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MATRIX PRODUCTION AND COLLAGEN STRUCTURE ARE ENHANCED IN TWO TYPES OF OSTEOGENIC PROGENITOR CELLS BY A SIMPLE FLUID SHEAR STRESS STIMULUS

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Abstract

Mesenchymal progenitor cells play a vital role in bone regenerative medicine and tissue engineering strategies. To be clinically useful osteoprogenitors should be readily available with the potential to form bone matrix. While mesenchymal stromal cells from bone marrow have shown promise for tissue engineering, they are obtained in small numbers and there is risk of donor site morbidity. Osteogenic progenitor cells derived from dermal tissue may provide a more abundant and easily expandable source of cells. Bone turnover *in vivo* is regulated by mechanical forces, particularly oscillatory fluid shear stresses (FSS), and *in vitro* osteogenic progenitors have been shown to be regulated by mechanical stimuli. The aim of this study was to assess what effect osteogenic media and FSS, generated by a simple rocking platform, had on cell behaviour and matrix production in human progenitor dermal fibroblasts (HDFs) and the embryonic stem cell-derived mesenchymal progenitor cell line (hES-MP).

Osteogenic media stimulated alkaline phosphatase activity (ALP) and calcium deposition in HDFs. The addition of FSS further enhanced ALP activity and mineralised matrix deposition in both progenitor cells cultured in osteogenic media. Both types of progenitor cell subjected to FSS showed increases in collagen secretion and apparent collagen organisation as imaged by second harmonic generation.

Keywords: Mesenchymal stem cells; dermal fibroblasts; fluid shear stress; second harmonic generation; osteogenesis; matrix production; collagen.

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Introduction

Mesenchymal progenitor cells play a vital role in bone regenerative medicine and tissue engineering strategies, and to be clinically useful they should be readily available with the potential to undergo osteogenesis. Mesenchymal stem or stromal cells (MSCs) harvested from bone marrow have shown great potential as an autologous bone cell source with self-renewing and multipotent properties capable of *in vitro* differentiation along the osteogenic lineage (Jaiswal *et al.,* 1997; Mauney *et al.,* 2004; Pittenger *et al.,* 1999). However, bone marrow extraction carries the risk of donor site morbidity and only a small number of MSCs are obtained from bone marrow, which are difficult to expand to sufficient numbers *in vitro*. This has led researchers to search for alternative multipotent cell reservoirs. Progenitor cells with similar phenotypic characteristics and differentiation capabilities have been obtained from a variety of other adult tissues including adipose (De Ugarte *et al.*, 2003; Zuk *et al.*, 2001), tendon (Rui *et al.*, 2011), and skeletal muscle (Asakura *et al.*, 2001; Bosch *et al.*, 2000), as well as foetal tissues such as umbilical cord blood (Erices *et al.*, 2000; Goodwin *et al.*, 2001) and amniotic fluid (Soncini *et al.*, 2007). Another recently identified tissue that might harbour a suitable cell source for bone repair is the dermis of skin. Dermal fibroblasts were initially thought to be terminally differentiated, but it has been reported that dermal fibroblasts may be more plastic than first thought and are able to switch their lineage preference (Rutherford *et al.*, 2002; Sommar *et al.*, 2009) while numerous studies report that multipotent progenitor cells reside in the dermal tissue of rodents and humans (Bartsch *et al.*, 2005; Chen *et al.*, 2007; Toma *et al.*, 2001; Xue and Li, 2011; Young *et al.*, 2001). Chen *et al.* (2007) established single cell clones from dermal foreskin fibroblasts and found that around 30 % upregulated alkaline phosphatase (ALP) and osteocalcin (OCN) mRNA along with strong staining of deposited calcium when cultured in osteogenic media. Others have observed osteogenic differentiation from a population of skin cells (Buranasinsup *et al.*, 2006; Lorenz *et al.*, 2008). Lorenz *et al.* (2008) observed an upregulation in OCN and osteonectin (ON) mRNA in cells derived from human juvenile foreskin when cultured in osteogenic media, while Buranasinsup *et al.* (2006) observed positive ALP and mineral staining in cells derived from middle-age human skin biopsies. This suggests that dermal tissue has the potential to be an easily accessible source of cells suitable for use in bone tissue engineering.

MSC behaviour and function can be controlled by biochemical stimuli such as growth factors, cytokines and signalling events (Augello and De Bari, 2010) as well as

physical and environmental cues (e.g. surface chemistry and topography) (Dalby *et al.*, 2007; Reilly and Engler, 2010). More recently, researchers have become aware that mechanical forces can also have an influence on MSC behaviour and may be important in directing their differentiation and maturation to obtain a fully-developed tissue engineered construct. It has been shown *in vivo* that mechanical forces regulate bone mass and strength (Bassey and Ramsdale, 1994; Janmey and McCulloch, 2007). Some of the major forces that contribute to the mechanical environment of bone cells are oscillatory fluid shear stresses (FSS) caused by fluid movement through interstitial bone space as a result of repetitive loading and unloading of the bone. It is also thought that oscillatory FSS would be experienced by cells in the bone marrow cavity. The magnitude of FSS in mature bone have been predicted to be in the range of 0.8-3 Pa (Weinbaum *et al.,* 1994) and although the magnitude of the FSS in bone marrow are not known, they are predicted to be much less due to the higher porosity and lower stiffness of the marrow (Gurkan and Akkus, 2008). Recent *in vitro* stimulation of osteoprogenitors using FSS have shown that osteogenic differentiation can be induced or enhanced on 2D substrates as well as 3D scaffolds, see recent reviews Delaine-Smith and Reilly (2011) and McCoy and O'Brien (2010). At present there are no studies that we know of that have looked at what effect the combination of osteogenic supplements and oscillatory FSS have on dermal fibroblasts.

While it is has been established that mechanical forces can influence MSC differentiation, it is still not clear what the best conditions are to achieve this. This is mainly due to the number of parameters that are relevant – the magnitude of the force, the number of cycles, the length of stimulation and the number of rest periods. Therefore, a simple system that is capable of mechanically stimulating large numbers of samples and testing a wide variety of parameters, which also allows for easy monitoring of cell differentiation and matrix production, would be ideal. It has been suggested that a rocking 'see-saw' system holding culture wells containing media is able to create FSS suitable for stimulating cells (Zhou *et al.*, 2010; Tucker *et al*., 2011). This simple system is an easily accessible device that has many advantages over other more commonly used apparatus including smaller amounts of medium per sample, cheap and easy operation, no special chambers required and high throughput. Therefore, this system seems ideal for testing a wide range of fluid flow regimes and how they influence the osteogenic differentiation of progenitor cells in a rapid and straightforward manner. The simplicity of the apparatus also allows for fast and easy monitoring of the production of the major bone matrix protein, collagen, by conventional methodologies and, as we assess in this study, by non-invasive monitoring using second harmonic generation (SHG). SHG is a multiphoton-based technique, which allows the imaging of non-centrosymmetric molecules such as collagen (Bayan *et al.,* 2009). The collagen molecule is excited by two near-infrared incident photons, which come together to produce a visible photon with exactly half the wavelength and twice the energy. This photon can be detected at half the wavelength of that used to excite the sample and the intensity of the resulting SHG image is influenced by the quantity of collagen present as well as the fibril organisation (Bayan *et al.,* 2009; Matcher, 2009).

In summary, this study had three objectives. The first was to use a simple platform rocking model to mechanically stimulate a mature osteoblastic cell line (MLO-A5) and osteogenic progenitor cells to see if the FSS created could influence their differentiation and matrix production. The second was to examine if osteogenic media and FSS were able to induce dermal fibroblasts to an osteogenic lineage. The third objective was to monitor the effect FSS had on the production and organisation of cell secreted tissue engineered collagen using SHG.

Methods

Cell culture

Three cell types were used in this study: primary human dermal fibroblasts (HDFs) isolated from dermal tissue taken from one consenting patient undergoing surgery (procedures were approved by the National Health Service Research ethics committee); the human embryonic cell-derived mesenchymal progenitor cell line hES-MP 002.5 (hES-MP) (Cellartis, Gothenburg, Sweden); and the late stage osteoblast/early stage osteocyte mouse cell line MLO-A5 kindly donated by Professor Lynda Bonewald (University of Missouri, Kansas City, MO, USA) under a Material Transfer Agreement with the University of Texas. HDFs were expanded in basal media, which consisted of Dulbecco's modified Eagle's medium (DMEM) (Biosera, Ringmer, UK) supplemented with 10 % foetal calf serum (FCS), 2 mM L-glutamine and 100 mg/mL penicillin and streptomycin (P/S). MLO-A5 and hES-MP cells were cultured in basal α-media, which consisted of Minimum Essential Alpha Medium (α-MEM) (Lonza, Verviers, Belgium) containing 10 % FCS, 2 mM L-glutamine and 100 mg/mL P/S and seeded onto gelatine coated T-75 flasks. All cells were incubated at 37 °C in the presence of 5 % CO_2 and fresh media changes were made every 2-3 d. For each experiment, hES-MPs were used between passages 3-7 and cultured in either basal α-media with 50 mg/mL ascorbic acid-2-phosphate (AA) and 5 mM β-glycerophosphate (βGP) (non-Dex containing media (NM)), or basal α-media with 50 mg/mL AA, 5 mM βGP and 100 nM dexamethasome (Dex) (osteogenic media (OM)). HDFs were used between passages 2-3 and were cultured either in the presence of fibroblastic media (FM) containing basal media with 50 µg/mL AA, or in OM. MLO-A5 cells were cultured in NM and used between passages 25-30. All reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

Application of fluid shear stress

For experiments, all cells were seeded onto gelatine-coated standard 6-well plates at a density of 10,000 cells per well in their respective basal media. Media with supplements were added 24 h after attachment. Cells of each media type were either cultured under static conditions (no forces) or subjected to fluid shear stresses (FSS) (also referred to as 'rocked' group) starting from day 4 of culture at 45 cycles/

min for 1 h per day, for 5 days per week. The rocking platform had a maximum tilt angle of 6 degrees and each well contained 2 mL of media. Bouts of rocking were performed outside of an incubator and static controls were also placed outside of the incubator for the same period of time. Media was changed every 2-3 days. The FSS generated were calculated for three separate points in space along the well bottom using a lubrication-based model previously described by Zhou *et al.* (2010) for a circular well. Values of FSS were obtained using the equation for calculating the characteristic shear stress (Eq. 1), where μ is the fluid viscosity (10^{-3} Pa s), θ_{max} is the maximal flip angle, δ is the ratio of the fluid depth to the well length, and T is the time for one cycle. Briefly, the model assumes that fluid movement is mainly driven by gravity, and that the fluid free surface remains horizontal. Secondly, the centrifugal forces acting on the fluid are neglected due to the low angular acceleration and velocity.

$$|\tilde{\tau}_w| = \frac{\pi\mu\theta_{max}}{2\delta^2 T} \quad \text{(Eq. 1)}$$

Cellular morphology

Cellular morphology was visualised at day 7 using fluorescence microscopy. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) (1 µg/mL) and phalloidin TRITC (phalloidin-tetramethylrhodamine B isothiocyanate) (1 µg/mL) (Sigma) staining were used for the cell nucleus and the cell actin-cytoskeleton, respectively, and images were captured using an Image Express™ fluorescent microscope (Axon Instruments, Wokingham, UK) using the built-in x20 objective and preset DAPI and Rhodamine filters. Images from DAPI and phalloidin TRITC channels were combined using ImageJ software.

Alkaline phosphatase activity and total DNA measurement

Total DNA was measured using a fluorescent Quant-iT™ PicoGreen® dsDNA reagent assay kit (Invitrogen, Paisley, UK) per the manufacturer's instructions. Briefly, cells were lysed in a carbonate buffer solution and freeze-thawed three times before a known volume of cell lysate was added to the provided Tris-buffered EDTA solution. The Quant-iT™ PicoGreen® reagent was then added, which binds to double-stranded DNA in solution, and fluorescence intensity was recorded using a FLx800 microplate fluorescence reader (BioTek, Potten, UK) using 485 nm excitation and 520 nm emission. Total DNA was converted to ng DNA/sample from a standard curve. ALP activity in the cells was assessed using a colorimetric assay; a known quantity of cell lysate was added to a p-nitrophenol phosphate substrate (Sigma) and the subsequent conversion to p-nitrophenyl was measured by recording the rate of colour change from colourless to yellow at 405 nm. ALP activity was calculated as nmol of substrate converted per minute using a standard curve and then normalised to total DNA.

Collagen and calcium staining

Total cellular collagen production was quantified at days 7, 14 and 21 by staining the deposited collagen using a 0.1 % Picrosirius red solution (Sigma) for 1 h on a platform shaker. The unbound Picrosirius red solution was washed away by three washes with deionised water and the resulting stain was removed with methanol:0.2 M NaOH (1:1) for 10 min on a platform shaker. The absorbance of the resulting solution was then measured at 490 nm on a 96-well plate reader. Calcium deposition by the cells was visualised at day 21 by staining with a 1 % Alizarin red solution for 15 min (Sigma). Excess Alizarin solution was removed by washing with deionised water five times and then deposited calcium was quantified by removing the Alizarin stain with 5 % v/v perchloric acid for 10 min and reading the absorbance of the resulting solution at 405 nm.

Second harmonic generation (SHG)

Deposited collagen fibres were visualised from SHG images obtained using a Zeiss Axioskop 2 FS MOT (Carl Zeiss MiroImaging, Jena, Germany) laser-scanning confocal microscope equipped with a tuneable Chameleon Ti:sapphire multiphoton laser (Coherent, Santa Clara, CA, USA). Excitation of the samples was performed at 940 nm and SHG emission was collected in the backwards scattering direction and filtered through a primary dichroic (HFT KP650) before entering a descanned LSM 510 Meta detector (Carl Zeiss MicroImaging) set with a narrow 10 nm bandpass filter centred around 469 nm with the pinhole set to maximum. Samples of all conditions were imaged at days 7, 14 and 21 of culture using a 40x NA 1.3 Plan Neofluar oil immersion objective (Carl Zeiss MicroImaging) focused on the central region of each sample with a power of 20 mW.

Statistics

All rocker experiments were performed two or three times with triplicate samples for each condition (n = 6 or 9). For collagen visualisation using SHG, one sample of each condition was imaged at each time point (n = 2-3), with images being obtained from the centre region approximately 10 µm into the sample. Cells of the same type and cultured using the same media conditions were compared for significant differences between statically cultured and rocked groups using an unpaired Student's t-test. All graphs are mean ± SD and significant differences are marked for $p < 0.05$ and $p < 0.01$.

Results

Fluid shear stress profiles

The FSS at the base of the culture wells were calculated at 3 separate points in space (x/L = 0.25, 0.5, 0.75, where x is the distance from the edge of the well and L is the diameter of the well) along the middle of the well parallel to the fluid movement during one rocking cycle. The FSS varied in a spatiotemporal fashion and were oscillatory in nature (Fig. 1a-b). Under the conditions used, the shear stress at the centre of the well (x/L = 0.5) was found to vary in a sinusoidal manner peaking at 0.041 Pa, while at the other two locations (x/L = 0.25 or 0.75) the stress peaked at 0.051 Pa and deviated from a typical sinusoidal wave. The stress profiles at locations x/L = 0.25 and 0.75 were identical except for a phase difference of 180 degrees.

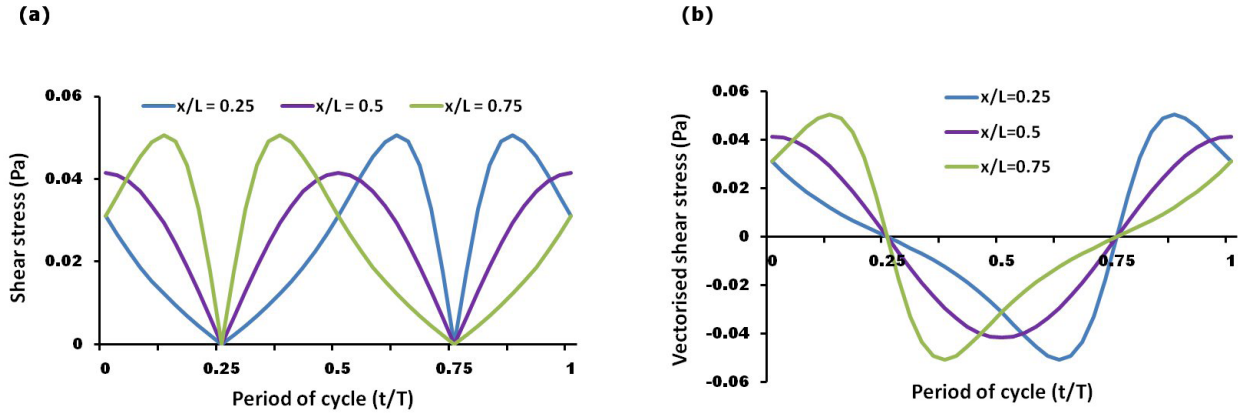

Fig. 1. (a) Calculated shear stress profiles for one cycle experienced at the base of a 6-well plate for three different locations, x/L = 0.25, 0.5, or 0.75, where x is the distance from the edge of the well and L is the diameter of the well. **(b)** The oscillatory nature of the fluid flow-induced shear stresses indicated by positive and negative stress.

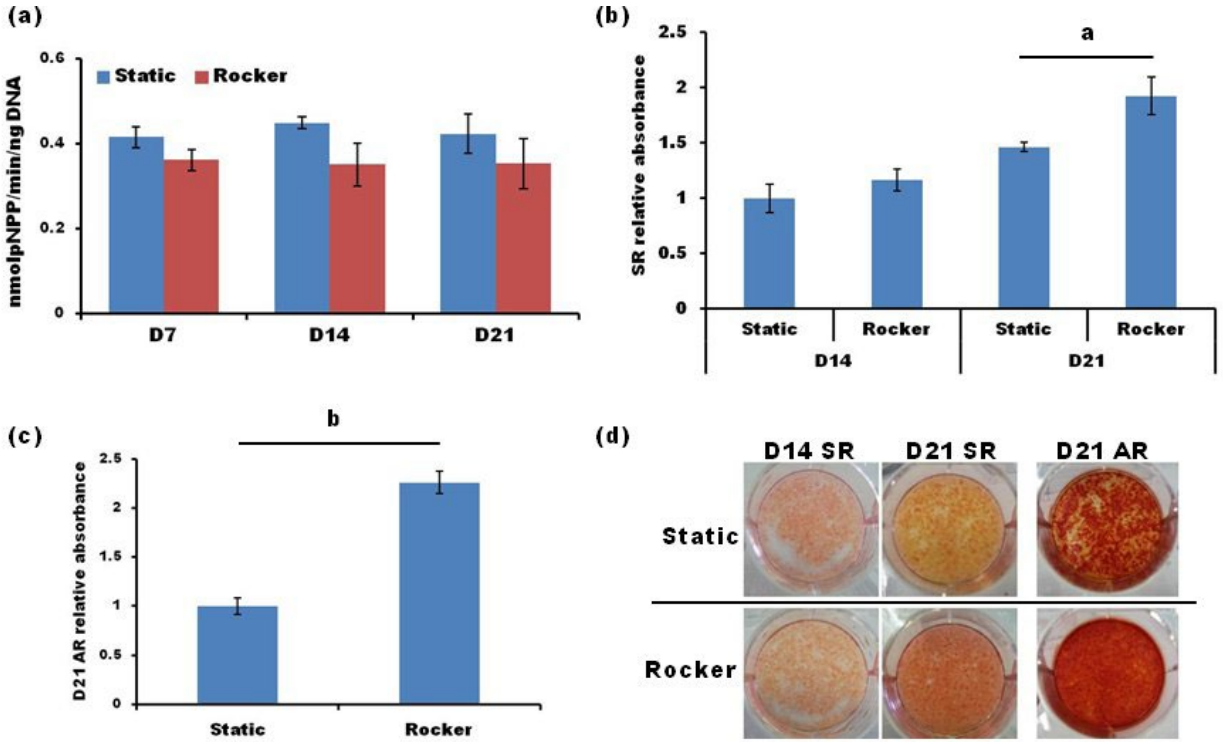

Fig. 2. The affect of FSS on MLO-A5s. **(a)** ALP activity normalised to total DNA; **(b)** total collagen production assayed by Picrosirius red staining; **(c)** calcium deposition assessed by Alizarin red; **(d)** Picrosirius red and Alizarin red staining of MLO-A5 cells at day 14 and 21 under static or rocked conditions (individual wells of a 6-well plate are shown). All bar graphs are mean ± SD (n = 9). Significant differences between static and rocked cells are a = $p < 0.05$ and b = $p < 0.01$.

The effect of FSS on matrix formation by MLO-A5 cells

The ALP activity of MLO-A5 cells peaked in both static and rocked groups at day 7 and remained constant up to days 14 and 21 (Fig. 2a). Cells exposed to rocking appeared to have 15 % lower ALP activity across all three time points but this was not statistically significant. Collagen production measured at day 14 was not affected by rocking, but by day 21 samples exposed to rocking had 25 % more secreted collagen than static controls (Fig. 2b). Calcium deposition assayed at day 21 was 2-fold higher when cells were subjected to rocking (Fig. 2c). At day 21 collagen and calcium staining in the rocked groups appeared more uniform across the culture dish, whereas statically cultured groups showed patchy staining (Fig. 2d).

The effect of FSS on the morphology of progenitor cells

hES-MP cells cultured under static conditions in non-Dex containing media (NM) had a fibroblastic, spindle-shaped morphology, whereas hES-MPs cultured in osteogenic media (OM) were larger and more cuboidal in shape, indicative of an osteoblastic cell (Fig. 3a). HDFs cultured in fibroblastic media (FM) showed a typical fibroblastic morphology, however when cultured in OM they showed a more cuboidal morphology (Fig. 3b) similar to the hES-MP

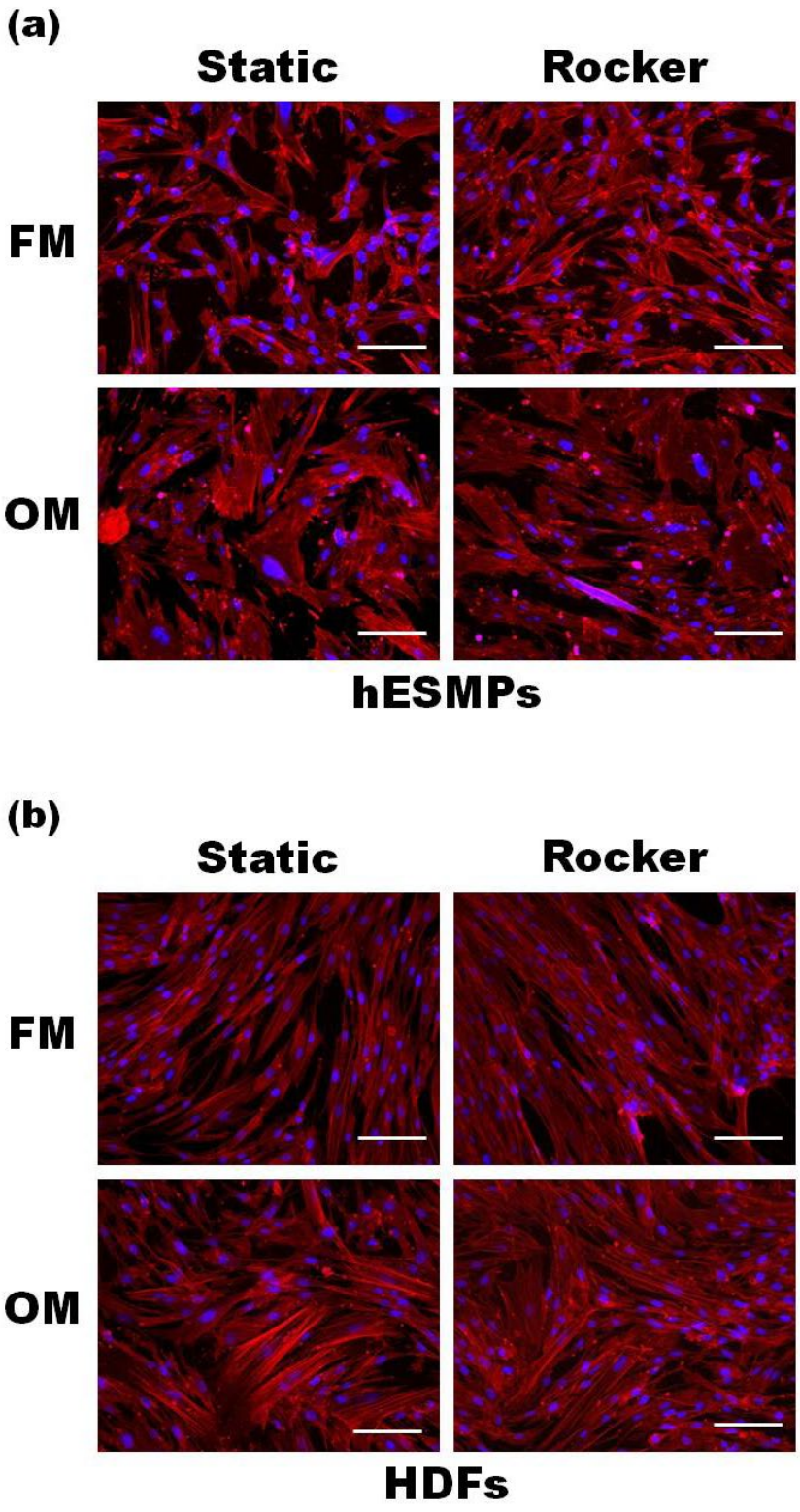

Fig. 3. Fluorescent DAPI staining of cell nucleus (blue) and phalloidin TRITC staining of cell actin cytoskeleton (red). **(a)** hES-MP cells cultured in either non-Dex containing media (NM) or osteogenic media (OM) under static or rocked conditions; or **(b)** HDFs cultured in either fibroblastic media (FM) or OM under static or rocked conditions. Cells cultured in FM or NM show a more fibroblastic morphology, whereas cells cultured in OM show a more osteoblastic morphology. (Scale bars are 100 μm).

cells and generally appeared larger than those cultured in FM. Cellular alignment did not appear to be influenced by fluid flow induced by rocking in any areas of the culture well for either cell type.

The effect of FSS on total DNA and ALP activity of progenitor cells

Total DNA content, an indicator of total cell number, increased for both cell types in all cell groups between days 7 and 14 and then remained constant up to day 21 (Fig. 4a-b). There were no statistically significant differences in total DNA between cells that were rocked or cultured under static conditions but it was noticed that hES-MP cells cultured in OM did have 20 % less DNA at days 14 and 21 compared with cells cultured in NM. Normalised ALP activity increased in both cell types for all cell groups up to day 21 (Fig. 4c-d), however no ALP activity was detectable in any HDF culture group at day 7. ALP activity in hES-MPs was an order of magnitude higher in OM cultured cells compared to those cultured in NM.

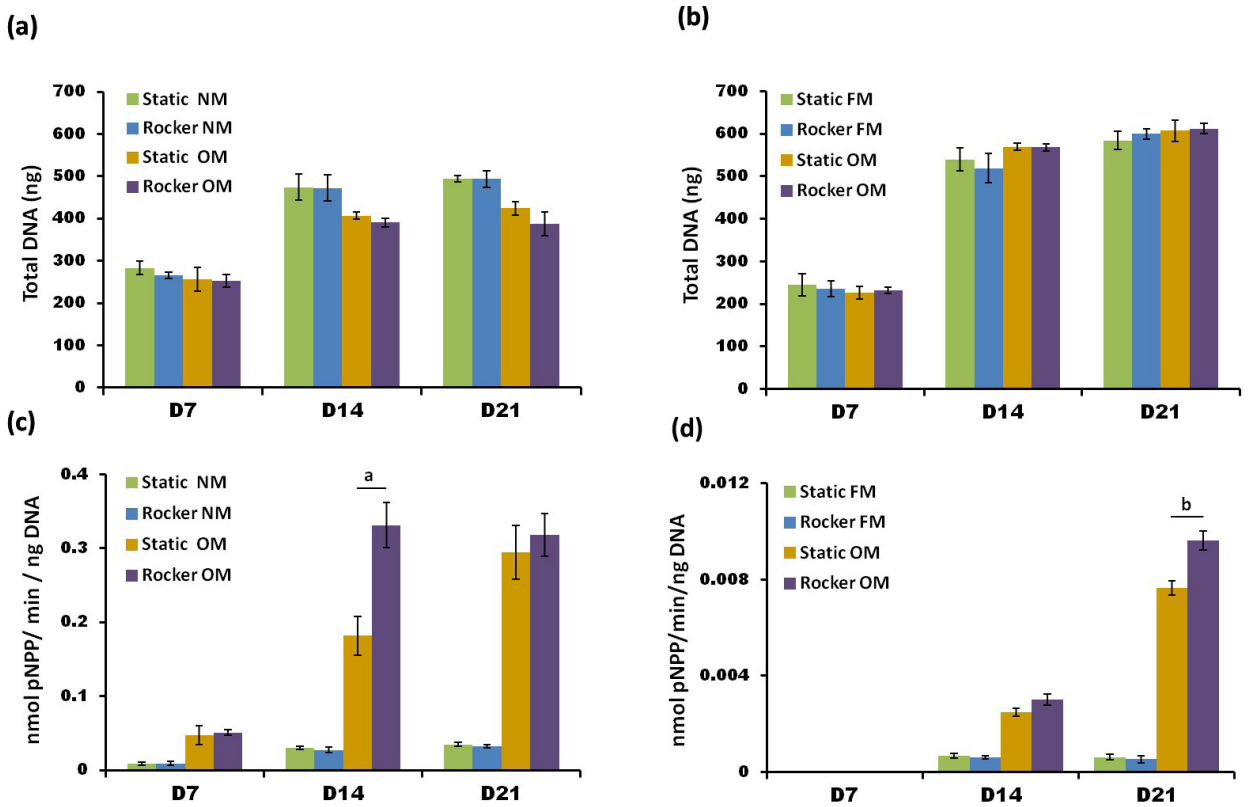

Fig. 4. The effect of FSS on total DNA content of hES-MPs **(a)** and HDFs **(b)** and ALP activity (plotted normalised to total DNA) for hES-MPs **(c)** and HDFs **(d)** measured at days 7, 14 and 21. FSS did not affect total DNA for any cell groups, but did cause statistically significant higher normalised ALP activity in Dex-treated cell groups. ALP activity in non-Dex-treated groups was minimal and FSS did not enhance this. Note the y axis range for HDFs is smaller than that for hES-MPs due to lower ALP activity. All bar graphs are mean ± SD (n = 9) and significant differences between static and rocked cells are a = $p < 0.01$ and b = $p < 0.05$.

When OM was combined with rocking, ALP activity in hES-MPs was 2-fold higher at day 14 compared with their static counterparts. It appears that the rocking accelerated the upregulation of ALP activity, as by day 21 the static controls were as high as the rocked samples. However, there was no effect seen when NM was combined with rocking. HDFs cultured in FM did not produce enough detectable ALP above baseline values at any time point; when cultured in OM they began producing detectable levels of ALP at day 14, and by 21 these levels had increased 4 times compared to day 14. When OM cultured HDFs were subjected to FSS, ALP activity increased by 20 % over static counterparts at day 21, which was statistically significant.

The effect of FSS on total collagen and calcium production by progenitor cells

Total collagen production quantified by Picrosirius red staining showed that cells cultured with Dex had produced less collagen at all time points, compared to those cultured without Dex (Fig. 5a-b). For hES-MPs cultured in either media group, the application of rocking caused the total amount of collagen deposited by day 21 to be 20 % higher ($p < 0.05$) (Fig. 5a). For HDFs subjected to rocking, significantly more total collagen ($p < 0.01$) was seen at days 14 and 21 for both media groups (Fig. 5b). Calcium deposition assayed at day 21 was 3-fold higher in Dex treated hES-MPs subjected to rocking compared to static counterparts and this staining was more uniform across the culture dish while static cells showed patchy staining (Fig. 5c). In comparison, HDFs cultured in OM showed a relatively small amount of calcium staining at day 21 but rocking significantly increased the amount deposited by 50 % (Fig. 5d). The Alizarin stain was seen to concentrate more around the centre of the wells and became fainter towards the outside of the well. Both hES-MP cells and HDFs cultured without Dex did not produce any calcium as visualised by the absence of Alizarin red stain.

Assessment of collagen production by second harmonic generation

The effect of FSS on collagen deposition and maturation was monitored in both HDFs and hESMP cells using SHG at days 7, 14 and 21 (Fig. 6a-b). Signal intensity increased for all samples from day 7 to 21, indicating an accumulation of collagen over the culture period. When both cell types were cultured in the presence of Dex, the signal intensity was lower at all time points compared to those cultured without Dex. When both cell types were cultured without Dex and subjected to rocking, an increase in SHG signal was seen at all time points compared with static counterparts. It was not until day 21 that a noticeable difference in SHG intensity was seen for both cell types when cultured in OM, with those subjected to rocking giving a stronger signal compared with statically cultured counterparts. Rocking appeared to have a smaller effect on

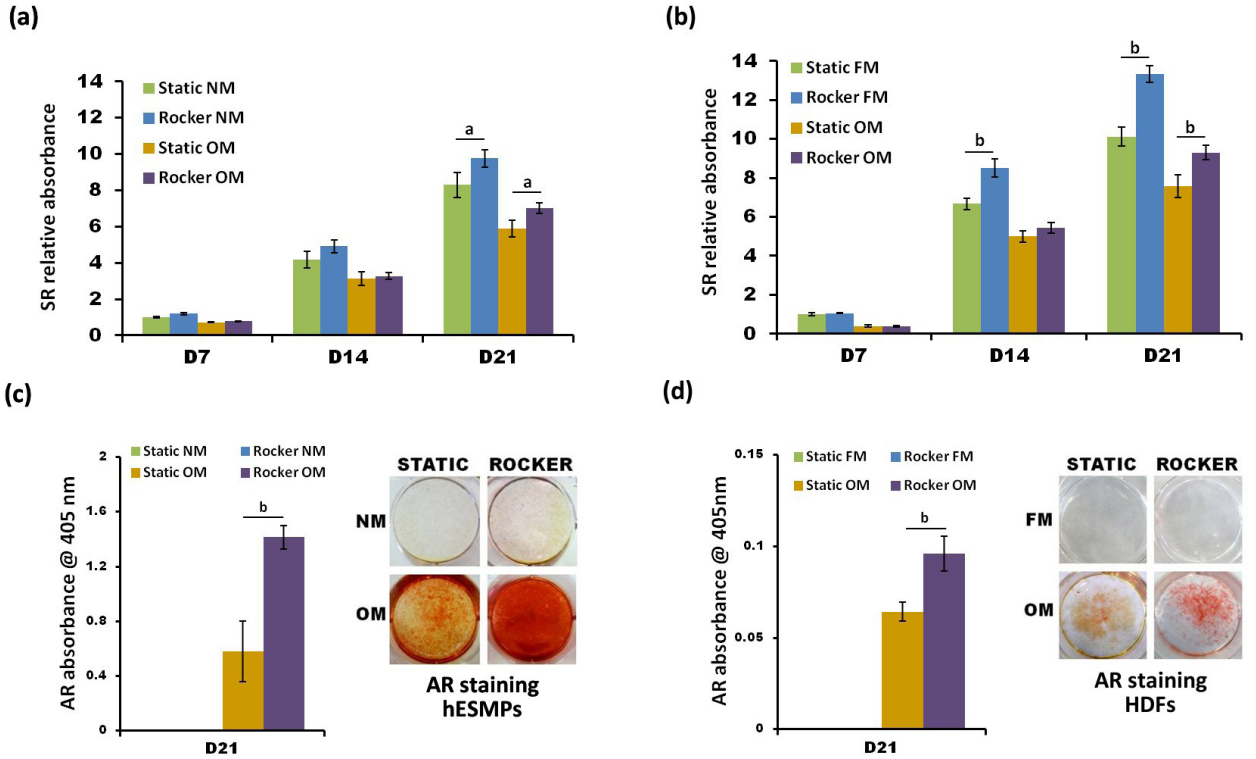

Fig. 5. Total collagen production at days 7, 14 and 21 as quantified by Picrosirius red staining for hES-MPs **(a)** and HDFs **(b)**. The application of FSS caused a statistically significant increase in collagen deposition for all cells at day 21, and cells treated with Dex produced less collagen than those without Dex. Calcium deposition was visualised at day 21 by Alizarin red staining for hES-MPs **(c)** and HDFs **(d)** and the application of FSS caused an increase in the amount of deposited calcium. Non-Dex-containing media (FM and NM) or FSS alone did not induce calcium deposition. Calcium was quantified from the Alizarin red stain. All bar graphs are mean ± SD ($n = 6$ for collagen; $n = 9$ for calcium) and significant differences between static and rocked cells are a = $p < 0.05$ and b = $p < 0.01$.

SHG intensity in hES-MP cells (Fig. 6a) than in HDFs (Fig. 6b) when compared with static counterparts. While rocking did not appear to induce a preferred direction of collagen orientation with either cell type in any media groups, rocking did appear to improve collagen organisation at day 14 and even more clearly at day 21. Statically cultured groups showed short and disorganised collagen fibres, whereas FSS groups had thicker and longer bundles of fibres and this effect was more evident in groups cultured without Dex.

Discussion

Our aims were to undertake research towards progressing tissue engineering of bone towards the clinic – examining a convenient possible source of osteogenic progenitor cells, assessing a simple methodology to apply mechanical stimulation to these cells, and monitoring collagen production and orientation using the minimally invasive technique of SHG.

This study was the first, to our knowledge, to use a simple platform rocking method to directly stimulate progenitor cells using oscillatory FSS for enhancing osteogenic differentiation. This is also the first study we know of to subject dermal fibroblasts and the hES-MP cell line to oscillatory FSS for the purpose of stimulating the production of a mineralised collagenous matrix. Although it has been demonstrated that osteoprogenitor MSCs respond to a variety of mechanical stimuli in a range of 2D and 3D bioreactor conditions (Delaine-Smith and Reilly, 2011), here we present the interesting result that dermal fibroblasts cultured in osteogenic media produce a mineralised collagenous matrix that is further enhanced by oscillatory FSS. The reasons for selecting HDFs for use in this study is that isolating progenitor cells from the dermis would have many advantages over other osteoprogenitor sources in that any donor will have large quantities of easily accessible skin and operations to remove it are simple and less painful than procedures to remove bone marrow. HDFs also have a high proliferative potential and can be expanded into large numbers *in vitro*.

The FSS calculated in this study were much lower than those estimated to occur within mature bone (0.8-3 Pa (Weinbaum *et al.*, 1994)), peaking at 0.041 Pa in the well centre, and are also much lower than the FSS generally used by others for mechanically stimulating osteoblastic cells, particularly in 2D (McCoy and O'Brien, 2010). However, the mature osteoblast MLO-A5 cell line responded to the shear forces with a noticeable increase in collagen and calcium production at day 21. Collagen production and organisation was improved in both the hES-MP cells and the HDFs when subjected to these FSS. When cultured in combination with osteogenic media, both cell types upregulated ALP activity and calcium production. Previous studies have also shown that hMSCs cultured in osteogenic supplements are mechanosensitive to relatively small shear

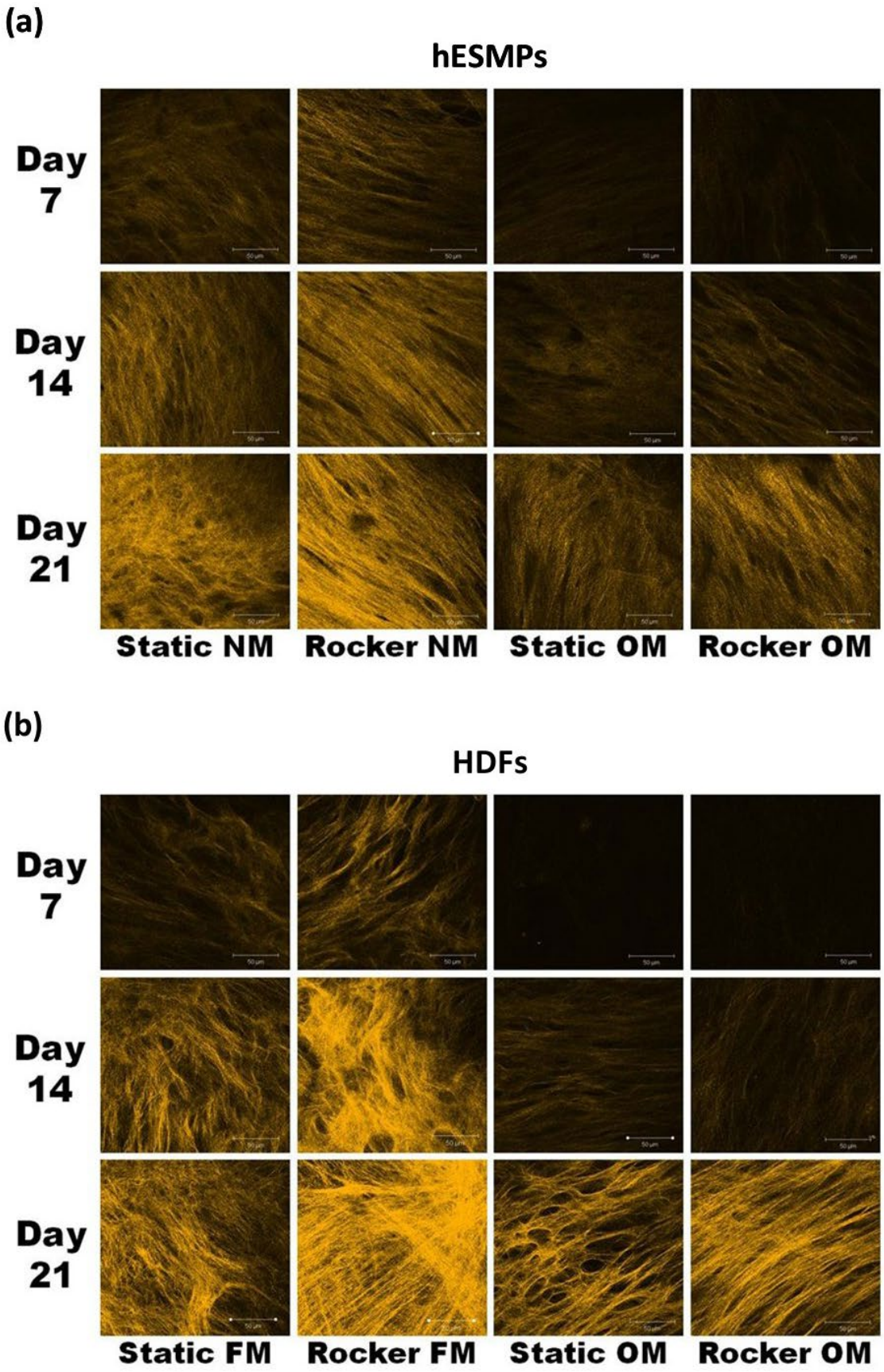

Fig. 6. Second harmonic generation (SHG) images of deposited collagen produced by hES-MPs **(a)** and HDFs **(b)** at days 7, 14 and 21. Increases in collagen deposition and organisation are indicated by an increase in SHG intensity and area coverage. A more organised collagen matrix can be observed in cells subjected to FSS, indicated by the appearance of thicker collagen bundles and more defined fibres. Images with a dark appearance did not produce enough detectable SHG signal (Scale bars are 50 μm).

forces (0.036 Pa) applied for only short periods of time (Kreke *et al.*, 2005). In a recent study, a T-75 flask rocking system was used to stimulate osteoblasts and osteocytes to condition media for MSCs (Hoey *et al.*, 2011), and although they did not calculate the FSSs present, it is likely they would have also been relatively low. It is unclear whether tissue engineers should be attempting to replicate the mature bone environment or rather a developmental or fracture-healing environment where bone cells differentiate *in vivo*. Immature, developing bone tissue resembles a healing wound and not a mature tissue and so the forces experienced are likely to be different from those of a fully developed tissue, although little is known about what these forces are (Willie *et al.*, 2010).

The shear stress profiles and the peak shear force varied for different locations within the well plate, but the resulting calcium staining for MLO-A5 cells and hES-MPs showed a rather uniform pattern across the well. This indicates that either the range of forces being experienced by the cells have a similar effect on their differentiation or that the cells are communicating with each other, such as via gap junctions (Donahue, 2000; Taylor *et al.*, 2007). Another contributing factor could be that the fluid flow is inducing chemotransport (Donahue *et al.*, 2003) and so biochemical factors regulating bone cell metabolism, such as prostaglandin E_2 (Genetos *et al.*, 2005), are released into the media by the cells and moved around due to mass transport. While we did not test the mechanisms by which fluid flow enhances osteogenic differentiation, the data presented combined with other studies suggests that osteoblastic differentiation may be guided by soluble factors that accumulate in the media from a combination of externally applied chemical stimulants and direct mechanical stress on the cells (Hoey *et al.*, 2011).

MLO-A5s are a late stage osteoblast/early stage osteocyte murine cell line that mineralise rapidly (8-10 d) even in the absence of βGP (Kato *et al.*, 2001) and they have been shown to respond to bouts of mechanical conditioning with enhanced matrix production (Morris *et al.*, 2010; Sittichokechaiwut *et al.*, 2010). MLO-A5s express high levels of ALP activity due to their advanced stage of maturity and FSS were not seen to have a significant effect on these levels at the selected time points, but calcium production was increased 2-fold. ALP activity is present in the early stages of osteogenesis and also plays a part in the initial stages of mineralisation via its enzymatic hydrolysis activity (Yadav *et al.*, 2011).

This study showed that hES-MPs and HDFs cultured in osteogenic media had significantly higher ALP activity than those cultured in non-Dex containing media and this level continued to rise up to day 21. Some authors report ALP activity as a biphasic process, rising to a peak level before gradually decreasing again (Bancroft *et al.*, 2002; Datta *et al.*, 2006), but this was not seen here. FSS increased ALP activity in both sets of progenitor cells when cultured in osteogenic media and both cells subsequently increased their calcium deposition. ALP activity in the hES-MPs was at least ten-fold higher than that in HDFs. The hES-MPs are a relatively homogeneous population of cells derived from a single source of embryonic stem cells already characterised as mesenchymal lineage specific and able to undergo osteogenesis in induction media (Karlsson *et al.*, 2009). However, HDFs are a much more variable cell population from a mature adult donor and it is likely that only a sub-population of the cells can undergo osteogenic differentiation, or that the cells have varying levels of differentiation potential (Chen *et al.,* 2007).

Cells derived from dermal tissue have previously been reported to show osteogenic differentiation potential (Bartsch *et al.*, 2005; Chen *et al.*, 2007) and this study showed that HDFs produced ALP and deposited calcium when cultured in osteogenic supplements. Previous studies have tended to culture HDFs under static conditions but this study showed that the application of oscillatory FSS could further enhance this osteogenic differentiation. In a previous study by Sommar *et al.* (2009), HDFs were cultured in a macroporous gelatine construct in the presence of osteogenic media and subjected to FSS in a rotating spinner flask. They noticed the formation of bone-like tissue, with further enhancements in the amount of deposited mineral in constructs cultured in spinner flasks. This observation, along with the present study, suggests that FSS can enhance mineralised matrix in HDFs cultured in osteogenic media. This revelation that HDFs can be induced towards an osteogenic phenotype using osteogenic supplements and FSS highlights their potential use in the repair of bone. A limitation of our study was that only HDFs from one patient were used and as with other progenitor populations, there will be cell variability resulting from variations in the source of tissue, such as tissue type and location within the body, donor characteristics, or *in vitro* passaging conditions.

Fibroblasts were only used to passage 3 because after this they did not consistently make calcium at day 21, although they did continue to up-regulate ALP activity to similar levels (data not shown). The majority of studies have used dermal fibroblast populations taken from foetal or juvenile skin (Lavoie *et al.*, 2009; Xue and Li, 2011), with the authors reporting loss of osteogenic potential or decreased potential at higher passage numbers. Some have reported that dermal progenitor populations display a delayed differentiation potential, often taking longer to mineralise than other MSC populations, anywhere between 4-8 weeks (Buranasinsup *et al.*, 2006; Jaager and Neuman, 2011; Lorenz *et al.*, 2008). However, there are a number of studies that have shown osteogenic differentiation to occur in cells from mature and aged dermis (Xue and Li, 2011; Young *et al.*, 2001). This loss of differentiation potential and donor variation could be a potential limitation with the future use of these cells for autologous bone repair, and so it is clear that more studies from a larger number of donors are required to assess their bone forming potential.

Monitoring matrix development by progenitor cells is very important for a successful tissue construct to be developed. Collagen type 1 fibres are the primary component of the organic portion of bone ECM and are well organised into orientated concentric layers in Haversian systems. This also provides the foundation for mineral deposition, and so it has a crucial role in bone formation. The amount of collagen present and how it is

organised plays a major role in determining the mechanical properties of the tissue. While some studies have shown that mechanical forces alone can induce osteogenic differentiation in MSCs (Chen *et al.*, 2008; Sumanasinghe *et al.*, 2006; Yourek *et al*., 2010), in this study FSS alone did not enhance calcium deposition. However, FSS did increase collagen production in all cells under all conditions and this has been seen in other studies using fluid flow as a stimulatory source (Augst *et al.*, 2008; Morris *et al*., 2010; Sharp *et al.*, 2009). When hES-MP cells and HDFs were treated with Dex, they produced less collagen at all time points, compared with those cultured without Dex. This is visualised very clearly from the SHG images (Fig. 6) and this is the first study that we know of to show the true extent of this effect of Dex on collagen production using SHG. It has been reported that MSCs treated with Dex *in vitro* show a reduction in collagen production (Leboy *et al.*, 1991; Ogston *et al.*, 2002) and large concentrations of Dex used to treat patients for various conditions can cause bone loss or impairment of bone formation leading to osteoporosis (Scutt *et al.*, 1996). Cells subjected to FSS also appeared to be more organised into thicker and longer bundles of fibres when imaged using SHG; information that could not be obtained from Picrosirius red staining. This enhanced collagen organisation suggests that cells subjected to these FSS would produce tissues with stronger tensile properties.

The process of converting mechanical stimulation into a biochemical response, mechanotransduction, is thought to occur through a number of mechanically-sensitive mechanisms including the cytoskeleton and integrins, ion channels, the glycocalyx and the primary cilia (Jacobs *et al.*, 2010; Morris *et al*., 2010; Reilly *et al.*, 2003; Weinbaum *et al.*, 2007). Through these mechanisms, the application of FSS initiates a number of signalling events, including the synthesis and release of nitric oxide and prostaglandins (Klein-Nulend *et al.*, 2005), a calcium signalling response and phosphorylation of the mitogen-activated protein (MAP) kinase ERK (You *et al.*, 2001). During osteogenic differentiation, the actin cytoskeleton in hMSCs remodels resulting in a morphological switch from a fibroblastic fusiform shape to a square shape which is more osteoblast-like. This was observed to happen with the hES-MP cells cultured in osteogenic media and a very similar morphological switch was observed when HDFs were cultured in osteogenic media. When subjected to flow, both cells appeared to be more elongated in either media condition. This is thought to be due to a stiffening of the cell cytoskeleton, and it has been seen that stiffer cells tend to become more mechano-responsive perhaps due to the forces being transmitted more efficiently (Yourek *et al.*, 2010). Previous studies have shown that actin cytoskeletal tension is required for the activation of mechanosensors or signalling mechanisms involved in the regulation of intracellular processes and protein expression resulting from FSS (Arnsdorf *et al.*, 2009). Also a number of studies have shown that remodelling of the cell cytoskeleton can induce changes in the organisation and distribution of deposited collagen (Brammer *et al*., 2009; Koepsell *et al*., 2011).

Conclusions

Isolating progenitor cells from the dermis for use as an autologous source of bone cells would have many advantages in that any donor will have large quantities of easily accessible skin and biopsies undertaken under local anaesthetic to remove it are straightforward. However, more work is needed to fully characterise this apparently diverse pool of multipotent cells in order to realise their full capabilities, including determining the effect that patient age and *in vitro* passaging conditions may have on their bone forming potential. The effect that mechanical forces have on progenitor cells is now a major research focus for musculoskeletal tissue engineers and their potential to aid healing and direct differentiation are being realised. The simple system employed here created FSS that enhanced osteogenic differentiation in mature bone cells and bone progenitor cells in the presence of osteogenic supplements. Using SHG, we saw an enhanced production and organisation of the major bone matrix protein collagen caused by FSS. This system has many advantages in that it is simple to use, can be used with many experimental samples, and could be easily scaled up for large defects. This system could be used for a number of tissue engineering strategies, such as pre-treating cells before injection into a scaffold or directly into a tissue defect or for stimulating cells cultured on thin scaffold sheets to be layered to form a 3D implantable tissue.

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Discussion with Reviewers

Reviewer I: Why use ESC derived MSC and not bone marrow/adipose derived? Are the authors satisfied that these are a good representation and what surface markers are they positive for?
Authors: The human embryonic stem cell-derived mesenchymal progenitor (hES-MP) cell line are free of undifferentiated hESC markers (Oct-4, TRA 1-60, TRA 1-81, SSEA-3, SSEA-4) but express the MSC markers (CD105, CD166, CD10, CD13) along with vimentin and desmin. hES-MP cells are readily available (Cellartis) and easy to culture and represent a relatively homogenous MSC cell line that can be passaged up to 20 times without any significant loss of proliferative capacity. They show similar differentiation potential to bone marrow-derived MSCs (Karlsson *et al*., 2009) but perform with a higher consistency. We used this cell line as model for MSCs where there would be less variability than with primary human MSCs, and suggest they are a good cell type to use for optimisation of mechanical stimulation parameters to subsequently be used for primary adult MSCs.

Reviewer I: Do the authors believe HDFs to have a good future in bone tissue engineering?
Authors: It is not currently known whether the cells derived from a skin biopsy which undergo osteogenesis are the HDFs themselves or progenitor cells residing amongst the HDFs. There are also relatively few studies looking at the osteogenic potential of HDFs compared with those of bone marrow or adipose-derived MSCs. Some of these studies suggest that they are a potential abundant source of autologous cells for bone tissue engineering but that more *in vivo* studies are needed to see how well they can incorporate into bone tissue. Certainly, autologous keratinocytes have proved very valuable for treating patients with extensive skin loss due to burns, wounds or chronic ulcers (MacNeil, 2007), and more recently dermal fibroblasts have been shown to have within them a population of cells which appear to be pre-angiogenic (Krajewska *et al*., 2011). The latter study suggests that the HDF cultures contain progenitor cells for several differentiation pathways.

Reviewer I: How specific is SHG to collagen detection and what else might be visualised (if anything)? Do the authors see this as a simple technique that could be picked up in different labs with the right kit?
Authors: Only molecules lacking a centre of symmetry can exhibit SHG, and this is amplified with increasing organisation of the structure. Collagen is a very strong emitter of SHG and is often very well organised, e.g. in tendon, so tissues or tissue-engineered constructs containing collagen can produce SHG. It is unsure whether other tissue components produce SHG, but if they do then it is not expected to be significant. When using multiphoton lasers to produce SHG, there is also the possibility of unwanted two-photon fluorescence from other tissue components spilling into the SHG emission window, however with the correct filters this can be minimised. We have carried out wavelength dependant studies and found that the selection of 940 nm as the excitation source not only increases SHG intensity at a given power compared with other excitation wavelengths (800-1000 nm) but also removes unwanted fluorescence from other components. The equipment required to visualise SHG is a confocal microscope and a multiphoton laser capable of excitation at wavelengths anywhere between 700 to 1060 nm. Using the right imaging conditions, any user familiar with confocal microscopy should be able to perform SHG easily, and as the sample requires no processing it is a very quick method for imaging collagen.

Reviewer II: With respect to the impact on the patient, how much skin would need to be removed to produce a clinically relevant number of low passage HDFs?
Authors: A small 3 mm skin biopsy is sufficient to extract and expand dermal fibroblasts. After 2-3 weeks of culture, tens of millions of cells will be available by passage 3.

Reviewer IV: The rocking system only allows for oscillatory flow, not for continuous flow. This may be a disadvantage for looking at different flow modes. Please elaborate.
Authors: Yes this is true. However, in mature bone (and many other tissues in the body, such as the bone marrow), fluid flow is oscillatory in nature and so we are interested in replicating this stimulus. It is true that one could not compare, for instance, unidirectional to oscillatory flow in this system. For that one could use the parallel plate flow chamber system. However, the rocker system has many advantages in that it has simple operation, a large number of samples can be stimulated at once, and it requires small volumes of media.

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