Gene Expression Responses to Mechanical Stimulation of Mesenchymal Stem Cells Seeded on Calcium Phosphate Cement

Borzo Gharibi, PhD,¹ Giuseppe Cama, PhD,² Marco Capurro, PhD,³ Ian Thompson, PhD,² Sanjukta Deb, PhD,² Lucy Di Silvio, PhD,² and Francis John Hughes, BDS, PhD¹

Introduction: The aim of the study reported here was to investigate the molecular responses of human mesenchymal stem cells (MSC) to loading with a model that attempts to closely mimic the physiological mechanical loading of bone, using monetite calcium phosphate (CaP) scaffolds to mimic the biomechanical properties of bone and a bioreactor to induce appropriate load and strain.

Methods: Human MSCs were seeded onto CaP scaffolds and subjected to a pulsating compressive force of 5.5 ± 4.5 N at a frequency of 0.1 Hz. Early molecular responses to mechanical loading were assessed by microarray and quantitative reverse transcription-polymerase chain reaction and activation of signal transduction cascades was evaluated by western blotting analysis.

Results: The maximum mechanical strain on cell/scaffolds was calculated at around 0.4%. After 2 h of loading, a total of 100 genes were differentially expressed. The largest cluster of genes activated with 2 h stimulation was the regulator of transcription, and it included *FOSB*. There were also changes in genes involved in cell cycle and regulation of protein kinase cascades. When cells were rested for 6 h after mechanical stimulation, gene expression returned to normal. Further resting for a total of 22 h induced upregulation of 63 totally distinct genes that were mainly involved in cell surface receptor signal transduction and regulation of metabolic and cell division processes. In addition, the osteogenic transcription factor *RUNX-2* was upregulated. Twenty-four hours of persistent loading also markedly induced osterix expression. Mechanical loading resulted in upregulation of Erk1/2 phosphorylation and the gene expression study identified a number of possible genes (*SPRY2*, *RIPK1*, *SPRED2*, *SERTAD1*, *TRIB1*, and *RAPGEF2*) that may regulate this process.

Conclusion: The results suggest that mechanical loading activates a small number of immediate-early response genes that are mainly associated with transcriptional regulation, which subsequently results in activation of a wider group of genes including those associated with osteoblast proliferation and differentiation. The results provide a valuable insight into molecular events and signal transduction pathways involved in the regulation of MSC osteogenic differentiation in response to a physiological level of mechanical stimulation.

Introduction

MECHANICAL FORCES ARE known to be critical in the regulation of bone homeostasis. Absence of mechanical stimuli has been implicated in loss of bone mass that is seen, for example, in bed rest¹ and during spaceflight.² In contrast, mechanical stimulation from increased physical activity increases the rate of bone formation, bone mineral density, bone mineral content, and bone strength.^{3–5} The majority of these effects have been attributed to the induction of osteoblast activity and function and increase in cell

numbers.^{6–9} Many transcription factors, growth factors, cell surface receptors, and signal transduction pathways have been implicated in the differentiation, proliferation, and functional activity of osteoblasts.^{10,11} However, the molecular and cellular events that occur during signal transduction of mechanical load are not fully understood, but are likely to be key factors that may govern bone formation. Altering fate decisions of the cellular components of bone is likely to be advantageous for the treatment of bone loss associated with reduced mechanical loading and for tissue engineering strategies in managing bone regeneration.

¹Department of Periodontology, Dental Institute, Kings College London, London, United Kingdom.

²Department of Dental Biomaterials and Tissue Engineering, Kings College London, London, United Kingdom.

³Department of Civil, Chemical and Environmental Engineering, University of Genoa, Genoa, Italy.

Various systems have been developed to mimic mechanical stimuli for investigating bone turnover *in vivo* and *in vitro*. Most of these have mainly focused on studying osteocytes as mechanosensors and osteoblasts as bone forming cells in their models. Commonly a gel based scaffold or tissue culture plastic has been used as the substrate on which cells were mechanically loaded, and the strain employed has been in the range of 2%–20%.^{6,12–20} Neither the scaffold nor the strain of this magnitude represents the true environment or magnitude of physiological or pathological loading of bone in humans *in vivo*. Further, a number of studies *in vivo* have been carried out in an attempt to overcome these obstacles, but these are limited to animal models and therefore could be associated with differences in responses to loading and mechanisms involved from one species to another.^{21,22} In addition, the presence of a mixed population of cells within these settings makes it difficult to study responses from specific cell types such as osteoblasts or mesenchymal stem cells (MSC).

Therefore, the aim of this study was to investigate the molecular response of MSCs to transient or continuous mechanical stimulation using a model that attempts to mimic the situation *in vivo*. A monetite calcium phosphate (CaP) scaffold was used to model the mechanical properties of bone and load and strain were induced using a bioreactor. A mathematical model was used to calculate a load required to mimic changes in strain equivalent to physiological-scale effects that have been reported previously.²³ A global gene expression microarray was carried out to identify candidate transcriptional regulators and signaling mechanisms induced by mechanical stimulation.

Materials and Methods

Cell culture

Commercially obtained primary human MSCs from three separate donors (Lonza), at passage 2 when purchased were expanded in culture and used at passage 3–5. Cells were maintained in normal growth medium consisting of α -Minimal Essential Medium (α -MEM), penicillin (50 U/mL), streptomycin (50 μ g/mL) (Sigma-Aldrich), Glutamax (2 mM) (Invitrogen), and 10% fetal bovine serum (FBS) (Sigma-Aldrich) at 37°C in a humidified 5% CO₂:95% air atmosphere.

MSCs were characterized for cell surface marker expression using four-color flow cytometry analysis as previously described.²⁴ To induce osteogenesis, MSCs were seeded at a density of 1.2×10^4 cell/cm² in 12-well plates and after 24 h were treated with osteogenic medium consisting of growth medium supplemented with 0.1 μ M dexamethasone, 0.05 mM ascorbic acid, and 10 mM β -glycerophosphate (Sigma-Aldrich). Mineralization was assessed by staining with alizarin red dye. Briefly, cells were fixed (15 min with 4% formaldehyde in phosphate-buffered saline [PBS]), stained for 10 min with alizarin red S (1:100 dilution in H₂O), washed in 50% ethanol, and air-dried. To induce adipogenic differentiation, cells were seeded at a density of 4×10^4 cell/cm² in 12-well plates (Nunc) and incubated for 24 h before switching to adipogenic medium (growth medium supplemented with 1 μ M dexamethasone, 0.25 mM isobutylmethylxanthine, 50 μ M indomethacin, and 10 μ g/mL insulin; Sigma-Aldrich). Adipogenesis was visualized by light microscopy following staining with Oil red O dye. Briefly, cells were fixed (15 min

with 4% formaldehyde in PBS), stained for 15 min, and washed with 60% isopropanol and with PBS.

Mechanical stimulation

Macroporous monetite cements were prepared with a solid phase made of equimolar quantities of β -tricalcium phosphate (β -TCP; assay >96%) (Fluka) and monocalcium phosphate monohydrate (MCPM; assay min. 98%) (Sharlau) with 60% w/w sodium chloride (NaCl) crystals in respect to the CaP powder were added as a water soluble porogen. The polygonal shaped salt crystals were sieved to achieve a diameter in the range of 150–600 μ m. The liquid phase was a solution consisting of 0.5 M of sodium dihydrogen citrate. The powder to liquid ratio (R) was fixed at a value equal to 3 (g·mL⁻¹). The mixture was manually stirred for $\sim$ 1.5 min. Following mixing, the paste was poured into two different Teflon moulds of cylindrical shape (one of length: $l=12$ mm, diameter: $d=6$ mm and the other of length: $l=4.2$ mm, diameter: $d=8.4$ mm) to make samples for the evaluation of the mechanical properties by uniaxial or diametral compression (“brazilian”) test in accordance to the ASTM F451 and C496 standard respectively. The samples were left to set for 24 h at ambient conditions (20°C–23°C and 50%–60% humidity) and then immersed in distilled water for 5 days to let the porogen dissolve. Identification of monetite was achieved by means of X-ray diffraction on powder samples using a Bruker D8 Advance diffractometer equipped with a Lynx Eye detector, in flat plate geometry using Ni-filtered Cu α radiation. The phase composition was checked using the reference patterns for β -TCP (PDF Ref. 09-0169), monetite (PDF Ref. 09-0080) and brushite (PDF Ref. 09-0077). The distribution of macropores and the volume of the pores on the cement were observed by SEM (Hitachi S-3500 machine) and micro-CT (Locus SP, microCT scanner; GE Healthcare) analysis set at a resolution of 7 μ m.

The mechanical tests were carried out by using a universal testing machine (Instron 5699) with a 500 N load cell and a cross-head speed of 1 mm/min. Twelve samples were used for each test. The tensile strength of the material from the diametral compression tests had an average of 0.54 ($\pm$ 0.01) MPa, corresponding to a maximum axial load of 30 N. This preliminary evaluation allowed us to define the dynamic load that can be applied in the bioreactor tests without risk of failure for the scaffolds.

MSCs were cultured to 80%–90% confluence in normal growth medium. Cells were then seeded on to the top of one side of the scaffold at a density of 1.5×10^5 in 70 μ L of media and allowed to attach for 30 min. The scaffold was then turned and the same number of cells were seeded on to the top of the other side and left undisturbed for a further 30 min for the cells to attach. Normal growth media was then carefully added to the culture plate and the cell/scaffold was incubated at 37°C in a humidified 5% CO₂:95% air atmosphere for 48 h prior to stimulation. Mechanical loading was performed using a BOSE bioreactor (ElectroForce BioDynamic test instrument; Bose) equipped with 200 N load cell (Fig. 1A). Seeded scaffolds were positioned between two loading plates inside the bioreactor chamber (Fig. 1A[iv]) and loaded in the diametral compression mode by a pulsating compressive force of 5.5 ± 4.5 N at a frequency of 0.1 Hz. The corresponding head displacement was 0.5–50 μ m. The test was performed in load control and the evolution of the head displacement versus the

FIG. 1. (A) Scheme of bioreactor machine. The bioreactor components are assembled in sterilized condition. The bioreactor chamber (i) is mounted on the load frame (ii). The displacement of the actuator (iii) produces a dynamic compressive force onto the calcium phosphate (CaP) scaffolds ($n=4$) placed inside the bioreactor chamber (iv). The force applied onto the scaffolds is measured by a cell load (v). The tests were performed keeping the machine inside an incubator at 37°C and 5% of CO₂. (B) SEM micrograph of the external surface of the macroporous monetite cement. (C) Compressive strength (σ_c), Young's modulus (E), and diametral tensile strength (σ_t) of the macroporous monetite cement. Color images available online at www.liebertpub.com/tea

pulsating compressive force was recorded by the bioreactor software (WinTest® controls). The stress distribution in the disk was analytically evaluated by the Timoshenko model.²⁵ For controls, cells were incubated for the same period of time on samples positioned between the loading plates of the bioreactor with no load applied.

The diametral compression test configuration was chosen because it generates a complex state of stress where all of the in-plane stress components (normal and shear stresses) are present. In this way the influence of the mechanical loading on cell behavior had a higher probability of being observed. The distributions of radial (σ_r), circumferential ($\sigma_{\theta\theta}$), and shear ($\tau_{\theta r}$) stress components shown in Figure 2A were calculated with reference to the load factor (Eq. [1], where P is the applied load, R the radius of the circular sample, and t its thickness):

$$\sigma_0 = \frac{2P}{\pi R t} = 0.198 \pm 0.162 \text{ MPa} \quad (1)$$

In the bioreactor tests, most of the cells were attached to the outer surface of the scaffold, where no stress is directly applied. Therefore, the situation is different from that of scaffolds submerged in a liquid current, where an effective shear stress is present at the surface and an increase of cell activity has been observed by others.^{26–28} In the present case however, a physical stimulation of the cells as a consequence of mechanical loading can occur on account of the stretching they undergo while following the elastic deformations of the scaffold. The maxi-

mum (ε_I) and minimum (ε_{II}) strains are related to the principal stresses σ_I and σ_{II} by Equations (2) and (3), respectively;

$$\varepsilon_I = \frac{1}{E}(\sigma_I - \nu \sigma_{II}) \quad (2)$$

$$\varepsilon_{II} = \frac{1}{E}(\sigma_{II} - \nu \sigma_I) \quad (3)$$

Accordingly, we have the distributions of ε_I and ε_{II} in the disk (referred to σ_0/E), which are represented in the plot of Figure 2B where, E is the Young's modulus and ν is the Poisson ratio of the scaffold respectively.

The averages of the maximum ($\bar{\varepsilon}_I$) and ($\bar{\varepsilon}_{II}$) minimum strain values over the whole disk were calculated from the Equations (4) and (5), respectively:

$$\bar{\varepsilon}_I = \frac{\pi \sigma_0}{4E} \left(1 - \nu + \frac{\pi}{2}(1 + \nu) \right) \quad (4)$$

$$\bar{\varepsilon}_{II} = \frac{\pi \sigma_0}{4E} \left(1 - \nu - \frac{\pi}{2}(1 + \nu) \right) \quad (5)$$

Microarray analysis

For total gene expression analysis, samples from three primary MSC lines were loaded or left unloaded for 2 h in

FIG. 2. The stress analysis of disk subjected to two concentrated diametral forces. **(A)** Distributions of **(i)** circumferential ($\sigma_{\theta\theta}$), **(ii)** radial (σ_{rr}), and **(iii)** shear ($\tau_{\theta r}$) stress components, referred to the load factor (σ_o) for different angular positions with respect to x -axis. Note the constant behavior of $\sigma_{\theta\theta}$ along the vertical radius ($\theta = \pi/2$) corresponding to the maximum tensile stress, which would eventually bring the material to failure. **(B, i)** Distribution of the maximum and minimum strain value in the whole disk referred to (σ_o/E) for different angles to x -axis. Color images available online at www.liebertpub.com/tea

the bioreactor chamber. Samples were then removed from the bioreactor and the cells were either lysed for RNA isolation or incubated for a further 6 or 22 h in normal culture medium at 37°C in a humidified 5% CO₂:95% air atmosphere prior to RNA isolation. Total RNA was extracted using TRI reagent (Ambion) and Phase Lock Gel Heavy tubes (5 prime; VWR) according to the manufacturer's instructions. RNA purity and quantity was assessed by nanodrop (Fisher Scientific) and Qubit 2.0 (Invitrogen). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent). An equal quantity of RNA from three separate experiments was pooled together for each MSC line and processed for microarray analysis. The gene expression analysis was performed using the Illumina whole-genome expression array HumanHT-12 v4.0 Expression BeadChip according to the manufacturer's instructions. Briefly, 500 ng of total RNA was processed and cRNA was hybridized to an Illumina whole genome expression chip. The Beadchips were scanned on the Illumina iScan System with iScan software. The raw data were processed for the background signal, normalized, and differences in gene expression were compared by the Differential Expression Algorithm using the Illumina custom error model of GenomeStudio software (Illumina, Inc.). Genes with a DiffScore of ± 13 , equivalent to $p < 0.05$, were considered as being significantly down- or upregulated; a DiffScore of ± 20 , ± 30 or ± 40 was equivalent to a p -value of < 0.01 , < 0.001 , or < 0.0001 , respectively. Gene Ontology (GO) analysis was

subsequently carried out on genes significantly up- and downregulated using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.7 Web tool to study associations corresponding to biological processes (BP-FAT). The software categorizes related genes into functionally related clusters and rank them based on the statistical calculations according to their cluster enrichment by EASE score, which is calculated as higher probability to get a GO term under the null hypothesis than would be randomly selected from the total number of gene on the microarray.

Gene Expression

cDNA was prepared from 1 μ g RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed on a rotor gene 6000 thermal cycler (Qiagen) using Brilliant III Ultra-Fast SYBR Green qPCR Master mix (Stratagene; Agilent Technologies) and primer pairs as listed in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea). PCR conditions consisted of 1 cycle of 95°C for 3 min and 40 cycles of 95°C for 10 s and 60°C for 10 s followed by melting analysis of 1 cycle with gradual increase from 65°C to 95°C. RPL13a was used as an invariant housekeeping gene. The relative quantitative expression of genes of interest, relative to housekeeping

RPL13a gene was calculated and compared with untreated cells that were assigned a value of one. RPL13a was used as it has been shown by our group and others to be a stable housekeeping gene for qPCR analysis of MSCs during *in vitro* characterization and differentiation.²⁹

Western blotting analysis

MSC/scaffolds were incubated with serum-free α -MEM for 6 h prior to mechanical stimulation. Cells were then loaded for the times indicated then immediately lysed and subjected to western blot analysis as described previously³⁰ using antibodies against pAkt (Ser473) and p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signalling; New England Biolabs). Proteins were visualized and photographed using ECL Plus detection reagent (GE Healthcare) and Molecular Imager Gel Doc XR+ documentation and analysis System with Image Lab Software (Bio-Rad). Equal sample loading was confirmed by probing with antibody to the housekeeping protein GAPDH.

Cell morphology and viability

Phalloidin-TRITC (Sigma) was used to stain for actin filaments. Briefly, MSC/scaffolds were fixed for 15 min with 4% formaldehyde, permeabilized for 10 min with 0.1% Triton X-100, and stained for 40 min with a 0.5- μ g/mL phalloidin-TRITC conjugate solution in PBS. Samples were washed with PBS and visualized and imaged using a fluorescence microscope. Cell viability was determined using the Live/Dead, viability/cytotoxicity assay (Invitrogen). Cells were stained with 2 μ M calcein AM and 4 μ M ethidium homodimer-1 (EthD-1) in PBS for 20 min. Samples were visualized using a fluorescence microscope with live and dead cells staining green (calcein AM) or red (EthD-1) respectively.

Data analysis

For qRT-PCR analysis, statistical comparisons between means were made by Student's *t*-test (SPSS 16; SPSS). A *p*-value of less than 0.05 was considered statistically significant. The data were pooled from duplicate tests carried out in three separate experiments.

Results

MSC characterization

Prior to mechanical loading experiments, MSCs were assessed for their cell surface marker expression and differentiation potential. Using four-color flow cytometry analysis of panels of known negative and positive markers we showed that MSCs were 90%–100% positive for CD105, CD90, CD44, and CD146 but lacked the HSC marker (0%–5%) CD34 and CD45 expression (Fig. 3A). We also demonstrated the potential of MSCs to differentiate toward osteoblasts and adipocytes, by the increase in specific expression of markers of each lineage, that is, *Runx-2*, *ALP*, *PPAR γ* , and *FAB-4* and accumulation of calcium deposits and lipid droplets by the cells following culture for 14 days in differentiating media. (Fig. 3B, C).

Monetite scaffold characterization and strain values over the disk

X-ray diffraction confirmed that the final composition of the cement was monetite.³¹ Figure 1B is an SEM image of the

fracture surface of macroporous monetite cement tested by the uniaxial compressive test showing the pores produced by the dissolution in distilled water of the NaCl crystals. The micro-CT analysis reported an average porosity of 45% with pore diameters between 200 and 650 μ m.³¹ The pores were distributed throughout the cement while the structure maintained sound continuity along its multiple isosurfaces in all directions (*X*, *Y*, *Z*).

The mechanical properties of the monetite cements are reported in Figure 1C as average and standard deviation values of the maximum compressive strength (σ_c), Young's modulus (*E*), and maximum diametral strength (σ_t). The calculated distributions of radial (σ_r), circumferential ($\sigma_{\theta\theta}$), and shear ($\tau_{\theta r}$) stress components are reported in Figure 2A. The average strain values in a loading cycle were calculated using Equations (4) and (5) above, where $\nu=0.3$ is the Poisson ratio of the ϵ material. Their values in a loading cycle were as follows:

$$\epsilon_I = 2.15 \frac{\sigma_o}{E} = 0.0047 \pm 0.0038 = (0.47 + 0.38)\%$$

$$\bar{\epsilon}_{II} = -1.05 \frac{\sigma_o}{E} = -0.0023 \pm 0.0018 = (-0.23 + 0.18)\%$$

The strain amplitudes in a cycle (0.38; 0.18)% are comparable to those strains that have been previously reported to act on long bones.²³

Effect of mechanical loading on MSC gene expression

Global gene expression analysis of MSCs showed a total of 100 differentially expressed genes compared with unloaded MSCs following 2 h of loading. Among these, 75 genes were significantly upregulated and 25 downregulated in loaded MSCs compared to unloaded cells (Supplementary Tables S2 and S3). When MSCs were cultured for a further 22 h after 2 h of loading, 63 genes were also differently expressed, of which 40 were upregulated and 23 were downregulated when compared to unloaded cells (Supplementary Tables S4 and S5). When MSCs were rested in culture for 6 h following the loading, there were no significant differences in the levels of gene expression compared to unloaded controls (Supplementary Tables S6 and S7). To predict better the cascade of events that takes place during mechanical stimulation and the signaling mechanisms involved, we categorized the differentially expressed genes based on the biological processes that they are involved in. As shown in Tables 1–4 genes upregulated following 2 h of loading were mainly involved in regulation of transcription, protein kinase cascade, regulation of MAP kinase cascade, and activity and metabolic processes. However, when cells were rested for 22 h after 2 h loading, the upregulated genes were predominantly associated with cell surface receptor-linked signal transduction, regulation of metabolic processes, and cell division. In contrast, genes that were downregulated with 2 h of loading or after 22 h of rest following stimulation were mainly negative regulators of cell proliferation and cell cycle. Genes involved in programmed cell death and regulation of apoptosis were also downregulated with 2 h loading.

qRT-PCR validation of microarray data

To confirm the data obtained by microarray analysis, we carried out qRT-PCR analysis of RNA samples used for the

FIG. 3. Characterization of mesenchymal stem cells (MSCs). **(A)** Expression of MSC surface markers (CD34, CD45, CD105, CD44, CD90, CD71, and CD146) was analyzed by flow cytometry. Differentiation to osteogenic and adipogenic lineages was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyzing of mRNA expression for **(B)** osteogenic markers (*Runx-2* and *ALP*) and **(C)** adipogenic markers (*PPAR γ* and *FAB-4*) following 14 days of differentiation. (mean $\pm$ SEM of three experiments of duplicates). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with undifferentiated MSCs. Phenotypic changes for osteoblast lineage **(B)** were assessed by analyzing the ability of the cells to undergo matrix mineralization by staining with alizarin red for calcium deposition and adipogenic phenotype **(C)** was determined by staining with Oil red O for lipid accumulation. Color images available online at www.liebertpub.com/tea

microarray experiments. We selected five differentially expressed genes, namely *FOSB*, *SPRY2*, *SERTAD1*, *CDKN1b*, and *CEBP δ* based on their known roles in physiological processes involved in regulation of cell fate (differentiation or proliferation). The changes in expression of these genes obtained by microarray were 5.4, 1.94, 1.65, 0.64, and 0.55-fold, respectively compared to 10.3, 2.98, 5.3, 0.75, and 0.63-fold changes observed with qRT-PCR. As illustrated here and in Figures 4 and 5 qRT-PCR analysis confirmed the changes in these selected genes, showing significant changes in expression in accordance with the array data. The high consistency between assays gives us confidence of the accuracy of our microarray data. In addition, we tested the expression of the osteoblast-related transcription factor *Runx-2*. Although the microarray results did not show any significant differences in expression, when tested by qRT-PCR *Runx-2* expression was significantly increased after 2 h of loading followed by a further 22 h resting (Fig. 6).

To determine the outcome of a longer period of mechanical stimulation, we loaded the cells continuously

with the same strain for 24 h and compared the changes in expression of selected genes from microarray studies. None of the selected genes that were influenced by 2 h of loading were affected in the same manner with either 24 h continuous loading or following 22 h of resting after the load (Figs. 4 and 5). However, continuous loading for 24 h resulted in a dramatic increase in expression of osterix (Fig. 6).

Effect of loading on ERK and AKT pathways

To investigate the signal transduction pathways involved, we assessed the alteration in phosphorylation of Akt and P44/P42 MAPK in response to stimulation with mechanical loading. Serum starved MSCs were loaded for 15 min, 2 and 24 h and protein phosphorylation was determined using phosphor-specific antibodies against Akt (Ser473) and p44/42 MAPK (Erk1/2) (Thr202/Tyr204). The Erk1/2 were phosphorylated within 15 min and the level remained consistently high for the duration of 2 h and even further

TABLE 1. CLUSTERS OF GENES UPREGULATED WITH 2 H LOADING, CATEGORIZED BASED ON THEIR BIOLOGICAL PROCESSES

<i>Term</i>	<i>Count</i>	<i>p-Value</i>	<i>Gene symbol</i>
Regulation of transcription	28	1.34716E-06	ZNF614, ZNF17, ZBTB9, SNIP1, ZNF800, ZNF35, TRIB1, TFAM, SERTAD1, PLAGL2, ZNF529, KLF13, GMEB2, ARID5B, TADA2B, ZNF791, FOSB, GTF2B, NRIP1, POGK, RIPK1, PPRC1, ZNF416, TGFBF3, ZIK1, HIVEP1, RBM15, MYNN
Transcription	22	6.89E-05	ZNF614, ZNF529, ZNF17, KLF13, GMEB2, ZBTB9, ARID5B, TADA2B, ZNF800, ZNF791, ZNF35, GTF2B, NRIP1, TFAM, PPRC1, ZNF416, ZIK1, HIVEP1, MYNN, RBM16, SERTAD1, PLAGL2
Protein kinase cascade	8	8.81E-04	CCL2, RIPK1, SNIP1, DUSP10, SPRED2, TGFBF3, RAPGEF2, TRIB1
Regulation of RNA metabolic process	18	9.35E-04	ZNF614, ZNF529, ZNF17, KLF13, ARID5B, SNIP1, ZNF791, ZNF35, FOSB, GTF2B, NRIP1, TFAM, POGK, ZNF416, HIVEP1, ZIK1, RBM15, PLAGL2
MAPKKK cascade	5	0.007466346	RIPK1, DUSP10, SPRED2, RAPGEF2, TRIB1
RNA biosynthetic process	6	0.008022828	TFAM, KLF13, GMEB2, GTF2B, MYNN, RBM16
Regulation of MAP kinase activity	4	0.021534877	SPRY2, RIPK1, SPRED2, TRIB1
Stress-activated protein kinase signaling pathway	3	0.028909664	RIPK1, DUSP10, TRIB1
Cellular macromolecular complex assembly	5	0.044741009	SFRS6, TFAM, NIP7, H3F3B, MIS12
Regulation of protein kinase activity	3	0.051956371	SPRY2, RIPK1, SPRED2, SERTAD1, TRIB1
Regulation of phosphorus metabolic process	6	0.052919519	SPRY2, PPP1R2, RIPK1, SPRED2, SERTAD1, TRIB1
Negative regulation of metabolic process	3	0.061842323	SPRY2, SPRED2, TRIB1
Negative regulation of transcription, DNA-dependent	5	0.062724017	ARID5B, HIVEP1, FOSB, RBM15, NRIP1
Cellular macromolecular complex subunit organization	5	0.063242385	SFRS6, TFAM, NIP7, H3F3B, MIS12
Negative regulation of RNA metabolic process	5	0.065868521	ARID5B, HIVEP1, FOSB, RBM15, NRIP1
Regulation of transcription factor activity	3	0.069938158	RIPK1, TGFBF3, TRIB1
Transcription from RNA polymerase II promoter	4	0.075728866	KLF13, GMEB2, GTF2B, RBM16
Intracellular signaling cascade	10	0.078258448	RND3, CCL2, RIPK1, SNIP1, DUSP10, SPRED2, TGFBF3, RAPGEF2, TRIB1, NRIP1

TABLE 2. CLUSTERS OF GENES UPREGULATED FOLLOWING RESTING 22 H AFTER 2 H OF LOADING, CATEGORIZED BASED ON THEIR BIOLOGICAL PROCESSES

Term	Count	p-Value	Genes
Cell surface receptor-linked signal transduction	9	0.011827	<i>MIB1, FGF7, CCL20, NDP, PDGFC, BDKRB1, TGFBRA1, TBL1</i> ×, <i>GPR1</i>
Generation of precursor metabolites and energy	4	0.017328	<i>LEP, GBE1, STEAP1, GLRX</i>
Rhythmic process	3	0.021401	<i>LEP, FGF7, SIRT1</i>
Sex differentiation	3	0.029092	<i>LEP, FGF7, SIRT1</i>
Homeostatic process	5	0.041279	<i>FGF7, FTHL3, BDKRB1, FAS, FTH1, GLRX</i>
Iron ion transport	2	0.050242	<i>FTHL3, STEAP1, FTH1</i>
Positive regulation of cell division	2	0.066999	<i>FGF7, PDGFC</i>
Energy reserve metabolic process	2	0.073622	<i>LEP, GBE1</i>
Reproductive developmental process	3	0.078031	<i>LEP, FGF7, SIRT1</i>
Regulation of cell division	2	0.0802	<i>FGF7, PDGFC</i>

induced by 24 h of stimulation. In contrast there was no change in Akt phosphorylation (Fig. 7A).

Effect of loading on MSC morphology and viability

Microscopic analysis (Fig. 7B) showed a homogeneous monolayer of MSCs covering the scaffold surface. Almost all the cells were alive and only a few dead cells were detected in the unloaded control and following 2 h of loading. However, the number of dead cells slightly increased when cell were rested for 6 and 22 h following the 2-h of loading. As Figure 7C shows the cytoskeletal organization of the cells displayed alterations, with MSCs taking a more rounded shape when loaded for 2 h, while cells appeared more flattened with a more prominent filamentous actin network when rested for 22 h.

Discussion

The role of mechanical loading in skeletal tissue homeostasis has received considerable attention in the recent past. Osteoblasts and chondrocytes have been the main focus of many of these studies, but it is also evident that MSCs are responsive to mechanical loading. A number of studies have been conducted to study the mechanisms involved in mechanically induced MSC differentiation to osteoblasts *in vitro*. However, many of these studies have used loads that substantially exceed the physiological or even pathological equivalent loads *in vivo* and have not been able to closely mimic the bone environment.^{13,17,18,32} It is well known that surface stiffness and composition is a key regulator of MSC behavior, with stiff substrates with bone-like properties shown to support osteogenesis.^{33,34} Strain measurements in human bones physiologically indicate a strain magnitude of between 0.2% and 0.4%.²³ Biological responses to this loading have been reported following activities of more than

30 min duration and particularly following high intensity exercise of longer periods (>2 h) such as marathon running.²³ Consequently, in the present experiments we have used a CaP scaffold to mimic the biomechanical properties of bone and applied a carefully controlled load to study the mechanisms of mechanical loading in inducing bone formation using a strain and duration that may closely mimic physiological conditions and has been shown to initiate early biological responses *in vivo*.

Dramatic alterations in cell structure and shape with excess strain may bring about a large body of nonspecific changes in pathways other than those involved in differentiation or proliferation such as cell stress and inflammatory responses. In fact, previous gene expression studies of cells subjected to large mechanical loads have reported regulation of stress and inflammatory genes.^{15,35,36} In comparison, there were no signs of upregulation of inflammatory or stress-related genes in the present study, supporting the suggestion of an appropriate level of strain being utilized. Our study also showed a markedly lower total number of changes in gene expression in response to a mechanical loading. A total of 100 genes were differentially expressed after 2 h stimulation and 63 genes when cells were subsequently rested for 22 h. In contrast, others have reported between 1540,²⁰ 5449,³⁷ 400–6000³⁶ 861³⁸ differentially expressed genes in response to mechanical stimulation depending on the duration of induction in osteoblasts or MSCs. This large number of changes might cast doubt on the specificity of these effects and makes it difficult to draw conclusions on the mechanisms involved.

The limited number of changes in this study allowed us to divide the differentially expressed genes into only a few clusters associated with their biological processes. The largest cluster of genes activated with 2 h stimulation mainly belonged to regulators of transcription. Among these, *FOSB* has been shown to enhance bone formation *in vivo* and

TABLE 3. CLUSTERS OF GENES DOWNREGULATED WITH 2 H LOADING, CATEGORIZED BASED ON THEIR BIOLOGICAL PROCESSES

Term	Count	p-Value	Gene symbol
Purine nucleotide metabolic process	3	0.013276	<i>NME3, ADM, GUK1</i>
Programmed cell death	4	0.01868	<i>CDKN1B, NME3, NGFRAP1, DDIT4</i>
Regulation of apoptosis and cell death	3	0.062389	<i>CDKN1B, NME3, NGFRAP1</i>
Negative regulation of cell cycle	2	0.075135	<i>CDKN1B, GMNN</i>

TABLE 4. CLUSTERS OF GENES DOWNREGULATED FOLLOWING 22 H RESTING AFTER 2 H OF LOADING, CATEGORIZED BASED ON THEIR BIOLOGICAL PROCESSES

Term	Count	p-Value	Genes
Negative regulation of cell proliferation	3	0.052303	<i>LEPRE1, JARID2, TGFB3</i>
Nuclear import	2	0.087358	<i>TGFB3, LOC728643</i>

in vitro, and its expression is enhanced in a differentiation-related manner and in response to mechanical stimuli in mouse osteoblasts.^{39,40} These findings imply that FOSB might be a mechanoresponsive gene that activates the osteogenic program in osteoblasts in response to mechanical stress. Recent *in vivo* expression analysis of mouse osteoblasts also indicates an increase in members of the FOS family early in loading.²² Our data concur with these findings and show that responses are not limited to mouse osteoblasts but also occur in human MSC. In addition to FOSB, which has also been associated with osteoblast proliferation, a group of genes was identified that function in the regulation of the cell cycle and proliferation. These included *SERTAD-1*, *CDKN1b*, *GMNN*, *WDR37*, *WDR43*, *TP53RK*, and *SNIP-1*. The changes in expression of these genes are likely to result in regulation of

early cell cycle processes. Members of the MAPK family such as Erk1/2 are involved in cell cycle progression and also implicated as an important mechanically induced signal pathway involved in osteogenesis.^{7,41} Our data also showed upregulation of Erk1/2 phosphorylation with loading and we identified a number of possible genes (*SPRY2*, *RIPK1*, *SPRED2*, *SERTAD1*, *TRIB1*, and *RAPGEF2*) that may regulate this process. In particular, upregulation of Sprouty (*SPRY*)2 is interesting as this gene and other members of the Sprouty family have been extensively studied as negative regulators of FGF-2/MAPK signaling.^{42,43}

Surprisingly, when cells were rested for 6 h after mechanical stimulation, gene expression returned to normal, although after further resting for a total of 22 h upregulation of a totally new group of genes was seen, which were mainly

FIG. 4. Evaluation of microarray data. qRT-PCR analysis was carried out on selected differently expressed genes (FOSB, SERTAD1, CDKN1B, and CEBP δ) on RNA samples used for the microarray experiments. A comparison was made between 2 h of loading, 6 h post 2 h loading (2+6), 22 h post 2 h loading (2+22), and following 24 h of constant loading. Each time point was normalized to housekeeping gene and relative expression was calculated in relation to appropriate unloaded samples and this ratio was assigned a value of one. (mean $\pm$ SEM of three experiments of duplicates). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ when compared with unloaded cells for the same time point.

FIG. 5. Effect of mechanical stimulation on FGF-2 and SPRY-2. Cells were loaded with 0.4% strain and analyzed after 2 h of loading, following 6 or 22 h rest post 2 h loading (2+6 and 2+22) and following 24 h of constant loading. mRNA expression for FGF-2 and SPRY-2 was determined by qRT-PCR and compared with unloaded cells after normalization to RPL13a. (mean $\pm$ SEM of three experiments of duplicates; ** p < 0.01 and *** p < 0.001 vs. untreated).

involved in cell surface receptor signal transduction and regulation of metabolic and cell division processes. The absence of any significant changes at 6 h may reflect the fact that gene expression profiles are dynamic, and the data overall suggest that 2 h mechanical loading induces an initial wave of expression of immediate-early response genes, which cascade to induce a second wave of later response genes many hours after the initial responses seen. These data are consistent with the biphasic effects of prostaglandin inhibition on bone following mechanical loading previously reported by Chow and Chambers.⁴⁴ However, additional studies would be useful to clarify this and to study further the possibility of gene responses at other time points within or after the times described here.

To study whether changes in expression of transcription factors and protein kinase cascades regulators could be

translated to physiological changes, we also analyzed the expression of some key genes involved in osteogenic differentiation. As might be predicted, 2 h was not long enough to alter the expression of *Runx-2* or *osterix* transcription factors. However, when cells were left for a further 22 h a significant increase in expression of *Runx-2* was seen by qRT-PCR. Although the change in *Runx-2* expression in the microarray was not statistically significant, it followed a similar trend in 22 h rested cells in comparison to unloaded samples. In general, there was a good correlation in trend of expression between microarray and qRT-PCR data; however, there were differences in level of expression and statistical significance that could be attributed to variation in detection method, data normalization, and analysis.

The transforming growth factor β (TGF- β) signaling pathway has also been shown to regulate bone formation in

FIG. 6. Effect of mechanical stimulation on transcriptional regulators of osteogenesis. Cells were loaded with 0.4% strain and analyzed after 2 h of loading, following 6 or 22 h rest post 2 h loading (2+6 and 2+22) and following 24 h of constant loading for expression of osteogenic (*Runx-2* and *osterix*) transcription factors. Data (mean $\pm$ SEM) from three experiments in duplicates. * p < 0.05 and ** p < 0.01 when compared with unloaded at the same time point after normalization to RPL13a.

FIG. 7. Signal pathways regulated by mechanical loading in MSCs. **(A)** MSCs were serum starved for 4 h, placed between plates of the bioreactor without loading for further 2 h without serum and subsequently loaded for 15 min, 2 and 24 h with 0.4% strain. Cells were lysed in RIPA buffer and analyzed for expression of phospho-p44/42 MAPK (Erk1/2) by western blotting. NS, no stimulation. Blots were then stripped and re-probed for GAPDH. Representative blots from three separate experiments and the densitometry analysis of the ratio of phosphorylated protein to the housekeeping are shown. **(B, C)** Cells were loaded with 0.4% strain and analyzed after 2 h of loading, following 6 or 22 h rest post 2 h loading (2+6 and 2+22). Changes in cell **(B)** morphology and **(C)** viability was assessed respectively by Phalloidin-TRITC staining for actin filaments (200 $\times$) and calcein AM and ethidium homodimer-1 (EthD-1) staining for live (green) and dead (red) cells (100 $\times$).

response to mechanical loading. Members of the TGF- β family, particularly TGF- β 1 and - β 3, have been shown to be differentially expressed and are involved in various stages of bone formation in response to loading.^{22,45,46} In the current studies, TGF- β receptor 3 upregulation was seen as an early response to loading while TGF- β 3 itself was downregulated following a 22-h resting period. Members of other growth factor families (PDGFC and FGF7), chemotactic factors (CCL2 and CCL20) and members of the collagen family were among other genes previously implicated in mechanical loading gene expression studies, which were also identified as being differentially regulated in our study.^{22,38,46}

In summary, we have used an *in vitro* model based on a CaP scaffold and induced mechanical loads relevant to physiological conditions to mimic *in vivo* mechanical stimulation responses in MSCs. We have shown that the early stage of mechanical stimulation is associated with upregulation of a group of transcription factors including FOSB, a regulator of bone cell proliferation and differentiation. Other genes involved in regulation of MAPK signal transduction cascade and cell cycle progression were also induced. Later responses to loading were very distinct, revealing new changes in expression patterns of genes involved in cell surface receptor signaling and metabolic

processes and osteogenic transcription factors. These data may provide a valuable insight into the early molecular mechanisms and intracellular signal transduction pathways involved in the regulation of MSC osteogenic differentiation in response to a physiological level of mechanical stimulation. In addition, our data show for the first time the use of mechanical load in a bioreactor for induction of MSC proliferation and differentiation on porous BCP scaffolds. This could be used as a bone-like model for future study of mechanical loading or as a novel method for preparation of *ex vivo* implants to use in tissue engineering approaches for treatment of bone defects.

Acknowledgments

This work was supported by research funding from the Guy's and St Thomas Charity. The authors acknowledge financial support from the Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust. We also acknowledge support from the Wellcome/EPSCRC Centre of Excellence in Medical Engineering.

Disclosure Statement

The authors indicate no potential conflicts of interest.

References

1. Armbrecht, G., Belavý, D.L., Backström, M., Beller, G., Alexandre, C., Rizzoli, R., *et al.* Trabecular and cortical bone density and architecture in women after 60 days of bed rest using high-resolution pQCT: WISE 2005. *J Bone Miner Res* **26**, 2399, 2011.
2. Lang, T., LeBlanc, A., Evans, H., Lu, Y., Genant, H., and Yu, A. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. *J Bone Miner Res* **19**, 1006, 2004.
3. Shah, K., Armamento-Villareal, R., Parimi, N., Chode, S., Sinacore, D.R., Hilton, T.N., *et al.* Exercise training in obese older adults prevents increase in bone turnover and attenuates decrease in hip bone mineral density induced by weight loss despite decline in bone-active hormones. *J Bone Miner Res* **26**, 2851, 2011.
4. Bass, S.L., Naughton, G., Saxon, L., Iuliano-Burns, S., Daly, R., Briganti, E.M., *et al.* Exercise and calcium combined results in a greater osteogenic effect than either factor alone: a blinded randomized placebo-controlled trial in boys. *J Bone Miner Res* **22**, 458, 2007.
5. Nilsson, M., Ohlsson, C., Odén, A., Mellström, D., and Lorentzon, M. Increased physical activity is associated with enhanced development of peak bone mass in men: a five-year longitudinal study. *J Bone Miner Res* **27**, 1206, 2012.
6. Tang, L., Lin, Z., and Li, Y.-M. Effects of different magnitudes of mechanical strain on Osteoblasts *in vitro*. *Biochem Biophys Res Commun* **344**, 122, 2006.
7. Kanno, T., Takahashi, T., Tsujisawa, T., Ariyoshi, W., and Nishihara, T. Mechanical stress-mediated Runx2 activation is dependent on Ras/ERK1/2 MAPK signaling in osteoblasts. *J Cell Biochem* **101**, 1266, 2007.
8. Dumas, V., Ducharme, B., Perrier, A., Fournier, C., Guignandon, A., Thomas, M., *et al.* Extracellular matrix produced by osteoblasts cultured under low-magnitude, high-frequency stimulation is favourable to osteogenic differentiation of mesenchymal stem cells. *Calcif Tissue Int* **87**, 351, 2010.
9. Steinmetz, N.J., and Bryant, S.J. The effects of intermittent dynamic loading on chondrogenic and osteogenic differentiation of human marrow stromal cells encapsulated in RGD-modified poly(ethylene glycol) hydrogels. *Acta Biomater* **7**, 3829, 2011.
10. Long, F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* **13**, 27, 2012.
11. Karsenty, G., Kronenberg, H.M., and Settembre, C. Genetic control of bone formation. *Annu Rev Cell Dev Biol* **29**, 629, 2009.
12. Hsu, H.J., Lee, C.F., Locke, A., Vanderzyl, S.Q., and Kaunas, R. Stretch-induced stress fiber remodeling and the activations of JNK and ERK depend on mechanical strain rate, but not FAK. *PLoS One* **5**, e12470, 2010.
13. Sarraf, C.E., Otto, W.R., and Eastwood, M. *In vitro* mesenchymal stem cell differentiation after mechanical stimulation. *Cell Prolif* **44**, 99, 2011.
14. Wright, V., Attia, E., Bohnert, K., Brown, H., Bhargava, M., and Hannafin, J.A. Activation of MKK3/6, SAPK, and ATF-2/c-jun in ACL fibroblasts grown in 3 dimension collagen gels in response to application of cyclic strain. *J Orthop Res* **29**, 397, 2011.
15. Sanchez, C., Gabay, O., Salvat, C., Henrotin, Y.E., and Benbaum, F. Mechanical loading highly increases IL-6 production and decreases OPG expression by osteoblasts. *Osteoarthritis Cartilage* **17**, 473, 2009.
16. Rath, B., Nam, J., Knobloch, T.J., Lannutti, J.J., and Agarwal, S. Compressive forces induce osteogenic gene expression in calvarial osteoblasts. *J Biomech* **41**, 1095, 2008.
17. Sittichokechaiwut, A., Edwards, J.H., Scutt, A.M., and Reilly, G.C. Short bouts of mechanical loading are as effective as dexamethasone at inducing matrix production by human bone marrow mesenchymal stem cell. *Eur Cell Mater* **20**, 45, 2010.
18. Sumanasinghe, R.D., Bernacki, S.H., and Lobo, E.G. Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression. *Tissue Eng* **12**, 3459, 2006.
19. Song, G., Ju, Y., Shen, X., Luo, Q., Shi, Y., and Qin, J. Mechanical stretch promotes proliferation of rat bone marrow mesenchymal stem cells. *Colloids Surf B Biointerfaces* **58**, 271, 2007.
20. Emani, S., Mayer, J.E., Jr., and Emani, S.M. Gene regulation of extracellular matrix remodeling in human bone marrow stem cell-seeded tissue-engineered grafts. *Tissue Eng Part A* **17**, 2379, 2011.
21. Luu, Y.K., Capilla, E., Rosen, C.J., Gilsanz, V., Pessin, J.E., Judex, S., *et al.* Mechanical stimulation of mesenchymal stem cell proliferation and differentiation promotes osteogenesis while preventing dietary-induced obesity. *J Bone Miner Res* **24**, 50, 2009.
22. Mantila Roosa, S.M., Liu, Y., and Turner, C.H. Gene expression patterns in bone following mechanical loading. *J Bone Miner Res* **26**, 100, 2011.
23. Al Nazer, R., Lanovaz, J., Kawalilak, C., Johnston, J.D., and Kontulainen, S. Direct *in vivo* strain measurements in human bone—a systematic literature review. *J Biomech* **45**, 27, 2012.
24. Gharibi, B., and Hughes, F.J. Effects of medium supplements on proliferation, differentiation potential, and *in vitro* expansion of mesenchymal stem cells. *Stem Cells Transl Med* **1**, 771, 2012.
25. Timoshenko, S.P., and Goodier, J.N. *Theory of Elasticity*, 3rd edition. Singapore: McGraw-Hill, pp. 166–167, 1982.
26. Zeng, Y., Qiao, Y., Zhang, Y., Liu, X., Wang, Y., and Hu, J. Effects of fluid shear stress on apoptosis of cultured human umbilical vein endothelial cells induced by LPS. *Cell Biol Int* **29**, 932, 2005.
27. Kamel, M.A., Picconi, J.L., Lara-Castillo, N., and Johnson, M.L. Activation of β -catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE2: implications for the study of mechanosensation in bone. *Bone* **47**, 872, 2010.
28. Teichmann, J., Morgenstern, A., Seebach, J., Schnittler H-J, Werner, C., and Pompe, T. The control of endothelial cell adhesion and migration by shear stress and matrix-substrate anchorage. *Biomaterials* **33**, 1959, 2012.
29. Curtis, K., Gomez, L.A., Rios, C., Garbayo, E., Raval, A.P., Perez-Pinzon, M.A., *et al.* EF1 α and RPL13a represent normalization genes suitable for RT-qPCR analysis of bone marrow derived mesenchymal stem cells. *BMC Mol Biol* **11**, 61, 2010.
30. Gharibi, B., Ghuman, M.S., and Hughes, F.J. Akt and Erk-mediated regulation of proliferation and differentiation during PDGFR β -induced MSC self-renewal. *J Cell Mol Med* **16**, 2789, 2012.

31. Cama, G., Gharibi, B., Sait, M.S., Knowles, J.C., Lagazzo, A., Romeed, S., *et al.* A novel method of forming micro- and macroporous monetite cements. *J Mater Chem B* **1**, 958, 2013.
32. Yanagisawa, M., Suzuki, N., Mitsui, N., Koyama, Y., Otsuka, K., and Shimizu, N. Effects of compressive force on the differentiation of pluripotent mesenchymal cells. *Life Sci* **81**, 405, 2007.
33. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677, 2006.
34. Winer, J.P., Janmey, P.A., McCormick, M.E., and Funaki, M. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A* **15**, 147, 2009.
35. Nam, J., Aguda, B.D., Rath, B., and Agarwal, S. Biomechanical thresholds regulate inflammation through the nf-kb pathway: experiments and modeling. *PLoS One* **4**, e5262, 2009.
36. McKenzie, J.A., Bixby, E.C., and Silva, M.J. Differential gene expression from microarray analysis distinguishes woven and lamellar bone formation in the rat Ulna following mechanical loading. *PLoS One* **6**, e29328, 2011.
37. Huang, A.H., Farrell, M.J., Kim, M., and Mauck, R.L. Long-term dynamic loading improves the mechanical properties of chondrogenic mesenchymal stem cell-laden hydrogel. *Eur Cell Mater* **19**, 72, 2010.
38. Haudenschild, A., Hsieh, A., Kapila, S., and Lotz, J. Pressure and distortion regulate human mesenchymal stem cell gene expression. *Ann Biomed Eng* **37**, 492, 2009.
39. Inoue, D., Kido, S., and Matsumoto, T. Transcriptional induction of FosB/ Δ FosB gene by mechanical stress in osteoblasts. *J Biol Chem* **279**, 49795, 2004.
40. Sabatakis, G., Sims, N.A., Chen, J., Aoki, K., Kelz, M.B., Amling, M., *et al.* Overexpression of [Delta]FosB transcription factor(s) increases bone formation and inhibits adipogenesis. *Nat Med* **6**, 985, 2000.
41. Boutros, T., Chevet, E., and Metrakos, P. Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol Rev* **60**, 261, 2008.
42. Hanafusa, H., Torii, S., Yasunaga, T., and Nishida, E. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nat Cell Biol* **4**, 850, 2002.
43. Sivak, J.M., Petersen, L.F., and Amaya, E. FGF Signal interpretation is directed by sprouty and spread proteins during mesoderm formation. *Dev Cell* **8**, 689, 2005.
44. Chow, J.W., and Chambers, T.J. Indomethacin has distinct early and late actions on bone formation induced by mechanical stimulation. *Am J Physiol* **267**, E287, 1994.
45. Sakai, K., Mohtai, M., and Iwamoto, Y. Fluid shear stress increases transforming growth factor beta 1 expression in human osteoblast-like cells: modulation by cation channel blockades. *Calcif Tissue Int* **63**, 515, 1998.
46. Xing, W., Baylink, D., Kesavan, C., Hu, Y., Kapoor, S., Chadwick, R.B., *et al.* Global gene expression analysis in the bones reveals involvement of several novel genes and pathways in mediating an anabolic response of mechanical loading in mice. *J Cell Biochem* **96**, 1049, 2005.

Address correspondence to:
 Francis John Hughes, BDS, PhD
 Department of Periodontology
 Dental Institute
 Kings College London
 Floor 21 Tower Wing
 Guy's Hospital
 Great Maze Pond
 London SE1 9RT
 United Kingdom

E-mail: francis.hughes@kcl.ac.uk

Received: October 19, 2012

Accepted: May 13, 2013

Online Publication Date: August 22, 2013