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Research paper

Cyclic tensile strain upon human mesenchymal stem cells in 2D and 3D culture differentially influences CCNL2, WDR61 and BAHCC1 gene expression levels

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ABSTRACT

It has been shown that tensile strain can alter cell behaviour. Evidence exists to confirm that human mesenchymal stem cells can be encouraged to differentiate in response to tensile loading forces. We have investigated the short-term effects of cyclic tensile strain (3%, 1 Hz) on gene expression in primary human mesenchymal stem cells in monolayer and whilst encapsulated in a self-assembled peptide hydrogel. The main aims of the project were to gain the following novel information: (1) to determine if the genes CCNL2, WDR61 and BAHCC1 are potentially important mechanosensitive genes in monolayer, (2) to determine if these genes showed the same differential expression in a 3D environment (either tethered to RGD or simply encapsulated within a hydrogel (with RGE motif)) and (3) to determine whether the mesenchymal stem cells would survive within the hydrogels over several days whilst enduring dynamic culture. In the monolayer system, real-time PCR confirmed CCNL2 was significantly downregulated after 1 h strain and 2 h latency (post strain). BAHCC1 was significantly downregulated after 1 h strain (both 2 h and 24 h latency). WDR61 followed the same trend in 2D culture. After 24 h strain and 2 h latency, BAHCC1 was significantly upregulated. We found that both types of peptide hydrogel supported viable mesenchymal stem cells over 48 h. Results of the 3D dynamic culture did not correspond with those of the 2D dynamic culture, where the BAHCC1 gene was not expressed in the 3D experiments. The disparity in the differential gene expression observed between the 2D and 3D culture systems may partly be a result of the different cellular environments in each. It is likely that cells cultured within an intricate 3D architecture respond to mechanical cues in a different and more complex manner than do cells in 2D monolayer, as is illustrated by our gene expression data.

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1. Introduction

It has been well documented that cell activity can be influenced by mechanical forces. It is possible to utilise mechanical forces, within the correct parameters, for tissue engineering purposes (Rathbone and Cartmell, 2011; Cartmell et al., 2005; Dobson et al., 2006; Cartmell et al., 2002). Mesenchymal stem cells (MSCs) have great potential for tissue engineering purposes (Hidalgo-Bastida and Cartmell, 2010), although how mechanical forces influence MSCs remains to be fully characterised. We have previously documented that mechanical forces affect MSC responses in the short term (Glossop and Cartmell, 2010, 2009a,b). Studies have also demonstrated that MSCs respond to tensile strain (of a physiological 3% strain magnitude), at least in part through activation of mitogen-activated protein kinase (MAPK) pathways (Simmons et al., 2003; Ward et al., 2007). The objective of this study was to investigate the short-term effects of tensile strain on gene expression in primary human MSCs, with particular emphasis on the role of 2D versus 3D culture. Our intention was to examine gene targets downstream of strain-induced mechanotransduction in order to further understand the gene expression response induced in MSCs. We previously published microarray data (Glossop and Cartmell, 2007) identifying approximately 200 genes that were differentially expressed in primary human MSCs exposed to 3% cyclical 1 Hz tensile strain. This current study further investigates three genes that were profiled in the aforementioned publication: CCNL2, WDR61 and BAHCC1. It is important to understand the response of MSCs in both the well characterised monolayer format but also in a 3D format which will ultimately be the end stage for tissue engineered products that may undergo mechanical stimulation. As such a self-assembled peptide hydrogel system has been utilised in this study with directed cell attachment (RGD) and unattached cell encapsulation (RGE) configurations.

Peptide-based hydrogels are hydrated structures that produce networks of nanofibres as they self-assemble upon gelation (Zhang et al., 1995; Zhou et al., 2009). Self-assembly (gelation) of a hydrogel is controlled by the environment, where specific pH or temperature can direct assembly into highly ordered structured gels known as supramolecules. This sensitivity to pH can cause ionic or covalent cross-linking to form, and changes in temperature can trigger the phase transition of the solution to a gel (Lee and Mooney, 2001). The literature indicates that self-assembling peptide scaffolds have enabled cell attachment and supported a variety of mammalian primary cells and cell lines including neurons, endothelial cells, bovine chondrocytes, human dermal fibroblasts and mouse 3T3 fibroblasts (Zhou et al., 2009; Holmes et al., 2000; Jayawarna et al., 2007, 2006, 2009; Kisiday et al., 2002). The mechanical properties of the hydrogel can affect cell adherence and gene expression, where chondrocytes have been reported to attach and proliferate to a greater extent on stiffer gels, with a compliance similar to cartilage (Jayawarna et al., 2009). The structural and physical properties of a peptide hydrogel depend upon the amino acid sequence of the peptide building blocks (Jayawarna et al., 2006), where their stiffness can be finely tuned to control the cell proliferation rate and differentiation (Wang et al., 2010). Cell adherence may be cell-specific depending upon interactions between the cell receptors and ligands within the hydrogel (Lee and Mooney, 2001).

Di- and tripeptide-based hydrogels modified with the aromatic stacking ligands 9-fluorenylmethoxycarbonyl (Fmoc) form nanometre-sized fibres within the gel when exposed to physiological conditions (Zhou et al., 2009; Jayawarna et al., 2007). The short peptide sequences, the simplest self-assembling moieties reported so far, self-assemble into anti-parallel β-sheets interlocked by π–π-stacking of the fluoronyl rings on the Fmoc groups (9-fluorenylmethoxycarbonyl) which generates cylindrical nanofibres interwoven within the gel (Zhou et al., 2009). Systems based on small peptides are advantageous because their behaviour is predictable, enabling easier design of the molecules with lower costs (Jayawarna et al., 2007).

Regarding the incorporation of biological ligands, aromatic oligopeptide derivatives such as Fmoc-FF (9-fluorenylmethoxycarbonyl-diphenylalanine) have been modified by incorporating bioactive ligands onto the terminus to make the molecule biologically active. Bioactive ligands commonly used include the RGD tripeptide sequence (Arg–Gly–Asp) which was identified in the 1980's (Pierschbacher and Ruoslahti, 1984) as a ligand specific to integrin receptors (located on the cell surface), enabling fibroblasts to bind to the extracellular matrix (ECM) (Zhou et al., 2009). The RGD sequence is found in fibronectin (an ECM protein) and is often used *in vitro* to enhance cell adhesion to a substrate, where it has recently been shown to improve cell adhesion of human dermal fibroblasts (hDF) within 3D Fmoc-peptide hydrogels (Zhou et al., 2009).

Structural analysis of a mixed system of Fmoc-protected peptides (Fmoc-FF and Fmoc-RGD) indicated that the FF and RGD peptide sequences not only produced cylindrical nanofibres interwoven within the hydrogel, but the RGD tripeptide also displayed the bioactive ligands on the fibre surface. It demonstrated that the RGD sequence not only acted as a biological ligand but also had a structural role, and overall, the hydrogel mimicked some essential features of the ECM (Zhou et al., 2009). Cellular tests have indicated that it promoted cell adhesion of encapsulated hDF through specific RGD-integrin binding (Zhou et al., 2009). Other studies have shown that Fmoc-peptide hydrogels containing an RGD sequence have promoted cell attachment of MSCs, where the self-assembled network of nanofibres were also reported to influence the attachment, proliferation and osteogenic differentiation of the cells (Hosseinkhani et al., 2006).

Although the literature indicates that various different cell types have been successfully cultured upon, or encapsulated within hydrogels (Zhou et al., 2009; Holmes et al., 2000; Jayawarna et al., 2007, 2006, 2009; Hosseinkhani et al., 2006), there is no published data which focuses upon hMSCs in 3D dynamic culture using the novel tripeptide hydrogels developed by Zhou and colleagues (Fmoc-FF:Fmoc-RGD/RGE; 2009). MSCs have the capacity to differentiate in response to specific stimuli and it has been shown that mechanical straining forces can provide the necessary signals to promote differentiation and upregulation of specific ECM components (Altman et al., 2001). Because hydrogels are flexible and can be strained, we wanted to extend our knowledge with the novel

peptide hydrogels and assess their suitability as substrates in a 3D dynamic environment (mechanical straining) and determine the expression of key genes in response to the mechanical stimuli.

The Fmoc-peptide hydrogels used in this report were based upon and developed from the reported Fmoc-FF system and its proposed supramolecular model (Smith et al., 2008), where the RGD-modified versions have been reported to support hDF (Zhou et al., 2009). They were selected based upon their unique properties and because of their proven efficacy in supporting cell viability for medical applications. Because their self-assembly is fast, this enables homogeneous cell distribution within the 3D gel, whilst the nanofibrous networks mimics part of the ECM structural architecture (Zhou et al., 2009), helping to provide a suitable or more natural environment for the cells. In practice, these peptide hydrogels are not only easy to prepare but also allow quick cell encapsulation, where structurally, the mechanical properties enable the stiffer and more stable gels to promote cell attachment and proliferation.

Fmoc-FF:Fmoc-RGD (50:50 molar ratio) was chosen mainly because the incorporated RGD sequence has been shown to promote cell adherence and proliferation at 50% Fmoc-RGD incorporation (M/M[1] of total peptide) than at lower ratios (Zhou et al., 2009). Fmoc-protected peptides are also common intermediates in peptide synthesis, with some possessing anti-inflammatory properties (Zhang et al., 2003). Such peptides are thus highly biocompatible with very little chance of causing an immune response, establishing these as suitable substrates for tissue engineering applications. Fmoc-FF/RGE was used in addition to Fmoc-FF/RGD to act as a negative control, where it alternatively contained glutamic acid (E), an analogue to aspartic acid (D).

2. Materials and methods

2.1. Peptide hydrogel synthesis

2.1.1. Fmoc-FF:Fmoc-RGD/RGE

The Fmoc-FF powder was purchased from Bachem, Switzerland and the Fmoc-FF:RGD/RGE peptide powders from Cambridge Research Biochemicals, UK, all with a purity of at least 97%. Synthesis has been previously described (Zhou et al., 2009). Briefly, the peptide powders (Fmoc-FF, Fmoc-RGD and Fmoc-RGE) were sterilised by ultraviolet light, sterile water was added to give a concentration of 20 mM for each peptide and the pH adjusted with 0.5 M NaOH or 0.5 M HCL (Fisher Scientific, UK and Sigma Aldrich, UK respectively). Fmoc-FF and Fmoc-RGD (or Fmoc-FF and Fmoc-RGE-control) were then mixed 50:50 to give a final concentration of 10 mM of each peptide in the solution, and the pH adjusted to 7.0.

2.2. Cell culture

2.2.1. Cell culture and subculture

Primary human mesenchymal stem cells (Lonza, UK) were cultured in Dulbecco's Modified Eagle's Medium containing 1% L-glutamine (DMEM; PAA, UK) in T75 tissue culture flasks (Nunc, Fisher Scientific, UK) supplemented with 10% foetal calf serum (Biosera, UK) and 1% Antibiotic–Antimycotic solution (PAA, UK). Cultures were incubated at 37 °C with 5% CO_2 humidity and the media was changed every 3 days. Cells were grown in monolayer to 80% confluency and trypsinised (1× trypsin-EDTA, Gibco, UK) for subculture. They were harvested at passage 5 and resuspended in supplemented DMEM to the desired concentrations for 2D and 3D culture experiments.

2.2.2. Cell seeding (2D cell culture)

Volumes of 350 μl of cell suspension were dispensed into the central rectangular straining region of each well of Flexcell uniflex culture plates (pronectin coated; Dunn Lab, Germany). This was spread evenly to ensure homogeneous distribution with coverage of approximately 1.5×10^4 cells/cm^2. After incubating for 2–3 h at 37 °C and 5% CO_2 humidity to allow cell attachment, the seeding media was removed and replaced with fresh supplemented media (DMEM), where the cells continued to incubate in monolayer (37 °C, 5% CO_2).

2.2.3. Cell encapsulation and formation of cell–gel constructs (3D cell culture)

The cell suspension (approximately $2.5–4.5 \times 10^5$ cells/ml) was mixed with the pre-warmed peptide hydrogel solution (using equal volumes of cell suspension to hydrogel). A computer-controlled vacuum was used to create loading channels (troughs) in each well of the tissue train culture plates (pronectin coated; Dunn Lab, Germany) (Fig. 1(a)). 200 μl of the cell–gel solution was dispensed into the linear trough of each well (approximately $2.5–4.5 \times 10^4$ cells/well; Fig. 1(b)), followed by incubation at room temperature. Linear cell–gel constructs were formed approximately 30 min thereafter. The vacuum was then released and the constructs maintained at 37 °C and 5% CO_2 humidity (Fig. 1(c)). After 24 h, the constructs were strained (for 2 or 24 h) whilst incubating at 37 °C and 5% CO_2 humidity (Fig. 1(d)), with subsequent analysis after the post strain latency period.

2.2.4. Dynamic cell culture—mechanical loading

Mechanical loading was applied 24 h post seeding. We used a commercial system, the Flexcell FX-4000T tension plus system (Flexcell International, USA), to apply a uniaxial cyclic tensile strain to cells in 2D monolayer and to the 3D cell–gel constructs. The loading regimens consisted of different straining durations followed by different latency periods, all with 3% strain at 1 Hz, during incubation at 37 °C with 5% CO_2 humidity. The corresponding static controls were not loaded. The loading regimens for 2D and 3D culture were as follows:

(1) 1 h loading, 2 h latency post straining;
(2) 1 h loading, 24 h latency post straining;
(3) 24 h loading, 2 h latency post straining.

2.3. Cell viability—live–dead assay

For 3D experiments, the viability of the encapsulated cells was tested at each time point (2 h and 24 h post straining, and corresponding static controls) using a

[1] M/M—molar ratio.

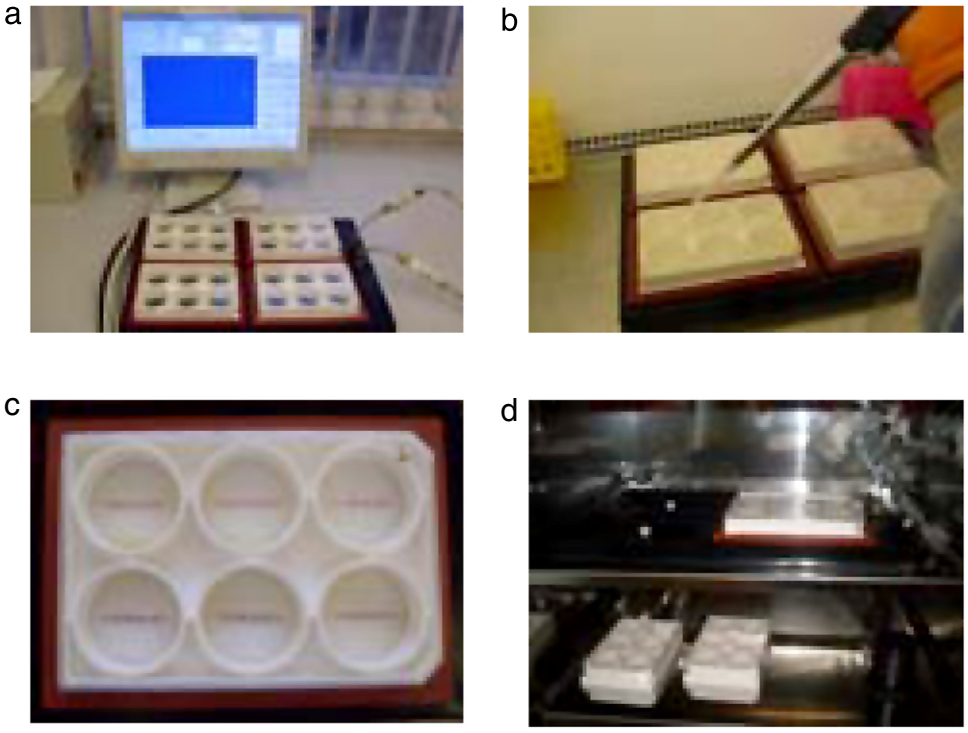

Fig. 1 – Cell seeding procedure. Loading channels (troughs) were created in each well using vacuum (a), where the gel-encapsulated cells were seeded into each trough (b), forming a strip of cell–gel construct (c), which were finally strained 24 h later (d).

Live–Dead staining kit (Molecular Probes, UK). Each cell–gel construct (sample/control) was placed into a glass bottomed dish (Wilco-dish) for viewing, where a 250 μl aliquot of the live–dead solution was added, containing concentrations of 4 μM EthD-1 (ethidium homodimer-1) and 2 μM calcein AM. This was incubated for 20–25 min, the stain was removed, and samples rinsed with sterile 1× phosphate buffered saline (PAA, UK). The samples were then viewed with a laser scanning confocal microscope (Leica SP5, Advanced Fluorescence software version 2.3.0) to obtain a 3D z-stack using a 10× objective, with excitation/emission filters 495/515 nm for calcein and 495/635 nm for ethidium homodimer-1. Viable cells stained green and necrotic cells red ($N = 1$).

2.4. *Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)*

In view of our previous microarray experiments investigating gene expression in MSCs exposed to tensile strain (Glossop and Cartmell, 2007), three genes of potential interest were selected for analysis herein. These genes were CCNL2 (cyclin L2), WDR61 (WD repeat domain 61) and BAHCC1 (BAH domain and coiled-coil containing 1). Quantitative real-time RT-PCR was used to determine the gene expression profiles from cells cultured in 2D and in 3D.

At each time point (2 h and 24 h post straining), RNA extraction and reverse transcription was carried out on the loaded samples and static controls before proceeding with real-time RT-PCR. Prior to RNA extraction from the 3D constructs, cell isolation was carried out by adding 1× sterile phosphate buffered saline (PBS (PAA, UK)) to each well and mixing with a pipette to disrupt the cell–gel constructs, where the solution of each sample/control was then transferred into 25 ml universal tubes and centrifuged at 1000 RPM for 6 min. The upper half of the supernatant was discarded and the gel debris retained in the lower portion of the tube. For 2D cell culture, cell lysis and RNA extraction was carried out using guanidine isothiocyanate and the standard RNA extraction protocol (phenol/chloroform), followed by reverse transcription (QuantiTect Reverse Transcription kit, Qiagen), whereas a MicroMacs one-step cDNA kit (Miltenyi Biotec Ltd, UK) was used for the 3D cell culture. Real-time RT-PCR was performed using TaqMan Gene Expression Assays specific for CCNL2, WDR61, BAHCC1 and the house keeping gene GAPDH (TaqMan, Applied Biosystems, UK) with a StepOne PCR system, model V2.1 (Applied Biosystems, UK). Relative gene expression was calculated using the delta–delta Ct method.

2.5. *Cell numbers and cell surface area quantification*

CellProfiler 2.0 (cell image analysis software, Broad Institute, USA) was used to determine cell numbers and semi-quantitatively analyse cell surface area. A primary object identifier function was used to select green objects above a specific threshold (viable cells), which excluded background fluorescence of the hydrogels, but include the individual cells, and calculated the cell number (number of objects present). A measurement function to estimate cell surface area was used by correlating the calculated pixel numbers to surface area of

cells in the 2D images, where the treated cells were compared with their corresponding controls and presented as a mean value.

2.6. Statistical analysis

Firstly, the data was tested for normality (Graphpad Instat, USA). Normally distributed data was tested with an unpaired two-tailed student *T*-test for each gene of interest between the loaded samples and static controls of each experiment, whereas the Mann–Whitney *U* test was performed on non-parametric data (version 2.7.7, StatsDirect Ltd, UK). The data were expressed as the mean ± standard error of the mean (SEM), where p-values <0.05 were considered to be statistically significant.

3. Results

3.1. Cell viability—live–dead assay (3D culture)

Fluorescent images of cells encapsulated in each hydrogel type (cell–gel constructs) are shown in Figs. 2 and 3. The images indicate that many viable cells were present in the loaded samples and static controls, demonstrating that the gel and mechanical straining forces were not detrimental to cell survival. There were no marked differences between the loaded samples and static controls, or between the two hydrogel types, where all the cells acquired the same rounded morphology.

3.2. Cell numbers and cell surface area quantification

Cell numbers for each gel sample were calculated (using CellProfiler software on the images presented in Figs. 2 and 3) and can be seen in Table 1. The number of cells encapsulated in the RGD and RGE gels were higher in static conditions (regardless of loading regime), except in the case of the RGE modified hydrogel (1 h of strain, 24 h latency) as can be seen in Table 1 (N.B. no statistics are available for this data as only one sample (the image presented in Figs. 2 and 3) for each experiment was used in this calculation). Differences in the individual cell size in these same images (where spreading cells may be assumed to have a greater surface area to those with a spherical morphology) can be seen in Fig. 4. Two of the samples analysed demonstrated a larger surface area in the static control cells (RGD—1 h strain, 24 h latency and RGE—1 h strain, 2 h latency, $p < 0.001$). One of the samples analysed showed a larger surface area in the loaded sample (RGE—1 h strain, 24 h latency, $p < 0.001$). The remaining samples analysed showed no significant difference in cell surface area between the strained samples and corresponding controls.

3.3. Quantitative real time RT-PCR

3.3.1. Dynamic cell culture (2D)

The loading regimens used for the 2D monolayer cell culture were the same as those used in 3D culture. Application of different loading regimens to hMSCs in monolayer revealed that the shortest straining duration (1 h) caused

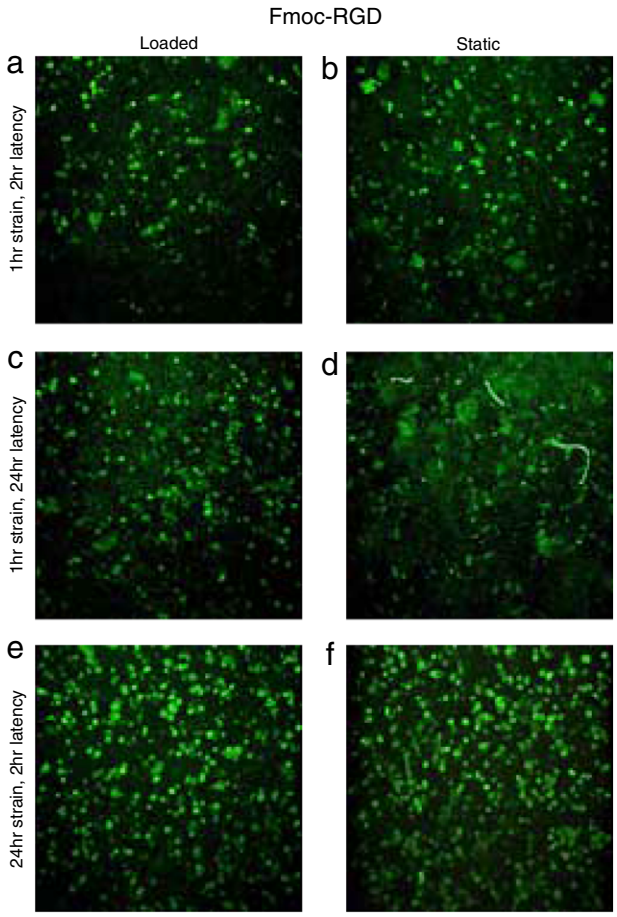

Fig. 2 – Cell viability in the modified tripeptide gel (Fmoc-FF:Fmoc-RGD) after 1 h strain, 2 h latency (a), 1 h strain, 24 h latency (c), 24 h strain, 2 h latency (e) and the corresponding static controls (b, d, f). Images are 3D z-stacks at 10× magnification. Viable cells are green, necrotic cells red. All cells were viable (colour image available online).

downregulation of all three genes in comparison with the static controls, regardless of the length of latency period. In contrast, the longer straining period (24 h) caused upregulation (Fig. 5). The only gene however, to consistently show significance ($p < 0.05$) was BAHCC1, where it was downregulated (3.0 and 2.6-fold) after 1 h of strain (2 and 24 h of latency respectively) and then became upregulated 5.4-fold after 24 h of strain. The CCNL2 gene showed significance in one experiment only where it was downregulated 3-fold (1 h of strain, 2 h latency).

3.3.2. Dynamic cell culture (3D)

The results revealed that the shortest loading duration followed by the shortest latency period produced the greatest effect upon the gene expression levels in both hydrogel types studied (Fig. 6).

Although there was some evidence for upregulation of WDR61 in both loaded hydrogels with the other two regimes analysed (1 h strain, 24 h latency; and 24 h strain, 2 h latency), these were not significantly different from the static

Table 1 – Viable cell numbers from fluorescent images (shown in Figs. 2 and 3) calculated by CellProfiler software.

Peptide hydrogel	Duration of straining period and latency period (h)	Cell number
Modified tripeptide hydrogel (Fmoc-FF:Fmoc-RGD)	1 h strain, 2 h latency	196
	Static	375
	1 h strain, 24 h latency	348
	Static	413
	24 h strain, 2 h latency	408
	Static	467
Modified tripeptide hydrogel (Fmoc-FF:Fmoc-RGE)	1 h strain, 2 h latency	171
	Static	267
	1 h strain, 24 h latency	208
	Static	185
	24 h strain, 2 h latency	148
	Static	304

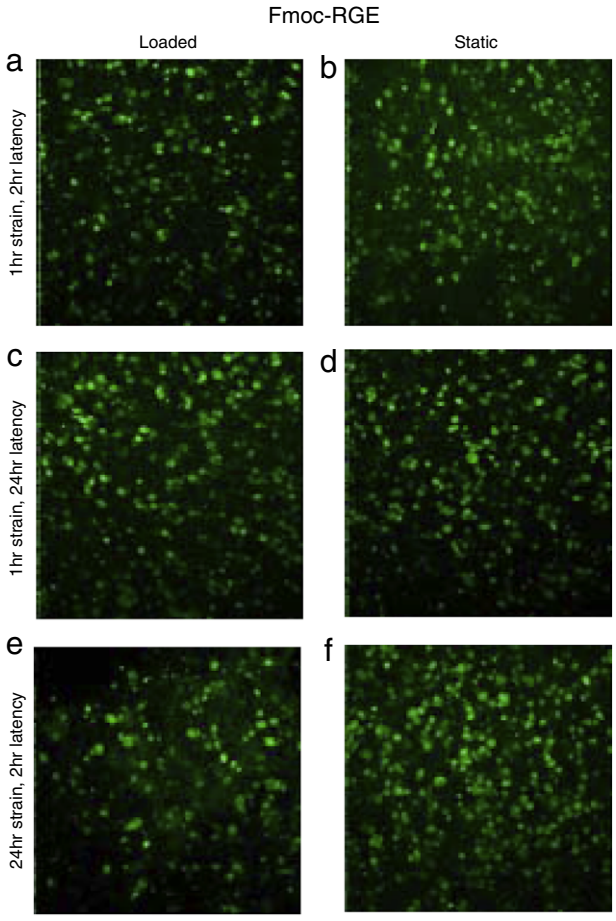

Fig. 3 – Cell viability in the modified tripeptide gel (Fmoc-FF:Fmoc-RGE) after 1 h strain, 2 h latency (a), 1 h strain, 24 h latency (c), 24 h strain, 2 h latency (e) and the corresponding static controls (b, d, f). Images are 3D z-stacks at 10× magnification. Viable cells are green, necrotic cells red. All cells were viable (colour image available online).

controls. In these later two straining regimes, CCNL2 was not expressed in the RGE-modified gel and BAHCC1 was not expressed in either type of hydrogel, whether static or strained and regardless of the loading regimen used.

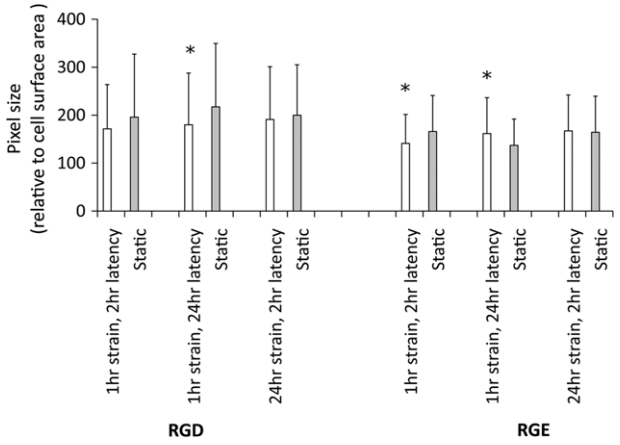

Fig. 4 – Mean cell surface area (pixels) of viable cells as imaged by fluorescent image (seen in Figs. 2 and 3), comparing loaded and static constructs for both the RGD and RGE-modified hydrogels. The data are expressed as the mean ± standard error (∗ $p < 0.001$).

4. Discussion

The main aims of this study were to gain the following novel information: (1) to determine if the genes CCNL2, WDR61 and BAHCC1 are sensitive to tensile strain in monolayer culture; (2) to determine if these genes showed the same differential expression pattern in 3D culture (either tethered to RGD or simply encapsulated within a hydrogel); and (3) to determine whether viability could be maintained within hydrogels over several days and whilst enduring dynamic culture.

In order to address these aims, we have studied the effects of mechanical loading upon cells cultured in both 2D monolayers and cells in a 3D environment—encapsulation in a hydrogel. We have compared the expression profiles of three specifically identified genes in both static and tensile strain loaded cells in a monolayer environment. As cell morphology is important in the response of a cell to mechanical deformation, we felt it was also important to elucidate the expression of these same three genes in a 3D environment. To this end, we have utilised a Fmoc based hydrogel to encapsulate the primary human mesenchymal stem cells and loaded the samples under the same conditions. This Fmoc gel contained either RGD or RGE sequences for the cell to adhere

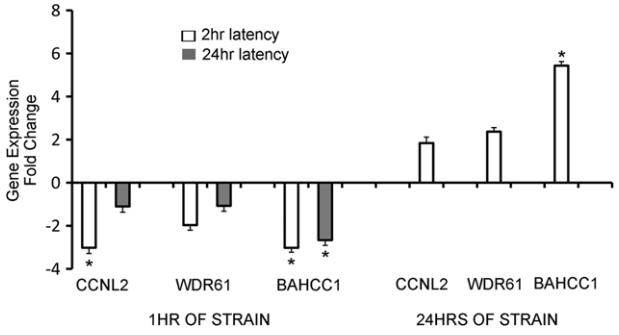

Fig. 5 – Results of cell monolayer gene expression analysis for the genes CCNL2, WDR61 and BAHCC1 showing relative fold changes between the loaded samples and static controls after loading for 1 h or 24 h, followed by a 2 h or 24 h latency period (3%, 1 Hz). The data are expressed as the mean ± standard error of the mean (SEM; $N = 6$). $* p \leq 0.05$.

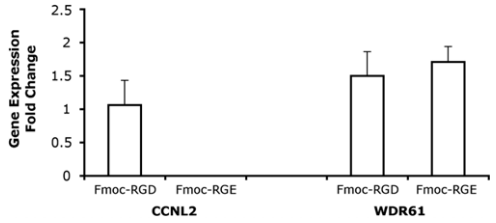

Fig. 6 – Gene expression analysis for the genes CCNL2 and WDR61 after loading for 1 h, followed by a 2 h latency period (3%, 1 Hz). Relative fold changes between the loaded samples and static controls are shown for both tripeptide hydrogels, Fmoc-FF:Fmoc-RGD and Fmoc-FF:Fmoc-RGE. The data are expressed as the mean ± standard error of the mean (SEM; $N = 5$). CCNL2 was not detected in the RGE-modified gel, and BAHCC1 was not detected in either hydrogel type.

to (or not) respectively. This allowed us further to speculate as to the mechanotransduction mechanism if any significant differences were seen (in terms of tensile strain sensing via integrins or indirectly sensed by shear stress initiated by hydrogel/extracellular fluid shift).

Our previous microarray-based study of hMSCs in 2D identified 229 and 269 genes, and 405 and 179 genes that were differentially expressed following 1 h and 24 h strain durations respectively (at 2 and 24 h latency post strain) (Glossop and Cartmell, 2007). From this data we focused on three genes of potential interest, namely CCNL2, WDR61 and BAHCC1. Although these genes have been identified and mapped, little is currently known about their functions. CCNL2 codes for cyclin L2, a recently identified member of the cyclin gene family (Yang et al., 2004), a family whose primary function is to regulate cell cycle progression. Cyclin L2 is thought to regulate the pre-mRNA splicing process, possibly in a cell/tissue-specific manner (Yang et al., 2004). Separately, inhibition of cellular differentiation and suppression of differentiation-related genes has been reported *in vitro* in a murine embryonic carcinoma cell line overexpressing cyclin L2 (Zhuo et al., 2009). These authors and Yang et al. (2004) also found evidence of growth arrest and apoptosis in cells overexpressing cyclin L2. WDR61 (also known as Ski8) is a subunit of the SKI complex. Although not well characterised in humans, yeast SKI complex (together with the exosome) is thought to mediate 3′-RNA degradation in the cytoplasm (Wang et al., 2005). It has been suggested that when human SKI interacts with the human PAF complex, the SKI complex becomes associated with transcriptionally active genes and the PAF complex coordinates transcriptional events (Zhu et al., 2005). Very little is known about the BAH and coiled-coil domain-containing protein 1 encoded by the BAHCC1 gene. It contains a BAH (bromo-adjacent homology) domain (Nicolas and Goodwin, 1996) and also a coiled-coil structural motif. There are 2 classes of coiled-coil proteins, short coiled-coil domains and long coiled-coil proteins. The short coiled-coil domains (leucine zippers) are often found as homo or heterodimerization motifs in transcription factors, whereas the long coiled-coil proteins function as anchors for the regulation of protein positioning in the cell and organising cellular processes such as cell division (Rose et al., 2004).

Gene expression analysis of the 2D dynamic experiments (RT-PCR) indicated that the shorter straining duration (1 h) produced downregulation of gene expression (for both short and longer latency periods), whereas the longer straining duration caused upregulation (at the shorter latency period). However, only BAHCC1 remained consistently significant throughout, showing downregulation (3.0 and 2.6-fold) and upregulation (5.4-fold) under different strain durations. This suggests that pathways associated with BAHCC1 gene expression may have become more or less active with different strain durations. Although the BAHCC1 protein product remains to be properly characterised, the BAH domain itself is thought to mediate specific protein–protein interactions (Callebaut et al., 1999). BAH domain-containing proteins include a number of proteins principally concerned with the regulation of gene transcription, gene repression and chromatin remodelling (Callebaut et al., 1999; Goodwin and Nicolas, 2001). What function the BAH and coiled-coil domain-containing protein 1 serves in relation to these processes is currently unknown but our data indicate that tensile strain exposure differentially regulates expression of this gene. Functional characterisation of this protein will help to establish the implications of this finding.

The application of mechanical stimuli is known to be an important factor in regulating gene expression and consequently MSC differentiation. The cellular micro-environment itself may also contribute in the regulation of cell behaviour. We investigated two novel tripeptide hydrogels for tissue engineering applications; Fmoc-FF modified with an RGD sequence, and Fmoc-FF modified with an RGE sequence, RGE representing a chemical analogue of and control for RGD. The hypothesis was that incorporation of an RGD sequence into the peptide hydrogel would encourage cell tethering to the gel. Initially, we needed to confirm that the hydrogels were biocompatible and would support human MSCs, but it was also important to demonstrate that the loading regimen was not detrimental to the cells whilst suspended within a 3D environment (for both the RGD and RGE gels).

To monitor the condition of the MSCs, we assessed cell viability during 3D culture using *in situ* fluorescent imaging. We quantified viable cell number in the loaded and static hydrogels to provide additional information as to whether mechanical loading was affecting viability or influencing proliferation. Cell numbers generally appeared lower in the loaded samples, which may indicate a transition towards differentiation, rather than proliferation.

The literature indicates that RGD-modified Fmoc peptide hydrogels are able to promote cell adhesion of MSCs (Hosseinkhani et al., 2006) and hDF through specific RGD-integrin binding within 24 h (Zhou et al., 2009). Semi-quantitative data was obtained from the confocal images presented in Figs. 2 and 3 to make comparisons in cell number and surface area between the loaded and static samples (where it was assumed that spread cells would have greater surface area than spherical-shaped cells as cell volume would be consistent). The surface area of each cell was estimated from the fluorescent images by calculating the pixel number and correlating it to the cell surface area. The demonstration of the majority of the samples cell size not differing is to be expected of this encapsulated gel system. The RGD in these gels is proposed to allow direct cell contact with a supporting substrate and hence efficient transfer of tensile strain directly to the cell. The RGE gels are used as a negative control to encapsulate the cells and hence provide a similar cell morphology/size but without direct tethering of the cells to a supporting substrate. Therefore the similarity in cell size as shown in Fig. 4 confirms the maintenance of cell surface area. Three samples did demonstrate a significant difference in cell surface area (RGD—1 h strain, 24 h latency; RGE—1 h strain, 2 h latency; and RGE—1 h strain, 24 h latency; all $p < 0.001$). However, these differences were not large fold changes (~10%–15% difference in total cell surface area). Further work investigating the role of WDR61 in cell spreading should now be performed as the same group that demonstrated a significant difference in expression of this gene at 1 h strain 2 h latency also showed an alteration in cell surface area. However, before this further work should commence, it would be wise to perform supplementary 3D cell morphology/size analysis as the data presented in this study was taken from single confocal z stacks of each sample rather than multiple imaging sets. As such, we recommend further visualisation techniques such as multiphoton imaging and 3D rendered microcomputed tomography scanning to accurately quantify cell size and shape and help to provide a better insight into the effects of mechanical straining upon cell morphology and corresponding gene expression. Although our results indicate that the MSCs remained viable for 48 h in both gel types, they retained a rounded morphology in the RGD-modified peptide gel, which contrasts with the current literature. Therefore, longer culture periods or modifications of the hydrogel may be necessary in order to better promote cell attachment and the development of a viable 3D hydrogel culture system for MSCs.

Analysis of the 3D dynamic experiments demonstrated that the shortest loading duration followed by the shortest latency period (1 h strain, 2 h latency) produced the greatest effect upon the gene expression levels of the cells in both hydrogel types studied. It is possible that during the longer straining durations, negative feedback mechanisms were activated, thereby inhibiting expression of the genes investigated. Genes may have become switched off at later time points after the straining ceased, suggesting that the changes in gene expression were transient and that mechanotransduction pathways had become inactive long before the data collection. The hydrogel itself could have also been a factor causing the genes to become switched off as demonstrated by gene expression in 2D monolayer culture compared to the absence of gene expression in 3D hydrogels (with the corresponding loading regimen). In the case of BAHCC1, this gene was not expressed at all in the 3D culture, but was in 2D, which further suggests that the 3D environment may have contributed to the silencing of gene expression observed. At this time it is difficult to speculate on the specific mechanotransduction pathways that regulate expression of this gene in MSCs and how these pathways are differentially influenced by 3D culture. Further investigation is currently underway to investigate if expression of this potentially important mechanosensitive gene is also absent in other 3D culture systems (such as collagen and fibrin gels). Gene expression of WDR61 and CCNL2 was retained when cells were cultured within the 3D hydrogel system. Of some interest was the trend observed with the shorter time points in the 3D hydrogels, where the mean expression of WDR61 was slightly elevated in both the RGD and RGE gels (although not reaching statistical significance). This suggests that engagement of integrin receptors on the cell surface may not always be required for mechanotransduction-induced differential expression of this and possibly other genes. This contrasts with our finding of a trend for CCNL2 upregulation ($p = 0.53$) present only in the RGD hydrogel and not in the RGE hydrogel, suggesting a key role for integrin binding in activation of this gene.

It was also noted that expression of CCNL2 in 2D culture was significantly downregulated following short-term tensile strain exposure, but with suggestion of the opposite response with long-term exposure. Given previous reports indicating that cyclin L2 can inhibit cellular proliferation (Yang et al., 2004; Zhuo et al., 2009), and that longer-term tensile strain application inhibits MSC proliferation (Simmons et al., 2003; Kearney et al., 2010), it is not without some justification to speculate that our finding of CCNL2 expression during long-term strain exposure may indicate a role for cyclin L2 in the strain-induced suppression of MSC proliferation. We are clear however, that our current data are unable to address this directly and that investigation of the molecular basis of MSC proliferation is warranted to explore this notion further.

We also considered the mechanical properties of the hydrogels and the flexible membranes of the tissue train plates, specifically the elastic modulus. Previous studies determined the modulus of elasticity for Fmoc-FF:Fmoc-RGD and Fmoc-FF:Fmoc-RGE hydrogels (50:50) to be approximately 0.85 kPa and 0.31 kPa respectively (Zhou et al., 2009), while Flexcell International Corporation, USA, indicate that the flexible membranes of their uniflex culture plates have a modulus of 930 kPa in the straining region. It is thus likely that the greater stiffness of the culture plates was also a factor influencing the gene expression we observed, eliciting different affects upon cells seeded directly onto the surface

of the plates (2D culture) in comparison to those suspended in the hydrogels (3D), which were far less stiff. It is worth noting however that the static controls used in this series of experiments were also performed in Flexcell plates (not in standard tissue culture plastic 6 well plates). As such, the cell response detected results from the imparted tensile strain of the Flexcell system, rather than a mechanical force sensed from the substrate to which the cells are attached.

As a consequence of its design, the system we used to apply tensile strain to our 2D and 3D cultures also imparted an unmeasured, nominal fluid shear stress through disturbance of the culture media. This is actually a feature common to other tensile strain systems also. Nevertheless, it remains possible that the genes examined herein may have been influenced, in part, by exposure to shear stress in addition to the intended tensile strain. To address this, we are currently conducting comparative 2D and 3D experiments in which we intend to characterise MSC gene expression responses following exposure to similar magnitude shear stresses. A further point of enquiry will be to specifically explore the mechanotransduction pathways that are activated following tensile strain exposure of MSCs cultured in our 3D system. Evidence from 2D culture indicates changes in gene expression and signalling via multiple pathways, including MAPK pathways, following exposure to tensile strain (Simmons et al., 2003; Ward et al., 2007; Kearney et al., 2010). However, whilst a small number of studies have investigated tensile strain application in 3D cultures of MSCs (Sumanasinghe et al., 2006, 2009), analysis of the mechanotransduction pathways involved remains to be addressed. Thus, examination of these in our 2D and 3D culture systems will help to unravel the biochemical interactions coupling strain exposure with cellular/nuclear responses, including changes in gene expression such as those observed herein. Although differential expression of the genes examined was not consistently significant in our 3D culture system at the time points investigated, further studies utilising differing strain modalities are warranted to more fully elucidate the role of these and other genes in tensile strain-induced mechanotransduction in MSCs.

5. Conclusion

We report the potential of human MSC encapsulation within novel tripeptide hydrogels that has not previously been reported. The peptide hydrogels studied demonstrated their ability to support viable human MSCs in a 3D environment over 48 h. We have corroborated in a 2D culture system the differential gene expression of three novel and potentially important mechanosensitive genes, namely CCNL2, WDR61, and BAHCC1. Investigation of these genes in 3D hydrogel constructs identified responses to tensile strain which were distinct from those observed with 2D culture. Further work is required to more fully characterise the role of these genes in MSC mechanotransduction and cell activity.

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