SHORT BOUTS OF MECHANICAL LOADING ARE AS EFFECTIVE AS DEXAMETHASONE AT INDUCING MATRIX PRODUCTION BY HUMAN BONE MARROW MESENCHYMAL STEM CELLS

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Abstract

Dexamethasone (Dex) is used widely to induce differentiation in human mesenchymal stem cells (hMSCs); however, using a pharmaceutical agent to stimulate hMSC differentiation is not the best choice for engineered tissue transplantation due to potential side-effects. The goal of the present study was to investigate the effects of dynamic compressive loading on differentiation and mineralized matrix production of hMSCs in 3D polyurethane scaffolds, using a loading regimen previously shown to stimulate mineralised matrix production of mature bone cells (MLO-A5). hMSCs were seeded in polyurethane scaffolds and cultured in standard culture media with or without Dex. Cell-seeded scaffolds were compressed at 5% global strain for 2 h on day 9 and then every 5 days in a media-filled sterile chamber. Samples were tested for mRNA expression of alkaline phosphatase (ALP), osteopontin (OPN), collagen type 1 (col 1) and runt-related transcription factor-2 (RUNX-212 h) after the first loading, cell viability by MTS assay and alkaline phosphatase activity at day 12 of culture and cell viability, collagen content by Sirius red and calcium content by alizarin red at day 24 of culture. Neither Dex nor loading had significant effects on cell viability. Collagen content was significantly higher ($p<0.01$) in the loaded group compared with the non-loaded group in all conditions. There was no difference in ALP activity or the amount of collagen and calcium produced between the non-loaded group supplemented with Dex and the loaded group without Dex. We conclude that dynamic loading has the ability to stimulate osteogenic differentiation of hMSC in the absence of glucocorticoids.

Keywords: Bone, repair/regeneration, bone - mineralization, bioreactors, stem cells, osteogenesis, collagen, biomaterials – scaffolds.

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Introduction

Tissue engineering, in which a patient's undifferentiated cells or stem cells are seeded into a biocompatible scaffold in controllable environments *in vitro* is the subject of much recent research focus. It is hoped that such techniques will allow the regeneration or replacement of ageing tissues without the need for human organs. To engineer a bone tissue replacement, cells need to be grown in a 3-D scaffold and provided with sufficient nutrients and stimuli. Bioreactors for cell culture that provide mechanical and chemical stimuli can be used to induce growth and differentiation of cells, and may enhance extracellular matrix deposition and mineralization of the tissue constructs (Cowie *et al.*, 2006; Huang *et al.*, 2004; Kreke *et al.*, 2008; Mauney *et al.*, 2004b; Meinel *et al.*, 2004; Simmons *et al.*, 2003; Sumanasinghe *et al.*, 2006).

Stem cells are an attractive source of cells for use in tissue engineering and regenerative medicine. They differ from progenitor cells in their theoretically limitless capacity for self-renewal and multilineage differentiation, whereas progenitor cells' self renewal capability is comparatively reduced (Weissman, 2000). In theory, this capacity for self renewal could provide an unlimited source of donor materials for transplantation (Heng *et al.*, 2004). Stem cells can be derived from the inner cell mass of an embryo blastocyst (embryonic stem cells (ESCs)) or from adult tissues (adult stem cells (ASCs)), such as bone marrow-derived mesenchymal stem cells (MSCs) (Heath, 2000). However, there is some controversy as to whether 'MSCs' are true stem cells or skeletal stem cells, able to regenerate only skeletal tissues such as bone, cartilage and marrow fat cells and having only limited self-renewal capacity (Bianco *et al.*, 2006; Robey and Bianco, 2006). Because of the doubts regarding the stem cell status of MSC the term 'multipotent mesenchymal stromal cells' has been suggested however for the purpose of this manuscript the term MSC will be retained (Dominici *et al.*, 2006). MSCs are easier to obtain and proliferate more rapidly than fully differentiated osteoblasts. Bone tissue engineering may require implantation of pre-differentiated osteogenic progenitor cells or osteoblasts, rather than undifferentiated stem cells, in order to prevent non-specific tissue differentiation of stem cells and accelerate tissue integration (Yoshikawa *et al.*, 1996).

To direct MSCs along the osteogenic lineage, non-protein-based chemical compounds such as Dexamethasone (Dex), a synthetic glucocorticoid, are widely used because they are cost-effective to produce

(Lecoeur and Ouhayoun, 1997; Porter *et al.*, 2003; Yoshikawa *et al.*, 1996). Dex has been shown to act at both early and late stages of osteogenic differentiation to accelerate osteoblastic maturation by mechanisms which are still unclear (Porter *et al.*, 2003), as well to stimulate adipogenesis and chondrogenesis *in vitro* depending on which co-factors are added to the media (Osyczka *et al*., 2002). In continuous treatment with Dex, MSCs have been shown to upregulate expression of osteocalcin and bone sialoprotein (Aubin, 1999), alkaline phosphatase activity (Peter *et al.*, 1998) and matrix mineralization (Dieudonne *et al.*, 1999; Maniatopoulos *et al.*, 1988). However, Dex has also been shown to downregulate expression of collagen type I and enhance maturation of adipocytes in culture (Beresford *et al.*, 1992) and glucocorticoids induce apoptosis of osteoblasts (Weinstein *et al*., 1998)

Mechanical forces have been shown previously *in vivo* to play an important role in bone formation by inducing osteoprogenitor cells of the marrow stroma to differentiate into osteoblasts (Holtorf *et al.*, 2005; Turner *et al.*, 1998). They are also well demonstrated to regulate bone growth *in vivo* (Klein-Nulend *et al.*, 2005). However, the effects of mechanical loading on differentiation of human MSC are not well understood due to the variety of mechanical stimuli and loading systems used. Previously, we demonstrated that mature osteoblasts from a mouse cell line respond to mechanical loading in an *in vitro* 3-D environment by increasing bone matrix production and upregulating matrix protein gene expression (Sittichockechaiwut *et al*., 2009). We predicted that the loading system and protocol used in that study would also have the potential to induce osteogenic differentiation and bone matrix production by hMSCs. Other studies using fluid flow as a stimulus have shown that mechanical stimuli can have a more potent effect than Dex on early osteogenic differentiation (Yourek *et al.*, 2010). The hypothesis in the present study is that short bouts of dynamic compressive loading, will have the ability to stimulate osteogenic differentiation of hMSC as a potential alternative to treatment with Dex, thereby avoiding the potential negative effects of Dex on long-term osteoblast differentiation.

Materials and Methods

Human mesenchymal stem cell preparation

Frozen human mononuclear cells from bone marrow aspirates were obtained from 5 donors (Table 1) from registered companies (StemCell Technologies Inc., Basel, Switzerland, denoted STEMCELL or Lonza Biologics, Cambridge, UK, denoted LONZA). Mononuclear cells were plated in T25 flasks with a minimum of 10^5 per flask. α-MEM media (Invitrogen, Paisley, UK) supplemented with 10% foetal calf serum (FCS), Penicillin/Streptomycin (100 μg/ml, respectively, Fungizone (25 μg/ml) and L-glutamine 100 μg/ml (all supplements from Sigma Aldrich, Dorset, UK). After the cells had adhered (7-10 days) the non-adherent hematopoietic cells were washed away leaving fibroblast-like cells with a spindle-like morphology. When the cells had reached 80% confluence, they were subcultured at a density of $5x10^5$ cells per scaffold. Cells were used at passage 2 for all experiments in this study.

Scaffold preparation

The polymer scaffolds used in this study were based on polyether polyurethane (PU) provided commercially by Caligen Foam Ltd, Lancashire, UK, foam reference: XE1700V (kindly donated by Professor Anthony J. Ryan, Department of Chemistry University of Sheffield, UK). The pore size of the foam varies between 150-1000 μm (mean 400 μm). The strut width varies from 43-96 μm (mean 65 μm). The Young's modulus of elasticity tested by a single cycle of loading to 50% strain at 0.2 mm/sec using mechanical testing machine ELF3200 (BOSE, USA) was 2.87±0.02 KPa (Sittichokechaiwut and Reilly, 2009). All samples were cut into cylinders with diameters of 10 mm and heights of 5 mm and were subsequently sterilized using 70% ethanol. Prior to cell seeding, the scaffolds were washed with phosphate buffered saline (PBS) and were then immersed in culture media for 10 minutes. The scaffolds were removed from the media, gently squeezed to remove excess media and placed in 1 cm internal diameter medical grade stainless steel rings (produced at the School of Medicine, University of Sheffield) which support the scaffolds while initial cell attachment occurs.

Cell seeding and culture in 3-D scaffolds

5×10^5 cells in 50 μl of media were seeded onto the top of each sterile scaffold in the steel ring. The scaffolds were then incubated for 1 h to allow the cells to attach after which sufficient basal culture media was added to the cultures to cover the scaffolds. After incubation overnight the cell-seeded scaffolds were removed from the ring and medical grade stainless steel wire holders made from LEOWIRE® (0.8 mm in diameter, Leone Orthodontic and Implantology, Florence, Italy) were placed over them (Fig. 1) to ensure the scaffolds were kept fully immersed in the media, fresh media was added to cover the scaffolds. 50 μg/ml Ascorbic acid-2-phosphate supplement was added on day 1 and 5mM βGP was added on day 4 to all samples, 10nM Dex was added on day 4 to selected samples to modulate MSCs differentiation. The cell-seeded scaffolds were cultured in the incubator for the experimental period and were supplied with fresh media and additional supplements every 3 days.

Dynamic cyclic compressive stimulation

Table 1. Information on the human bone marrow mononuclear cells obtained from five different donors.

Donor	Company	Age	Gender
A	STEMCELL	20	Female
B	LONZA	44	Male
C	STEMCELL	23	Male
D	STEMCELL	28	Male
E	STEMCELL	23	Male

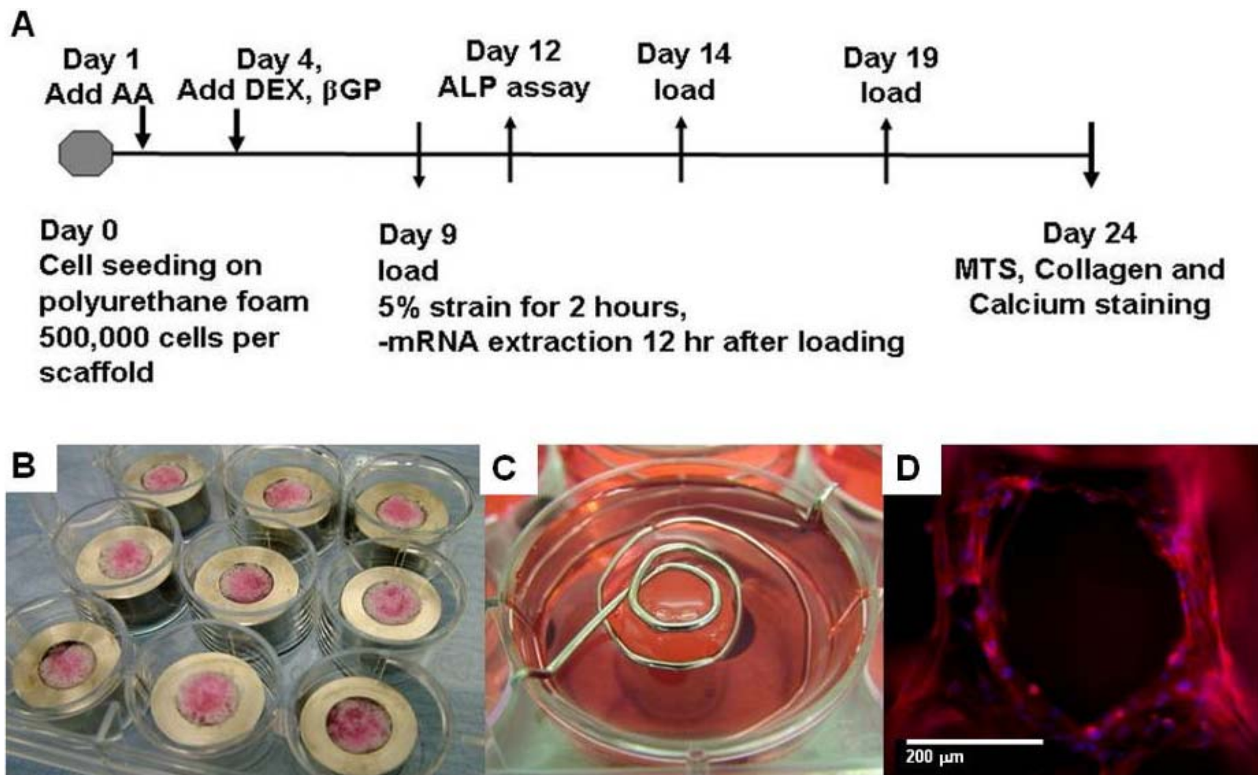

Fig. 1. (**A**) Summary of experimental timeline and conditions. Cells were seeded in a small volume of media into PU scaffolds contained in steel rings in 12 well plates (**B**) and topped up with media after 1 h. On day 1 of culture the rings were removed and scaffolds moved to 6 well plates where they were held immersed in media by steel wire (**C**). (**D**) Fluorescent micrograph of human MSCs (the cells are hES-MP™ 002.5 from Cellartis (Gothenburg, Sweden) not from the experiments described) attached to the struts of a polyurethane scaffold 24 h after seeding, stained with TRITC phalloidin (actin cytoskeleton) and DAPI (nucleus) the scaffold displays red autofluorescence.

Cyclic compression was performed in a BioDynamic™ chamber mounted on a ELF3200 mechanical testing machine (BOSE, Eden Prairie, MN, USA). The biodynamic chamber and all circuit components were sterilized by autoclave. Under the laminar flow hood, the sample to be loaded was removed from the well plate and placed into the chamber, between two compressive platens. The chamber was filled with 200 ml of loading media and then was mounted onto the mechanical testing machine. The cell-seeded scaffolds were dynamically loaded in compression using a sine wave at 1Hz, 5% strain on day 9 and then every 5 days up to and including day 19. The regimen was based on that which previously upregulated mineralised matrix in mature osteoblasts (Sittichockechaiwut *et al.*, 2009), but with a longer pre-loading culture period. We previously showed that neither 0.5 or 1 h of loading upregulated collagenous matrix in mature osteoblasts and that 2.5% strain caused less matrix upregulation while 10% reduced cell number (Sittichokechaiwut and Reilly, 2009). The experiment was repeated 3 times (N=2 per individual experiment). 6 samples from 3 donors (donor A, B and D; detail of donors shown in Table1) were tested for cell viability by MTS assay, collagen by Sirius red and calcium by alizarin red at day 24 of culture. In separate experiments 6 samples from 3 donors were used to assay the gene expression of matrix protein type I collagen (Col1), osteopontin (OPN), alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx-2), 12 h after a single bout of 2 h of loading on day 9 (donor C, D and E). ALP activity was measured on day 12, 3 days after the first bout of loading (donor C, D and E). The experimental timeline and conditions are summarised in Fig. 1. The loading force and displacement data were automatically recorded by WinTest software (BOSE). During loading, a paired-non-loaded sample was kept in a sterile media-filled T75 flask in the same conditions as the loaded sample, with the exception of mechanical stimulation, at room temperature. Between loading cycles, both loaded and non-loaded control samples were cultured in an incubator under standard conditions.

MTS assay for cell viability

MTS assay is a colourimetric method to determine the number of viable cells in culture (Ng *et al.*, 2005). The yellow MTS tetrazolium compound is reduced by live cells into a pink formazan product that is soluble in culture medium. The cell-seeded scaffolds were washed with PBS until there was no colour in the solution and were then placed in the 10 mm diameter stainless steel rings. Assays were performed by adding 0.5 ml of 1:10 MTS (Promega, Southampton, UK) in PBS, directly to the scaffolds and to an empty scaffold for the blank control, incubating for 3 h at 37ºC. The solution was removed and its absorbance read at 490nm with a 96-well plate reader. The quantity of formazan product as measured by the plate reader is

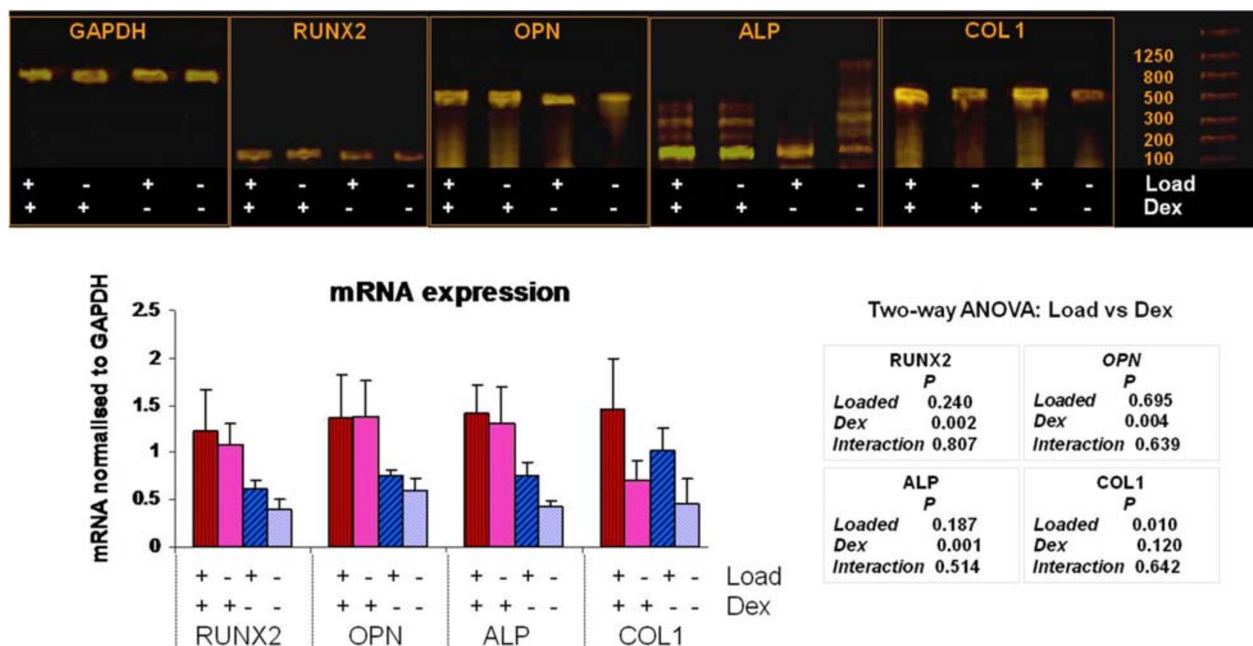

Fig. 2. mRNA expression of RUNX2, OPN, ALP and COL1 12 h after a single bout of 2 h of loading. Top: an example gel of 3 experiments, bottom: mean ± standard deviation (S.D.) for band density for the mRNA of interest normalised to GAPDH within the same experiment. Loading had a statistically significant effect on COL1 expression but Dex had no significant effect whereas RUNX2, OPN and ALP were upregulated significantly by Dex treatment compared to no-Dex treatment. ($p<0.05$, Two-way ANOVA, N=3)

directly proportional to the number of metabolically active cells that were present in the culture.

Calcium and collagen staining

After the MTS assay, scaffolds were washed with PBS, then fixed with 10% formalin for 10 min at room temperature. The solution was removed and scaffolds were washed with PBS, cut into 5-6 pieces and all pieces from a single scaffold placed in a well of a six-well plate. Alizarin Red (Sigma Aldrich, Dorset, UK), a dye that combines with calcium to form a bright red colour (Gregory *et al.*, 2004), was dissolved in distilled water 1 mg/ml, adjusted to pH 5.5 with ammonium hydroxide and added to each well, samples were placed under mild shaking for 30 min at room temperature. The dye was then removed and samples were washed with distilled water. The cultures were observed qualitatively under light microscopy. For quantitative analysis, the samples in each well were destained with 5% perchloric acid, under mild shaking for 15 minutes. Optical density was then measured at 490 nm using a 96-well plate reader. After alizarin Red destaining, all samples were washed with distilled water and air-dried. 1 mg/ml of Sirius red dye (Sigma Aldrich), a strong anionic dye used for measuring collagen (Tullberg-Reinert and Jundt, 1999), in saturated picric acid solution was added to each well and placed under mild shaking for 18 h. The dye solution was removed and washed with distilled water to remove unbound dye. The bound dye was observed qualitatively under light microscopy. For quantitative analysis, the scaffolds in each well were destained with 0.2 M NaOH/methanol, in a 1:1 ratio, under mild shaking for 15 minutes. Optical density was then measured at 490 nm.

Messenger ribonucleic acid (mRNA) isolation and reverse transcriptase polymerase chain reaction (RT-PCR)

12 h after loading, cellular mRNA was extracted using a Dynabeads® mRNA DIRECT™ kit (Invitrogen) according to the manufacturer's instructions. The isolated mRNA was reversely transcribed and amplified using the JumpStart™ RED HT RT-PCR kit (Sigma Aldrich) using primer sequences (MWG Biotech, London, UK) as in Table 2. The conditions were RT – 50°C for 30 min, RT inactivation – 94°C for 3 min, denaturation – 94°C for 15 s, annealing – 58.5°C for 30 s, extension – 72°C for 1 min, final extension – 72°C for 10 min for 28 amplification cycles, for all primer sets. RT-PCR products were visualized on a FlashGel™ system (Lonza, Berkshire, UK) and the relative band density of the RT-PCR products was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Alkaline phosphatase (ALP) activity

Total ALP was measured spectrophotometrically with p-nitrophenol phosphate as a substrate and normalized to relative cell number by MTS assay. Cell assay buffer was made up by mixing 1.5 M Tris (pH 9.0), 1 mM $ZnCl_2$ and 1mM Mg Cl_2 in ddH_2O. Samples were washed with PBS and then digestion buffer (1:10 of cell assay buffer in ddH_2O and 1% Triton X-100) was added to the samples. The scaffold samples were squeezed for 1 min, then the extracted solution was transferred to a 1.5 ml microcentrifuge tube and incubated for 30 min at 37°C and then overnight at 4°C. 10 µl of extracted solution was mixed with 190 µl of ALP solution containing 37.1 mg p-nitrophenol phosphate (Sigma Aldrich) in 20 ml of cell

assay buffer. The mixture was transferred into a well of a 96 well plate and incubated for 10 min at room temperature. The plate was read at a wavelength of 410 nm at 1 min and 5 min, ALP levels were expressed as nmol of p-nitrophenol.

Statistical analyses

Statistical analyses were performed using Minitab™ software. Two-way ANOVA was used to test differences between multiple treatments followed by a Tukey's post-hoc pair-wise comparison or a Student's paired *t*-test for non paired groups (Dex *vs.* no-Dex treatment) and paired groups (loaded *vs.* non-loaded) respectively. Differences were considered statistically significant if the *p*-value was less than 0.05.

Results

mRNA expression

Dex treatment significantly upregulated ALP, Runx-2 and OPN mRNA expression (Fig 2). In contrast, loading alone had no significant effect despite small apparent effects of loading in the no-Dex groups (Fig. 2). Interestingly, Col1 mRNA showed a different pattern of expression with loading having a significant effect on mRNA production but Dex having no significant effect either by two-way ANOVA or pair-wise comparisons within the treatment groups. Col1 mRNA in the loaded group was significantly higher than in the non-loaded group with no-Dex treatment, by paired *t*-test, although this was not the case within the Dex treated groups.

Alkaline phosphatase activity

The relative number of metabolically active cells in each scaffold was analysed by MTS assay prior to alkaline phosphatase assay and there were no differences between treatment groups (Fig. 3a). There was little difference in ALP activity between the 3 donors assayed (Fig. 3b) and all followed the same pattern with respect to the treatment conditions so were pooled for statistical analysis (Fig. 3c). As would be expected the enzyme activity of ALP measured on day 12 (3 days after the first bout of loading)

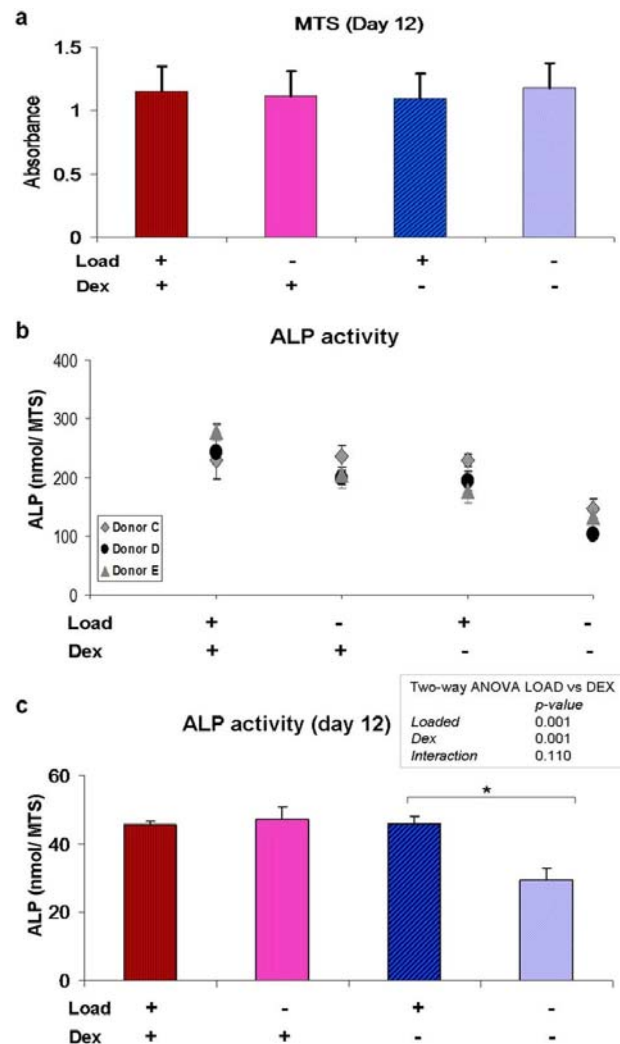

Fig. 3. Cell viability (**A**), ALP activity per donor (**B**) and ALP activity per viable cell (**C**) at day 12 of culture (3 days after first bout of loading), mean ± S.D. Samples subjected to mechanical loading, treated with Dex or both exhibited significantly higher ALP activity compared with static non-loaded controls in basal medium with no-Dex ($p<0.05$, Two-way ANOVA, N=6, 2 samples per donor). The effect of loading was only apparent in the no-Dex treated group (*$p<0.05$, Tukey's post-hoc pair-wise comparison).

Table 2. Human primer sequences for RT-PCR.

mRNA	Base pairs	Primer sequences	
GAPDH	702	Forward	5'-GGG CTG CTT TTA ACT CTG GT-3'
		Reverse	5'-TGG CAG GTT TTT CTA GAC GG -3'
Osteopontin	416	Forward	5'-AGC CAG GAC TCC ATT GAC TCG AAC-3'
		Reverse	5'-GTT TCA GCA CTC TGG TCA TCC AGC-3'
RUNX2	125	Forward	5'-AGA TGA TGA CAC TGC CAC CTC TG-3'
		Reverse	5'-GGG ATG AAA TGC TTG GGA ACT GC-3'
Alkaline phosphatase	162	Forward	5'-ACC ATT CCC ACG TCT TCA CAT TTG-3'
		Reverse	5'-AGA CAT TCT CTC GTT CAC CGC C-3'
Type I collagen	461	Forward	5'-GGA CAC AAT GGA TTG CAA GG-3'
		Reverse	5'-TAA CCA CTG CTC CAC TCT GG-3'

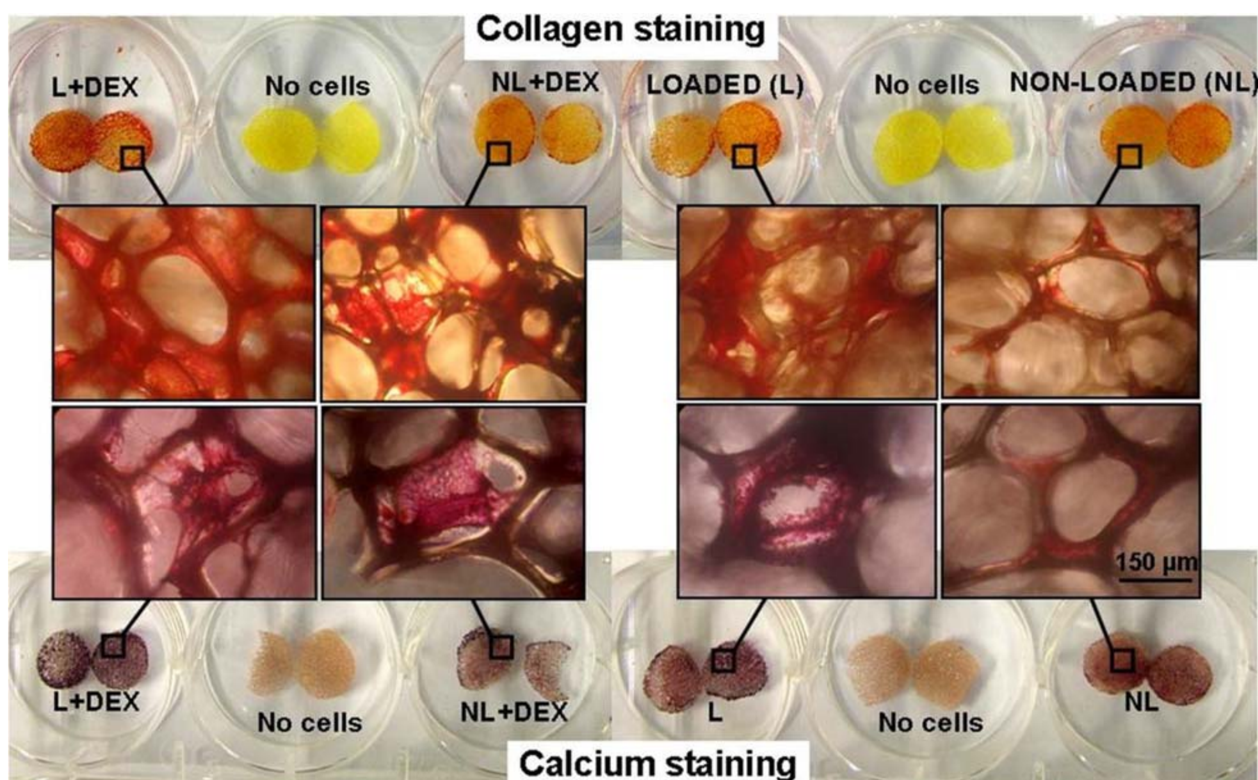

Fig. 4. Sirius red (collagen) and alizarin red (calcium) staining of samples. Images show representative stained scaffolds (one scaffold per well, cut into cross sections) and light micrographs of random areas of scaffolds. The amount of collagen in scaffolds appeared higher in loaded samples supplemented with Dex compared to other groups.

followed similar trends to the mRNA expressed on day 9. Samples subjected to mechanical loading, treated with Dex or Dex and loading exhibited significantly higher ALP activity compared with static non-loaded controls in basal medium with no-Dex. The effect of loading was significant only in the no-Dex treated group.

Cell viability, collagen and calcium production

Staining of scaffolds after 24 days of culture showed that although there was some extra deposition of matrix on the outside of the scaffold, as is commonly seen in 3D culture of MSCs, collagen and calcium were present throughout the scaffold in each section (Fig. 4). Examination of the scaffolds by light microscopy showed qualitative differences in both Sirius red staining for collagen and alizarin red staining for calcium. Prior to staining the relative number of metabolically active cells was analysed by MTS (Fig. 5a) and as for the day 12 assay on a different group of cells (Fig. 3a) there were no significant effects of the treatments on relative viable cell number.

Samples were analysed by individual donor and there were differences between different donors (Figs. 5a-c) as has been previously shown in the alkaline phosphatase responses of human MSCs to BMP-2 (Diefenderfer *et al.*, 2003) and bioactive glass (Reilly *et al.*, 2007). In particular cells from donor A had fewer metabolically active cells at day 24 and a very low calcium content, below the limit at which the plate reader measures absorbance linearly. However, all donors showed similar relative differences between treatment groups. Data were normalised to an internal control for statistical analyses and the results for the donor cells with low calcium values were not included in the calcium analyses.

Collagen per viable cell was higher in all treatment groups with loading (2 fold), Dex (1.5 fold) or both treatments (2.5 fold) compared to non-loaded without Dex (Fig. 5d). A two-way ANOVA confirmed that both the effect of Dex and the effect of loading were statistically significant and there was no interaction between them, suggesting that the two effects are independent. Paired *t*-tests indicated that collagen content was significantly higher in the loaded group compared with the non-loaded group regardless of whether Dex was added to the media. Similarly to Col 1 mRNA there was no significant effect of Dex treatment alone within the non-loaded group. Calcium content per cell followed the same trend as collagen content with the effect of loading being statistically significant although in this case the overall effect of Dex was not statistically significant. Within the no-Dex treated groups, loading caused a 2 fold increase in calcium deposition, having an effect as large as that seen with Dex and loading combined.

Discussion

Scaffold materials

In the present study we used an inert polyurethane foam as a scaffold and demonstrated that it supports bone marrow

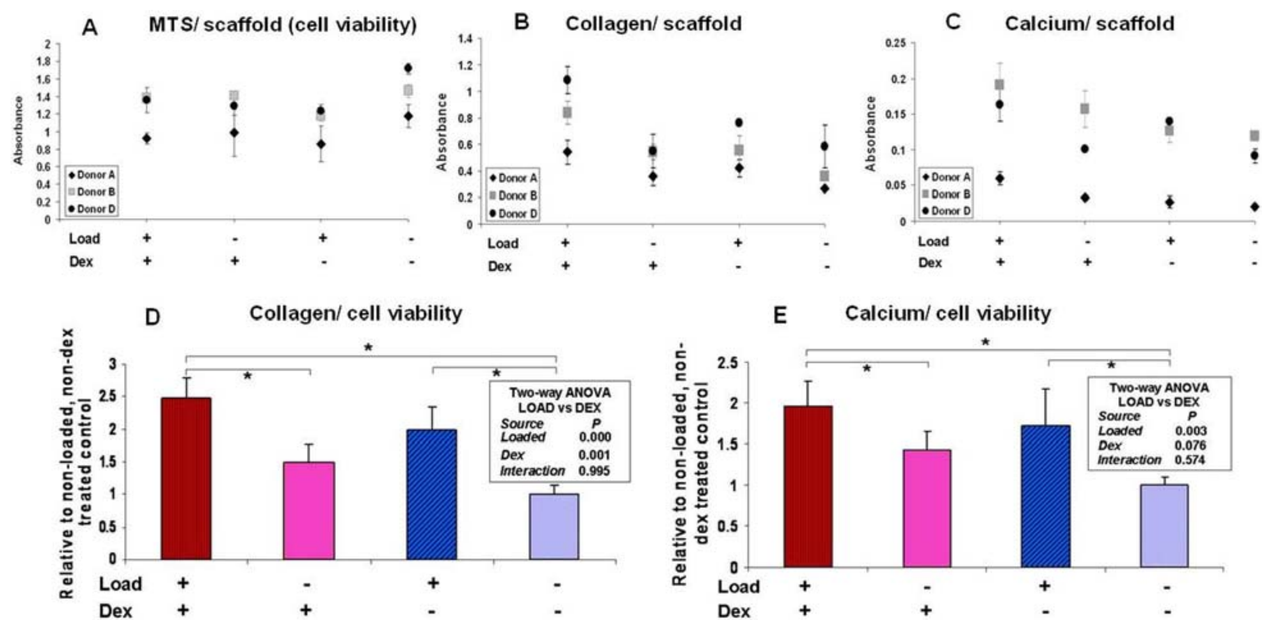

Fig. 5. Cell viability (**A**), total collagen content (**B**), total calcium content (**C**), collagen per viable cell (**D**) and calcium per viable cell (**E**) at day 24 of culture, mean ± S.D. All donors had similar relative differences between treatment groups. Collagen and calcium per viable cell were higher in all treatment groups (*p<0.05, Tukey's post-hoc pair-wise comparison or paired *t*-test, N=6). Dex had an overall significant effect on collagen content by two-way ANOVA but no statically significant effect within treatment groups.

derived MSC differentiation along the osteogenic lineage in both chemical and mechanical induction of MSCs. Using a loading regimen that had previous been shown to induce increases in calcium and collagen production by mature bone cells we demonstrated that both early markers of MSC differentiation such as ALP activity and late markers such as collagen and calcium production, can be induced by short bouts of mechanical loading, to levels as high as those seen with Dex treatment in static conditions.

PU scaffolds were chosen for use in the present study because of their elasticity, resilience and stiffness which allows them to withstand repeated *in vitro* mechanical loading. Because all scaffolds were cut from a single industrially processed block they were highly reproducible and cost-effective. It has also been reported that PU foams can support human MSC adhesion, proliferation and osteogenic differentiation *in vitro,* in static culture (Zanetta *et al.*, 2009) and that small fragments of PU foam incorporated into a fibrin gel are a good carrier for chondrocytes (Mauth *et al.*, 2009). The PU foam used in this study is suitable for *in vitro* use only, however there is much on-going work in the development of degradable PU foams for clinical tissue engineering purposes (Guelcher *et al.*, 2006).

Osteogenic differentiation of MSCs

Previous studies have demonstrated that pre-induced osseointegration of MSC-seeded scaffolds, once implanted *in vivo,* accelerates bone defect repair by delivering a more mature osteogenic population capable of immediate bone formation (Bruder *et al.* 1998; Schliephake *et al.*, 2001; Yoshikawa *et al*., 1996). Osteogenic differentiation of MSCs can be induced by protein-based cytokines and growth factors such as BMP (Canalis *et al.*, 2003; Lecoeur and Ouhayoun, 1997) and Interleukin-6 (Taguchi *et al.* 1998), as well as chemical compounds such as dexamethasone (Dex) (Lecoeur and Ouhayoun, 1997) and Vitamin D (Calcitriol) (van Leeuwen *et al.*, 2001). For this study Dex was chosen because it is the most commonly used chemical agent to induce hMSCs differentiation *in vitro*. Ascorbic acid (Asc) and Beta glycerolphosphate (βGP) were added in all conditions so we could investigate only the effect of Dex and not the overall effect of 'osteogenic media', which is the term usually applied to media containing those three supplements.

In this system, the expression of typical markers of osteogenesis such as Runx-2, OPN and ALP mRNA were upregulated in MSCs on day 9 of culture when subjected to Dex from day 4. In addition ALP enzyme activity was elevated at day 13 and collagen and calcium production at day 24. However, while the effects of Dex on mRNA expression for 'osteogenic' genes was strong, with the exception of Col 1 the effect on the long-term outcomes of matrix production were smaller and not statistically significant. This is interesting in the light of the work of Beresford *et al.* (1992) which showed that Dex can inhibit expression of collagen type I and enhance maturation of adipocytes in culture.

Mechanical stimulation of MSCs

It has been well demonstrated that osteoblasts respond to mechanical forces with an upregulation of many signalling factors involved in bone formation and regulation of matrix proteins (Rubin *et al.*, 2006). Mechanical loading has been shown previously to play an important role in bone formation *in vivo* by inducing osteoprogenitor cells in the marrow stroma to differentiate into osteoblasts at the cortical bone surface (Turner *et al.*, 1998). These and

Table 3. Example of previous studies of osteogenic responses of MSCs to mechanical stimulation in 3-D environments.

Reference	Type of stimulation	Loading procedure	Culture period	Outcome (loaded samples compared with non loaded
Cartmell *et al.* (2003)	Continuous flow	Flow induced shear stress on cell-seeded human trabecular bone scaffold.	7 days	Upregulation of RUNX2, OCN, ALP mRNA expression
Grayson *et al.* (2008)		Flow induced shear stress on cell-seeded decellularized bone matrix.	35 days	Greater bone volume by µCT. Increasing of total protein, ALP, BSP and OPN
Datta *et al.* (2005)		Flow induced shear stress on cell-seeded titanium fiber mesh scaffold.	16 days	Higher calcium content
Jagodzinski *et al.* (2008)	Continuous flow and intermittent strain	Continuous flow through cell-seeded demineralised bone matrix with some constructs subjected to additional cyclic scaffold compression	21 days	Higher cell number (both loading conditions) maintenance of osteocalcin production (cyclic compression only).
Sumanasinghe *et al.* (2006)	Intermittent strain	Uniaxial cyclic tensile strain on cell-seeded collagen gels.	14 days	Upregulation of BMP-2 gene expression.
Byrne *et al.* (2008)		Uniaxial cyclic tensile strain on cell-seeded collagen-glycosaminoglycan scaffolds	7 days	Upregulation of OPN gene expression.
Mauney *et al.* (2004)		4-point bending on cell-seeded demineralised bone matrix	16 days	Higher ALP activity.
Sittichokechaiwut *et al.* (Current results)		Cyclic dynamic compressive strain on cell-seeded polyurethane scaffolds	24 days	Higher ALP activity, COL1 gene expression, collagen and calcium content.

related findings led researchers to hypothesise that osteogenic precursors respond to mechanical loading and much recent research supports this. For example in cell monolayers, oscillatory fluid flow (Arnsdorf *et al.*, 2009) has been shown to induce the upregulation of transcription factors involved in the osteogenic differentiation pathways of MSCs, and laminar flow to upregulate BMP-2 and OPN mRNA (Yourek *et al.*, 2010). Cyclic stretching of hMSCs on a flexible membrane resulted in upregulation of both bone transcription factors, matrix proteins and ALP (Diederichs *et al.*, 2009; Jagodzinski *et al.*, 2004) in addition to markers of cartilage (Friedl *et al.*, 2007).

However, it is becoming increasingly clear that mechanotransduction pathways operate differently in 2D compared to 3D culture (Reilly and Engler, 2009) and understanding 3D culture is not only important to better mimic the physiological environment but also necessary to produce tissue in bulk for tissue engineering. Recent efforts to study the mechanical effects of osteogenesis in 3D have led to the development of multiple models which

can be broadly divided into continuous loading systems such as perfusion flow bioreactors, or short term stimulation systems such as stretch, compression and bending of cell seeded constructs, or short periods of fluid flow through constructs. Examples of such studies are summarised in Table 3, though this is not intended to be an extensive review of all previous research in this area. Here we examined the short term effects of a single loading bout and the long term effects of repeated loading within the same system.

For continuous perfusion systems, it is difficult to separate the effects of improved nutrient perfusion from the effects of the shear stress induced by fluid flow, in fact both effects appear to be involved in osteoblast responses to fluid flow in monolayer (Donahue *et al*., 2003). In this system we chose to use an open cell highly porous foam so that our cells could survive well enough in long term static culture to see the effects of a specific loading regimen. While our loading regimen would have temporarily improved nutrient perfusion in the loaded scaffold, this occurred for only 6 h within a 576 h (24 day) culture period therefore, we believe nutrient perfusion played only a minor role in this system.

mRNA expression and ALP activity

RUNX2 (Cbfa1) gene expression was performed in this study as it is specific to the early differentiation of MSCs into the osteogenic lineage, and is a modulator of bone formation by fully differentiated osteoblasts (Karsenty, 2001). Osteopontin is a bone matrix protein that is upregulated during human MSC differentiation *in vitro* (Lian *et al.,* 1998) and responsive to mechanical loading in mature bone cells (You *et al.*, 2001) and human MSCs (Sharp *et al.*, 2009; Yourek *et al.*, 2010). ALP has been widely used as a marker of MSCs differentiation toward the osteogenic lineage, as increases in enzymatic activity and expression of both the gene and protein correspond to an osteoblastic phenotype (Grayson *et al.*, 2008; Mauney *et al.*, 2004a; Wozniak *et al.*, 2000). It peaks during the matrix formation phase and declines thereafter, while OPN and OCN peak in the late maturation or early mineralization phases (Lian *et al.*, 1998). For each of the three genes there appeared to be more mRNA in loaded than non-loaded samples within the no-Dex treatment group however the band density measurement did not show a statistically significant difference. Previous studies in which these genes have been shown to be upregulated by mechanical stimulation have usually used higher strains or longer loading periods then used here (Table 3). For example Runx2 has been shown to be upregulated by mechanical loading of hMSCs after cyclic loading at 8% strain in tension or 10% in compression (Friedl *et al.*, 2007; Jagodzinski *et al.*, 2008; Jagodzinski *et al.*, 2004).

Two hours of dynamic compressive loading at 5% strain doubled the expression of Col1 mRNA in both Dex and non-Dex treated groups compared to non-loaded constructs suggesting that mechanical loading could be used to stimulate collagen synthesis in situations where it is preferable not to use Dex. This suggests that Col1 mRNA expression is more sensitive to loading and less sensitive to Dex than the other 'ostoegenic' genes investigated. However, these responses are likely to be specific to cell source, scaffold and loading regimen for instance Friedl *et al.* (2007), Jagodzinski *et al.* (2008) and Jagodzinski *et al.* (2004) also saw a strong effect of cyclic stretching of hMSCs (monolayer) on Col1 expression however in their case the effect was much stronger in Dex treated cultures, while Byrne *et al.* (2008) saw no effect of cyclic stretching on Col1 expression by rat MSCs in collagen-glycosaminoglycan porous scaffolds when all cells were treated with Dex, but Grellier *et al.* (2009) saw a decrease in Col1 expression together with an increase in ALP expression after a fluid flow stimulus.

ALP activity was measured 3 days after the loading bout and in this case the loading-induced difference was larger than that for ALP mRNA (40% higher) and statistically significant. ALP activity induced by just 2 h of compression loading was as high as that induced by 8 days of Dex treatment (culture days 4 to 12). The effects of Dex in our 3D scaffold (50% higher ALP) are comparable with its effects on the same source of human MSCs in standard 2D tissue culture plastic (40% higher in Dex treated cultures by day 14, data not shown). Mauney *et al.* (2004a) have shown that ALP activity *in vitro* was elevated by cyclic mechanical loading for cells cultured in 10nM Dex, but this effect was abolished with 100nM concentration, suggesting that Dex can inhibit this mechanism at high concentrations.

Matrix production and mineralization

Collagen and calcium were distributed over the scaffold struts, although cell bodies were not localised in these experiments, it is likely there were distributed throughout this deposited ECM as in our previous study on mature osteoblasts (Sittichockechaiwut *et al*., 2009). In agreement with the ColI mRNA data, collagen and calcium deposition doubled in mechanically stimulated MSCs when compared to non-loaded samples with no Dex (Fig. 2). Although there was some effect of Dex, constructs treated with Dex only had no higher collagen and calcium content than constructs subjected to loading only (no-Dex). This indicates that matrix synthesis and mineralization can be elevated by mechanical stimulation independent of Dex. There is some indication of an additive effect of the two stimuli with the highest production being seen in constructs subjected to both loading and Dex though this is not significantly higher than loading treatment alone. Others have shown that perfusion culture improves matrix production (Table 3) and implied that some of the reason for this is the production of flow induced shear stresses, but here we show that continuous matrix perfusion is not necessary to induce a robust matrix forming response. Interestingly, in a study in which continuous perfusion and dynamic compression were applied to 3D hMSC constructs in the same bioreactor conditions, perfusion upregulated cell proliferation only and bouts of dynamic compression combined with perfusion were required to maintain production of the bone matrix gene osteocalcin (Jagodzinski *et al.*, 2008). There is also evidence that when MSC-containing constructs are implanted *in vivo,* short bouts of compression loading enhance mineralisation (Duty *et al.*, 2007), including in a scaffold implanted

without cells and infiltrated by the animal's precursor cells (Roshan-Ghias *et al.*, 2010). This implies that bone differentiation will be stimulated if hMSC seeded constructs are implanted into load bearing sites *in vivo*.

A limitation of our scaffold is that although we apply a global strain of 5%, individual cells will experience very different substrate strain (tensile, compressive shear) and fluid flow regimens depending on how well they are attached to the scaffold and the scaffold strut orientation in relation to the loading direction, while other cells are stress-shielded. This may not be of high importance when optimising conditions for growth of tissue engineered bone, where as long as the construct as a whole produces matrix this is a positive result. However, it does make difficult to elucidate the mechanotransduction mechanisms that trigger the response. Models such as those being developed to better understand the strains and shear stresses experienced within porous scaffolds (Lacroix *et al.*, 2006; O'Brien *et al.*, 2007) would help to elucidate what mechanical forces cells are experiencing within this complex system.

In conclusion, this study has shown that a combination of short bouts of cyclic loading and rest periods can improve matrix production by human bone marrow derived MSCs in engineered bone constructs *in vitro.* The data suggest that a short mechanical stimulus is an additional or alternative tool for establishing precultivation conditions prior to clinical implantation of tissue engineered bone, which has implications in the design of mechanical loading regimens in bioreactor culture. However, given the variety of scaffolds and loading regimens under current investigation and the contrasting stimulus-specific results presented in the literature further research is needed to understand how cell-lineage specific effects on differentiation can be consistently obtained using mechanical stimuli.

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Discussion with Reviewer

Reviewer I: Describe the hypothesised biological mechanism of how loading is able to induce osteogenic differentiation in a similar manner to, but in the absence of glucocorticoids.

Authors: At this stage it is not possible to tell if loading and Dex initiate differentiation via the same pathways or different pathways as multiple pathways are involved in both stimuli. There is some evidence of an additive effect of Dex and loading (Fig. 5) indicating that there is probably more than one pathway involved. If Dex and loading do partially operate via the same mechanism a strong candidate for the signalling pathway mediating this is the extracellular signal regulated pathway 1 and 2 (ERK1/2). ERK1/2 has been consistently shown to be upregulated by a range of mechanical loading stimuli in both osteoblasts (You *et al*., 2001; text reference) and MSCs (Simmons *et al*., 2003, text reference; Glossop and Cartmell, 2009) including by cyclic hydrostatic pressure in a 3D PLGA constructs (Kim *et al*., 2007). In preliminary experiments we detected increased ERK1/2 phosphorylation in cells subjected to the loading conditions described here (data not shown). However, the contribution of ERK1/2 signalling to osteoblast differentiation is very complex. While ERK1/2 has been shown to stimulate the dexamethasone-induced differentiation of MSCs into osteoblasts, via phosphorylation of the osteogenic transcription factor RUNX2/Cbfa1 in monolayer culture (Jaiswal *et al.*, 2000), and 3D culture (Farrell *et al.*, 2006) it has also been shown to inhibit BMP-2 induced differentiation in monolayer (Osyczka and Leboy, 2005) and collagen induced osteogenic differentiation in 3D gels

(Lund *et al*., 2009). Therefore, there is likely to be a complex relationship between the temporal phosphorylation of ERK1/2, the co-stimulation of other pathways and the ultimate fate of human MSCs which warrants further and detailed investigation.

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