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Experimental and Toxicologic Pathology 1 (111) 111–111

**EXPERIMENTAL
AND
TOXICOLOGIC
PATHOLOGY**www.elsevier.de/etp

Cyclic strain induces FosB and initiates osteogenic differentiation of mesenchymal cells

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Received 12 November 2007; accepted 27 November 2007

Abstract

Mechanical loading is crucial for bone remodeling and osteoblast differentiation. FosB belongs to the AP-1 family of transcription factors, a group of proteins known to regulate osteoblast differentiation and bone formation. In mice, FosB is rapidly induced by mechanical stress at the transcriptional level. The aim of this study was to determine the effect of different mechanical stretch patterns on FosB gene expression and on osteogenic differentiation of human osteoblast precursor cells. Human bone-marrow-derived mesenchymal precursor cells were grown in flexible silicone dishes and stimulated by a daily application of three rounds of 2 h of cyclic stretch of either 2% or 8% elongation at 1 Hz on 3 consecutive days using a special motor-driven apparatus. By real-time PCR, we quantified FosB mRNA and the expression of genes involved in osteoblast differentiation such as Runx2 and collagen 1 to determine the osteogenic effect of mechanical stretch. Stretching induced FosB transcription and the expression of osteoblast markers in partly committed human mesenchymal precursor cells in a stretch- and time-dependent manner. We conclude that cyclic stretch-induced FosB expression and the upregulation of osteoblast genes plays a role in osteogenic differentiation of human mesenchymal precursor cells.

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Keywords: Mesenchymal cells; Mechanical strain; Osteogenic differentiation; Transcriptional regulation; Real-time PCR

Introduction

Bone is a highly dynamic tissue that undergoes continuous remodeling involved in the maintenance of its architectural integrity and metabolic activity, two critical and competing functions of bone. The micro-structure of bone depends on genetic determinants and

on the continuous response of the skeleton to mechanical cues, adding new bone to withstand increased amounts of loading, and removing bone due to unloading or disuse during immobilization or in space (Suva et al., 2005). Mechanical stress influences bone homeostasis in post-natal remodeling processes (McNamara and Prendergast, 2007), fracture healing (Gardner et al., 2006), and in osseointegration of orthopedic implants (Leucht et al., 2007). This strain-induced adaptation and remodeling ability is frequently disturbed in low bone-mass diseases such as osteoporosis and it is therefore clinically important (Sterck et al., 1998). Thus, it is

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doi:10.1016/j.etp.2007.11.013

Please cite this article as: Haasper C, et al. Cyclic strain induces FosB and initiates osteogenic differentiation of mesenchymal cells. *Exp Toxicol Pathol* (2008), doi:10.1016/j.etp.2007.11.013

crucial to understand mechanisms that form a mechano-molecular interface that couples physical stimuli to intracellular molecular events, thereby modulating the genetic program and the fate of the target cells. A better understanding of the mechanisms by which mechanical strain controls skeletal remodeling and induces bone formation could be very helpful in developing novel therapeutic strategies for treating low-bone mass diseases using pharmacological approaches.

The prevailing model suggests that a key factor of bone remodeling is a strain-driven oscillatory flow of extracellular fluid in the lacunar–canalicular network in bone that is sensed by osteocytes, the terminally differentiated osteoblasts buried in extracellular matrix. Osteocytes are metabolically active and highly interconnected with each other and with other types of cells via numerous dendrites, allowing osteocytes to communicate over a distance within the bone (Bonewald, 2005, 2007). However, evidence exists that osteoblasts and even their precursors, the mesenchymal bone marrow stromal cells (BMSCs), also perceive and translate mechanical stimuli (Jagodzinski et al., 2004; Mauney et al., 2004; Inoue et al., 2004; Ignatius et al., 2005).

Different types of mechanical stimulation, such as fluid-shear stress, compression, and axial longitudinal stretch, are widely used in studies investigating the effect of mechanical stimulation on cell behavior. Although it is not clear yet what the specific differences or similarities are between different stimuli, all types of mechanostimulation have been shown to activate a divergent array of key anabolic intracellular effectors such as nitric oxide (NO) (Klein-Nulend et al., 1998), prostaglandins (Siddhivarn et al., 2006), and intracellular calcium (Chen et al., 2000), and stimulate signaling events in the cilium (Xiao et al., 2006). Most of these signaling cascades promote cell proliferation and differentiation and activate members of the mitogen-activated protein kinase (MAPK) family, including the extracellular signal-regulated kinases (ERK)-1 and 2 (Simmons et al., 2003). These signaling events activate a variety of osteoblast-related genes, including Runx2, the master regulator of osteoblast differentiation that facilitates osteoblast lineage commitment (Ducy et al., 1997; Xiao et al., 2000; Ge et al., 2007).

Activator protein-1 (AP-1) transcription factors are dimers of Fos (c-Fos, FosB, DFosB, Fra-1, Fra-2) and Jun (c-Jun, JunB, JunD) leucine zipper-containing proteins. Early activation of some AP-1 factors, particularly c-Fos, is known to occur in response to mechanical stimulation (Kletsas et al., 2002). Members of the AP-1 family of homo-/heterodimeric transcription factors bind a consensus sequence in the promoters of several genes that are essential for osteoblast differentiation and function, such as alkaline phosphatase, collagen type 1, osteopontin, osteocalcin, and Runx2 (Ducy and Karsenty 1995; Ducy et al., 1997).

Inoue et al. demonstrated that mechanical strain rapidly induces the transcription of the FosB gene *in vivo*, using an unloading mouse model and *in vitro*, using mouse calvarial osteoblasts (Inoue et al., 2004). The mechano-induced FosB transcription was caused by the activation of ERK1/2 by a Ca^{2+} influx. These data strongly suggest that FosB transcription participates in linking mechanical cues via transcriptional machinery into activation of an osteogenic program in bone cells of mice.

The potential physiological and pharmacological relevance of this observation led us to determine whether early immediate induction of the FosB gene expression by mechanical stimulation also occurs in mesenchymal progenitor cells of human origin. In addition, we examined the effect of different degrees of stress on induction of FosB expression and whether the rise in FosB levels was accompanied by an activation of the osteogenic program in mesenchymal precursor cells of human nature.

Materials and methods

Sample acquisition

Human bone marrow aspirates were obtained during routine orthopedic surgical procedures involving exposure of the iliac crest. The institutional ethical committee approved the procedure and written informed consent was obtained from each patient. Bone marrow aspirates (20–80 ml) were harvested from seven healthy donors using a bone marrow biopsy needle inserted through the cortical bone of the iliac crest. Four donors were female and three donors were male. The average age was 32.5 \pm 6.2 years. Aspirates were cooled on ice immediately and processed within 1 h.

Primary cell culture

Human bone marrow aspirates were divided in aliquots of 25 ml, transferred into 50 ml conical tubes, and mixed with 25 ml standard tissue culture medium (DMEM/Ham's F-12 1:1 with L-glutamine, 10% human serum, 20 mg/ml amphotericin B, 100 U/ml penicillin, 100 mg/ml streptomycin) (Invitrogen, Karlsruhe, Germany). After centrifugation at 400 g for 5 min, the pellet was transferred onto 45 ml Percoll-Paque^s (Pharmacia, Freiburg, Germany) density gradient solution and centrifuged for 15 min at 400 g. Fourteen ml of the nucleated cell-containing supernatant were mixed with 35 ml of tissue culture medium and centrifuged at 400 g for 5 min. The supernatant was removed and the pellet was suspended in 20 ml of tissue culture medium, plated in a 75 cm² tissue culture flask, and incubated for 5 days in a humidified atmosphere with 5% CO₂ at

37°C. Tissue culture medium was replaced at day 4 and every other day thereafter. Cells were grown to subconfluency, enzymatically detached using 0.25% Trypsin/1 mM EDTA (Invitrogen). The cells were then aliquoted, frozen, and stored in liquid nitrogen for future use. After thawing, the multi-colony-derived hBMSCs from seven donors of similar age but different sexes were combined and sub-cultured two times at a 1:4 ratio in 75 cm² tissue culture flasks to minimize variations caused by the heterogeneous nature of hBMSCs.

Stimulation system

Human BMSCs were mechanically stimulated using a custom-made multiple station apparatus to apply repetitive cycles of longitudinal stretch by an electric motor (Fig. 1). Briefly, the system consisted of rectangular deformable silicone dishes fixed at one end and held in a movable clamp at the other end. The variable electric motor driving the movable end allowed for variations in magnitude (0.5–10%) and frequency (0.5–2 Hz) in a microprocessor-controlled fashion. Silicone dishes were molded of a two-component silicone elastomer containing Silicosehl RTV 270 and cross-linker A 47 (Rhône-Poulenc, Lübeck, Germany) at a 10:1 ratio. After molding, dishes were cured in the oven for 2 days at 37°C. The rectangular dishes measured 8 × 3 × 1 cm (length × width × height), the actual cell culture surface was 5 × 2.3 cm (length × width). Prior to use, the dishes were autoclaved and conditioned for 7 days in tissue culture medium.

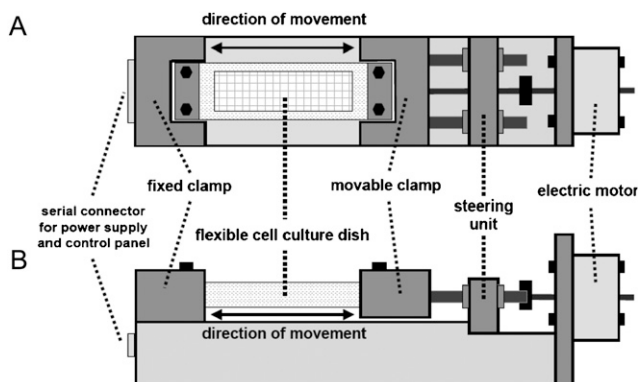


Fig. 1. The custom-made cell strain system consists of two clamps holding the flexible silicone cell culture dish (A, view from top; B, lateral view). One clamp is fixed and the other clamp can be moved using an electric motor in a controlled manner. Repetitive reciprocal movement of the moveable clamp driven by the electric motor generates cyclic longitudinal stretch. These movements elongate the cell culture dish for a pre-defined percentage (between 0.5% and 10%) of its total length and at a defined frequency (between 0.5 and 2 Hz) and return to its starting position.

Cell stretching experiments

2.5 × 10⁵ hBMSCs were seeded in conditioned silicone dishes and incubated for 3 days in 4 ml of tissue culture medium containing osteogenic supplements (50 mg/ml ascorbic acid, 2.55 mM dexamethasone, and 10 mM β-glycerophosphate) (Sigma-Aldrich, Steinheim, Germany), to induce osteogenic differentiation and to allow for cell attachment and cell spreading. Twenty-four hours prior to initiation of treatment, serum concentration was reduced to 1% to synchronize cells in the G₀ phase of the cell cycle. Stimulation experiments were performed in a tissue culture incubator (5% CO₂, 37°C) without serum starvation. Flexible silicone dishes were subject to cyclic mechanical stretch at 1 Hz for either 2% or 8%, referring to the total length of the tissue culture surface in the silicone dish. Stretch was applied in a cumulative manner for 2 h one, two and three times on day 1 and three times on day 2 and on day 3. Cells were analyzed at different times after each individual round on day 1 and after all three rounds on day 2 and day 3. Cells that were analyzed by day 2 or day 3 had also been treated on the days prior to analysis. Multiple stretch applicators were used to treat all samples of one group simultaneously. A break of 1 h was given between strain cycles. Controls were treated identically but were kept under static conditions. Cells were harvested for analysis immediately after the final period of stretch application.

RNA isolation

Total RNA was extracted from cell layers using TRIzol Reagent (Invitrogen), and digestion with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany) was applied to eliminate genomic DNA prior to RNA purification using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The total RNA concentration was measured fluorometrically by the use of RiboGreen RNA Quantitation Kit (MoBiTec, Göttingen, Germany).

Reverse transcription

Synthesis of cDNA was performed using 1 mg of total RNA and 3 ml oligo-dT₂₀ oligonucleotides (25 pmol/ml) (MWG, Ebersberg, Germany). After incubation at 70°C for 10 min, a reaction mixture containing 4 ml dNTPs (5 mmol/ml of each dNTP) (Roche Diagnostics), 8 ml 5 SuperScript II Buffer, 4 ml DTT (0.1 mmol/ml), and 1 ml SuperScript II (200 U/ml) (Invitrogen) was added and incubated for 50 min at 42°C followed by a last incubation for 10 min at 70°C. The final volume was adjusted to 100 μl using tRNA solution (0.25 mg/ml) (Roche Diagnostics).

Quantitative real-time PCR

Real-time PCR analysis was performed using Light Cycler (Roche Diagnostics). Expression of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal positive control in each assay performed. The reaction mixture contained 2.5 ml cDNA, 2 ml sense/antisense oligonucleotide (10 pmol/ml) (MWG), 2 ml hybridization probe (4 pmol/ml) (for sequence of all oligonucleotides and probes see Table 1), 2 ml Fast Start DNA Master Hybridization Mix, 3.2 ml MgCl₂ (25 mmol/l), and 1 ml DMSO (Roche Diagnostics). The volume was adjusted to 20 ml using DEPC-ddH₂O. For denaturation, a temperature of 95 °C was maintained for 15 min. Amplification was performed without color compensation mode using a slope of 20 °C/s. Reaction parameters specific for each sequence such as temperature for strand dissociation (T_{Diss}), annealing temperature (T_{Ann}), temperature for strand extension (T_{Ext}), and the respective times used for each step as well as the number of cycles are listed in Table 1. Reactions either lacking template or containing RNA without reverse transcription served as negative controls and to exclude non-specific signals due to potentially remaining genomic DNA. After measurement of the relative fluorescence intensity (channel F3/F1) for each sample, a proportional baseline adjustment for sample quantification was carried out by second derivative maximum calculation. Values of each target gene were normalized to the values of GAPDH, correlated to non-stimulated controls for each time point, and presented as relative expression.

For each sequence, a standard calibration curve was constructed to determine the assay sensitivity and to absolutely quantify the samples. In brief, a PCR product of each gene of interest was generated from a cDNA library derived from human osteoblasts. The cDNA was amplified using Taq polymerase and sequence-specific oligonucleotides (Table 1) in a standard PCR reaction. The amplicon contained the binding sites of the sense and antisense oligonucleotides and hybridization probes to be used in the real-time PCR assay (Table 1). The amplified cDNA fragments were cloned into the pCR2.1-TOPO vector according to the manufacturer's protocol (TOPO TA Cloning Kit; Invitrogen). Chemically competent one shot *Escherichia coli* were transformed with the pCR2.1-TOPO vector and streaked out on agar plates containing ampicillin, IPTG (100 mM), and X-gal (40 mg/ml). Positive colonies appeared white and a single colony for each gene of interest was grown in 200 ml LB-media containing ampicillin. Plasmid-DNA was extracted using the Plasmid Isolation Kit (Qiagen). Isolated plasmid DNA was tested for the presence of the right insert by PCR. Vectors were linearized using appropriate restriction enzymes. Serial

10-fold dilutions were prepared and used as standards for quantification by real-time PCR.

The standard curves of all sequences yielded a linear response over ranges of 10⁷–10¹ initial cDNA copies (GAPDH, Runx2), or 10⁸–10² initial cDNA copies (FosB, collagen 1) (Fig. 2A–D). In summary, all assays were highly sensitive and able to detect small changes in gene expression.

Statistical analysis

Stretch experiments were performed two or three times. Each group consisted of three samples and each sample was analyzed in triplicate. Statistical analysis was carried out by analysis of variance (ANOVA) with pairwise comparison and Mann–Whitney test (SPSS 10.0; SPSS Inc., Chicago, IL, USA). Results are expressed as mean values \pm 7 standard deviations. Statistical significance was assumed if the p-value was 0.05 or below.

Results

Mechanical stretch induces osteogenic differentiation

Several studies reported the promotion of osteogenic differentiation of hBMSCs by mechanical stimulation (Jagodzinski et al., 2004; Ignatius et al., 2005; Zhao et al., 2007). To determine the osteogenic response of hBMSCs in our model, we analyzed the expression of Runx2 and collagen type 1, two marker genes for osteoblast differentiation, following treatment with two different degrees of stretch and after different durations of treatment.

Briefly, cells plated in flexible silicone dishes were subjected to progressively greater periods of cyclic mechanical stretch at a frequency of 1 Hz for either 2% or 8% elongation of the total length of the tissue culture surface as described in Materials and methods. This regimen of treatment and analysis was chosen to determine the rapid osteogenic response of hBMSCs to mechanical stimulation since it was appropriate to stimulate osteoblasts in an in vitro system (Neidlinger-Wilke et al., 2001).

Mechanical stretch immediately induced Runx2 expression in a stretch-dependent manner (Fig. 3A). Runx2 expression in cells subjected to 8% stretch increased significantly after the first round of stretching on day 1 (119.0 \pm 7.1%) and further increased after a second period of stretch to a maximum (127.5 \pm 8.3%) that remained after the third stimulation on day 1 (127.8 \pm 9.3%). Expression of Runx2 decreased significantly (110.7 \pm 5.5%) after three cycles of 8% strain on day 2, and was indistinguishable from controls after another three rounds of 8% stretch on day 3

Table 1. Synopsis of oligonucleotides used for cloning and amplification and hybridization probes for product detection

Gene (accession no.)	Name	Sequence (5'-3')	T _m (IC)	Product (bp)	T _{Dis} (IC)/ time (s)	T _{Ann} (IC)/ time (s)	T _{ext} (IC)/ time (s)	Cycles
GAPDH M33197	Cloning							
	GAPDH-clon-for	CAGGAGCGAGATCCC	71	465				
	GAPDH-clon-rev	TTCAGCTCAGGGATGACC	71					
	Amplification							
	GAPDH-for	TGCTGAGTATGTCGTGGAGTC	59	361	95/15	59/12	72/10	50
	GAPDH-rev	GGATGCAGGGATGATGTTCT	60					
	Hybridization							
GAPDH-FITC	GACAACTTTGGTATCGTGGAAAGGACTCATGACCACA-FITC	77						
FosB NM_006732	GAPDH-Cy5.5	Cy5.5-CCATGCCATCACTGCCACCCAGAAAGACT-Ph	76					
	Cloning							
	FosB-clon-for	CTACTCCACACCAGGCATGA	59	340				
	FosB-clon-rev	CACCAGCACAAACTCCAGAC	59					
	Amplification							
	FosB-for	GGAGAGCTCACCCCCAGA	62	125	95/20	60/30	72/30	40
	FosB-rev	GATCTGTCTCCGCCTGGA	60					
Hybridization								
FosB-FITC	GGGTGCGCCGGGACGAAATAAAA-FITC	73						
FosB-Cy5.5	Cy5.5-AGCAGCAGCTAAATGCAGGAAACCCGG-Ph	72						
Runx2 NM_004348	Cloning							
	Runx2-clon-for	CGGAATGCCCTCTGCTGTAT	60	708				
	Runx2-clon-rev	GACTGGCGGGGTGAAGTAA	60					
	Amplification							
	Runx2-for	AACCCAGAAGGCACAGACAG	60	192	95/20	59/30	72/30	40
	Runx2-rev	GCCTGGGGTCTGTAATCTGA	60					
	Hybridization							
Runx2-FITC	TGTTCTCTGACCCCTCAGTGATTTAGG-FITC	70						
Runx2-Cy5.5	Cy5.5-TGTTCTCTGACCCCTCAGTGATTTAGG-Ph	70						
Collagen1 NM_000088	Cloning							
	Col1-clon-for	GGACTAGACATTGGCCCTGTC	62	849				
	Col1-clon-rev	GTCTTCAAGCAAGAGGACCA	60					
	Amplification							
	Col1-for	ACAGTTGGCCCTGTCGTCTC	60	250	95/20	58/30	72/20	50
	Col1-rev	GTAAGTTGAAATGCACCTTTGG	57					
	Hybridization							
Col1-FITC	CCCTTGCATTCATCTTTCAAAACCTTAGTTTTTA-FITC	68						
Col1-Cy5.5	Cy5.5-CTTTGACCAACTGAACATGACCAAAA-Ph	68						

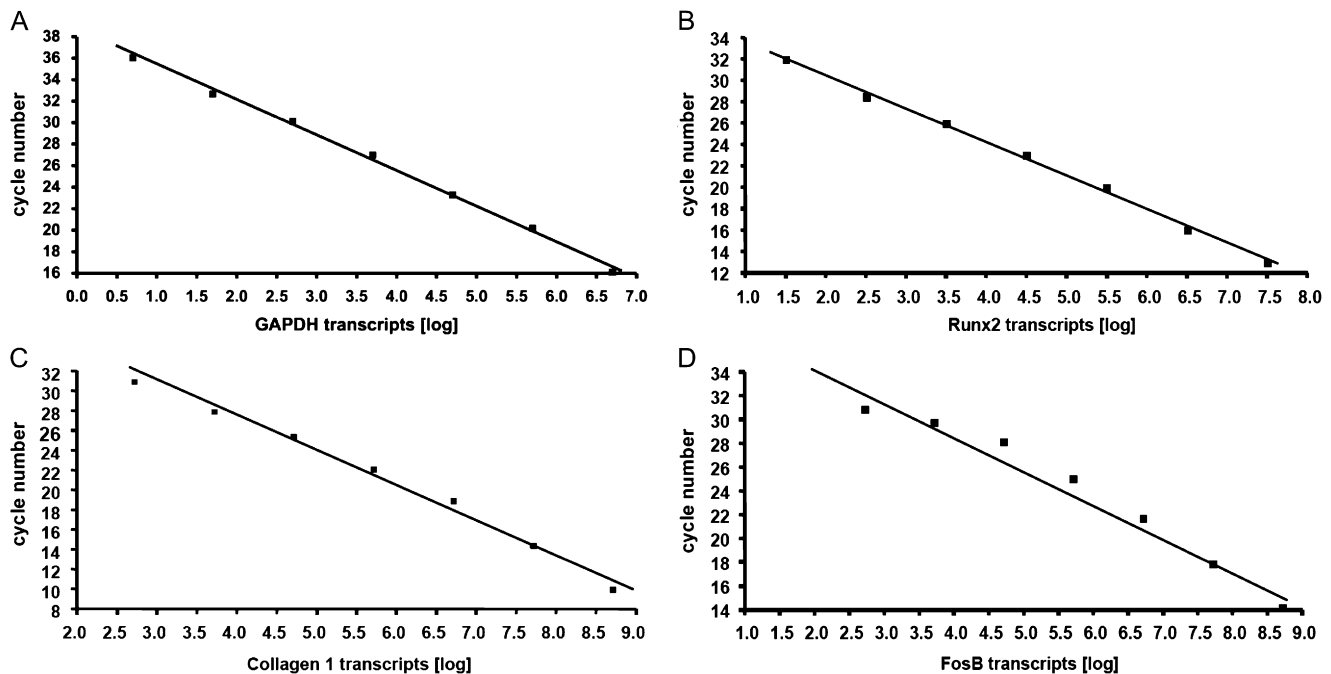


Fig. 2. Establishment of standard curves to determine the sensitivity of the developed real-time PCR assays and to quantify the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A), Runx2 (B), collagen 1 (C), and FosB (D). After cloning of each target sequence, serial 10-fold dilutions were prepared and quantified by real-time PCR. All standard curves revealed a linear regression over a range of 10^7 – 10^1 initial cDNA copies for GAPDH and Runx2. The standard curves for FosB and collagen 1 were linear over the range of 10^8 – 10^2 initial cDNA copies.

(105.67 2.9%). Runx2 expression responded less strongly to stimulation with 2% stretch, attaining a significant increase (116.07 5.1%) only after the third cycle on day 1. Runx2 expression returned to near-control levels after three rounds of 2% stretch on day 2 (103.57 1.8%) and three (104.07 2.4%).

Collagen type 1 expression, like Runx2 expression, increased with length of treatment. However, in contrast to Runx2, the responses to 8% and 2% stretch were not significantly different among treatment groups at any point of analysis (Fig. 3B). In detail, mechanical stimulation progressively increased the level of collagen 1 mRNA after one (8% stretch – 113.77%; 2% stretch – 118.74%), two (8% stretch – 124.74%; 2% stretch – 123.76%), or three (8% stretch – 128.79%; 2% stretch – 134.78%) rounds of mechanical stimulation on day 1. The level of collagen 1 mRNA progressively decreased after three cycles of mechanical stretch on day 2 (8% – 113.75%; 2% – 110.73%) and day 3 (8% – 109.73%; 2% – 111.74%) but remained significantly up-regulated compared to non-stretched control samples.

In summary, cells stimulated with either 2% or 8% stretch demonstrated a stretch- and time-dependent characteristic expression of Runx2 and collagen 1 mRNA in a transient manner, indicating that our system successfully initiates an osteogenic program in human mesenchymal precursor cells.

Effect of mechanical strain on FosB expression

Using a murine system, Inoue et al. (2004) observed that mechanical stress, both in vitro and in vivo, induced an increased level of expression of FosB, an AP-1 transcription factor that promotes osteogenesis (Sabatakos et al., 2000). We therefore determined if mechanical stress that induced the expression of the osteoblast marker genes Runx2 and collagen 1 in human mesenchymal precursor cells would also cause increased FosB expression.

A single round of 8% stretch for 1 h on day 1 was sufficient to significantly increase FosB expression (107.87 0.5%) (Fig. 3C) which did not change further after the second round of stretch (109.37 1.1%), but reached its maximum after the third round of cyclic stretching (118.97 2.1%), and remained elevated until the end of the experiment (day 2 – 118.67 3.1%, day 3 – 121.77 4.0%). In contrast, FosB expression in cells subjected to 2% stretch did not increase until the third round of treatment on the first day (106.47 1.4%). FosB expression in the cells subjected to 2% stretch peaked after the second day of treatment (110.87 2.6%) and returned to normal by day 3 (102.47 1.3%). At all time points, FosB expression in cells subjected to 8% stretch was significantly higher than both the 2% strain group and the non-stimulated controls.

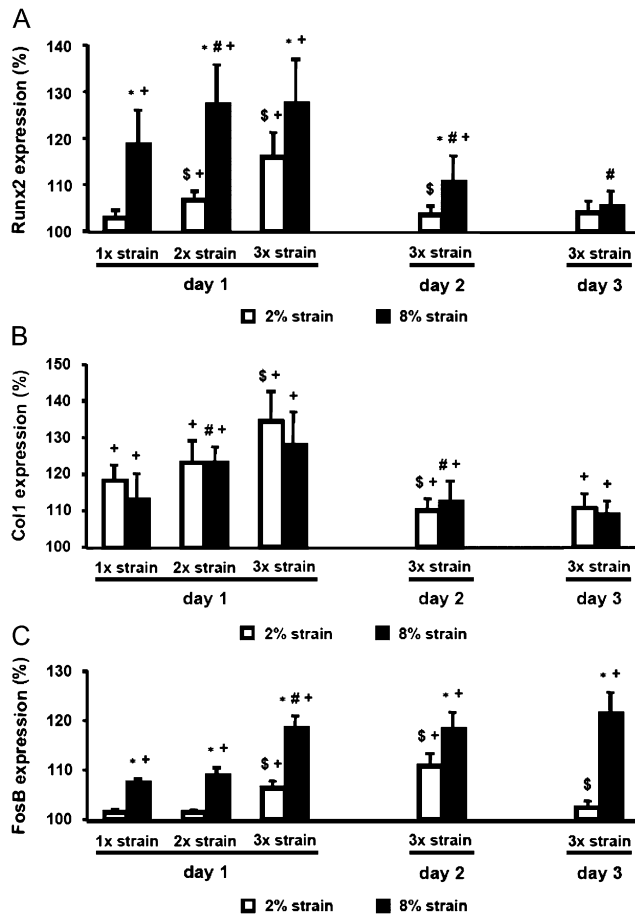


Fig. 3. Expression of mRNA for Runx2 (A), collagen 1 (B), and FosB (C) quantified by real-time PCR. Human bone marrow mesenchymal cells were stimulated by either 2% or 8% cyclic longitudinal stretch at a frequency of 1 Hz three times for 1 h on day 1, day 2, and day 3 of the experimental course. Values of expression of each target gene were normalized to GAPDH, and expressed as a percentage of non-stimulated controls. Data points represent the means and standard deviations of two to three independent experiments in which each sample was quantified in triplicate. Statistical significance ($p < 0.05$) is indicated as (+) compared to control, (*) group of 8% strain compared to the group of 2% strain, (#) group of 8% strain compared to the group of 8% strain at the previous point of quantification, (\$) group of 2% strain compared to the group of 2% strain at the previous point of quantification.

Discussion

Mechanical loading of the skeleton *in vivo* is known to stimulate bone formation and to positively regulate bone remodeling and skeletal strength (Vainionpaa et al., 2005, 2007). *In vitro*, mechanical stimulation has been reported to promote osteogenic lineage commitment of mesenchymal precursor cells and to improve osteoblast differentiation (Mauney et al., 2004; Jagodzinski et al., 2004; Ignatius et al., 2005).

FosB is an AP-1 transcription factor that is known to participate in the regulation of osteoblast differentiation and bone formation (Sabatakos et al., 2000). In mice, FosB transcription is induced by mechanical stimulation, providing a potential mechanism by which physical stimulation of bone cells activates an osteogenic program (Inoue et al., 2004).

Primary BMSCs are variable because it is a cell population that is more heterogeneous in terms of phenotype, functional, and differentiation stage, both between subjects and within the same culture, than the more established and well characterized mesenchymal- or osteoblastic cell lines used in other studies (Ignatius et al., 2005). However, we combined cells derived from several individuals to reduce variations caused by cell heterogeneity.

In this study, we investigated the influence of different patterns of cyclic uniaxial mechanical stretch on primary human osteoblast precursors. Two different amplitudes of cyclic longitudinal stretch of the total length of the tissue culture surface (either 2% or 8%) were applied at a fixed frequency of 1 Hz to hBMSCs in three successive rounds of stretch on each of 3 consecutive days in a controlled fashion using a custom-made stretch application device. A magnitude of 2% and 8% strain was chosen because this regimen has previously been proven to stimulate osteoblasts and progenitor cells in an *in vitro* stimulation system (Neidlinger-Wilke et al., 2001; Jagodzinski et al., 2004), although the magnitude of strain might be different in *in vivo* models. Our results demonstrate that mechanical stimulation of hBMSCs grown on flexible silicone culture dishes induced a rapid and progressive time- and intensity-dependent induction of FosB transcription, which was accompanied by an increased expression of the osteoblast marker genes collagen 1 and Runx2, providing direct evidence of a regulated early initiation of an osteoblast-specific transcriptional program in a more immature pool of mesenchymal precursor cells.

Runx2 is known to bind numerous transcription factors and other transcription-regulating proteins including the AP-1 family transcription factors c-Fos, c-Jun, and JunD and the BMP-responsive R-Smads and Smad4 (D'Alonzo et al., 2002). Although the possibility of independent effects of Runx2 and AP-1 on osteoblast lineage commitment cannot be ruled out, it is possible that both factors synergize in the regulation of downstream target gene activation (Komori, 2006).

In mice, mechanical loading increased the expression of FosB mRNA (Inoue et al., 2004). Since overexpression of DFosB, a Fos-related protein generated from alternative splicing of FosB transcripts, is known to increase bone formation and osteoblastogenesis in mice (Sabatakos et al., 2000), it is possible that FosB only indirectly contributes to an enhanced osteogenic differentiation via generation of DFosB. Splicing of

FosB to produce DFosB may occur at a fixed rate and the more FosB is expressed, the more gets spliced. Alternatively, mechanostimulation may increase the amount of DFosB relative to FosB, ultimately leading to an improved osteoblast function. However, examining this hypothesis will be the focus of future research.

In summary, we have shown that mechanical stretch induces FosB gene expression in human mesenchymal precursor cells in a time- and amplitude-dependent manner. In agreement with other studies (Jagodzinski et al., 2004; Ignatius et al., 2005), physical forces enhanced the progression of the progenitor cell population towards an osteogenic fate, demonstrated by the increased levels of Runx2 and collagen 1 expression. Although the mechanism by which FosB promotes osteoblast lineage commitment and bone formation remains to be elucidated, this system could provide novel and important insights into osteoblast differentiation and bone formation.

Acknowledgments

We gratefully acknowledge W.C. Horne for helpful discussions and for critical reading of the manuscript. We are indebted to B. Luens for constant technical assistance. This study was supported in part by the Deutsche Forschungsgemeinschaft (E.H.), by a Hannelore Munke Research Grant from Hannover Medical School (E.H.), and by an institutional Grant (HilF) from Hannover Medical School (M.J.).

References

- Bonewald LF. Summary – osteocytes and mechanotransduction. *J Musculoskelet Neuronal Interact* 2005;5:333–4.
- Bonewald LF. Osteocytes as dynamic, multifunctional cells. *Ann NY Acad Sci*. 2007.
- Chen NX, Ryder KD, Pavalko FM, Turner CH, Burr DB, Qiu J, et al. Ca(2+) regulates fluid shear-induced cytoskeletal reorganization and gene expression in osteoblasts. *Am J Physiol Cell Physiol* 2000;278:C989–97.
- D'Alonzo RC, Selvamurugan N, Karsenty G Partridge NC. Physical interaction of the activator protein-1 factors c-Fos and c-Jun with Cbfa1 for collagenase-3 promoter activation. *J Biol Chem* 2002;277:816–22.
- Ducy P, Karsenty G. Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol Cell Biol* 1995;15:1858–69.
- Ducy P, Zhang R, Geoffroy V, Ridall AL Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 1997;89:747–54.
- Gardner MJ, van der Meulen MC, Demetrakopoulos D, Wright TM, Myers ER Bostrom MP. In vivo cyclic axial compression affects bone healing in the mouse tibia. *J Orthop Res* 2006;24:1679–86.
- Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol* 2007;176:709–18.
- Ignatius A, Blessing H, Liedert A, Schmidt C, Neidlinger-Wilke C, Kaspar D, et al. Tissue engineering of bone: effects of mechanical strain on osteoblastic cells in type I collagen matrices. *Biomaterials* 2005;26:311–8.
- Inoue D, Kido S, Matsumoto T. Transcriptional induction of FosB/DeltaFosB gene by mechanical stress in osteoblasts. *J Biol Chem* 2004;279:49795–803.
- Jagodzinski M, Drescher M, Zeichen J, Hankemeier S, Krettek C, Bosch U, et al. Effects of cyclic longitudinal mechanical strain and dexamethasone on osteogenic differentiation of human bone marrow stromal cells. *Eur Cell Mater* 2004;7:35–41 [discussion 41].
- Klein-Nulend J, Helfrich MH, Sterck JG, MacPherson H, Joldersma M, Ralston SH, et al. Nitric oxide response to shear stress by human bone cell cultures is endothelial nitric oxide synthase dependent. *Biochem Biophys Res Commun* 1998;250:108–14.
- Kletsas D, Basdra EK, Papavassiliou AG. Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. *J Cell Physiol* 2002;190:313–21.
- Komori T. Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem* 2006;99:1233–9.
- Leucht P, Kim JB, Wazen R, Currey JA, Nanci A, Brunski JB, et al. Effect of mechanical stimuli on skeletal regeneration around implants. *Bone* 2007;40:919–30.
- Mauney JR, Sjostrom S, Blumberg J, Horan R, O'Leary JP, Vunjak-Novakovic G, et al. Mechanical stimulation promotes osteogenic differentiation of human bone marrow stromal cells on 3-D partially demineralized bone scaffolds in vitro. *Calcif Tissue Int* 2004;74:458–68.
- McNamara LM, Prendergast PJ. Bone remodelling algorithms incorporating both strain and microdamage stimuli. *J Biomech* 2007;40:1381–91.
- Neidlinger-Wilke C, Grood ES, Wang J-C, Brand RA, Claes L. Cell alignment is induced by cyclic changes in cell length: studies of cells grown in cyclically stretched substrates. *J Orthop Res* 2001;19:286–93.
- Sabatokos G, Sims NA, Chen J, Aoki K, Kelz MB, Amling M, et al. Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat Med* 2000;6:985–90.
- Siddhivarn C, Banes A, Champagne C, Riche EL, Weerapradist W, Offenbacher S. Prostaglandin D2 pathway and peroxisome proliferator-activated receptor gamma-1 expression are induced by mechanical loading in an osteoblastic cell line. *J Periodontol Res* 2006;41:92–100.
- Simmons CA, Matlis S, Thornton AJ, Chen S, Wang CY, Mooney DJ. Cyclic strain enhances matrix mineralization by adult human mesenchymal stem cells via the extracellular signal-regulated kinase (ERK1/2) signaling pathway. *J Biomech* 2003;36:1087–96.
- Sterck JG, Klein-Nulend J, Lips P, Burger EH. Response of normal and osteoporotic human bone cells to mechanical stress in vitro. *Am J Physiol* 1998;274:E1113–20.
- Suva LJ, Gaddy D, Perrien DS, Thomas RL, Findlay DM. Regulation of bone mass by mechanical loading:

- microarchitecture and genetics. *Curr Osteoporos Rep* 2005;3:46–51.
- Vainionpaa A, Korpelainen R, Leppaluoto J, Jamsa T. Effects of high-impact exercise on bone mineral density: a randomized controlled trial in premenopausal women. *Osteoporos Int* 2005;16:191–7.
- Vainionpaa A, Korpelainen R, Sievanen H, Vihriala E, Leppaluoto J, Jamsa T. Effect of impact exercise and its intensity on bone geometry at weight-bearing tibia and femur. *Bone* 2007;40:604–11.
- Xiao G, Jiang D, Thomas P, Benson MD, Guan K, Karsenty G, et al. MAPK pathways activate and phosphorylate the osteoblast-specific transcription factor, Cbfa1. *J Biol Chem* 2000;275:4453–9.
- Xiao Z, Zhang S, Mahlios J, Zhou G, Magenheimer BS, Guo D, et al. Cilia-like structures and polycystin-1 in osteoblasts/osteocytes and associated abnormalities in skeletogenesis and Runx2 expression. *J Biol Chem* 2006;281:30884–95.
- Zhao F, Chella R, Ma T. Effects of shear stress on 3-D human mesenchymal stem cell construct development in a perfusion bioreactor system: experiments and hydrodynamic modeling. *Biotechnol Bioeng* 2007;96:584–95.