

● *Original Contribution*

EFFECTS OF LOW-INTENSITY PULSED ULTRASOUND, DEXAMETHASONE/ TGF- β 1 AND/OR BMP-2 ON THE TRANSCRIPTIONAL EXPRESSION OF GENES IN HUMAN MESENCHYMAL STEM CELLS: CHONDROGENIC VS. OSTEOGENIC DIFFERENTIATION

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Abstract—The effects of low-intensity pulsed ultrasound (LIPUS) on the differentiation of human mesenchymal stem cells (hMSCs) were investigated in this study. hMSCs were subjected to LIPUS with or without dexamethasone/transforming growth factor- β 1 (TD) or bone morphogenetic protein-2 (BMP-2) and the effects of this treatment were assessed. TD-treated hMSCs exhibited characteristic chondrogenic morphology and increased messenger RNA (mRNA) expression of chondrogenic markers and LIPUS enhanced the chondrogenic differentiation of hMSCs treated with TD. The expression of Runx2, an osteogenic transcription factor was not altered in either TD treatment group; however, a significant increase was detected in the LIPUS only group. The osteogenic appearance exhibited 3 days after LIPUS and/or BMP-2 treatment. Increases in the mRNA expression levels of osteogenic markers, Runx2 and ALP were also detected. There was no additive or altered effect with combined LIPUS and BMP-2 treatment. LIPUS alone can increase osteogenic differentiation of hMSCs and LIPUS enhances TD-mediated chondrogenic differentiation of hMSCs. Clinically, LIPUS may differentially influence bone vs. cartilage repair. (E-mail: whchang@cycu.edu.tw or laitw@tmu.edu.tw or tlai@mclean.harvard.edu) © 2010 World Federation for Ultrasound in Medicine & Biology.

Key Words: Human mesenchymal stem cells, Low-intensity pulsed ultrasound, Transforming growth factor, Chondrogenic differentiation, Osteogenic differentiation.

INTRODUCTION

Cartilage is an avascular musculoskeletal tissue that possesses little capacity for self-repair after damage (Buckwalter and Mankin 1998). Various treatments for cartilage regeneration including the use of carbon (Brittberg et al. 1994a), periosteum (Hoikka et al. 1990), perichondrium (Homminga et al. 1990), autologous chondrocyte transplantation (Brittberg et al. 1994b; Peterson

1996) and subchondral drilling have failed to achieve much success (Bouwmeester et al. 2002; Kon et al. 2008; Minas and Nehrer 1997).

Mesenchymal stem cells (MSCs) are thought to have significant potential for facilitating tissue repair (Bruder et al. 1998; Goshima et al. 1991; Maniopoulos 1988; Nakahara et al. 1990). MSC tissue repair occurs either as a result of MSC migration from bone marrow or by MSC transplantation to the injured area where new cartilage or bony reparative tissue is formed (Cheung et al. 1980; Furukawa et al. 1980). However, because implantation of undifferentiated MSCs for cartilage regeneration may pose a high risk of undesirable effects (such as bone spur formation), this therapeutic approach should be applied cautiously (van der Kraan and van den Berg 2007). In addition, morbidity is frequently associated with failure of bone regenerative techniques and

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complications from bone grafts (Griffin *et al.* 2008; Kwong and Harris 2008). Therefore, an optimal therapy for promoting cartilage and bone regeneration is still being sought.

Attempts have already been made to enhance chondrogenic and osteogenic differentiation by treating MSCs with cytokines such as bone morphogenetic proteins (BMPs) (Lecanda *et al.* 1997; Selvamurugan *et al.* 2007) and dexamethasone/transforming growth factor- β 1 (TD) (Chen *et al.* 2005; Johnstone *et al.* 1998). In addition, stimulation with mechanical stresses such as cyclic hydrostatic pressure and noninvasive low-intensity pulsed ultrasound (LIPUS) have shown regenerative potential (Linkhart *et al.* 1996; Sant'Anna *et al.* 2005).

LIPUS has been reported to have various biologic effects and triggers different signaling events involved in bone formation (Azuma *et al.* 2001; Takayama *et al.* 2007; Warden *et al.* 2001) and cartilage regeneration (Choi *et al.* 2006; Cook *et al.* 2001; Parvizi *et al.* 1999; Zhang *et al.* 2003) *in vivo* and *in vitro*. Findings from previous studies, for example, have suggested that exposure to LIPUS enhances aggrecan synthesis in rat chondrocytes (Parvizi *et al.* 1999). Early studies have shown that LIPUS can accelerate bone healing in animal models (Pilla *et al.* 1990) and in humans (Heckmann *et al.* 1994). Recent studies continue to confirm the positive effects of LIPUS on healing of bone (Esteki *et al.* 2010; Rutten *et al.* 2008), articular cartilage (Jia *et al.* 2005) and the tendon graft-bone interface (Papatheodorou *et al.* 2009). Few studies, however, have investigated the effects of LIPUS on the differentiation of human mesenchymal stem cells (hMSCs). It has been recently reported that chondrogenic differentiation of both animal and human MSCs is enhanced by the application of LIPUS in studies utilizing *in vitro* and three-dimensional (3-D) models (Ebisawa *et al.* 2004; Lee *et al.* 2006, 2007; Schumann *et al.* 2006). However, there have been no reports describing how LIPUS regulates the signaling of chondrogenic vs. osteogenic differentiation in hMSCs. We hypothesized that LIPUS may differentially modulate the signaling of chondrogenic and osteogenic differentiation in hMSCs *in vitro*.

The aim of this study was to investigate the regulatory effects of LIPUS on chondrogenic and osteogenic differentiation of hMSCs induced by cytokines such as transforming growth factor (TGF)- β 1, dexamethasone and bone morphogenetic protein-2 (BMP-2). We first examined the effects of these cytokines on differentiation by observing morphologic changes in hMSCs. Second, we used quantitative real-time polymerase chain reaction (PCR).

PCR to measure levels of chondrogenic and osteogenic markers, such as Sox9, Runx2, aggrecan and type

I and II collagen, expressed by hMSCs after exposure to cytokines. Finally, we examined the effect of combined LIPUS and cytokine treatment on chondrogenic and osteogenic differentiation of hMSCs.

MATERIALS AND METHODS

Subjects

Bone marrow was harvested from individuals undergoing surgical treatment of femur fractures in the Orthopedic Section of Taipei Municipal Chung-Hsin Hospital, Taipei, Taiwan. Exclusion criteria included any history of endocrine disease or hormone replacement therapy. The study was explained to the patients and all patients provided informed consent for harvesting of bone marrow during their surgical procedure. Bone marrow was harvested from femur fracture sites by proximal femur aspiration during surgical treatment procedures. The study was approved by the Institutional Review Board of our hospital.

Isolation and culture of MSCs

MSCs were isolated from human bone marrow. Bone marrow was mixed with sodium-heparin and diluted with five volumes of phosphate buffered saline. The cell suspension was fractionated on a Percoll gradient (60% initial density, Pharmacia, Sweden). The MSC-enriched interface fraction was collected and cultured in Dulbecco's modified eagle medium with 1 g/mL glucose (DMEM/LG: Sigma, St. Louis, MO, USA), 10% fetal bovine serum (FBS), 1% penicillin streptomycin ampicillin (PSA), 0.2% amphotericin B and 1% gentamycin. The culture medium was changed every 3 days. Adherent cell colonies formed during primary culture were passaged when cells exhibited subconfluent proliferation. Second-passage cells were selected to identify the mechanisms of differentiation, as reported previously (Chen *et al.* 2005). The hematopoietic portion of the bone marrow was depleted, as described previously (Chen *et al.* 2006; Jiang *et al.* 2002).

Analysis of chondrogenic differentiation in monolayer culture

The hMSCs were seeded onto 48 35-mm plates after primary culture. The medium was changed after 24 h. The 48 plates were divided into four groups: (1) control group: hMSCs cultured in basic medium, without TGF- β 1, dexamethasone, or ultrasound (US); (2) LIPUS group: hMSCs cultured in basic medium with LIPUS treatment every 24 h; (3) TD group: hMSCs cultured in chondrogenic medium containing TGF- β 1 (10 ng/mL) and dexamethasone (10^{-7} M) as previously described (Chen *et al.* 2005); and (4) TD/LIPUS group: hMSCs cultured in

chondrogenic medium containing TGF- β 1 (10 ng/mL) and dexamethasone (10^{-7} M) with LIPUS treatment every 24 h.

There were 12 plates for each group and three plates for each condition. The basic medium contained DMEM/LG (Sigma D5523), 0.2% FBS, 1% PSA, 0.2% amphotericin B and 1% gentamycin. All samples were cultured at 37 °C with 5% CO₂. Culture medium was changed every 3 days until the samples were harvested. Cells exposed to LIPUS were treated with a single 20 min exposure every 24 h over periods of 1, 2, 3 and 4 weeks. Cells not exposed to LIPUS treatment were exposed to sham ultrasound stimulation (the LIPUS generator was not turned on). Samples from the four groups were harvested after 1, 2, 3 and 4 weeks.

Analysis of osteogenic differentiation in monolayer culture

After primary culture, hMSCs were seeded into 12 35-mm plates, which contained basic medium as described above. The 12 plates were divided into four groups: (1) control group: hMSCs cultured in basic medium without BMP-2 (recombinant human BMP-2: Wyeth Research, Cambridge, MA, USA) or LIPUS; (2) LIPUS group: hMSCs cultured in basic medium with LIPUS treatment every 24 h; (3) BMP group: hMSCs cultured in osteogenic medium containing BMP-2 (100 ng/mL) (Lecanda et al. 1997); and (4) BMP/LIPUS group: hMSCs cultured in osteogenic medium with LIPUS treatment every 24 h. There were three plates for each condition. LIPUS treatment involved a single 20 min exposure every 24 h for three days of culture. This treatment was chosen as previous studies have demonstrated that the expression levels of osteogenic genes are maximal on day three (Gleizal et al. 2006; Sant'Anna et al. 2005).

Application of LIPUS

A modified clinical LIPUS device (Sonopuls 490; Delft Instruments, Enraf-Nonius, The Netherlands) was used as previously described (Li et al. 2002, 2003). A diagram of the device is presented in Figure 1. The device consists of a circular surface transducer (T) with a diameter of 2.6 cm, 5.8 cm² circular surface area and an effective radiating area of 5.0 cm² that was placed at the bottom of a glass tank (GT) filled with distilled water (DW) and a culture well with an inner diameter of 33 mm located 30 mm above the transducer (Fig. 1a). A special absorption chamber (SAC) on top of the culture well directly coupled to the culture medium was used to minimize reflections of the LIPUS signal. The SAC was as described in our previous study (Li et al. 2002). Briefly, the chamber consists of a 32 mm diameter covering film (CF) attached to the culture medium (M). This was tightly sealed with an acrylic base covering (AC), which was

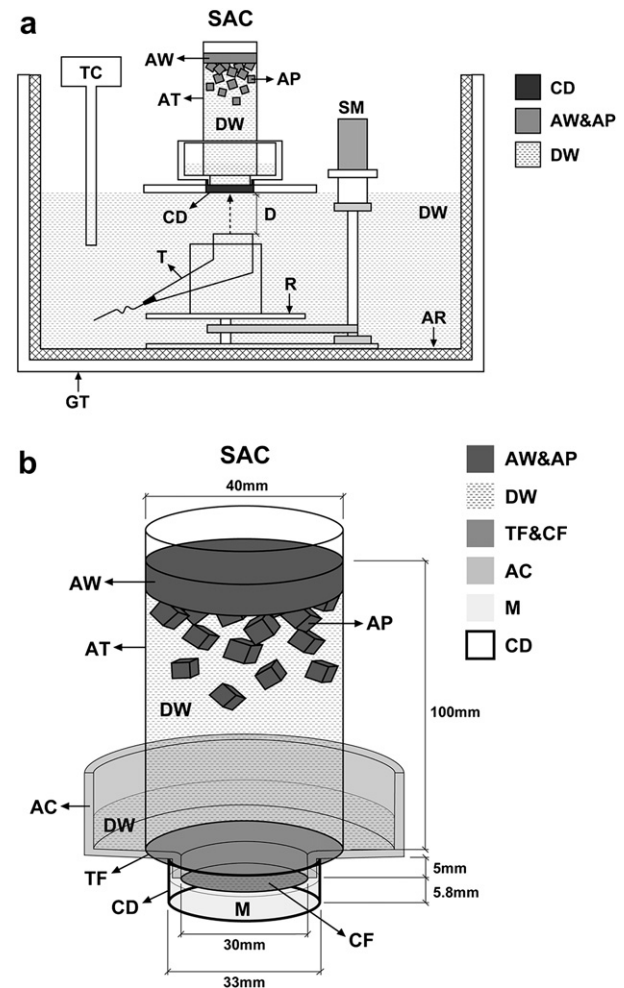


Fig. 1. (a) Diagram of the experimental set-up for exposure of mesenchymal stem cells (MSCs) grown as a monolayer to low-intensity pulse ultrasound (LIPUS). (b) An enlarged diagram of culture dish (CD) covered by specific absorption chamber (SAC) of the LIPUS exposure system. AP = absorption particle; AW = absorption wall; AT = acrylic base absorption tube; SAC = special absorption chamber; AR = absorption rubber; CD = culture dish; D = distance from transducer to cell flask, 30 mm; DW = distilled water; R = x-y rotator; SM = stepping motor; T = transducer; TC = temperature controller; M = culture medium; CF = covering film; AC = acrylic base covering; TF = absorption tube film.

sustained by the wall edge of the culture dish (CD). A 40-mm diameter absorption tube film (TF) was in contact with distilled water (DW) and tightly sealed with one end of an acrylic absorption tube (AT). The other end of the AT was sealed with an absorption wall (AW) and the AT contained 5-mm² absorption particles (APs). Both the AW and APs were made from sound-absorbing rubber foam (CALMFIEX F-6, Pineer Conductor, Taiwan) (Fig. 1b). The acrylic base covering and film were sterilized with 70% alcohol and then exposed to ultraviolet rays for at least 24 h before the experiments. This

absorption chamber did not reflect any ultrasound at the bottom of the film as detected by hydrophone (TNU 001A; NTR Systems, Seattle, WA, USA). The water temperature was modulated with a temperature controller and then degassed using a degassing tank. A stepping motor (SM) and rotator system (R) rotated the US transducer within a 10 mm radius in a horizontal circular plane around the axis of the culture dish at 0.1 Hz to increase the effective exposure area and avoid the production of standing waves (Fig. 1a). The water bath was filled with distilled, deionized and demineralized (3d) water, which was changed before each experiment. The inferior and side walls of the tank were covered with US-absorbing rubber (AR).

The apparatus was set to 1 MHz, pulsed 1:4 (2 ms “on” and 8 ms “off”) and the intensity was set to 200 mW/cm² (spatial-average temporal peak intensity) with a repetition rate of 100 Hz. The acoustic output power was measured and calibrated using a precision LIPUS power meter (UPM-DT-10; Ohmic instruments Co., St. Michaels, MD, USA). Findings from our previous study indicated that 1 MHz, pulsed 1:4 (2 ms “on” and 8 ms “off”) with a repetition rate of 100 Hz had an optimal effect on osteoblast differentiation (Li *et al.* 2002, 2003). In contrast to our findings, Ebisawa *et al.* reported that a 200 μ s tone burst repeating at 1.0 kHz, 30mW/cm² may better stimulate aggrecan synthesis (Ebisawa *et al.* 2004). In addition, other studies found that an intensity of 200 mW/cm² resulted in more pronounced MSC chondrogenesis *in vivo* and *in vitro* (Cui *et al.* 2006, 2007; Lee *et al.* 2006). Based on these reports, we chose to use a higher intensity (200 mW/cm²) to promote MSC chondrogenesis and a repetition rate of 100 Hz to optimally stimulate osteoblast differentiation and MSC osteogenesis.

Calibration was considered to be in the acceptable range if the error accuracy of the output readings was $\leq 10\%$. The intensity was previously determined using a calibrated needle hydrophone (TNU 001A, NTR Systems) at the Biomedical Engineering Center, Industrial Technology Research Institute, Taiwan. The output intensity varied with axial distance from the transducer head surface as shown in Figure 2. The output intensity varied with axial distance from the transducer head surface as shown in Figure 2. The distance from the face of the transducer to the bottom of the culture dish is 30 mm. At this distance, the power intensity is 164.93 mW/cm² before the culture dish and is 126.40 mW/cm² after the culture dish. The thickness of the bottom of the culture dish is 1 mm, thus, multiple reflections within the bottom of the dish may affect the ultrasound exposure of the cells. The field distribution from the transducer after passing through the culture well bottom was measured as previously described (Li *et al.* 2002).

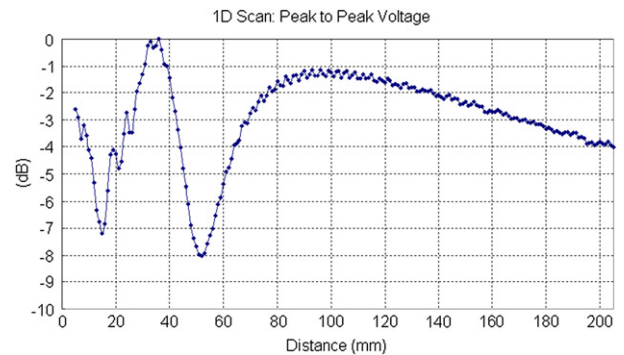


Fig. 2. Measurement of axial attenuation of the transducer transmission of acoustic waves (1 MHz, 200 mW) through distilled water.

Real-time PCR

Total RNA was extracted from each subconfluent monolayer culture (approximately 10⁶ MSCs) at the end of the incubation period using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Trizol extracts for the subconfluent monolayer cultures for each treatment group were collected separately. Total RNA was reverse-transcribed using the Superscript II System (Invitrogen Life Technologies) to yield complementary DNA (cDNA), which served as the template for real-time PCR to determine the messenger RNA (mRNA) expression levels of chondrogenesis- and osteogenesis-related genes. Primer sequences used in the experiment were as follows: Runx2, 5'-TTA CTT ACA CCC CGC CAGTC-3' and 5'-CAG CGT CAA CAC CAT TC-3' (Cho *et al.* 2005); collagen type II, 5'-TGGTCTTGGTGGAACTTTGC-3' and 5'-GCC CATTGGTCCTTGGATTA-3'; aggrecan, 5'-TGCATTC CACGAAGCTAACCT-3' and 5'-CGCCTCGCCTTCTT GAAAT-3'; Sox9, 5'-CAGTACCCGCACTTGCACAA-3' and 5'-CTCGTTTCAGAACTCTCCAGAGCTT-3' (Yagi *et al.* 2005); type I collagen, 5'-TTCCCCAGCCA-CAAAGAGTC-3' and 5'-CGTCATCGCACAAACACCT-3' (Miosge *et al.* 2004); β 1 integrin, 5'-AGTGAATGG GAACAACGAGGTC-3' and 5'-CAATTCCAGCAAC CACACCA-3' (Chen *et al.* 2006); and alkaline phosphatase (ALP) 5'-ACCATTCACGTCTTCACATTG-3' and 5'-AGACATTCTCTCGTTACCGCC-3' (Diefenderfer *et al.* 2003).

Real-time quantification was performed with the LightCycler assay, using a fluorogenic SYBR Green I reaction mixture with the LightCycler instrument (Roche, Mannheim, Germany). Expression levels of all genes were normalized to levels of β -actin mRNA within the same sample. The β -actin primer was designed using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) with published sequence data from the NCBI database (GeneBank NID:

NM_001101). Primer sequences for β -actin were 5'-GCA TCCCCAAAGTTCACAA-3' and 5'-AGGACTGGGC CATTCTCCTT-3'.

Statistical analysis

Continuous variables are presented as mean and standard deviation and were compared between groups by one-way analysis of variance (ANOVA). To identify significant between group differences compared, Bonferroni post-hoc tests were performed. Statistical significance was set at 0.05 for one-way ANOVA and for Bonferroni post-hoc tests, a corrected α (α') = 0.0083 (i.e., 0.05/6) was used. Statistical analyses were performed using SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphologic changes associated with chondrogenic differentiation of hMSCs

Control hMSCs exhibited a fibroblast-like morphology at the end of 1 week (Fig. 3a) whereas a less dense cell morphology was noted among the other three groups (Fig. 3b-d). Compared with the other conditions, more cuboidal cells were observed in the TD groups, both with and without LIPUS exposure (Fig. 3c and d).

The hMSCs retained fibroblast-like morphology after 2 weeks (Fig. 3e). A lower density of cells with increased intercellular space was evident after LIPUS treatment (Fig. 3f). In comparison with the observations from the first week, cells treated with TD exhibited less of a spindle-shaped morphology and a lower cell density (Fig. 3g). The cells treated with TD and LIPUS attained a mildly rectangular and nodular-like morphology (Fig. 3h).

After 3 weeks of culture, the spindle-shaped morphology of hMSCs was retained and cells were present at a higher density (Fig. 3i). After LIPUS treatment, hMSCs appeared loosely arranged, with increased intercellular space, similar to that seen in the second week (Fig. 3j). After TD treatment, hMSCs displayed a mild to moderate rectangular and nodular shape. A more pronounced nodular shape was noted after applying LIPUS to the TD treated cells (Fig. 3k and l).

After 4 weeks of culture, hMSCs retained a spindle-shaped fibroblastic morphology (Fig. 3m). After LIPUS treatment, cells also exhibited a spindle-shaped fibroblastic morphology and grew at a lower density (Fig. 3n). Cells treated with TD were of a somewhat more rectangular-like shape compared with those at week three (Fig. 3o). Rectangular- and nodular-like morphology was apparent with abundant extracellular matrix in cells exposed to TD with LIPUS (Fig. 3p).

Real-time PCR to detect changes in mRNA levels of genes associated with chondrogenic differentiation of hMSCs

To further support the chondrogenic differentiation of hMSCs induced by applying LIPUS after exposure to cytokines, the expression levels of genes involved in chondrogenic differentiation were analyzed by real-time PCR. The genes analyzed included integrin β 1, Sox9, aggrecan, type I collagen, type II collagen and Runx2. Table 1 presents a comparison of the mean expression levels of these genes. Expression levels were measured in hMSCs cultured for 1, 2, 3 and 4 weeks, for each of the four treatment groups (control, LIPUS, TD and TD/LIPUS).

A significant difference in integrin β 1 mRNA expression levels was noted between the four groups at the second and third weeks (both $p < 0.001$). Integrin β 1 expression in the TD and TD/LIPUS group were significantly higher compared with in the LIPUS and control groups. Integrin β 1 expression levels were optimal in all groups by the second week and were significantly lower at week four ($p < 0.001$). The integrin β 1 expression levels detected at week four were similar to those measured at week one (Fig. 4a).

Expression levels of Sox9 mRNA were significantly different between the four groups at each week analyzed (all $p < 0.001$), with the exception of week one. In the TD and TD/LIPUS groups, levels of Sox9 mRNA were significantly higher compared with the LIPUS and control groups. Within groups, the mean expression levels during the 4 weeks showed an increasing trend between weeks one and two and then decreased by week four in all groups. Significant differences existed between weeks in the LIPUS group ($p = 0.031$), the TD group ($p < 0.001$) and the TD/LIPUS group ($p < 0.001$) (Fig. 4b).

Levels of aggrecan mRNA expression differed significantly between groups at weeks three ($p < 0.001$) and four ($p = 0.002$). However, there was no between group differences at weeks one and two. Aggrecan mRNA expression levels in the TD and TD/LIPUS groups were significantly higher than levels in the LIPUS and control groups. Significant differences existed between weeks in all four groups ($p < 0.05$). There were increasing trends from week one to week four within all four groups, with the highest levels of aggrecan mRNA detected at week four (Fig. 4c).

Type II collagen mRNA levels were significantly different between groups at weeks three and four ($p < 0.001$). Levels of type II collagen mRNA were significantly higher in the TD and TD/LIPUS groups compared with the LIPUS and control groups. Significant differences existed between weeks in all four groups ($p < 0.001$). The mean expression levels of type II collagen within groups showed increasing trends from weeks one to three, with a slight decrease at week four. In contrast, minimal expression of type I collagen was apparent during weeks one and two. Mean type I collagen mRNA levels in the LIPUS

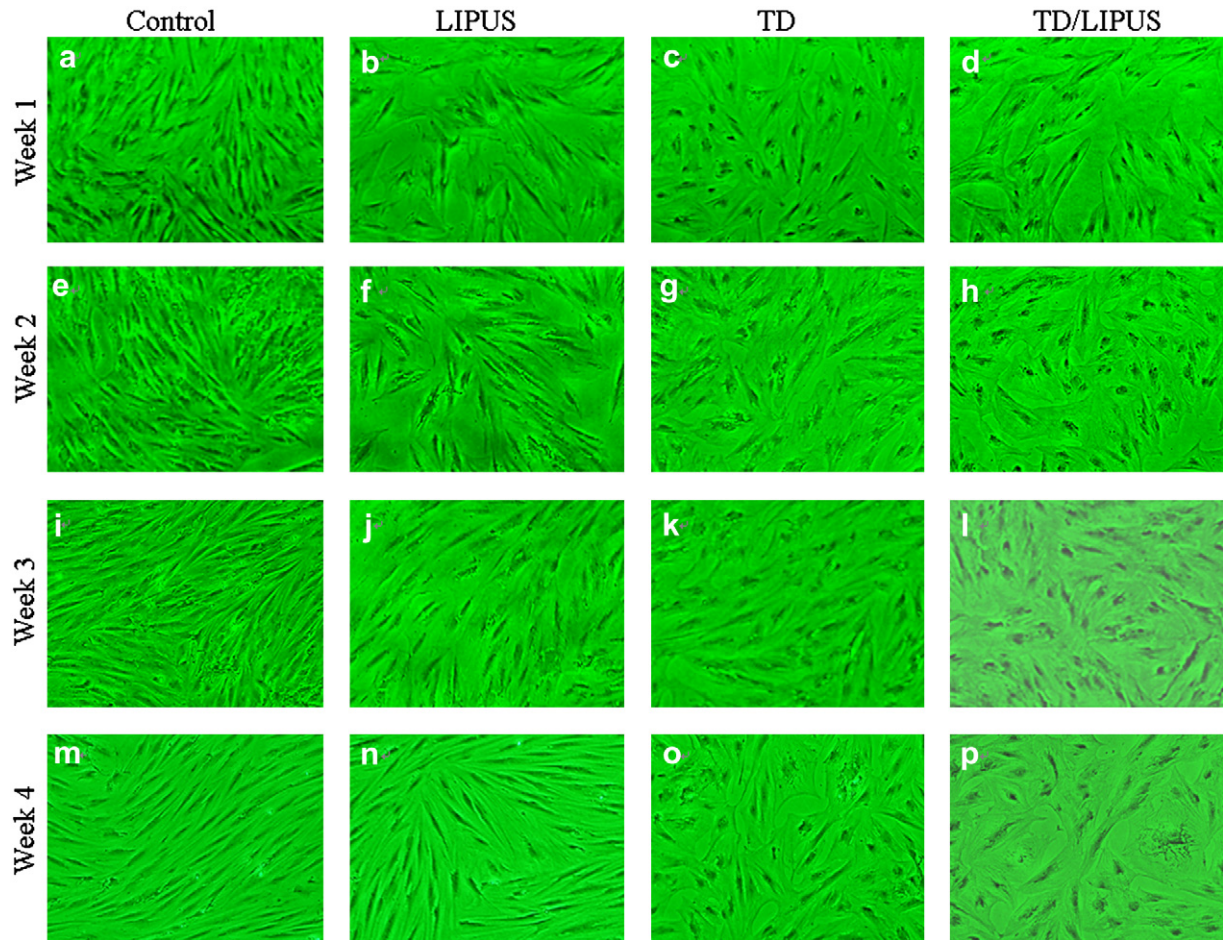


Fig. 3. Morphologic changes in the chondrogenic differentiation of human mesenchymal stem cells (hMSCs) in monolayer culture ($\times 100$). Cellular morphology and cell density were analyzed by microscopy and representative images of hMSCs grown in monolayer cultures for 1 week (a-d), 2 weeks (e-h), 3 weeks (i-l) and 4 weeks (m-p) are shown. The images represent four conditions analyzed for each week. hMSCs received either no treatment (control, a, e, i, m), low-intensity pulsed ultrasound (LIPUS) only (b, f, j, n), dexamethasone/transforming growth factor $\beta 1$ (TD) only (c, g, k, o) or combined TD/LIPUS treatment (d, h, l, p). In general, hMSCs displayed fibroblast-like morphology (a, e, i, m). When hMSCs were treated with only LIPUS, the cells appeared spindle-shaped but had increased intracellular space relative to the control cells. In addition, a lower density of cells was noted (b, f, j, n). Cells treated with TD (c, g, k, o) and TD/LIPUS (d, h, l, p) displayed a more cuboidal shape; this characteristic became more noticeable with time.

group were significantly higher than in the TD and TD/LIPUS groups at each week except for week two. In addition, significant differences were detected between weeks in each group (all $p < 0.05$). There was a significant trend for the mean levels of type I collagen mRNA to increase from week one to week four in each group (Fig. 4d and e).

Interestingly, expression levels of Runx2 mRNA were not altered in the TD groups, with or without LIPUS. Significant between group differences were apparent at every week ($p < 0.001$). The mean expression level of Runx2 mRNA in the LIPUS group was significantly higher than in the other three groups at each week. There was a trend for Runx2 expression levels to increase from week one to week four, however, the differences were not statistically significant (Fig. 4f).

Morphologic changes associated with osteogenic differentiation of hMSCs

hMSCs exhibited a fibroblast-like morphology after being cultured for 3 days with basic medium while cell density decreased after treatment with LIPUS (Fig. 5a and b). hMSCs treated with BMP and LIPUS exhibited cuboidal morphology (Fig. 5d), with a lower density and increased intercellular space compared with cells in the BMP only group (Fig. 5c).

Real-time PCR results regarding the osteogenic differentiation of hMSCs

To investigate osteogenic differentiation induced by LIPUS and BMP-2 treatment, 12 hMSCs samples were collected and divided into four groups: Control, LIPUS,

Table 1. Summary of the mRNA expression levels of integrin $\beta 1$, Sox9, aggrecan, type I collagen, type II collagen and Runx2

			Group				p value
			Control	LIPUS	TD	TD/LIPUS	
Integrin $\beta 1$	Week	1	0.367 (0.058)	0.5 (0.1)	0.5 (0.1)	0.533 (0.058)	0.137
		2	0.5 (0.1)	1.1 (0.1) ^{a†}	1.067 (0.115) ^{a†}	1.133 (0.058) ^{a†}	<0.001*
		3	0.467 (0.058)	0.8 (0.1) ^a	0.833 (0.058) ^a	0.933 (0.058) ^a	<0.001*
		4	0.4 (0.1)	0.5 (0.1) [‡]	0.467 (0.058) [‡]	0.567 (0.058) ^{‡§}	0.17
Sox9	Week	1	0.012 (0.001)	0.015 (0.001)	0.015 (0.002)	0.017 (0.002)	0.067
		2	0.016 (0.003)	0.022 (0.003)	0.034 (0.004) ^{a†}	0.049 (0.005) ^{ab†}	<0.001*
		3	0.015 (0.003)	0.021 (0.003)	0.03 (0.003) ^{a†}	0.048 (0.004) ^{abc†}	<0.001*
		4	0.014 (0.001)	0.017 (0.002)	0.024 (0.002) ^a	0.033 (0.003) ^{ab†‡§}	<0.001*
Aggrecan	Week	1	0.027 (0.006)	0.03 (0.01)	0.027 (0.006)	0.03 (0.01)	0.916
		2	0.053 (0.006)	0.063 (0.021)	0.067 (0.012)	0.077 (0.015)	0.33
		3	0.107 (0.015) [‡]	0.143 (0.021) [‡]	0.173 (0.023) ^{†‡}	0.257 (0.025) ^{abc†‡}	<0.001*
		4	0.113 (0.032) [‡]	0.157 (0.038) [‡]	0.197 (0.032) ^{†‡}	0.273 (0.032) ^{a†‡}	0.002*
Type I collagen	Week	1	0.049 (0.008)	0.066 (0.006)	0.032 (0.003) ^b	0.03 (0.003) ^b	<0.001*
		2	0.113 (0.015)	0.14 (0.02)	0.087 (0.021)	0.083 (0.025)	0.032*
		3	0.233 (0.065)	0.52 (0.053) ^{†‡}	0.153 (0.025) ^{b†}	0.16 (0.03) ^{b†}	<0.001*
		4	0.343 (0.09) ^{†‡}	0.567 (0.09) ^{†‡}	0.23 (0.026) ^{b†‡}	0.23 (0.036) ^{b†‡}	0.001*
Type II collagen	Week	1	0.005 (0.001)	0.007 (0.002)	0.006 (0.001)	0.007 (0.003)	0.525
		2	0.29 (0.053) [‡]	0.387 (0.095)	0.42 (0.095) [‡]	0.543 (0.186)	0.147
		3	0.467 (0.055) [‡]	0.683 (0.146) [‡]	1.087 (0.099) ^{a†‡}	1.733 (0.134) ^{abc†‡}	<0.001*
		4	0.423 (0.078) [‡]	0.593 (0.122) [‡]	0.967 (0.131) ^{†‡}	1.667 (0.2) ^{abc†‡}	<0.001*
Runx2	Week	1	0.00065 (0.00011)	0.00156 (0.00012) ^a	0.00053 (0.00014) ^b	0.00048 (0.0001) ^b	<0.001*
		2	0.00069 (0.00013)	0.00157 (0.00015) ^a	0.00052 (0.00008) ^b	0.00049 (0.00014) ^b	<0.001*
		3	0.00083 (0.00011)	0.00167 (0.00014) ^a	0.00067 (0.00011) ^b	0.00073 (0.00014) ^b	<0.001*
		4	0.00097 (0.00017)	0.00163 (0.00019) ^a	0.00067 (0.00013) ^b	0.00074 (0.00013) ^b	<0.001*

* $p < 0.05$ as determined by one-way ANOVA. †Significant difference between the indicated week and week 1 for a given group as determined by Bonferroni post hoc test. ‡Significant difference between the indicated week and week 2 for a given group as determined by Bonferroni post hoc test. §Significant difference between the indicated week and week 3 for a given group as determined by Bonferroni post hoc test. ^aSignificant difference between the indicated group and the control group for a given week as determined by Bonferroni post-hoc test. ^bSignificant difference between the indicated group and the LIPUS group for a given week as determined by Bonferroni post-hoc test. ^cSignificant difference between the indicated group and the TD group for a given week as determined by Bonferroni post-hoc test.

BMP and BMP/LIPUS. For each treatment group, the mRNA expression levels of four osteogenic markers were measured by real-time PCR. These results are depicted in Figure 6. The osteogenic markers analyzed were alkaline phosphatase (ALP), Runx2, type I collagen and type II collagen. There were significant between group differences in expression levels of ALP ($p = 0.004$) and Runx2 ($p = 0.001$). The increase in ALP mRNA expression was slightly higher in the BMP/LIPUS group compared with both the LIPUS and BMP groups (Fig. 6a). The mean expression level of Runx2 mRNA in both the LIPUS and BMP groups was significantly increased compared with the control group, however, these levels were slightly lower compared with in the LIPUS/BMP co-treatment group (Fig. 6b). There were no significant between group differences in either type I collagen (Fig. 6c) or type II collagen (Fig. 6d) mRNA expression levels. There was low expression of type I collagen and almost no expression of type II collagen in all groups.

DISCUSSION

There is an extensive body of literature reporting on the chondrogenic and osteogenic differentiation of MSCs (Maniatopoulos 1988; Nakahara et al. 1990). Factors

promoting chondrogenic or osteogenic differentiation include the extracellular matrix (ECM) (Chen et al. 2005; Ou et al. 2009), local cytokines (Johnstone et al. 1998) and mechanical stress (Friedl et al. 2007). These factors enable local MSCs to accumulate, proliferate and terminally differentiate into chondrocytes or osteocytes. The use of MSCs in tissue repair has great potential clinical significance. Cell culture studies have shown that LIPUS can stimulate expression of Runx2, collagen, alkaline phosphatase, osteocalcin, IGF-1 and TGF- $\beta 1$ in fibroblasts, osteoblasts and bone marrow stromal cells (Azuma et al. 2001; Li et al. 2002; Sant'Anna et al. 2005; Warden et al. 2001).

LIPUS has been reported to have various biologic effects and triggers different signaling events involved in bone formation (Azuma et al. 2001; Takayama et al. 2007; Warden et al. 2001) and cartilage regeneration (Choi et al. 2006; Cook et al. 2001; Parvizi et al. 1999; Zhang et al. 2003) *in vivo* and *in vitro* and is currently used clinically for stimulation of bone healing. In addition, there is also considerable interest in the clinical application of BMPs for bone healing. As early as the 1990s LIPUS was shown to accelerate bone healing in rabbit models (Pilla et al. 1990) and in a randomized, double-blind study accelerated healing of tibial shaft

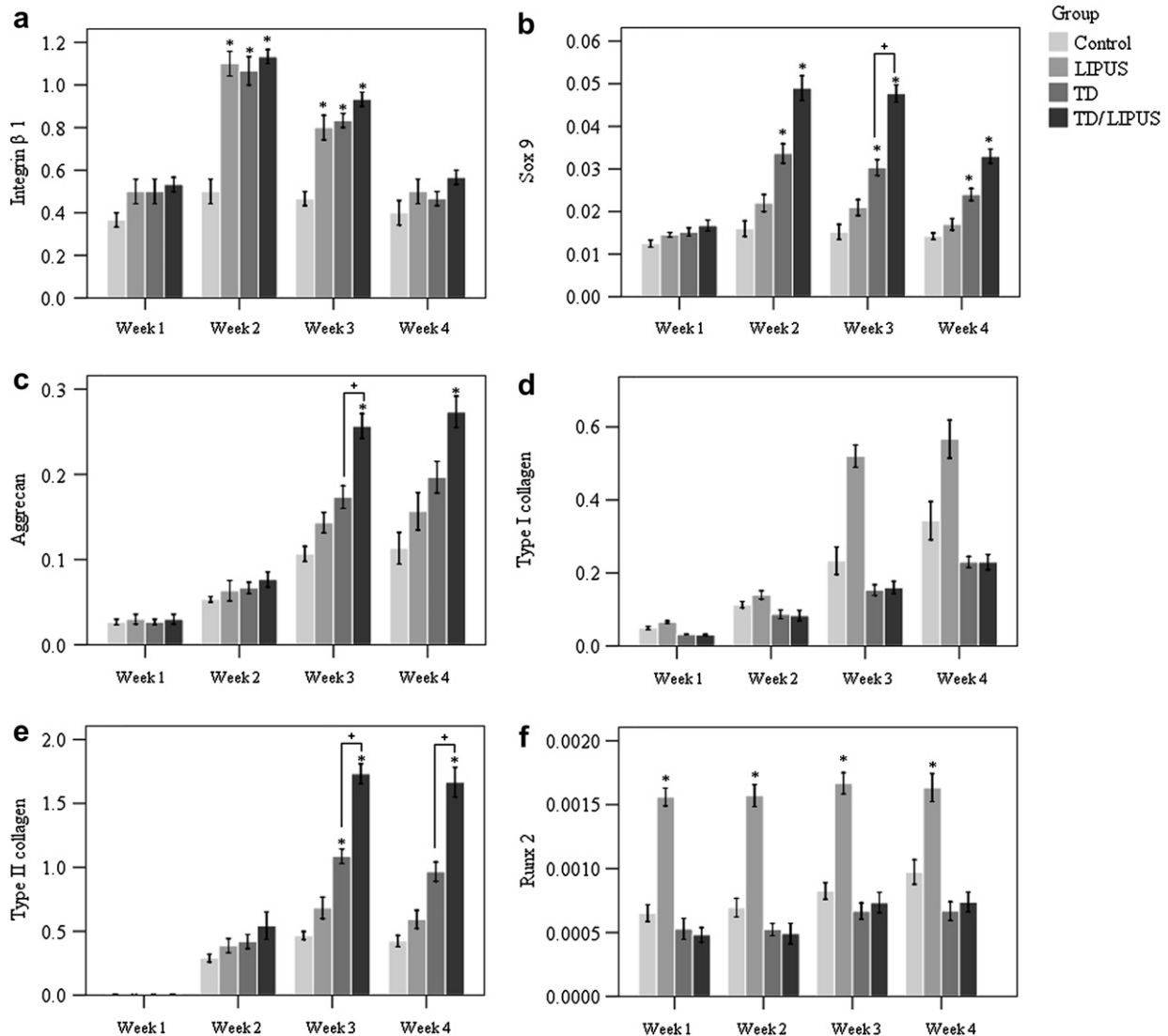


Fig. 4. Box plots of mRNA expression levels of integrin $\beta 1$, Sox9, aggrecan, type I collagen, type II collagen and Runx2 under different treatment conditions (graphic presentation of Table 1 data).

fractures in humans (Heckmann *et al.* 1994). More recently, LIPUS was shown to accelerate clinical fracture healing of delayed unions of the fibula (Rutten *et al.* 2008). The authors found that LIPUS increased osteoid thickness, mineral apposition rate and bone volume at the front of new bony callus formation, which indicated increased osteoblast activity. Other authors have reported accelerated healing of articular cartilage (Jai *et al.* 2005) and tendon graft-bone interface (Papatheodorou *et al.* 2009) in rabbit models. Few studies, however, have investigated the effects of LIPUS on the differentiation of human MSCs (hMSCs). We hypothesized that a combination of these two therapies, LIPUS and BMPs, may provide better results than either treatment alone.

This is also true regarding cartilage regeneration research (Jia *et al.* 2005). If damage to the articular

cartilage extends to within the bone, pluripotential mesenchymal cells migrate to the site of injury (Buckwalter and Mankin 1998; O'Driscoll *et al.* 1988). The present study attempted to characterize the effects of LIPUS alone and in combination with cytokines TD and BMP-2 on chondrogenic and osteogenic differentiation and has potential clinical significance given that LIPUS has already been demonstrated to accelerate bone growth during fracture healing and distraction osteogenesis (Azuma *et al.* 2001; Reuter *et al.* 1984; Sant'Anna *et al.* 2005; Takikawa *et al.* 2001; Warden *et al.* 2001).

This study examined the effect of combined TD and LIPUS treatment to induce chondrogenic differentiation of hMSCs. The results demonstrated that TD and TD/LIPUS induced morphologic changes associated with chondrogenic differentiation (Fig. 2). In addition, we detected

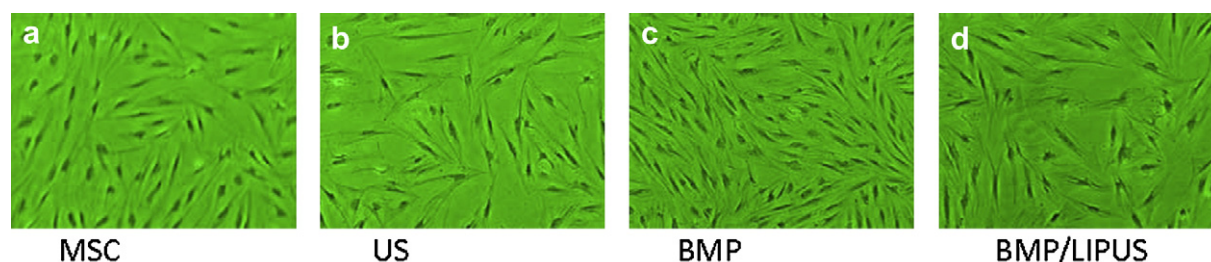


Fig. 5. Morphologic changes in the osteogenic differentiation of human mesenchymal stem cells (hMSCs) in monolayer culture ($\times 100\times$). hMSCs exhibited fibroblast-like morphology after three days of culture in basic medium and became less dense after treatment with LIPUS (a, b). When supplemented with bone morphogenetic protein (BMP) and LIPUS, cells exhibited cuboidal morphology (d), with a lower density and more intercellular space compared with cells in BMP only group (c).

increases in the mRNA expression levels of chondrogenic markers such as Sox9, aggrecan and type II collagen in hMSCs treated with TD and TD/LIPUS (Table 1). Our findings are in accordance with what has been reported in the literature. Chondrogenesis of bone marrow progenitors was reported to be stimulated in the presence of the TD (Chen et al. 2005; Grigoriadis et al. 1996) and in a prior study, we found that levels of type II collagen and Sox9 mRNA increased when hMSCs were treated with TD (Chen et al. 2005).

This study provides evidence of an enhanced effect of LIPUS and TD on chondrogenic differentiation of

hMSCs. Our data indicate that LIPUS enhanced chondrogenic differentiation of TD-treated hMSCs but not of non-TD treated cells. This finding is in agreement with that reported by Ebisawa and colleagues. Combined, our findings indicate that the TGF- $\beta 1$ signal may be mediated by specific membrane receptors and Smad signaling (Attisano and Wrana 2002; van der Kraan et al. 2009). Our data suggests that the effect observed during LIPUS treatment occurs via an integrin-mediated mechanotransduction pathway. Specifically, in our three experimental groups, we observed a significant increase in the mRNA expression of integrin $\beta 1$ after 2 and 3 weeks of hMSC

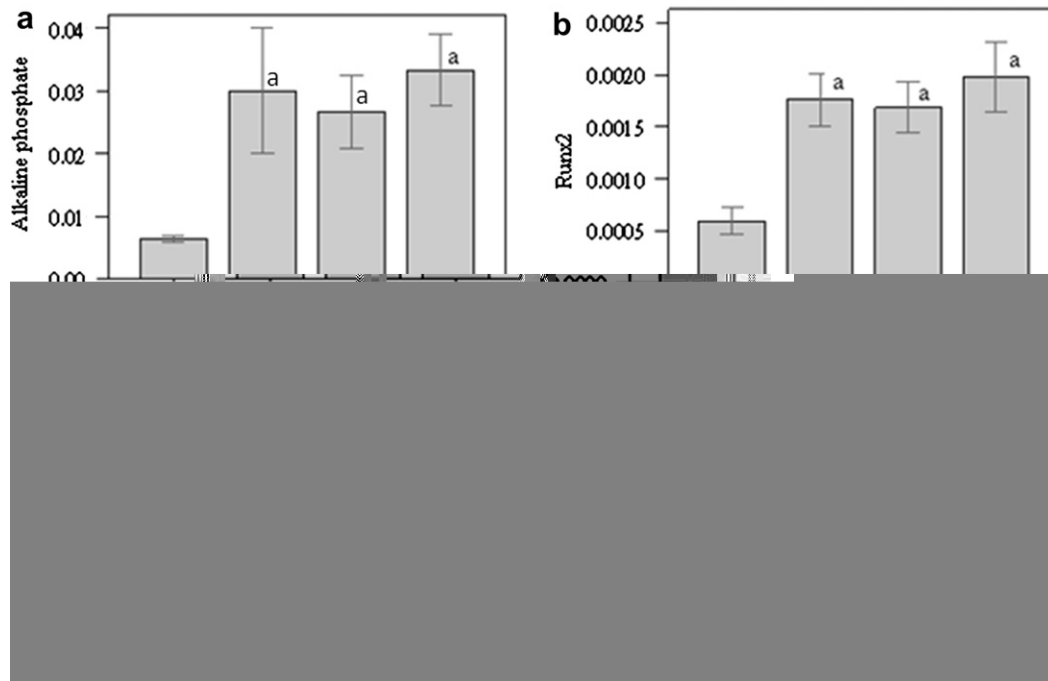


Fig. 6. Effect of low-intensity pulsed ultrasound (LIPUS) and/or bone morphogenetic protein (BMP) treatment on expression of genes related to osteogenic differentiation of human mesenchymal stem cells (hMSC). Quantitative real-time PCR was performed to determine the effects of various treatments on the expression of mRNAs encoding (a) alkaline phosphatase (ALP), (b) Runx2, (c) type I collagen and (d) type II collagen. Cells were treated for three days under the following conditions: A nontreated control, LIPUS only, BMP only and LIPUS/BMP. The data represent mRNA levels for each gene that have been normalized to β -actin mRNA levels for each condition.

treatment. The increase was followed by a dramatic decrease in integrin $\beta 1$ mRNA expression at week four to a level similar to that assessed at week one. These results also agree with previous studies reporting that mechanical signaling was mediated by integrins (Lee *et al.* 2006; Zhou *et al.* 2004).

Integrins are heterodimeric transmembrane glycoproteins consisting of one α - and one β -subunit. The $\beta 1$ subfamily of integrins consists of many ECM receptors, including those for fibronectin, collagens and laminin. For example, integrin $\alpha 2\beta 1$ is the preferential receptor for type II collagen (Loeser *et al.* 2000). Integrin binding stimulates intracellular signaling which can affect gene expression and alter cellular expression and affinity of integrins (Humphries 1990; Hynes 1992; Loeser *et al.* 2000). The current hypothesis is that integrins function by inducing a conformational change which results in repositioning of the ligand binding site to a more accessible position away from the cell surface. This conformational change also triggers intracellular signaling. This hypothesis is supported by data demonstrating that, in addition to playing a role promoting adhesion to ECM ligands or counter-receptors on adjacent cells, integrins serve as a transmembrane mechanical link between extracellular contacts and the cytoskeleton (Hynes 2002). In addition, many integrins are not constitutively active; in fact, they are normally expressed on the cellular surface in an inactive or "OFF" state, a state in which they do not bind ligands and do not signal (Hynes 2002). We hypothesize that LIPUS must somehow activate integrins, perhaps by facilitating a mechanical change in the conformation of the integrin molecule. The mRNA expression levels of integrin $\beta 1$ and the chondrogenic transcription factor, Sox9, were significantly increased at week two. Aggrecan mRNA and type II collagen mRNA levels were increased at weeks three and four. The mRNA levels of integrin $\beta 1$ (the receptor) and Sox9 (a transcription factor) were up-regulated more rapidly in this study than mRNA levels of aggrecan and type II collagen.

Our studies revealed that mRNA expression levels of aggrecan and type II collagen were increased by LIPUS treatment. These data confirm findings from previous studies reporting that LIPUS treatment enhanced aggrecan mRNA expression and proteoglycan synthesis of chondrocytes cultured in a monolayer (Parvizi *et al.* 1999). Our 2-D data showed that aggrecan and type II collagen did not increase in the presence of LIPUS, with or without TD, until week three. This may be explained by the fact that ECM synthesis is followed by cytokine or mechanical stimulation.

In our study of osteogenic differentiation of hMSCs, mRNA levels of Runx2, an osteogenic transcription factor, did not significantly increase from week one to

four in any group except the LIPUS group. Runx2 mRNA expression levels in the LIPUS treatment group were increased at week one. Expression of type I collagen mRNA did not increase until weeks three and four in the LIPUS group. These results indicate that in hMSCs, LIPUS treatment can only stimulate the expression of genes associated with osteogenesis. Levels of Runx2 mRNA were upregulated at week one while type I collagen mRNA levels did not increase until week three in this study.

It has previously been reported that LIPUS and BMP-2 individually increase Cbfa-1 and ALP mRNA expression, with maximal levels being attained on day three (Sant'Anna *et al.* 2005). However, combined treatment with LIPUS and BMP-2 did not result in a synergistic effect. In another study, it was found that BMP-2 enhanced the osteogenic differentiation of human bone marrow stromal cells in a dose-dependent manner (Lecanda *et al.* 1997). In this study, we observed a morphologic shift from elongated fibroblast-like cells to shorter and shaped cuboidal cells 3 days after LIPUS and BMP treatment. In addition, we observed increases in the mRNA levels of osteogenic markers such as Runx2 and ALP. This is in keeping with findings from previous reports. We found that there were no significant differences in mRNA expression of Runx2 and ALP among the LIPUS, BMP-2 and LIPUS/BMP-2 groups. BMP-2 receptors act through the Smads signaling proteins, which influence the function of Runx2 (Hanai *et al.* 1999; Zijnsen *et al.* 2003).

We acknowledge that our study has several limitations. It was difficult to compare week four-induced-chondrogenesis to day three-induced-osteogenesis. In particular, we need to provide additional evidence that cytokines can increase the production of integrins at the cell surface before LIPUS is applied. It appears that LIPUS treatment can somehow activate integrins by an unknown mechanism.

In conclusion, LIPUS enhances chondrogenic differentiation of hMSCs treated with TD. The combined treatment of LIPUS and TD induced integrin $\beta 1$, Sox9, aggrecan and type II collagen mRNA expression. LIPUS alone can increase osteogenic differentiation. LIPUS with BMP induced ALP and Runx2 mRNA expression. Clinically, LIPUS may differentially influence bone vs. cartilage repair.

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