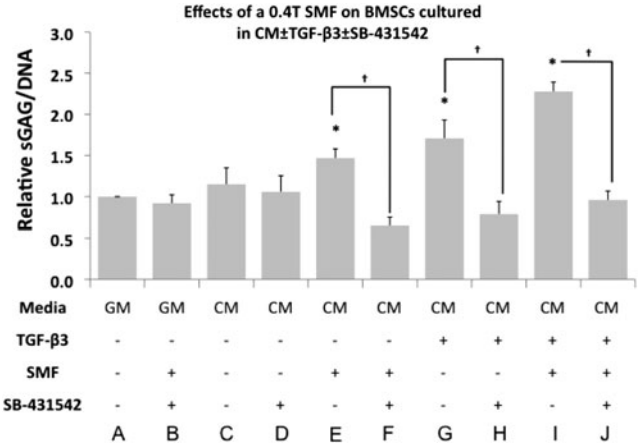

FIG. 7. Effects of a TGF-β inhibitor SB-431542 on 0.4 T SMF-mediated chondrogenic differentiation of BMSCs. sGAG levels in BMSC pellets cultured in GM ± SB-431542 or CM ± TGF-β3 ± SB-431542 ± 0.4 T SMF for 2 weeks were examined. The plotted quantities are the sGAG/DNA ratios, normalized to values observed from cells cultured in GM, defined as 1.0. $^{*}p < 0.05$ compared with GM; $^{\dagger}p < 0.05$ between conditions with and without SB-431542.

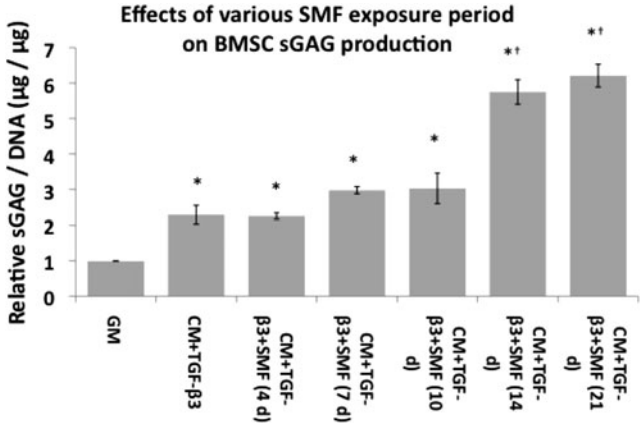

FIG. 8. Effects of various 0.4 T SMF exposure durations on BMSC chondrogenic differentiation. BMSC pellets were cultured for 21 days in GM, CM + TGF-β3, or CM + TGF-β3 + SMF. For cells exposed to SMF, the exposure duration was 4, 7, 10, or 14 days, starting at day zero. The amounts of sGAG in pellets were quantified and divided by DNA quantity in order to examine the extent of terminal chondrogenic differentiation of BMSCs. The plotted quantities are the sGAG/DNA ratios normalized by the value for cells cultured in GM, defined as 1.0. $^{*}p < 0.05$ compared with GM; $^{\dagger}p < 0.05$ compared with CM + TGF-β3.

CM + TGF-β3 + SMF (bar I), where the addition of SB-431542 (bar J) caused a 58% reduction in sGAG levels ($p < 0.05$). In contrast, little, if any, change in sGAG levels was observed when BMSCs were cultured in GM + SB-431542 (bar B), compared with GM (bar A). Similarly, no marked difference in sGAG levels was seen when BMSCs were cultured in CM + SB-431542 (bar D), compared with CM (bar C). These results suggest that SMF-induced up-regulation of TGF-β3 production plays a role in SMF-induced chondrogenic differentiation.

Effects of various SMF exposure time lengths on BMSC chondrogenic differentiation

Application of a 0.4 T SMF for 4, 7, or 10 days, followed by further culture in CM + TGF-β3 for the remaining culture period (total of 21 days), did not appear to cause any change in sGAG levels, compared with cells cultured for 21 days in control condition of CM + TGF-β3. In contrast, when SMF was applied for 14 days followed by a further 7 days of culture in CM + TGF-β3, sGAG levels of BMSCs were significantly higher (251% increase; $p < 0.05$) compared with CM + TGF-β3 (Fig. 8). Similarly, when BMSCs were exposed to 0.4 T SMF for the full period of 21 days, a 271% increase in sGAG levels was observed compared with CM + TGF-β3 ($p < 0.05$). Notably, no marked difference was observed between cultures exposed to SMF for 14 and 21 days, suggesting that the SMF up-regulates chondrogenic differentiation of BMSCs within the first 14 days of exposure, after which further SMF exposure may not be required.

Discussion

Our results show that a moderate-strength SMF promotes chondrogenic differentiation of MSCs. This result is supported by data gathered from several outcome measures: increased mRNA transcript levels of the early chondrogenic transcription factor *SOX9* and the late marker genes *ACAN* and *COL2A1*; production of the cartilage-specific macromolecules sGAG, *COL2*, and proteoglycans. *SOX9* up-regulation is one of the pivotal up-stream chondrogenic events that are responsible for the up-regulation of *COL2*, *ACAN*/proteoglycans, and sGAG production,[37] while secretion of such matrix molecules is one of the hallmarks of terminally differentiated/mature chondrocytes.[7,9,37] The present study, therefore, suggests that SMF-mediated induction of BMSC chondrogenic differentiation is *SOX9* dependent. The gene expression study cited earlier was carried out using a conventional semi-quantitative RT-PCR (as described in Materials and methods); thus, the quantitative gene expression method (quantitative real-time PCR) would be more useful in future studies.

This effect appears to be dose dependent, with maximal response observed in the presence of a 0.4 T SMF and no further increase in sGAG levels in the presence of 0.6 T SMF. Although > 0.6 T SMF intensities were not tested here, a study by Hsieh *et al.* showed that 3.0 T SMF abrogated chondrocyte proliferation and induced p53-, p21-, and p27-mediated apoptosis, suggesting that higher SMF intensities could have deleterious effects on human chondrocytes.[38] Jaberi *et al.* reported a histological improvement in the rate of matrix formation in rabbit hyaline cartilage treated with a 40 mT SMF.[26] This is a lower field strength than we found to be effective, which may reflect differences in experimental design, that is, production of mature cartilage vs. chondrogenic differentiation of MSCs.

A time course study showed that the most profound effect of SMF on BSMCs occurred after 14 days of magnetic field exposure. Notably, SMF exposure periods of 10 or fewer days did not induce BMSC chondrogenic differentiation to a greater extent than CM conditions alone. These results, therefore, suggest that SMF is required throughout the early, intermediate, and late phases of BMSC chondrogenic

differentiation to achieve highest chondrogenic induction, after which further SMF exposure may not be required.

Further, the outcomes of terminal differentiation assays (using BMSCs between passage 2 and 5) discussed earlier were internally consistent. This suggests that regardless of BMSC passage number (within passages 2 and 5), the exposure of a 0.4 T SMF consistently stimulates BMSCs to undergo chondrogenic differentiation, showing the efficacy of SMF to promote chondrogenic differentiation of BMSC.

Thus, as per the earlier studies, two parameters appear to be crucial in SMF-mediated terminal chondrogenic differentiation: (1) the SMF exposure period for BMSCs—a minimum of 10 days is needed; and (2) the SMF field strength experienced by the cells—in our studies, a 0.4 T magnet positioned 3 mm from the cells was optimal.

Role of TGF-β

The pro-chondrogenic effects of a 0.4 T SMF were observed when cells were cultured in CM + TGF-β3 (an environment facilitating chondrogenic differentiation), but not in nonselective growth conditions. This suggests that an environment favoring chondrogenesis, for example, one in which TGF-β3 is present, may be essential for SMF-induced chondrogenic differentiation. We, therefore, considered whether up-regulation of BMSC chondrogenic differentiation by SMF is, at least, partly due to a synergistic interaction between TGF-β3 and SMF. Our results showed for the first time that a biochemical cue (TGF-β3) + a biophysical cue (0.4 T SMF) elicited the highest BMSC chondrogenic differentiation, compared with the biochemical cue alone or the biophysical cue alone, suggesting that SMF and TGF-β3 synergistically promote BMSC chondrogenesis.

Generally speaking, the molecular mechanisms involved in biophysical stimuli-mediated cell functions are unclear. For purposes of this work, we studied the possible involvement of the TGF-β3 pathway by examining (1) TGF-β3 production by BMSCs in the presence of a 0.4 T SMF, and (2) functional consequences of the TGF-β antagonist SB-431542. Our results clearly show that SMF has the ability to stimulate TGF-β3 production by BMSCs cultured in the absence of exogenous TGF-β3. Since our TGF-β3 ELISA used acidified supernatants, we measured total TGF-β3 levels (latent + active forms). Therefore, further studies are required to determine (1) levels of latent and active forms of TGF-β3 peptides in the supernatant of BMSCs treated with SMF, and (2) the effects of SMF on TGF-β3 peptide activation mechanisms (including αV integrin-dependent and integrin-independent).

Moreover, when TGF-β receptors were blocked by SB-431542, the chondrogenic effects of SMF on BMSCs cultured in CM were completely abrogated. Similar observations were noted when BMSCs were cultured in full chondrogenic conditions (+TGF-β3). In passing, we note that BMSCs cultured in CM ± TGF-β3 + SMF + SB-431542 exhibited sGAG levels which were lower than those found in cultures in GM, although these reductions in sGAG levels were not statistically significant, compared with cultures in GM. The reason for this effect is not yet clear, and may warrant further investigation. Nonetheless, these observations together suggest that stimulation of TGF-β3 secretion by a 0.4 T SMF is an important mechanism by which an SMF affects the complex cellular process of BMSC chondrogenic differentiation.

Aaron *et al.* showed that pulsed EMF led to increased levels of TGF-β1 mRNA transcript and protein in ossicles of rats which were injected with decalcified bone matrix (to induce endochondral ossification).[39] Although the present study did not study the effects of SMF on BMSC TGF-β1 levels, the work of Aaron *et al.* motivates further investigations to determine whether TGF-β1 increases are observed in SMF-mediated chondrogenic differentiation of BMSCs.

Implications

Regenerative medicine-based OA solutions that rely on the *in situ* differentiation of adult human precursor cells (i.e., cartilage cells, MSCs) are attractive. However, intra-articular inflammation is observed in OA or mechanical trauma, and in such an environment, there may be limited success in eliciting terminal chondrogenic differentiation to regenerate cartilage tissue. Strategies involving *in vitro* stimulation of chondrogenic differentiation and production of cartilage-specific ECM of human precursor cells, followed by implantation of preconditioned cells to repair damaged cartilage, are thus appealing. Tissue engineering-based solutions involving transplantation of preconditioned terminally differentiated chondrocytes require large number of precursor cells with the ability to undergo chondrogenic differentiation. Notably, articular chondrocytes have limited ability to proliferate and have been shown to undergo cell senescence/de-differentiation after ~15 population doublings,[6] making large scale-up of chondrocytes a technically difficult task. Alternatively, BMSCs have been shown to have extensive proliferation capacity and the potential to differentiate into multiple lineages. Moreover, BMSCs have been studied extensively for their stem cell-like characteristics,[40,41] and research tools are abundantly available that scale up such a cell population to either carry out large experiments or use them for cartilage regeneration after chondrogenic preconditioning. Although the present study suggests that SMF could be a valuable chondrogenic differentiation stimulus for such approaches, whether terminally differentiated chondrocytes de-differentiate in the absence of SMF has not been determined here. Thus, further studies are warranted to (1) examine whether terminally differentiated chondrocytes de-differentiate in the absence of SMF; and (2) dissect SMF-mediated molecular mechanisms up-stream of TGF-β3 signaling to better understand the mode of action of this biophysical cue in chondrogenesis.

Future applications

An SMF-mediated BMSC chondrogenic preconditioning method could also be used with appropriate biodegradable polymer scaffolds providing a three-dimensional environment/meshwork that mimics cartilage tissue. Such an approach would be consistent with the tissue-engineering paradigm, namely a suitable cell source, scaffold, and differentiation signals. This approach can be combined with a novel bioreactor developed by our group, capable of applying multiple biophysical (SMF and mechanical) cues, to further investigate and engineer functional replacement tissue for the treatment of OA.[42]

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