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Interactive Effects of Mechanical Stretching and Extracellular Matrix Proteins on Initiating Osteogenic Differentiation of Human Mesenchymal Stem Cells

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ABSTRACT

Human mesenchymal stem cells (hMSCs) are characterized by their abilities to differentiate into different lineages, including osteoblasts. Besides soluble factors, mechanical strain and extracellular matrix (ECM) proteins play important roles in osteogenic differentiation of hMSCs. However, interactions between them are still not fully understood. The purpose of this study was to investigate the combined effects of insoluble chemical and mechanical factors (ECM proteins vs. cyclic stretching) in driving hMSCs into osteogenic differentiation. To avoid the influence from osteogenic supplements, hMSCs were cultured in regular medium and subjected to cyclic mechanical stretching using a Flexcell Tension system (3% elongation at 0.1 Hz) when they were grown on substrates coated with various ECM proteins (collagen I (Col I), vitronectin (VN), fibronectin (FN), and laminin (LN)). Using alkaline phosphatase (ALP) activity and mineralized matrix deposition as respective indicators of the early and late stages of osteogenesis, we report herein that all of the ECM proteins tested supported hMSC differentiation into osteogenic phenotypes in the absence of osteogenic supplements. Moreover, cyclic mechanical stretching activated the phosphorylation of focal adhesion kinase (FAK), upregulated the transcription and phosphorylation of core-binding factor alpha-1 (Cbfa1), and subsequently increased ALP activity and mineralized matrix deposition. Among the ECM proteins tested, FN and LN exhibited greater effects of supporting stretching-induced osteogenic differentiation than did Col I and VN. The ability of ECM proteins and mechanical stretching to regulate osteogenesis in hMSCs can be exploited in bone tissue engineering via approximate matrix design or application of mechanical stimulation. J. Cell. Biochem. 108: 1263–1273, 2009.

KEY WORDS: HUMAN MESENCHYMAL STEM CELLS (HMSCS); CYCLIC MECHANICAL STRETCHING; EXTRACELLULAR MATRIX; OSTEOGENIC DIFFERENTIATION

Human mesenchymal stem cells (hMSCs) are known to be multipotent cells capable of differentiating into various mesenchymal phenotypes, including osteogenic [Bruder et al., 1997, 1998], myogenic [Ferrari et al., 1998], chrondogenic, and adipogenic lineages [Pittenger et al., 1999; Franceschi and Xiao, 2003]. hMSCs can be an excellent cell resource to regenerate damaged or diseased bone tissues because they can be obtained from bone marrow aspirate of adult individuals and amplified in vitro [Pittenger et al., 1999; Franceschi and Xiao, 2003]. Therefore, understanding the factors controlling hMSCs' osteogenic differentiation is critical for their use in bone tissue engineering. In addition to osteogenic supplements, such as ascorbic acid, beta-glycerophosphate, and dexamethasone, some insoluble factors also play critical roles in driving hMSCs to commit to an osteogenic phenotype.

The influences of insoluble factors arise primarily from cellular binding to extracellular matrix (ECM) proteins. The engagement of ECM proteins by cell adhesion receptors can activate intracellular signaling cascades, and subsequently upregulate the expressions of alkaline phosphatase (ALP) and osteocalcin (OCN) in hMSCs [Kundu and Putnam, 2006; Klees et al., 2007; Salasznyk et al. 2007]. ECM proteins include collagen and non-collagenous glycoproteins (e.g., fibronectin (FN), vitronectin (VN), and laminin (LN)). Collagen type I

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(Col I) is the most abundant ECM protein in connective tissues and also a major constituent of the bone matrix. FN plays important roles in cell adhesion, migration, growth, and differentiation. VN is also involved in cell adhesion and spreading, and LN is the major non-collagenous component of basal lamina. Both Col I and VN were shown to support the osteogenic differentiation of hMSCs, and the initial cell–ECM interaction is an important regulator of hMSC fate [Salasznyk et al., 2004]. It was recently reported that the signaling pathway regulating osteogenic differentiation of hMSCs on laminin-5, in the absence of any soluble osteogenic supplements, was via focal adhesion kinase (FAK) activation [Klees et al., 2007]. Cellular binding to ECM proteins mainly occurs via integrin receptors, which play a critical role in the mechano-transduction process by connecting the cytoskeleton to the ECM and converting mechanical stimuli into biochemical signals [Huang and Ingber, 1999; Katsumi et al., 2005].

Mechanical loading plays important roles in regulating the development, function, and repair of musculoskeletal tissues. Similar to fibroblasts and osteoblasts, hMSCs can sense substrate deformation and respond differentially to mechanical stretching depending on its magnitude, duration, and frequency [Sai et al., 1999; Chen et al., 2008]. Mechanical stretching was shown to promote osteogenic differentiation of osteoblasts by increasing the tyrosine phosphorylation of FAK [Yano et al., 1996; Sai et al., 1999] and activating the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways [Wang et al., 2001], thus upregulating the expressions of core-binding factor alpha-1 (Cbfa1), ALP, and OCN [Owen et al., 1990]. Many researchers investigated the effects of mechanical stretching on cultured cells by deforming the growth substrates coated with Col I [Simmons et al., 2003; Chen et al., 2008; Lee et al., 2007; Ward et al., 2007]. However, interactions of ECM proteins and mechanical stretching in driving hMSCs to commit to an osteogenic phenotype are still not fully understood. The purpose of this study was to investigate the effects of mechanical stretching on the phosphorylation of FAK and ERK, on the transcription and phosphorylation of Cbfa1, on ALP activity, and on matrix mineralization in hMSCs grown on various ECM proteins.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), PBS lacking Ca^{2+} and Mg^{2+} (PBS(−)), trypsin–EDTA, and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Penicillin G-streptomycin sulfate was purchased from Gibco-BRL Life Technologies (Paisley, UK). Purified human plasma FN, LN, VN, and a mouse monoclonal immunoglobulin G (IgG) antibody against phospho-specific anti-FAK (pY397) were from BD Biosciences (Franklin Lakes, NJ). The actin cytoskeleton staining kit, phosphor-serine, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-rabbit IgG secondary antibodies were purchased from Chemicon International (Billerica, MA). Rabbit monoclonal IgG antibodies against ERK1/2 and phopho-p44/42 MAP kinase (p-ERK) were purchased from Cell Signaling Technology (Danvers, MA). Cbfa1 was from MBL International (Woburn, MA). The Catch and Release1 v2.0 reversible immunoprecipitation system was from Millipore (Billerica, MA). The TRIzol reagent was purchased from Invitrogen (Carlsbad, CA), and real-time reverse-transcriptase polymerase chain reaction (RT-PCR) kits and probes were purchased from Applied Biosystems (Carlsbad, CA). The protein assay kit was purchased from Pierce (Rockford, IL).

CELL CULTURE

To isolate hMSCs, individual bone marrow aspirates of 10–20 ml were taken from the iliac crests of three patients ranging in age from 55 to 65 years. Institutional Review Board approval was obtained. The hMSCs were purified by density centrifugation [Pittenger et al., 1999], cultured in DMEM with 10% FBS and antibiotic/antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. After 24 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with PBS. The culture medium was changed twice a week, and non-adherent cells were discarded until the hMSCs had reached confluence. Cells were harvested with 0.05% trypsin–0.53 mM EDTA for 5 min at 37°C.

APPLICATION OF MECHANICAL STRETCHING TO CULTURED CELLS

hMSCs in the 3rd to 6th passages were used in this study, and cells for the control and experimental conditions were at the same passage numbers. Flexible-bottomed culture plates (Flexcell International, Mckeesport, PA) were coated overnight at 4°C with Col I, VN, FN, or LN. Coated plates were washed twice with PBS and treated with 1% bovine serum albumin (BSA) for 30 min prior to cell seeding at a density of 1.5×10^4 cells/cm^2. To reduce the effects of FBS, cells were maintained in low-serum medium (2% FBS) for the first 24 h, which was replaced by medium with 10% serum in subsequent culture.

The Flexcell Tension Plus system 3000 (Flexcell International) was used to apply cyclic mechanical stretching with 3% surface elongation at 0.1 Hz for 1, 3, or 5 days. hMSCs seeded onto various ECM-coated surfaces were allowed to completely spread for 24 h before being subjected to mechanical stretching for the experiments described below. Cells grown on similarly coated plates but without mechanical stretching served as the controls.

METABOLIC ASSAY

Cells with functional mitochondria reduce the tetrazolium salt, 3,[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to the insoluble purple formazan. The cellular activities of hMSCs grown on various ECM proteins, without or with mechanical stretching for 1, 3, and 5 days, were measured by an assay based on the reduction of MTT. Briefly, cells were incubated with fresh medium containing 0.5 mg/ml MTT for 2 h, and the medium was aspirated. The cell layer with formazan was dissolved with dimethyl sulfoxide. The optical density at 570 nm was measured with a Dias microwell plate reader (Dynatech Medical Products, St. Peter Port, UK), and the absorbance was taken as proportional to the number of viable cells present.

IMMUNOFLUORESCENCE STAINING

The F-actin cytoskeleton in hMSCs was checked by immunofluorescence staining. After being subjected to mechanical stretching for 1, 3, and 5 days, hMSCs were fixed with 4% paraformaldehyde for 20 min, washed with PBS containing 0.05% Tween-20 twice, then permeabilized with 0.1% Triton X-100 for 5 min, and blocked with 1% BSA for 30 min. TRITC-conjugated phalloidin at 1:1,000 was used as the primary antibody and was incubated with cells for 1 h. Finally, 4′,6-diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei. Cells were visualized with a fluorescence microscope (Zeiss AXIOSVERT 200M, Göttingen-Vertrieb, Germany).

ELECTROPHORESIS AND WESTERN ANALYSIS

Detection of activated or total levels of FAK, ERK, and Cbfa1 in hMSCs was performed by electrophoresis and Western blot analysis. hMSCs were seeded onto various ECM-coated surfaces with low-serum medium (2% FBS) and allowed to completely spread for 24 h before being subjecting to mechanical stretching, at which time, medium with 10% FBS was used. After six different periods of mechanical stimulation (1, 4, and 8 h and 1, 3, and 5 days), whole-cell extracts were prepared by harvesting cells in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 1 mM sodium vanadate, 1 mM EDTA, and 0.05% sodium dodecylsulfate (SDS); pH 7.5) with sonication for 10 s. Lysates were then centrifuged at 15,000 rpm for 20 min at 4°C. The total protein concentration of the resulting supernatant was determined using a bicinchoninic acid (BCA) protein assay kit. For immunoprecipitation of Cbfa1, the Catch and Release® v2.0 Reversible immunoprecipitation system was used. Briefly, 500 μg of cell lysate, 2 μg of the Cbfa1 antibody, and 10 μl of the antibody capture affinity ligand were added to a spin column, and sufficient wash buffer was added to a final volume of 500 μl. Following an overnight incubation on a rotator at 4°C, the columns were centrifuged at 5,000 rpm for 30 s to discard all non-labeled proteins. The denatured Cbfa1 protein was eluted by centrifugation after adding 70 μl of denaturing elution buffer containing β-ME. Then the proteins (whole cell extract or immunoprecipitate of Cbfa1) were diluted in 5× Laemmli's sample buffer, denatured at 95°C for 5 min, resolved by 10% SDS–PAGE, and electrophoretically transblotted onto polyvinylidene difluoride (PVDF) microporous membranes. The membranes were incubated with blocking solution (10% non-fat dried milk in PBS) overnight, then probed with various primary antibodies (1 μg/ml Cbfa1, 2.5 μg/ml phosphor-serine, 0.25 μg/ml p-FAK, and 1:1,000 diluted ratios of FAK, ERK, and p-ERK) for 1 h at room temperature. After three washes with PBST (0.5% Tween 20 in PBS), membranes were incubated with HRP-conjugated secondary mouse IgG1 (1:10,000) to p-FAK, Cbfa1, and phosphor-serine, and rabbit IgG1 (1:10,000) to FAK, ERK1/2, and p-ERK/1/2 for 1 h, followed by another three washes with PBST. Immunoreactive bands were detected by Immobilon Western Chemiluminescent HRP Substrate® (Millipore) and quantitatively analyzed in triplicate by normalizing the band intensities to the controls on scanned films with Alpha Image® software. Blots to be re-probed were stripped with stripping buffer (15 g glycine, 1 g SDS, and 10 ml Tween 20, to a final volume of 1 L with ultrapure water at pH 5.5) for 30 min and then treated as described above. All blots were re-probed for total levels of these proteins confirming equal protein loading. All blot data were confirmed by repeating experiments to assure the repeatability.

RNA ISOLATION AND REAL-TIME QUANTITATIVE RT-PCR

Total cellular RNA was extracted from hMSCs using the Trizol reagent according to the manufacturer's instructions. After being lysed, cells were transferred to centrifuge tubes. Chloroform was added and mixed with the samples. Samples were incubated at room temperature for 5 min, and centrifuged at 12,000*g* for 15 min at 4°C. The aqueous phase was transferred to new tubes, each containing 500 μl isopropanol, and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was removed, and the RNA pellet was washed with 500 μl 75% ethanol. The RNA pellet was then dissolved in 20 μl water containing 0.01% DEPC and stored in a −80°C freezer for further use.

Complementary (c)DNA was prepared from RNA using a reverse-transcription kit. A real-time PCR was performed using an ABI Prism 7900 Sequence Detection System (Carlsbad, CA). Cbfa1 and glyceraldehyde phosphate dehydrogenase (GAPDH) genes were detected by a Quantitative TaqMan reverse-transcriptase (RT)-PCR with pre-designed assays (assay ID: Hs00758162_m1 and Hs99999905_m1, Applied Biosystems). The annealing and extension temperatures were set to 60°C, and the cycle number was 40. Data were collected with instrument spectral compensations by the Applied Biosystems SDS 2.1 software, and analyzed using the threshold cycle-relative quantification method. Measurements are expressed as the threshold of the cycle number (Ct) at which the fluorescence generated by cleavage of the probe passes a fixed threshold above the baseline. The reference gene, GAPDH, was used as an internal control to calculate the difference in the Ct value of the target gene (Cbfa1) and control. To compare differences among various experimental groups, the value obtained from unstretched hMSCs grown on Col I for 1 day was used as the reference group. Then the data were expressed as multiples of Cbfa1 mRNA expression in each group relative to the reference group (hMSCs grown on Col I on day 1).

MEASUREMENT OF ALKALINE PHOSPHATASE ACTIVITY

At the end of the appropriate experimental periods, hMSCs were washed with PBS and lysed with triton X-100 (0.5%) for 30 min. Sample solutions of 25 μl were taken to determine the protein concentration using a BCA assay kit. An aliquot of the working reagent containing equal parts of 1.5 mol/L 2-amino-2-methly-l-propanol, 20 mmol/L *p*-nitro-phenyl phosphate, and 1 mmol/L magnesium chloride was used to incubate cells for 30 min at 37°C. The reaction was stopped with 3 N sodium hydroxide. ALP activity was determined from the absorbance of *p*-nitrophenol at 405 nm, and results are expressed as nanomoles of *p*-nitrophenol/mg protein/30 min.

ALIZARIN RED S STAINING AND QUANTITATIVE ASSESSMENT

To determine mineralized matrix deposition, hMSCs which had been subjected to cyclic mechanical stretching for 5 days were incubated for an additional 9 days, and calcium deposition in the mineralized matrix was detected using 2% ARS staining for 5 min.

Unstretched hMSCs grown on various ECM proteins were run in parallel and served as the controls. The reddish-orange staining of calcium was observed, and an alizarin red-based spectrophotometric method was used for the quantitative assessment [Gregory et al., 2004]. Briefly, stained cultures were collected with 10% acetic acid and transferred to a 1.5-mL microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 500 μl mineral oil, heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000*g* for 15 min, and 500 μl of the supernatant was placed in a new tube. Then 200 μl of 10% ammonium hydroxide was added to neutralize the acid (pH 4.1). Aliquots of the supernatant were read at 405 nm.

STATISTICAL ANALYSIS

All experiments were repeated two or three times on each hMSC pool from three individual donors, and the representative data are presented as the mean and standard deviation from three independent replicates. Statistical analysis was performed using one-way ANOVA and Scheffe's test to check differences among various ECM groups or the change over time. As to the data presented for each kind of ECM, unpaired Student's *t*-test was used to compare differences between stretched and unstretched groups. A *P*-value of <0.05 was considered statistically significant.

RESULTS

CHARACTERISTICS OF HMSCS

The characteristics of hMSCs used in our study were checked according to the criteria proposed for defining multipotent mesenchymal stromal cells [Dominici et al., 2006]. The hMSCs expressed the CD29, CD44, CD71, and CD105 surface antigens; but lacked expression of CD18, CD31, CD34, and CD45, as revealed in our previous study [Chen et al., 2008]. The ability of hMSCs to differentiate in vitro to osteogenic and adipogenic lineages was also confirmed (data not shown). As reported by previous investigators [Tropel et al., 2004; Kundu and Putnam, 2006], two morphologically distinct kinds of cells, spindle-shaped cells and large flattened cells, were observed in hMSC cultures.

EFFECTS OF MECHANICAL STRETCHING ON MTT ACTIVITY

The time course of MTT activity of hMSCs in response to mechanical stretching is shown in Figure 1. In the unstretched group, MTT activity on VN was lower, compared to the other ECM proteins, at the earlier time point post-seeding (day 1). The MTT activity of unstretched hMSCs had a trend of increasing with culture time, but only the change in the VN group reached statistical significance. Contrary to the unstretched control, the MTT activity of stretched

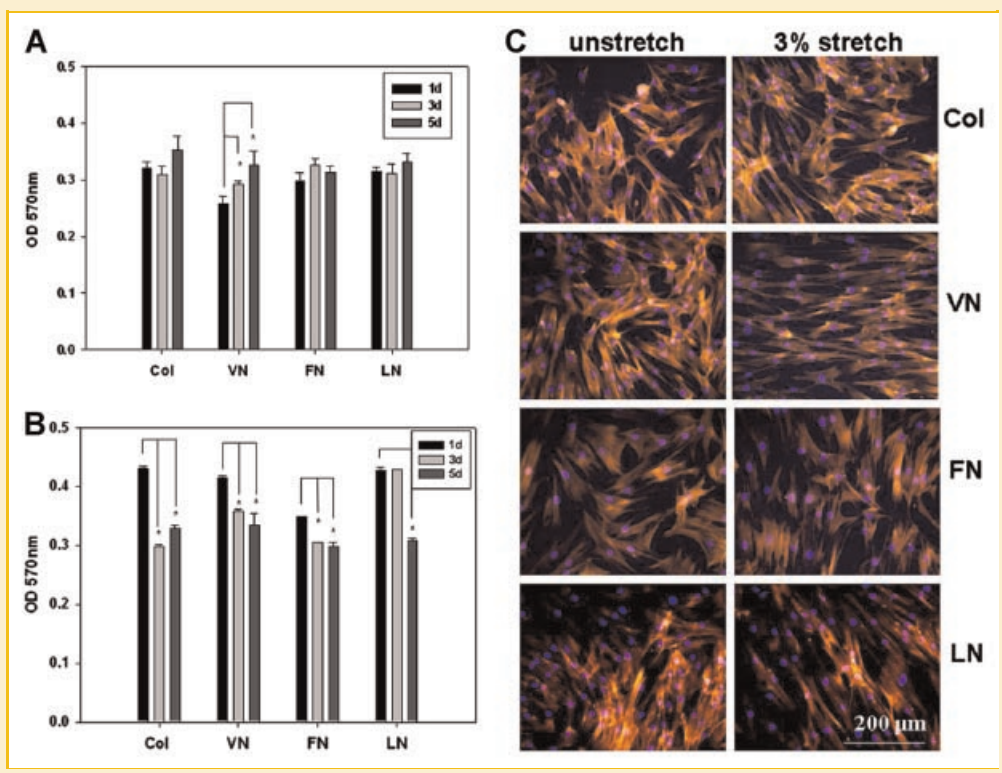

Fig. 1. Mechanical stretching increases MTT activity and induces re-alignment of F-actin filaments. Human mesenchymal stem cells (hMSCs) were cultured on substrates coated with collagen I (Col), vitronectin (VN), fibronectin (FN), and laminin (LN) without or with mechanical stretching. A,B: The metabolic activities of unstretched and stretched hMSCs were measured on days 1, 3, and 5 using an assay based on the ability of functional mitochondria to reduce the tetrazolium salt, 3,[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). The change over time was analyzed for each extracellular matrix (ECM) group, using ANOVA followed by Scheffe's test. Asterisks indicate a significant difference ($P<0.05$) compared to the value on day 1 (mean ± SD) ($n=3$). C: Immunofluorescence staining for actin filaments in hMSCs subjected to mechanical stretching for 24 h (200× magnification). The re-orientation of the cytoskeleton in stretched hMSCs was obvious compared to unstretched cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

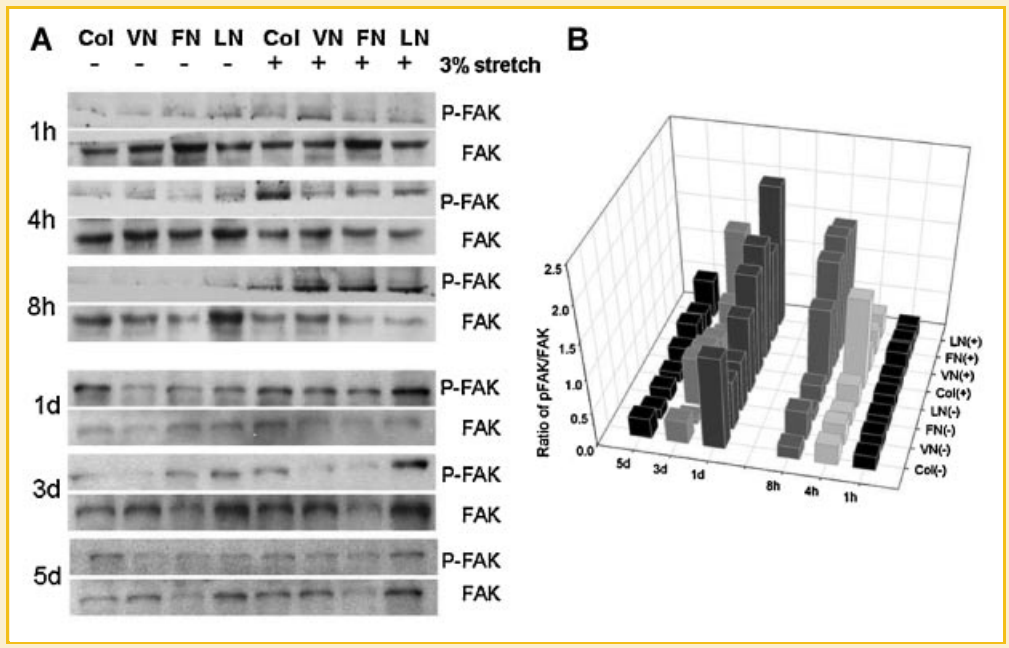

Fig. 2. Mechanical stretching differentially regulates focal adhesion kinase (FAK) phosphorylation of human mesenchymal stem cells (hMSCs) grown on various extracellular matrix (ECM) proteins. hMSCs cultured on collagen I (Col), vitronectin (VN), fibronectin (FN), and laminin (LN) without or with mechanical stretching for 1, 4, and 8 h, and 1, 3, and 5 days were lysed. Equal amounts of protein from cell lysates were then subjected to electrophoresis and probed for activated FAK by Western blots with a phosphor-specific antibody. Total FAK was detected by Western blotting with a non-phospho-specific antibody. Blots representative of multiple repeated experiments are shown (A). Densitometric measures of the band intensity are expressed as signal ratios (p-FAK/FAK) indicating the level of FAK phosphorylation (B).

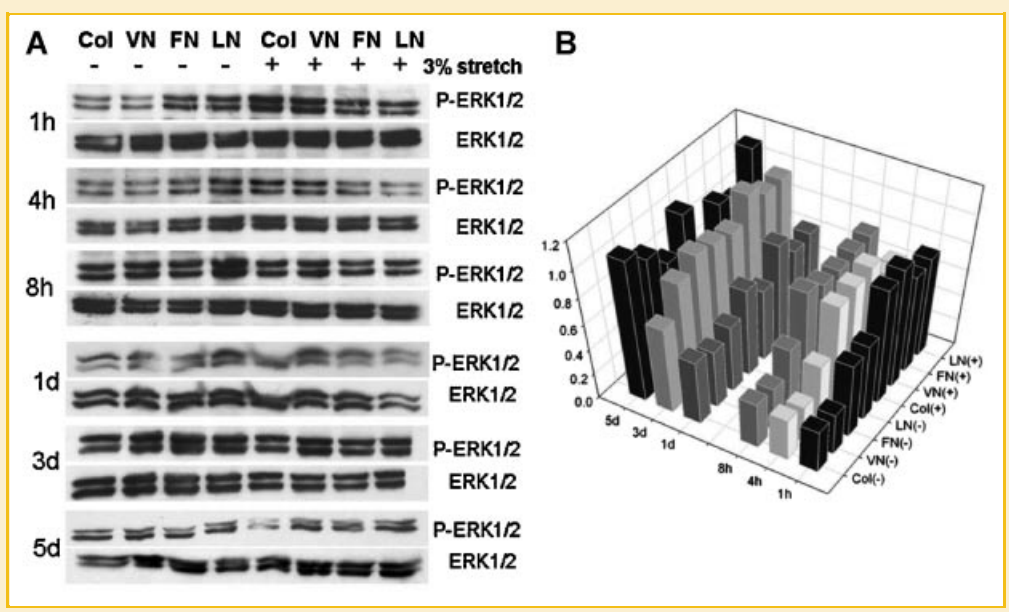

Fig. 3. Expression and phosphorylation of extracellular signal-regulated kinase (ERK) in both unstretched and stretched human mesenchymal stem cells (hMSCs) were noted throughout the 5-day experimental period. hMSCs cultured on collagen I (Col), vitronectin (VN), fibronectin (FN), and laminin (LN) without or with mechanical stretching for 1, 4, and 8 h, and 1, 3, and 5 days were lysed. Equal amounts of protein from cell lysates were then subjected to electrophoresis and probed for activated ERK by Western blots with a phosphor-specific antibody. Total ERK was detected by Western blotting with a non-phospho-specific antibody. Blots representative of multiple repeated experiments are shown (A). Densitometric measures of the band intensity are expressed as the signal ratios (p-ERK/ERK) indicating the level of ERK phosphorylation (B).

hMSCs exhibited a significant increase on day 1, followed by a decrease, regardless of the kind of ECM protein tested. Moreover, there was no significant difference in MTT activities of stretched hMSCs grown on different ECM proteins for 5 days.

Immunostaining of F-actin filaments showed the re-orientation of the cytoskeleton in stretched hMSCs compared to unstretched cells. Only pictures on day 1 are presented here because the patterns were similar at the different observation time points (Fig. 1C).

PHOSPHORYLATION OF FAK AND ERK 1/2

Focal contact formation is accompanied by increased phosphorylation of FAK, which activates multiple signaling pathways to regulate cell migration, survival, proliferation, and differentiation [Wang et al., 2001; Schlaepfer and Mitra, 2004; Salasznyk et al., 2007]. In this study, phosphorylation of FAK was not obvious at 1 h in either unstretched or stretched cells (Fig. 2). In the unstretched group, hMSCs grown on Col I expressed a higher level of p-FAK than on other ECM proteins on day 1, and no significant group difference was noted among the different ECM proteins before this time point. However, the increased level of p-FAK in stretched hMSCs at 4 h was noted on all ECM proteins, especially on Col I. Interestingly, FAK activation in hMSCs grown on Col I was significantly higher at 4 h, but lower at 8 h, than those on VN, FN, and LN. The stretch-induced FAK activation of hMSCs grown on VN, FN, and LN was not noted until 8 h, subsequently followed by a decreasing trend through a period of 5 days, at which time, FAK activation remained only with LN. The statistical significance of these results was confirmed by one-way ANOVA followed by Scheffe's tests. Taken together, these results indicate that activation of FAK signaling is regulated by the combined effects of mechanical stretching and ECM proteins. The timing of stretch-induced phosphorylation of FAK was dependent on the type of ECM protein.

ERK1/2 is one of the FAK downstream signaling targets, and this pathway is responsible for Cbfa1 activation in mechanically loaded osteoblastic cells [Wang et al., 2001; Kanno et al., 2007]. In our study, p-ERK1/2 was detected in both stretched and unstretched hMSCs at as early as 1 h and was maintained throughout the 5-day experimental period (Fig. 3). Although a statistical analysis was

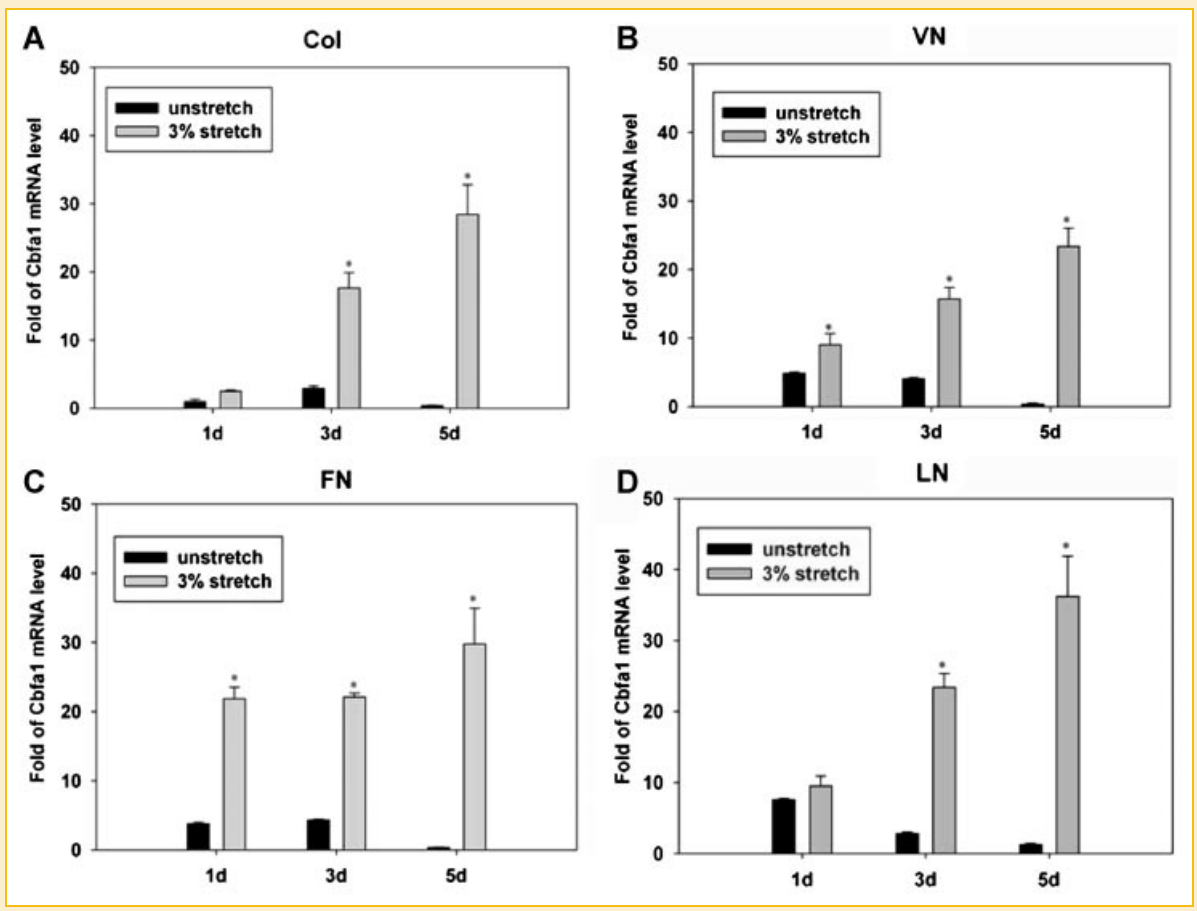

Fig. 4. Mechanical stretching upregulates core-binding factor alpha-1 (Cbfa1) mRNA expression of human mesenchymal stem cells (hMSCs) grown on various extracellular matrix (ECM) proteins. hMSCs cultured on substrates coated with (A) collagen I (Col), (B) vitronectin (VN), (C) fibronectin (FN), and (D) laminin (LN) without or with mechanical stretching for 1, 3, and 5 days were lysed. Complementary DNA was prepared as described in Materials and Methods Section. Cbfa1 and glyceraldehyde phosphate dehydrogenase (GAPDH) genes were detected by Quantitative TaqMan RT-PCR with pre-designed assays. GAPDH was used as an internal control to calculate Cbfa1 expression using the threshold cycle-relative quantification method. Data were expressed as multiples of Cbfa1 mRNA expression in each group relative to the reference group (hMSCs grown on Col I on day 1). Asterisks indicate a significant difference between the stretched group and unstretched control (unpaired Student's *t*-test, $P < 0.05$).

performed, no conclusive difference could be delineated for ERK1/2 phosphorylation among the various ECM groups.

EXPRESSION AND PHOSPHORYLATION OF Cbfa1

Figure 4 shows the stretch-induced upregulation of Cbfa1 mRNA in hMSCs grown on various ECM proteins. The Cbfa1 mRNA levels of hMSCs grown on LN, VN, and FN increased at earlier time points, but not at the end of the experimental period. In contrast, mechanical stretching induced a dramatic increase in the mRNA expression of Cbfa1, regardless of the kind of ECM protein tested. This promoting effect was sustained throughout the period of stretching; thus the Cbfa1 mRNA level in hMSCs grown on LN was elevated by approximately 35-fold on day 5, and 25-fold on Col I and VN compared to unstretched cells. Interestingly, the Cbfa1 mRNA level of stretched cells on FN on day 1 was the highest among the various ECM proteins tested. As shown in Figure 5, Cbfa1 phosphorylation of hMSCs on day 5 varied with the kind of ECM protein tested, and the level on Col I was significantly higher than on FN, whereas, Cbfa1 phosphorylation of stretched hMSCs grown on Col I and FN was lower than that on VN and LN. This implies that mechanical stretching and ECM proteins exhibit differential impacts and may interact to affect the expression and phosphorylation of Cbfa1.

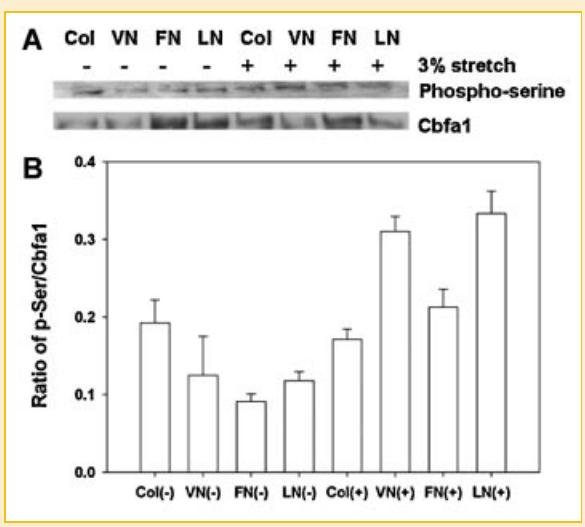

Fig. 5. Mechanical stretching differentially regulates core-biding factor alpha-1 (Cbfa1) phosphorylation of human mesenchymal stem cells (hMSCs) grown on various extracellular matrix (ECM) proteins. hMSCs cultured on collagen I (Col), vitronectin (VN), fibronectin (FN), and laminin (LN) without or with mechanical stretching for 1, 4, and 8 h, and 1, 3, and 5 days were lysed. Equal amounts of protein from whole-cell lysates were then subjected to immunoprecipitation with an antibody against Cbfa1. Eluted Cbfa1 was probed for activated Cbfa1 by Western blots with a phosphor-specific antibody (61-kDa bands). Total Cbfa1 was detected by Western blotting with a non-phospho-specific antibody. Blots representative of multiple repeated experiments are shown (A). Densitometric measures of the band intensity are expressed as signal ratios (p-Cbfa1/Cbfa1) indicating the level of Cbfa1 phosphorylation (B).

ALP ACTIVITY AND ARS STAINING

Figure 6 shows that on day 1, higher ALP activity was noted in all stretched hMSCs than unstretched controls for each ECM protein group, especially for FN. ALP activity of stretched hMSCs exhibited a tendency to increase with the period of stretching applied. Similar to ALP activity, a positive result of ARS staining was detected, but was not too strong (Fig. 7A). Figure 7B shows the quantitative assessment of ARS staining, which revealed enhanced mineralized matrix deposition in long-term cultures of stretched hMSCs. In the unstretched group, the level of matrix mineralization was highest on FN. In the stretched groups, the levels on FN and LN were similar and higher than those on the other two ECM proteins. Our observations demonstrate that mineralized matrix formation in long-term culture of hMSCs, in the absence of osteogenic supplements, was dependent on the ECM protein used and mechanical stretching.

DISCUSSION

Using ALP activity and mineralized matrix deposition as respective indicators of the early and late stages of osteogenesis, we report here that all ECM proteins (Col I, FN, VN, and LN) supported hMSC differentiation into an osteogenic phenotype, and this effect was overwhelmed by mechanical stimulation. Cyclic mechanical stretching dominantly promoted osteogenic differentiation of hMSCs on substrates coated with various ECM proteins, in the absence of osteogenic supplements. This implies that cues from contact with the ECM alone may be sufficient to drive hMSCs into osteogenic differentiation, which was promoted by mechanical strain via activation of FAK signaling and phosphorylation of Cbfa1.

Medium with osteogenic supplements (OSs), such as ascorbic acid, beta-glycerophosphate, and dexamethasone, is commonly used to induce the osteogenic differentiation of hMSCs via activating signal transduction pathways and upregulating osteogenic marker genes (e.g., Col, ALP, and OC) that lead to extracellular matrix mineralization [Bruder et al., 1997; Pittenger et al., 1999; Salasznyk et al., 2005; Kanno et al., 2007]. However, differentiated hMSCs cultured with OS medium may be in different transitional stages relative to fully differentiated osteoblasts. It was reported that ALP expression was found only in OS-driven differentiated hMSCs, but not in hMSCs cultured in regular medium [Salasznyk et al., 2005]. To avoid the influence of osteogenic supplements, we cultured hMSCs in regular medium and investigated the combined effects of insoluble chemical and mechanical factors (ECM proteins vs. cyclic stretching) in driving hMSCs toward osteogenic differentiation. We independently confirmed that hMSCs do not express ALP activity when grown on tissue culture plates without an ECM coating (data not shown). In fact, the expressions of ALP and matrix mineralization of hMSCs in the absence of OS induction have not been extensively explored. The use of regular medium without OS may allow greater differences between culture substrates to be revealed. There is at least one previous study showing that osteogenic differentiation occurred in 16-day culture of hMSCs plated on VN and Col I in the absence of OS, while almost no

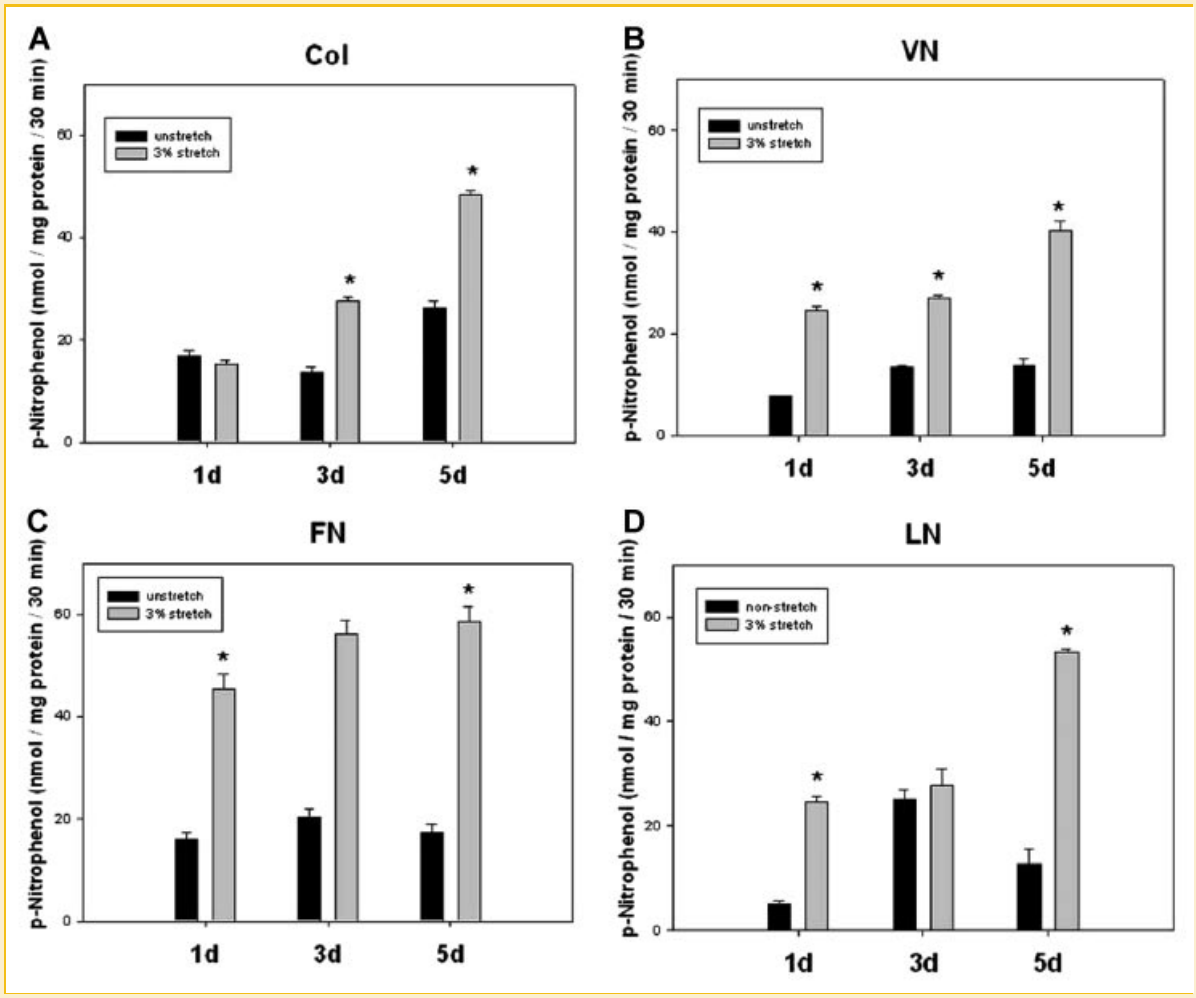

Fig. 6. Mechanical stretching increases alkaline phosphate (ALP) activities of human mesenchymal stem cells (hMSCs) grown on various extracellular matrix (ECM) proteins. hMSCs cultured on substrates coated with (A) collagen I (Col), (B) vitronectin (VN), (C) fibronectin (FN), and (D) laminin (LN) without or with mechanical stretching for 1, 3, and 5 days were lysed. ALP activities were measured using a spectrophotometric assay based on the hydrolysis of *p*-nitro-phenyl phosphate. The protein concentration was determined using a BCA assay. Representative data from three experiments are shown as the mean ± SD of independent triplicate cultures. Asterisks indicate a significant difference between the stretched group and unstretched control (unpaired Student's *t*-test, $P < 0.05$).

differentiation took place on uncoated plates [Salasznyk et al., 2004].

Mineralized bone needs an organized collagenous matrix for deposition of calcium phosphate and formation of hydroxylapatite. Col I is the most abundant protein in bone, and thus may be the major ECM entity responsible for directing the fate of hMSCs into an osteogenic lineage. However, the results of our study did not reveal the superiority of Col I relative to other ECM proteins tested, in supporting the osteogenesis of hMSCs, regardless of whether mechanical stretching was applied or not. Previous studies reported that 3–5% tensile strain to a collagen I substrate stimulated osteogenesis in attached hMSCs [Ward et al., 2007; Chen et al., 2008]. In the present study, 3% cyclic mechanical stretching was applied to hMSCs grown on Col I, VN, FN, and LN; and the results showed that FN was the most potent ECM protein supporting the osteogenic differentiation in stretched hMSCs, as shown by ALP activity on day 5 and matrix mineralization in 14-day culture. Further study is needed to explore why FN on stretched substrate is the ECM entity with the greatest effect in promoting osteogenic differentiation of hMSCs.

Interestingly, MTT activity in stretched hMSCs was significantly higher on day 1, but was followed by a decreasing trend to a level similar to that of unstretched cells on day 5 (Fig. 1). The MTT assay is based on the reducing activity of functional mitochondria and is often used as a measure to indicate the number of living cells. In our study, there were no detached, floating, or dying cells detected throughout the experimental period. Thus, the increase in MTT activity on day 1 might possibly reflect an increase in metabolic activity in stretched hMSCs rather than cell proliferation. Cells resist mechanical stresses and transmit signals by remodeling cytoskeletal filaments. Thus, ATP-producing mitochondria are needed to support the energy requirements of cytoskeletal structural dynamics [de Cavanagh et al., 2009]. Reciprocally, mitochondria need to interact with cytoskeletal elements to maintain organelle motility, localization, and function [Boldogh and Pon, 2006]. It is possible that the MTT activity of hMSCs in our study reflects mitochondrial energy

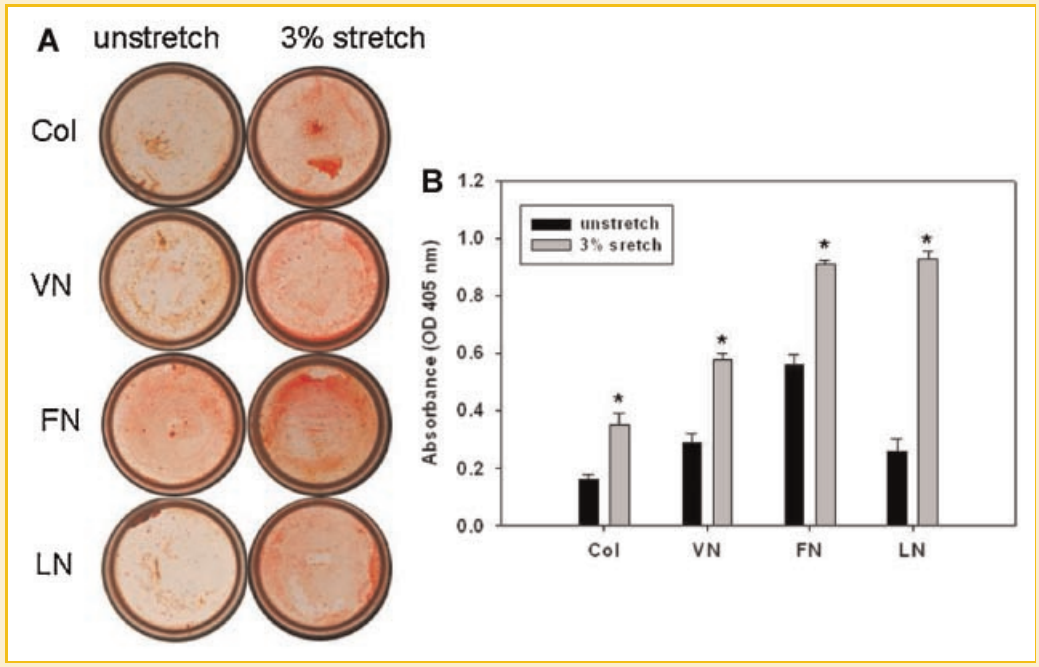

Fig. 7. Mechanical stretching increases extracellular matrix (ECM) mineralization in long-term cultures of human mesenchymal stem cells (hMSCs). A: hMSCs grown on various ECM proteins, including collagen I (Col), vitronectin (VN), fibronectin (FN), and laminin (LN), after being subjected to mechanical stretching for 5 days, were cultured an additional 9 days, and stained with 2% Alizarin red S (ARS). Although the intensity of ARS staining was low, especially in the unstretched group, it was above the limit of detection. B: Quantification of ARS staining based on spectrophotometric measurements at 405 nm. Representative data of three independent experiments are shown as the mean ± SD of independent triplicate cultures. Asterisks indicate a significant difference between the stretched group and unstretched control (unpaired Student's *t*-test, $P < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activity, which is associated with rearrangement of the cytoskeleton and which diminishes as cells adapt by orienting themselves perpendicular to the strain axis.

As a central regulator of integrin-mediated signaling, FAK is present in focal adhesions, mediates cellular attachment to ECM, and promotes actin polymerization [Burridge and Chrzanowska-Wodnicka, 1996; DeMali et al., 2003; Burridge et al., 2006]. Salasznyk et al. [2007] reported that FAK signaling pathways provide a link of the ECM to activation of ERK1/2 and induce subsequent phosphorylation of Cbfa1 to regulate the osteogenic differentiation of hMSCs. Our results showed that the p-FAK levels of hMSCs on static ECM substrates (unstretched) could not be detected until day 1. In contrast, stretched hMSCs showed an increase in the p-FAK level, which peaked with Col I at 4 h, and with other ECM proteins (FN, VN, and LN) at 8 h. Interestingly, the p-FAK level in stretched hMSCs on LN was sustained for an extended period of 5 days. This finding is consistent with the higher expression of Cbfa1 mRNA, phosphorylation of Cbfa1, ALP activity, and matrix mineralization of hMSCs on LN, compared to other ECM proteins.

It was recently reported that 15% mechanical stretching mediated the phosphorylation of MAPKs (ERK1/2 and p38) in rat osteoblasts grown on FN at 60–90 min [Kanno et al., 2007]. Another study showed a peak increase in p-ERK1/2 by hMSCs subjected to a 5%, 0.5-Hz cyclic strain for 30–120 min [Ward et al., 2007]. The difference in the timing of ERK1/2 phosphorylation might have been due to different protocols of mechanical stretching in the different studies. In our study, the basal level of p-ERK1/2 was high and was maintained throughout the experimental period. This result may be partially explained by the regular medium with 10% FBS being used during the period of mechanical stretching. Thus, directly comparing our results to those with serum starvation is not valid. To reduce the effects of FBS, we seeded and cultured hMSCs with low-serum medium (2% FBS) for 24 h, which was replaced by medium with 10% serum in subsequent cultures.

Cbfa1/Runx2 plays a central role in coordinating multiple signals involved in osteoblast differentiation [Franceschi and Xiao, 2003]. Phosphorylated Cbfa1 operates by binding to the osteoblast-specific *cis*-acting element 2 (OSE2), which exists in the regulatory region of osteoblast-related genes [Franceschi and Xiao, 2003; Kanno et al., 2007; Ziros et al., 2008]. In a study on osteoblast-like MC3T3-E1 cells, mechanical stress regulated Cbfa1 activation and favored osteoblast differentiation [Kanno et al., 2007]. Using the real-time RT-PCR technique, we confirmed the stretch-induced upregulation of the Cbfa1 gene, and this effect was sustained throughout the stretching period of 5 days, especially on FN. Contrary to the long-lasting effect due to cyclic mechanical stretching, Cbfa1 mRNA levels on static substrates decreased with culture time. Osteogenic cues from ECM proteins possibly diminished after cells achieved complete attachment to the ECM. Thus, signals from ECM proteins tend to not be sustained as long as cyclic mechanical stretching. Furthermore, an increased level of p-Cbfa1 was noted on day 5 in stretched hMSCs, especially when grown on VN and LN. Although the p-Cbfa1 levels on Col I and FN were not as high as those on VN and LN, the ALP activity on day 5 was higher on FN than on other

ECM proteins. It is possible that the peak level of p-Cbfa1 on FN occurred before day 5. Further study is needed to check the time course of Cbfa1 expression and phosphorylation to delineate the possibility of the involvement of other signaling pathways.

It is well known that ALP activity of osteoblasts cultured with OS medium is cell density dependent and increases with the progressive maturation of the ECM [Owen et al., 1990]. In our study, hMSCs were cultured in regular medium, and mechanical stretching progressively increased ALP activity during the period of stretching. No similar effect was demonstrated for hMSCs grown on static ECM substrates. Although the ALP activity was detectable in the unstretched group for 5 days, there was no notable increasing trend. Again, this phenomenon implies that osteogenic cues from ECM proteins may diminish with culture time. The promoting effect of mechanical stretching on osteogenic differentiation was confirmed in long-term culture of hMSCs by quantitatively assessing mineralized matrix deposition. Among the ECM proteins tested, FN and LN exhibited greater effects of supporting stretch-induced osteogenic differentiation than did Col I and VN. Such a phenomenon indicates that mechanical stretching promotes the early expression of upstream signaling proteins and also the mature expression of an osteoblastic lineage, and this effect was ECM dependent.

In conclusion, cyclic mechanical stretching increased the phosphorylation of FAK, upregulated the transcription of Cbfa1, and increased ALP activity and mineralized matrix deposition in hMSCs grown on various ECM proteins. Taken together, mechanical stretching speeded up ECM-induced osteogenic differentiation in addition to promoting its overall expression. As critical factors in regulating the osteogenesis of hMSCs, the identity of ECM proteins and cyclic mechanical stretching can be exploited in bone tissue engineering via approximate matrix design or application of mechanical stimulation.

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