DIFFERENCES IN HUMAN MESENCHYMAL STEM CELL SECRETOMES DURING CHONDROGENIC INDUCTION

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Abstract

Mesenchymal stem cells (MSCs) can be induced towards chondrogenesis through the application of chondrogenic stimuli such as transforming growth factor-β (TGF-β) or by multiaxial mechanical load. Previous work has showed that the chondrogenic effect of multiaxial load on MSCs is mediated by the endogenous production of TGF-β1 by stimulated cells. This work compared the effects of TGF-β1 stimulation and multiaxial mechanical load on the secretomes of stimulated cells. MSCs were seeded into fibrin-poly(ester-urethane) scaffolds and chondrogenically stimulated with either TGF-β1 or mechanical load. The culture media was collected and analysed for 174 proteins using a cytokine antibody array. The results of the secretome analysis were then confirmed at a gene expression level by real-time PCR. As results implicated nitric oxide (NO), the media nitrite content was also determined as an indirect measurement of media NO levels. Results showed that TGF-β1 stimulation and mechanical load lead to similar changes in factors such as BLC, VEGF and MMP13, whilst differences in detected levels were seen for factors including leptin, MDC, MIP3α and LAP. Gene expression analysis confirmed significant changes in four factors: angiopoietin 2, GROα, MMP13 and osteoprotegerin. After one week in culture the media nitrite content was significantly higher in loaded groups than both control and TGF-β1 stimulated groups, suggesting this may be a major therapeutic target. These data show that despite clear similarities, TGF-β1 stimulation and load have distinct effects on MSCs and are not analogous. This study has identified a number of potentially novel targets for tissue engineering, these data may also be useful for improving rehabilitation protocols *e.g.* after microfracture.

Keywords: Secretome, paracrine signalling, regenerative medicine, cartilage repair, cytokines.

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Abbreviations

ALCAM – Activated leukocyte cell adhesion molecule
αMEM – Alpha minimal essential medium
BLC – B lymphocyte chemoattractant
BMP – Bone morphogenic protein
DMEM – Dulbecco's modified Eagle medium
DR6 – Death receptor 6
FBS – Foetal bovine serum
GRO – Growth related oncogene
hBMSC – Human bone marrow derived stem cell
LAP – Latency associated peptide
MDC – Macrophage derived chemokine
MIF – Macrophage migration inhibitory factor
MIP3α – Macrophage inflammatory protein 3 alpha
MMP13 – Matrix metalloproteinase 13
MSC – Mesenchymal stem cell
NO – Nitric oxide
OPG – Osteoprotegerin
PCR – Polymerase chain reaction
PDGF – Platelet derived growth factor
TGF-β – Transforming growth factor beta.
TIMP – Tissue inhibitor of metalloproteinase
uPAR – Urokinase receptor
VEGF – Vascular endothelial growth factor

Introduction

Currently, standard protocols for the induction of chondrogenesis in human mesenchymal stem cells (MSCs) *in vitro* involve the culture of cells in a 3D environment (*e.g.* in a pellet/micromass culture or encapsulated with in a hydrogel) coupled with the exogenous application of an isoform of TGF-β (Barry *et al.*, 2001; Johnstone *et al.*, 1998). This chondrogenic response is induced by TGF-β and mediated by SMAD signalling proteins, which results in an increase in the expression of chondrogenic markers such as the transcription factor Sox9 and the matrix molecules aggrecan and type II collagen (Furumatsu *et al.*, 2005; Hellingman *et al.*, 2011; Johnstone *et al.*, 1998). Following chondrogenesis, both *in vitro* (Johnstone *et al.*, 1998) and *in vivo* (Mueller and Tuan, 2008), MSCs have a tendency to progress towards hypertrophy, which is marked by the expression of molecules such as collagen type X and MMP13 (D'Angelo *et al.*, 2001; D'Angelo *et al.*, 2000; Johansson *et al.*, 1997). This progression of MSCs from a cartilage producing phenotype into a hypertrophic phenotype is reminiscent of the behaviour of mesenchymal progenitor cells during bone formation through endochondral ossification and presents a major barrier to the use of MSCs for the clinical repair of cartilage

tissue (Goldring *et al.*, 2006; Mackie *et al.*, 2011; Mueller and Tuan, 2008; Sheehy *et al.*, 2015).

Previous work has shown that chondrogenesis of human bone marrow derived MSCs can be induced in the absence of exogenous recombinant TGF-β through the application of multiaxial load (Li *et al.*, 2010b; Schatti *et al.*, 2011). In this system multiaxial load refers to a combination of shear and compression, which mimics the loading of a diarthrodial joint *in vivo* (Grad *et al.*, 2005; Wimmer *et al.*, 2004). This combination of mechanical stimuli provides an *in vitro* model for studying the response of constructs designed to facilitate cartilage repair in a simulated joint mechanical environment. Further investigation showed that the induction of chondrogenesis in this system is linked to the TGF-β signalling pathway (Li *et al.*, 2010a). The application of multiaxial load to MSCs seeded within fibrin-poly(ester-urethane) scaffolds leads to the endogenous production of TGF-β1 by the cells, which then drives the chondrogenesis of cells within the scaffold. Blocking TGF-β receptor 1 signalling prevents this induction of chondrogenesis in response to multiaxial mechanical load, showing that the endogenously produced TGF-β1 acts through TGF-β1R to induce chondrogenesis in response to load (Li *et al.*, 2010a). However, it is not currently clear if load induced chondrogenesis is exclusively TGF-β mediated.

MSCs are known to respond to specific stimuli by generating matrix molecules and adopting cellular characteristics associated with tissues such as bone, cartilage and adipose. In addition to these matrix molecules, MSCs also secrete a large number of bioactive factors (Caplan, 2007; Czekanska *et al.*, 2014). These factors provide information on the phenotype of the cells producing them, as well mechanistic information about changes that occur in cells in response to certain stimuli. Crucially, a cell's secretome provides an indication of what effect the cell might have on endogenous cells in a clinical situation. The secretomes of MSCs are increasingly being studied (Stoddart *et al.*, 2015) and the secretomes of MSCs from various sources have been investigated in a number of culture conditions in recent years (Haynesworth *et al.*, 1996; Kinnaird *et al.*, 2004; Liu and Hwang, 2005; Rehman *et al.*, 2004; Sze *et al.*, 2007). These studies have focused on different aspects of MSC biology including; angiogenesis, myogenesis and osteogenesis (De Lisio *et al.*, 2014; Hoch *et al.*, 2012; Oskowitz *et al.*, 2011). A number of papers have also investigated the effects of chondrogenic stimulation on the MSC secretome (Arufe *et al.*, 2011; Bara *et al.*, 2014; Grassel *et al.*, 2009; Rocha *et al.*, 2014; Rodriguez *et al.*, 2015). These studies have demonstrated the upregulation of factors such as VEGF, MMP13 and TIMP1 and 2. To the authors knowledge, no investigations have been made into the secretome of MSCs undergoing chondrogenesis in response to multiaxial load, or compared the secretomes of MSCs responding to mechanical load and TGF-β1 stimulation.

The aim of this study was to compare the secretomes of unstimulated hBMSCs, hBMSCs cultured with TGF-β1 in order to induce chondrogenesis, and hBMSCs cultured in the absence of TGF-β1 but with multiaxial mechanical load in order to induce chondrogenesis. An early time point was investigated as it is relevant during the period of cell engraftment and the initiation of healing. It was hypothesised that by analysing the secretomes of MSCs cultured in different chondrogenic culture conditions, and in different mechanical environments, it would be possible to compare the effects of mechanical load and TGF-β1 stimulation on the chondrogenic induction of hBMSCs in order to gain greater understanding of the response of cells to these two different stimuli. This knowledge could also be used to identify interesting and potentially novel factors for MSC based tissue engineering.

Materials and Methods

MSC isolation, culture and scaffold seeding

MSCs were harvested from bone marrow aspirates collected during routine operations with full-ethical approval (KEK-ZH-NR: 2010-0444/0). Mononuclear cells were isolated from whole bone marrow of three donors (males aged 22, 38 and 77 years old) collected from vertebral bodies using a Ficoll density separation method (Histoplaque-1077, Sigma-Aldrich, Buchs, Switzerland). Mononuclear cells were then seeded at a density of 50,000 cells/cm^2 and left to attach for 96 h in expansion medium consisting of alpha Minimum Essential Medium (αMEM) (Gibco, Carlsbad, USA), 10 % MSC screened foetal bovine serum (FBS) (Pan Biotech, Aidenbach, Germany), 5 ng / mL basic fibroblast growth factor (bFGF) (Peprotech, Rocky Hill, USA) and 1 % penicillin/streptomycin (Gibco, Carlsbad, CA). Passage 1 MSCs were frozen and stored in liquid nitrogen. Proliferation of MSCs after storage was carried out using αMEM 10 % FBS with 5 ng / mL bFGF. Passage 3 or 4 cells were seeded into 8 mm × 2 mm fibrin-poly(ester-urethane) scaffolds (fibrin from Baxter, Vienna, Austria) as previously described (Li *et al.*, 2009). Briefly, 2 million MSCs per scaffold were resuspended in 37.5 μL of fibrin (33 mg/mL). The fibrin/cell mixture was then mixed with 37.5 μL of thrombin (0.5 U/mL) before being infused in to a poly(ester-urethane) sponge and then left at 37°C 90 % humidity and 5 % CO_2 to cure.

Culture conditions

Three different culture conditions were used for this experiment. Constructs were either cultured in a free swelling environment with chondropermissive medium (consisting of Dulbecco's modified Eagle medium 4.5 g/L glucose (Gibco, Carlsbad, USA), sodium pyruvate 0.11 g/L (Sigma-Aldrich, Buchs, Switzerland), L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate 50 μg/mL (Sigma-Aldrich, Buchs, Switzerland), dexamethasone 1×10^{-7} M (Sigma-Aldrich, Buchs, Switzerland), insulin transferrin and selenium 1 % (Cyangen, Guangzhou, China), Non-essential amino acids 1 % (Gibco, Carlsbad, USA), Primocin 0.1 % (Invitrogen, San Diego, USA) and 6-aminocaproic acid 5 μM (Sigma-Aldrich, Buchs, Switzerland) to prevent fibrin degradation (Kupcsik *et al.*, 2009), a free swelling environment with chondropermissive medium supplemented with 1 ng/mL active TGFβ1 (Fitzgerald, Acton, USA), or with exposure to multiaxial loading in TGFβ free chondropermissive media. A

concentration of 1 ng/mL was chosen for the TGF-β1 stimulated group as media collected from mechanically loaded scaffolds consistently contain approximately 1 ng/mL of endogenously produced TGF-β1 (Li *et al.*, 2010a). The medium was refreshed on day 2, 4 and 6.

Mechanical loading

Multiaxial shear (± 25 at 1 Hz) and compression (10 % compression superimposed on top of a 10 % pre-strain at 1 Hz) loading was applied for one hour a day six times over 8 d (on days 2-7) (Li *et al.*, 2010a).

Sample collection

The work presented here represents data from two sets of experiments, each with the same experimental design. The first set of experiments consisted of three biological repeats, each using MSCs from a different donor (donors were aged 22, 27 and 77 y). In the first set of experiments the culture media were collected on day 2, 4, 6 and 8 for TGF-β1 quantification and secretome analysis. The second set of experiments was performed in order to collect additional data to supplement and confirm the results of the first set of experiments. This second set of experiments replicated the first, using cells from the same three MSC donors as the first experiment with the same passage number at the time of seeding. Culture media were collected on day 2, 4, 6 and 8 for nitrite quantification and on day 8 the scaffolds were homogenised in TRI reagent (Molecular Research Centre Inc., Cincinnati, OH, USA) and stored at −80°°C for RNA isolation and real-time PCR analysis.

Culture media analysis

Media collected on day eight of culture were further analysed to determine the presence of 174 different cytokines within each sample using the RayBio Human Cytokine Antibody Array G-Series 2000 protein array according to the manufacturer's instructions, for relevant abbreviations see Table 1. The slides were sent to THP Medical Products Vertreibs Gmbh (Vienna, Austria) for analysis.

The total amount of TGF-β1 in the culture media collected between day 2 and day 8 was determined using the human TGF-beta 1 DuoSet ELISA (R&D systems, Minneapolis, USA) according to the manufacturer's instructions

The Griess reaction to detect nitrite in collected culture media was performed using Griess reagent (modified) (Sigma) according to the manufacturer's instructions.

Table 1. The results of statistical comparisons made between groups. All factors that showed at least one significant change are included. A *p*-value displayed in red represents a decrease in expression in the group the comparison was made to (*e.g.* a red *p*-value in the 'TGF-β1-load' column indicates a decrease in the TGF-β1 stimulated group compared to the loaded group) whilst green represents an increase in expression in the group the comparison was made to (*e.g.* a green *p*-value in the 'TGF-β1-control' column indicates an increase in the TGF-β1 stimulated group compared to the control group). Abundance demonstrates the overall amount of protein in the medium based on the strength of signal detected during measurement, this is only arbitrary and acts as a general indicator of the amount of a particular protein relative to the others detected (the units are arbitrary and represent fluorescence intensity, Very low < 100, low 100-500, moderate 500-1000, high 1000-4000 and very high > 4000).

Factor name:	Abundance	TGF-β1-Control *p*-value	Load-Control *p*-value	TGF-β1-Load *p*- value
Factors with a Significant Difference in One Comparison				
Leptin	Low	0.0246	0.4757	0.6992
Leptin receptor	Low	0.0144	0.9999	0.1721
Macrophage derived chemokine (MDC)	Very Low	0.0378	0.3897	0.9813
Chemokine (CC motif) ligand 20 (CCL20), macrophage inflammatory protein 3 α (MIP3α)	Very Low	0.2550	0.032	0.9999
Chemokine (CXC motif) ligand 1,2 and 3 (CXCL1,2 and 3), Growth related oncogene α, β and γ (GROα, β and γ)	Moderate-High	0.0749	0.0272	0.9999
Urokinase receptor (uPAR)	Low	0.1820	0.0358	0.9999
Latency associated peptide (LAP)	Moderate	0.1968	0.0001	0.0667
Angiogenin	High-Very High	0.0526	0.0019	0.8959
Angiopoietin 2 (ANG2)	High	0.7034	0.1869	0.0061
Osteoprotegrin (OPG)	Moderate-High	0.9999	0.1573	0.0113
Tumour necrosis factor receptor superfamily 21 (TNFRSF21), death receptor 6 (DR6)	Low-Moderate	0.4128	0.8959	0.0348
Factors with a Significant Difference in Two Comparisons				
Transforming growth factor β 1 (TGF-β1)	Low	0.0157	0.9999	0.0411
Cluster of differentiation 166 (CD166), activated leukocyte cell adhesion molecule (ALCAM)	Low	0.9999	0.0226	0.0378
Chemokine (CXC) motif ligand 13 (CXCL13), B lymphocyte chemoattractant (BLC)	Low-Moderate	0.032	0.0002	0.4884
Chemokine (CC motif) ligand 7 (CCL7), Monocyte specific chemokine 3 (MCP3)	Low	0.0012	0.0089	0.9999
Macrophage migration inhibitory factor (MIF)	High	0.0328	0.0001	0.2883
Vascular endothelial growth factor (VEGF)	Moderate-High	0.0091	0.0091	0.9999
Matrix metalloproteinase 13 (MMP13), collagenase 3	Low-Moderate	0.0189	0.0010	0.9999
Platelet derived growth factor AA (PDGFAA)	Low	0.004	0.0055	0.9999

Briefly, 100 µL of Griess reagent was added to 100 µL of standards and samples. The reaction was allowed to proceed for 15 min in the dark before the absorbance was measured at 530 nm.

Reverse transcription and real-time PCR

Total RNA was isolated on day 8 from samples in TRI reagent as *per* the manufacturer's instructions. Reverse transcription was performed using random hexamer primers and TaqMan reverse transcription reagents (Applied Biosystems, Carlsbad, CA, USA).

Real-time PCR was performed using the applied biosciences StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primers for VEGF mRNA were synthesised by Microsynth AG (Balgach, SG, Switzerland). Primers for angiogenin, angiopoietin 2, BLC, GROα, leptin, MCP3, MIF, MIP3α, MMP13, osteoprotegerin, PDGFA and ribosomal 18s RNA were purchased from Applied Biosystems, Switzerland. The level of gene expression for each gene was determined relative to 18s rRNA using a ΔΔCT comparison.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, USA). Results of TGF-β1 quantification and secretome analysis represent data from three different MSC donors with each group containing three technical repeats. Results of nitrite quantification and real-time PCR analysis represent data from three biological repeats, with each group containing four technical repeats.

The fluorescent intensity levels recorded for each sample from the cytokine array were adjusted to remove background interference. Outliers were then removed using the ROUT method, normality was determined using the D'Agostino-Pearson omnibus normality test and the statistical difference was then determined between control and TGF-β1 cultured groups, between load and control and between load and TGF-β1 cultured groups using Kruskal-Wallis test and Dunn's multiple comparison test.

The results of TGF-β1 quantification, nitrite quantification and real-time PCR analysis were tested for normality using the D'Agostino-Pearson omnibus normality test. Statistical differences of media TGF-β1, nitrite ions and all genes analysed by real-time PCR (except leptin and MMP13) were determined using the Kruskal-Wallis test and Dunn's multiple comparison test. Statistical significance for leptin and MMP13 was determined using the Mann-Whitney test. $p \leq 0.05$ was considered to be significant.

Results

TGF-β1 quantification

On day 2 the amount of TGF-β1 measured in the culture medium of TGF-β1 stimulated scaffolds was higher than both control and loaded groups ($p = 0.0378$) (Fig. 1**A**). This was expected, as the TGF-β1 cultured group was receiving TGF-β in the media at this point whilst the other two groups were not. At day 4 of culture (Fig. 1**B**), as mechanical loading began to induce the production of TGF-β1 by the cells within the scaffold, the amount of TGF-β1 in the media collected from TGF-β1 stimulated scaffolds was significantly higher than that of the control group ($p = 0.0017$) but not significantly different from the loaded group. By day 6 of culture, multiaxial mechanical loading had led to a significant increase in TGF-β1 production compared to control scaffolds and the level of TGF-β1 in the media of both TGF-β1 stimulated and loaded groups was significantly higher than that of the control group ($p = 0.0001$ and 0.0207 respectively). Both TGF-β1 stimulated and load media also contained significantly more TGF-β1 than control samples on day eight ($p = 0.0002$ and 0.0060 respectively).

Cytokine detection

All 174 factors were detected at varying levels of intensity in all samples. An average fluorescence intensity value was calculated for each factor at day 8 from all three conditions (control, load and TGF-β1 stimulated) in order to provide a guide to the level of detection of the different factors across the groups, rather than in specific conditions. 55 factors had an average fluorescence intensity across the three groups of less than 100 including; leptin (93.79 ± 81.83) and MDC (54.81 ± 9.98). The average intensity of 94 factors was between 100 and 500 including; BLC (389.19 ± 557.10), MCP3 (201.30 ± 115.94), ALCAM (141.57 ± 37.54), uPAR (286.63 ± 109.39), leptin receptor (110.07 ± 29.35), MMP13 (422.77 ± 512.66) and PDGFaa (171.75 ± 76.35). The intensity of 11 factors was between 500-1000 including; osteoprotegerin (986.16 ± 560.48) and VEGF (938.11 ± 397.31). 12 factors had an average intensity between 1000 and 4000 including; angiopoietin-2 (1341.87 ± 937.81), GRO (1326.36 ± 1011.64) and LAP (1302.15 ± 752.86). The intensity of two factors was above 4000; angiogenin (40273.68 ± 7037.95) and TIMP2 (6778.68 ± 2231.98). The factor with the highest recorded intensity, by a factor of 10, was angiogenin and the lowest was BMP6 (20.50 ± 18.21).

For each of the 174 factors analysed, three sets of comparisons were performed (between control and TGF-β stimulated scaffolds, control and loaded scaffolds and TGF-β1 stimulated and loaded scaffolds) and the significance of statistical differences between the groups determined. Analysis showed that 19 factors changed significantly in at least one of these comparisons. The three volcano plots in Fig. 2 graphically represent the results of these three comparisons. These plots were produced by plotting the Log10 of the fold change for a factor between one condition and another (*e.g.* control and TGF-β1) on the X-axis, against the −Log10 of the *p*-value generated when testing the difference between the two conditions on the Y-axis. Therefore, the further a factor is away from zero on the X-axis the greater the fold change up or down. The further a factor is up the Y-axis the lower the *p*-value, a *p*-value of 0.05 equates to 1.30 on the Y-axis. Therefore, any factors above this value underwent a significant change.

Fig. 2**A** shows a volcano plot generated based on the comparison between media conditioned by TGF-β1 stimulated scaffolds and control medium, factors that

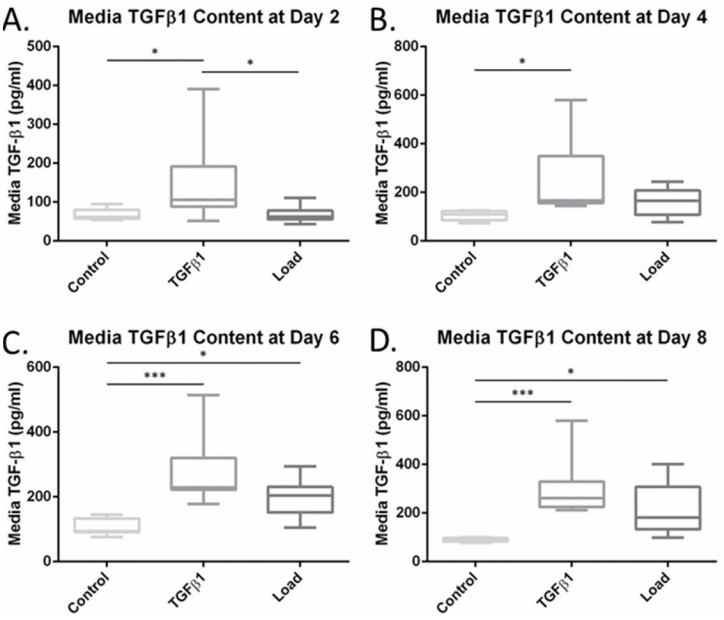
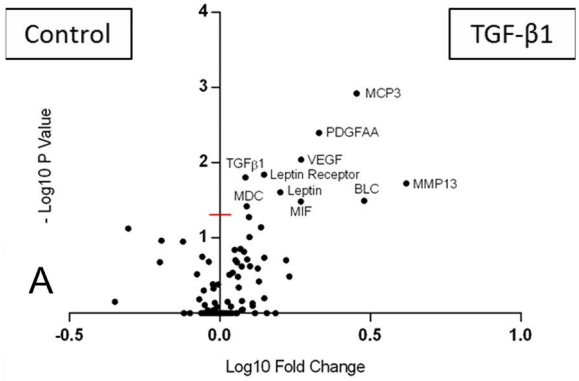
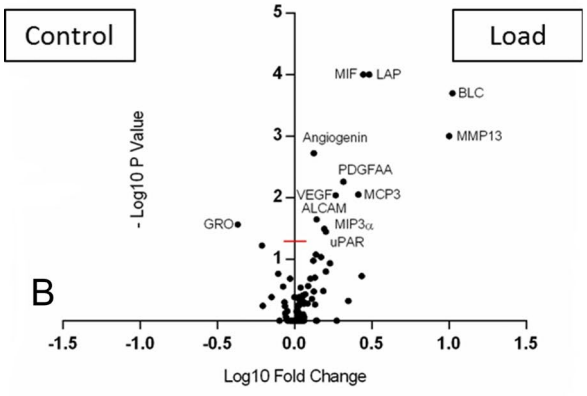


Fig. 1. Quantification of total TGF-β1 in the culture media of control, TGF-β1 stimulated and loaded scaffolds by week, over four weeks of culture. Statistical significance was defined as $p \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $p \leq 0.05$, ** represents $p \leq 0.001$ and *** represents $p \leq 0.0001$.





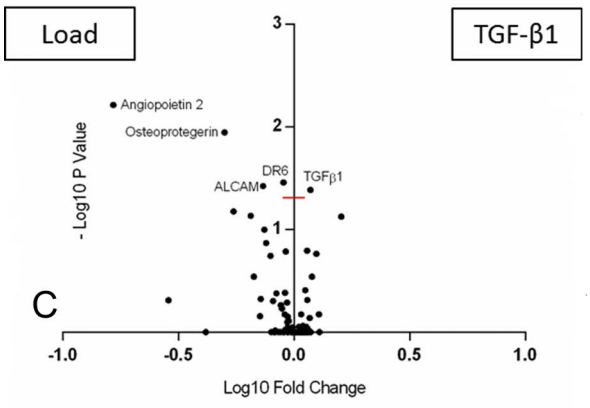


Fig. 2. Volcano plots showing the results of the three sets of statistical comparisons made between groups. These plots have −Log10 *p*-value of the comparison on the Y-axis and Log10 fold change of the comparison for each factor on the X-axis. As a result the greater the fold change the further a factor is away from zero on the X-axis and the lower the *p*-value of a comparison the further away from zero on the Y-axis. Factors that underwent a significant change have been labelled. The red line on the Y-axis represents a −Log10 *p*-value of 1.3 this is equivalent to a *p*-value of 0.05, factors above this line underwent a significant change.
Plot **A**. represents TGF-β1 stimulated samples compared to controls. Factors on the left-hand side of the Y-axis were higher in controls than TGF-β1 stimulated samples and factors on the right-hand side were higher in TGF-β1 stimulated samples than controls.
Plot **B**. represents loaded samples compared to controls. Factors on the left had side of the Y-axis were higher in controls than loaded samples and factors on the right hand side were higher in loaded samples than controls.
Plot **C**. represents TGF-β1 stimulated samples compared to loaded samples. Factors on the left hand side of the Y-axis were higher in loaded samples than TGF-β1 stimulated samples and factors on the right hand side were higher in TGF-β1 stimulated samples than loaded samples.

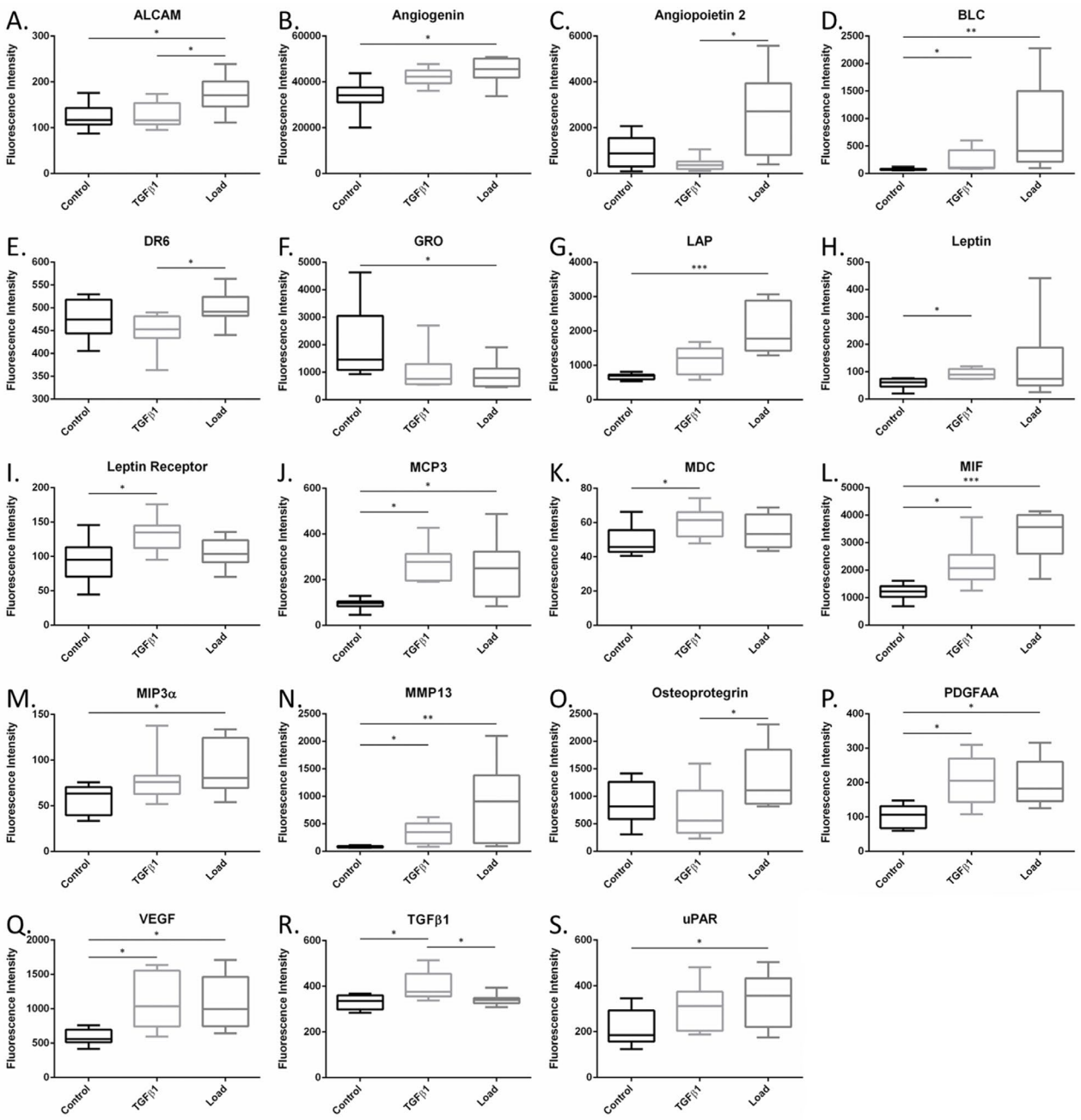


Fig. 3. Box plots showing the results of cytokine antibody array analysis. This figure contains the 19 factors that showed a significant change between in at least one comparison (the three comparisons were between control and TGF-β1, between load and controls and load and TGF-β1). Statistical significance was defined as $p \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $p \leq 0.05$, ** represents $p \leq 0.001$ and *** represents $p \leq 0.0001$.

appear on the left side of the Y-axis were detected at a higher level in control medium, and factors on the right hand side of the axis at a higher level in TGF-β1 stimulated medium. Factors which are significantly different between control and TGF-β1 stimulated medium have been labelled. Fig. 2**B** shows a volcano plot constructed in the same way as Fig. 2**A,** using data from the comparison between control medium and medium conditioned by cells exposed to multiaxial load. Factors that appear on the left side of the Y-axis were detected at a higher level in control medium and factors on the right hand side of the axis at a higher level in medium from loaded samples.

The volcano plots for control-TGF-β1 media and control-load media show both similarities and differences between the effects of load and TGF-β1 stimulation compared to control conditions. In medium conditioned by both TGF-β1 stimulated and loaded scaffolds there was a significant upregulation (found on the right hand side of the Y-axis and above 1.30 on the Y-axis) of BLC, MCP3, MIF, VEGF, MMP13 and PDGFaa compared to control (Fig. 2**A**&**B**). No factors were significantly down regulated in both TGF-β1 stimulated and loaded groups compared to controls. Leptin, leptin receptor and MDC were upregulated in medium from TGF-β1 stimulated scaffolds

Table 2. The factors identified by this work as undergoing a significant change in media content in response to load or TGF-β1 stimulation and a description of their known links to cartilage/chondrogenesis.

Factor name	Known links to chondrogenesis/cartilage	References
Leptin	Localised to the growth plate and osteoarthritic cartilage. Knock out mice have disrupted growth plates. Leptin induces GAG loss from cartilage plugs and activates MMPs.	(Dumond *et al.*, 2003; Hui *et al.*, 2012; Iliopoulos *et al.*, 2007; Kishida *et al.*, 2005; Kume *et al.*, 2002; Zeddou *et al.*, 2012)
Leptin Receptor	Localised the growth plate and to osteoarthritic cartilage. Expressed by chondrocytes in monolayer culture.	(Kishida *et al.*, 2005; Ohba *et al.*, 2010; Simopoulou *et al.*, 2007)
MDC	Expressed in the synovial tissue of arthritic joints.	(Flytlie *et al.*, 2010)
MIP3α	Induced MMP13 expression in articular chondrocytes in monolayer culture. Increased levels found in rheumatic joints.	(Lisignoli *et al.*, 2006; Mazzetti *et al.*, 2004)
GRO	Found in arthritic joint and expressed by normal chondrocytes. Capable of inducing hypertrophy in chondrocytes in vitro.	(Chen *et al.*, 2013a; Merz *et al.*, 2003; Olivotto *et al.*, 2007; Wenke *et al.*, 2011)
uPAR	Capable of activating MMPs and associated with plasmin, an activator of TGF-β.	(Beaufort *et al.*, 2004; Blasi and Carmeliet, 2002; Montuori *et al.*, 2005; Schwab *et al.*, 2004)
LAP	Released during TGF-β activation.	(Maeda *et al.*, 2002)
Angiogenin	Found in diseased and arthritic joints.	(Liote *et al.*, 2003)
Angiopoietin 2	Destabilises blood vessel formation by acting as a TIE2 antagonist.	(DeBusk *et al.*, 2003; Horner *et al.*, 2001)
Osteoprotegerin	Found in normal and arthritic cartilage. Chondroprotective in a murine OA model.	(Komuro *et al.*, 2001; Shimizu *et al.*, 2007)
DR6	-	-
TGF-β	Induces chondrogenesis in mesenchymal stem cells.	(Johnstone *et al.*, 1998)
ALCAM	Used to identify chondroprogenitor cells. Expressed by chondrocytes from diseased and normal joints.	(Alsalameh *et al.*, 2004; Arai *et al.*, 2002; Chen *et al.*, 2013b)
BLC	Expressed by chondrogenically differentiated MSCs. BLC neutralisation reduced the severity within a murine autoimmune arthritis model.	(Cristino *et al.*, 2008; Zheng *et al.*, 2005)
MCP3	-	-
MIF	Detected in RA joints and associated with inflammation and MMP expression. Neutralisation reduced the severity of a murine autoimmune arthritis model.	(Morand and Leech, 2005; Santos *et al.*, 2001)
VEGF	Expressed by normal and hypertrophic chondrocytes. Important in hypertrophy, chondrocyte specific inactivation leads to failed endochondral ossification.	(Bluteau *et al.*, 2007; Dai and Rabie, 2007)
MMP13	Involved in cartilage turnover and acts as a marker of MSC and chondrocyte hypertrophy.	(Mueller and Tuan, 2008)
PDGF	Important in chondrogenesis during development.	(Ataliotis, 2000)

compared to controls but did not change in medium from loaded scaffolds compared to controls. MIP3α, uPAR, LAP and angiogenin were detected at significantly levels in response to load compared to control, but did not change in TGF-β1 compared to controls, whilst the concentration of GRO was significantly lower in response to load but did not change significantly in response to TGF-β1 stimulation.

The third volcano plot (Fig. 2**C**) shows the results of a direct comparison between media from TGF-β1 stimulated scaffolds and loaded scaffolds. In this plot, factors detected at a higher level in media collected from loaded scaffolds are found on the left hand side of the Y-axis and factors found at a higher level in media from TGF-β1 stimulated scaffolds are found on the right hand side of the Y-axis. This plot clearly shows that the majority of factors measured were found at a higher level in the media collected from loaded scaffolds. Angiopoietin 2, osteoprotegerin, ALCAM and DR6 were found at higher levels in loaded media than TGF-β1 stimulated media whilst TGF-β1 was found at higher levels in TGF-β1 stimulated medium than loaded medium.

The comparisons made between the three groups have been provided in the form of box plots (Fig. 3) as well as in the volcano plots (Fig. 2) in order to display the results in a factor by factor manner. Six factors (BLC, MCP3, MIF, VEGF, MMP13 and PDGFaa) were found to change significantly in both TGF-β1 and loaded groups compared to controls. In all of these cases the factors were significantly higher in TGF-β1 and loaded groups than controls (Table 1, Fig. 3**D**, **J**, **L**, **N**, **P** and **Q**). The similarities in the responses of these factors indicate similarities in the effects of these two forms of stimulation, potentially due to the effect of TGF-β1 signalling in both systems.

ALCAM was significantly increased in the loaded group over both control and TGF-β1 stimulated groups showing a clear difference in effect between load and TGF-β1 stimulation (Table 1, Fig. 3**A**).

Table 2 provides information on the interactions that are described in the literature between the factors that changed significantly in this study with/on cartilage/chondrogenesis.

Real-time PCR gene expression analysis

Real-time PCR was performed in order to determine the relative gene expression levels of factors that demonstrated significant changes between groups in the secretome analysis. Of the 19 factors that underwent significant changes, 9 were chosen for real-time PCR analysis based on their expression as soluble and not membrane bound proteins, and evidence for a potential link to

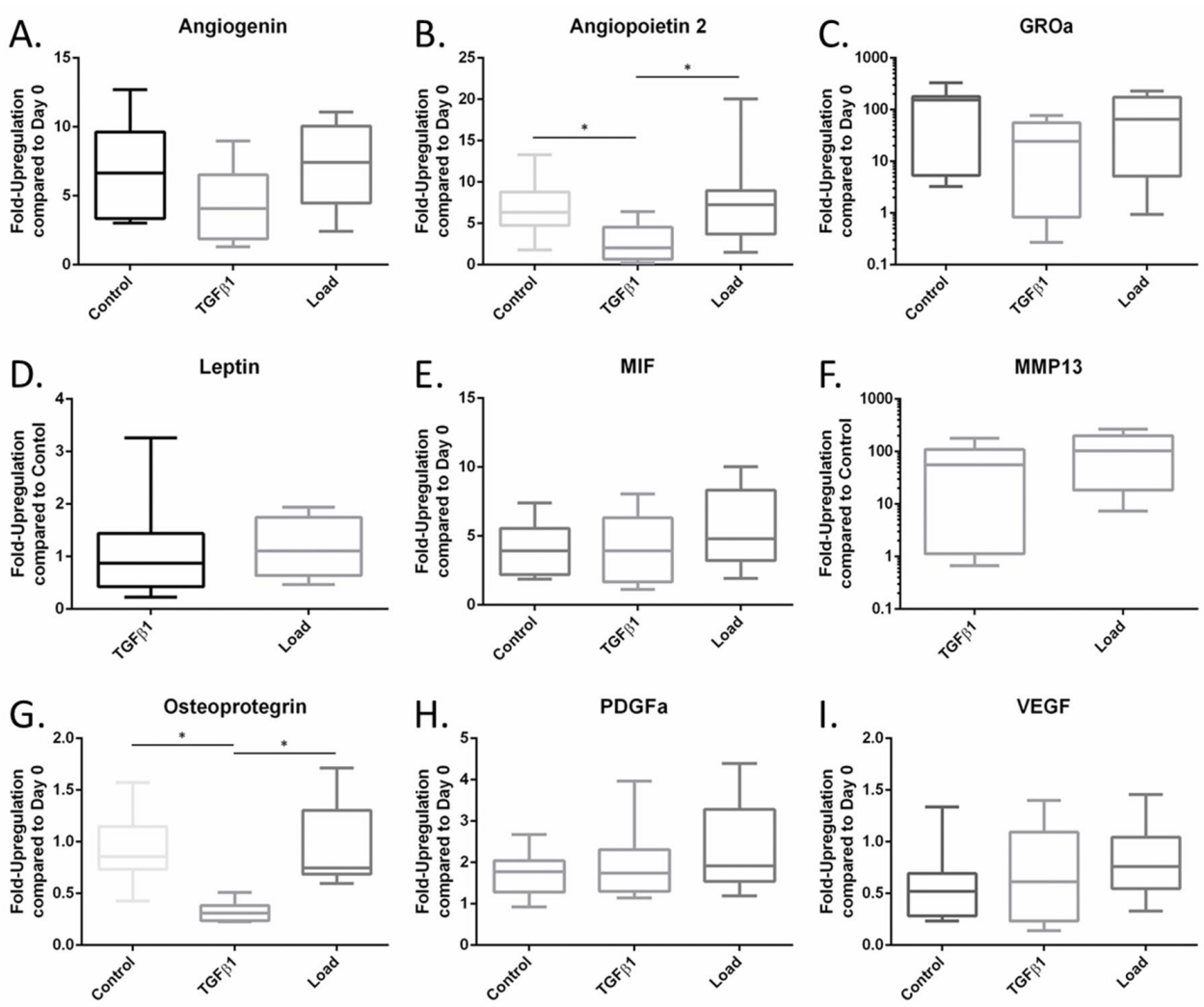


Fig. 4. Box plots showing the results of real-time PCR. This figure contains shows the results of the 12 factors investigated, each of which had showed a significant change between in at least one comparison made between groups during secretome analysis (the three comparisons were between control and TGF-β1, between load and controls and load and TGF-β1). Statistical significance was defined as $p \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $p \leq 0.05$, ** represents $p \leq 0.001$ and *** represents $p \leq 0.0001$.

chondrogenesis, hypertrophy or mechanical load in the literature. Membrane bound proteins were not analysed as changes in receptor turnover and shedding mean that comparison between media protein levels and mRNA levels difficult to reconcile. The factors were; angiogenin, angiopoietin 2, GROα, leptin, MIF, MMP13, OPG, PDGFa and VEGF.

Results of real-time PCR for angiopoietin 2 and osteoprotegerin show that as in the secretome analysis the expression in the TGF-β1 group is significantly lower than in the loaded group, in the PCR analysis the expression level is also seen to be lower in the TGF-β1 stimulated group than in control scaffolds (Fig. 4**B** and **G**). The expression of MMP13 was normalised to the control group as the expression of MMP13 in cells at day 0 was donor dependent. Real-time PCR shows that the expression of MMP13 is similar in TGF-β1 stimulated and loaded groups and that also the expression in both groups is much higher than control groups (Fig. 4**F**). The expression of GRO was also lower than controls in both stimulated groups (Fig. 4**C**), although not as strongly as suggested by the secretome data or other results collected by this group (data not shown).

Secretome analysis showed that leptin was detected at a higher level in media from TGF-β1 stimulated scaffolds than control samples, whilst angiogenin was detected at a higher level in loaded samples than controls (Fig. 3**B** and **H**). For both of these factors real-time PCR analysis showed no difference between stimulated groups in the case of leptin (which as with MMP13 was normalised to the control groups due to donor dependent expression at day 0) or stimulated groups and controls in the case of angiogenin (Fig. 4**A** and **D**).

The results of secretome analysis showed that MIF, PDGFaa and VEGF were all detected at higher levels in both TGF-β1 stimulated and loaded groups than controls, whilst not being significantly different from each other (Fig. 3**L**, **P** and **Q**). This was not reflected in gene expression analysis where neither stimulated group was found to be significantly different from controls (Fig. 4**E**, **H** and **I**).

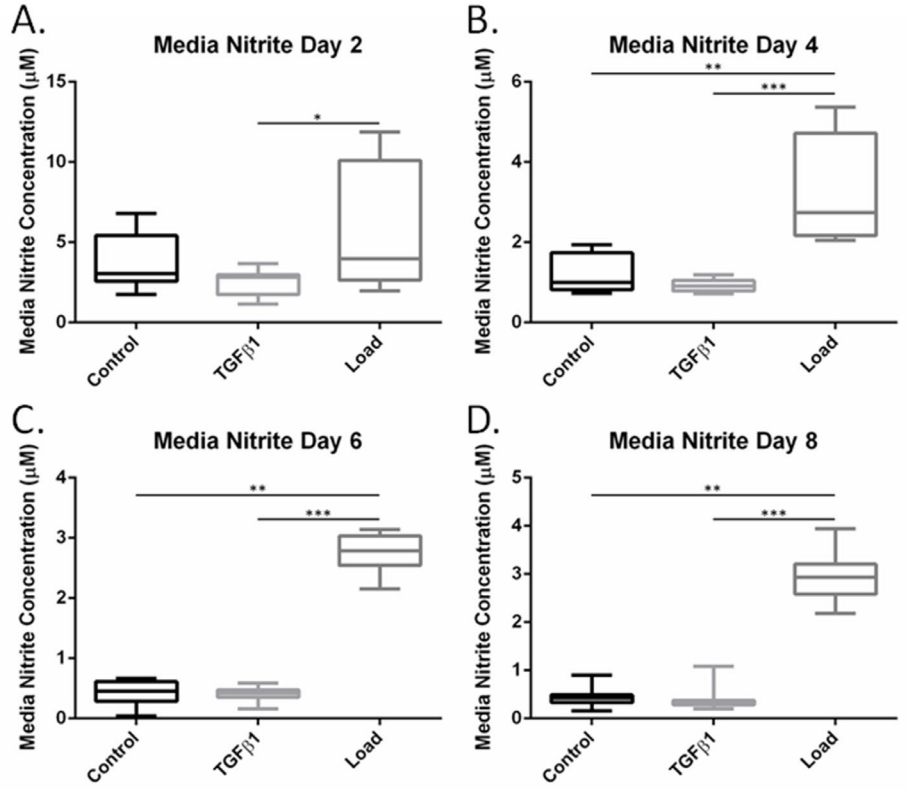


Fig. 5. Quantification of nitrite (as an indirect marker measure of nitric oxide (NO)) in the culture media of control, TGF-β1 stimulated and loaded scaffolds by week, over four weeks of culture. Statistical significance was defined as $p \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $p \leq 0.05$, ** represents $p \leq 0.001$ and *** represents $p \leq 0.0001$.

Media nitrite quantification

A number of the factors identified by the initial secretome analysis are known to respond to shear forces in other model systems (angiopoietin-2, PDGF and MCP3). Another factor commonly associated with shear loading, particularly in vascular cells, is nitric oxide. In order to investigate the effect of load on nitric oxide in this system, the presence of inorganic nitrite was detected in the culture media using the Griess reaction. Results showed that at all four time points (day two, four, six and eight) the level of nitrites detected in the TGF-β1 stimulated group was significantly lower than that of the loaded group, but not significantly different from the control group (Fig. 5**A**, **B**, **C** and **D**). There were significantly higher levels of nitrites in the culture media of loaded scaffolds compared to both control and TGF-β1 stimulated scaffolds on days four, six and eight (Fig. 5**A**, **B**, **C** and **D**). No significant difference was detected between loaded scaffolds and controls on day 2 (Fig. 5**A**).

Discussion

Currently accepted methods for the induction of chondrogenesis in MSCs use a combination of 3D culture and the exogenous administration of an active recombinant isoform of TGF-β (Barry *et al.*, 2001; Johnstone *et al.*, 1998). Recent work has shown that chondrogenesis can be induced in MSCs using multiaxial mechanical load in the absence of exogenous TGF-β (Li *et al.*, 2010a; Li *et al.*, 2010b; Schatti *et al.*, 2011). The purpose of this investigation was to compare the secretomes MSCs stimulated either with exogenous TGF-β or multiaxial load with each other and with unstimulated control scaffolds, to highlight similarities and differences between these two forms of chondrogenic induction and determine interesting or potentially novel factors for MSC based cartilage tissue engineering.

BLC, MCP3, MIF, VEGF, MMP13 and PDGF were upregulated in response to both load and TGF-β1 stimulation and may represent a core of factors that are responsive to TGF-β1, which is known to be involved in the induction of chondrogenesis in the loaded scaffolds as well as the TGF-β1 stimulated scaffolds (Li *et al.*, 2010a). MMP13 is a well characterised and described marker associated with chondrocyte and MSC hypertrophy (D'Angelo *et al.*, 2000). The secretion of BLC in to the culture media has previously been described on day seven of the TGF-β driven chondrogenesis of MSCs. However, little information is available to suggest a role for BLC in MSC chondrogenesis (Cristino *et al.*, 2008). MCP3 has been shown to be induced by TGF-β in fibroblasts in a SMAD dependent manner and in response to mechanical stimulation in osteocytes (Ong *et al.*, 2009). The production of both PDGFaa and VEGF is known to be responsive to TGF-β (Jeon *et al.*, 2007; Tanabe *et al.*, 2006; Wang *et al.*, 1997). These factors therefore have clear links to TGF-β1 or TGF-β1 driven chondrogenesis and this may explain the similarities between loaded and TGF-β1 stimulated scaffolds when compared to control scaffolds. The expression of all six of these factors has also previously been described in MSCs (Cristino *et al.*, 2008; Grassel *et al.*, 2009; Hoch *et al.*, 2012; Kinnaird *et al.*, 2004; Palumbo *et al.*, 2014; Rehman *et al.*, 2004; Ribeiro *et al.*, 2012; Sze *et al.*, 2007). Some of these factors have also previously been associated with similar responses to TGF-β in a

range of cell types *e.g.* BLC, MCP3, VEGF, MMP13 and PDGFaa (Cristino *et al.*, 2008; Fitzgerald *et al.*, 2008; Jeon *et al.*, 2007; Ong *et al.*, 2009; Tanabe *et al.*, 2006; Wang *et al.*, 1997).

As well as factors that responded similarly to both forms of chondrogenic induction there were a number that underwent different responses. Leptin has been localised to the synovial fluid of arthritic joints and is found at a higher level in cartilage from arthritic joints than normal cartilage (Kume *et al.,* 2002). A role for leptin has also been suggested in the growth plate where leptin knock out leads to disrupted column formation and reduced collagen type X production (Kishida *et al.,* 2005). These observations *in vivo* suggest that leptin is involved in hypertrophy and may act as a marker of hypertrophy. This is supported by work by Iliopoulos *et al.* (2007), who showed that leptin could stimulate MMP13 expression in articular chondrocytes *in vitro* and that siRNA knock down of leptin expression leads to a reduction in MMP13 production. This suggests that the increase in leptin, in response to TGF-β stimulation, may be indicative of a more hypertrophic phenotype than that induced by load. However, further work would be required to investigate this. Should leptin's hypertrophic effects on MSCs be verified then it would provide and interesting avenue for reducing hypertrophy within cartilage tissue engineering.

Little is known regarding the role of or effect on chondrogenesis of MDC. However, its expression has been detected in the synovial tissue of patients with rheumatic and psoriatic arthritis. The presence of MDC in these tissues may be due to its role in inflammation and further work is required to determine its effect of chondrogenesis (Flytlie *et al.*, 2010).

LAP is released from the mature TGF-β1 peptide during activation. Mechanical loading and, in particular, shear loading has been shown to activate TGF-β, an effect also seen within the bioreactor culture system presented in this paper (Paper in review) (Ahamed *et al.*, 2008; Albro *et al.*, 2012; Annes *et al.*, 2004; Wipff *et al.*, 2007). The increased presence of LAP in the culture media of loaded scaffolds is, therefore, likely to be due to activation of endogenously produced pro-forms of TGF-β.

The expression of GRO has been detected in osteoarthritic chondrocytes, and has is capable of inducing the expression hypertrophic markers such as MMP13 and collagen type X, as well as mineralisation in chondrocytes (Endres *et al.*, 2010; Merz *et al.*, 2003; Olivotto *et al.*, 2007). These results suggest that the reduction in GRO expression in response to TGF-β1 stimulation and mechanical load expression marks an anti-hypertrophic effect. This may be an interesting avenue for cartilage repair. Hypertrophy is a major bar on the use of MSCs clinically and targeting factors such as GRO that are involved in hypertrophy may provide ways of circumventing this issue.

Both angiopoietin-2 and osteoprotegerin have been shown to be induced by the application of shear forces which may be linked to their increased presence in media from loaded scaffolds over TGF-β1 stimulated scaffolds (Goettsch *et al.*, 2008; Kim *et al.*, 2006; Li *et al.*, 2014).

The amount of TGF-β1 detected in the culture media by cytokine array was significantly higher at day eight in TGF-β1 stimulated samples than both control and loaded samples. This is at odds with the result of the ELISA quantification of day eight media, which showed that there was no significant difference in the media TGF-β1 concentration of loaded and TGF-β1 stimulated groups. The ELISA data also indicated that both TGF-β1 stimulated groups and loaded groups were significantly higher than the control group on day eight. However, the results of the cytokine array also demonstrated that the overall fold change between TGF-β1 and load was 1.17 and between TGF-β1 and control 1.2, suggesting very little overall difference the total amounts of TGF-β1 present. These changes are lower than the 1.5-fold change used by Rodriguez *et al.* to denote significance and the 2-fold change used to class a factor as physiologically relevant by Grassel *et al.* (Grassel *et al.*, 2009; Rodriguez *et al.*, 2015). This result highlights the fact that this form of analysis is extremely valuable for identifying the presence of potential factors of interest within samples. But, more precise techniques incorporating standard curves, such as ELISA, should be performed for absolute quantification.

Following the results of secretome analysis, this work was repeated to confirm that the changes seen at a protein level were also detectable at a gene expression level using real-time PCR. The results of real-time PCR analysis confirmed some of the changes seen in the secretome data *e.g.* for angiopoietin 2 and osteoprotegerin, but did not support the changes seen in all the factors *e.g.* VEGF and PDGFa. The detection of similar changes in secretome analysis and gene expression suggests that the changes seen in these factors are robust; this highlights angiopoietin 2, osteoprotegerin, MMP13 and GROa as factors of particular interest. The difference in the gene expression and protein analysis for these genes may relate to the time points of sample collection. The media used for the secretome analysis were conditioned between days 6 and 8 of culture, whilst the mRNA collection represents a single time point on day 8. As a result, the gene expression profiles of these factors may have risen and fallen again before the sampling on day 8, and may explain the difference in mRNA and protein profiles. Alternatively, this may be due to the small fold changes seen in the secretome results for these factors, which may explain the lack of a significant change in gene expression levels despite a statistically significant change in the amounts of protein detected.

Study of the literature associated with the factors identified during the secretome analysis highlighted similar responses to shear force with regards to factors such as angiopoietin-2, PDGF and MCP3 in other cell types and model systems. The literature also highlighted the increase in nitric oxide (NO) production in response to shear loading in endothelial cells in response to mechanical shear forces (Bao *et al.*, 1999; Goettsch *et al.*, 2008). In MSCs, NO production has previously been shown to be induced in response to fluid flow induced shear loading (Knippenberg *et al.*, 2005), and also has been shown to play a role in MSC immunomodulation through the suppression of the T-cell response (Ren *et al.*, 2008; Sato *et al.*, 2007). The clear changes in NO production in response to joint-like multiaxial load suggest that NO production mechanisms could be targeted for therapeutic benefit. This work is

the first to implicate NO as a potential influencing factor during the multiaxial load applied after marrow stimulation techniques (which result in the formation of a fibrin clot containing MSCs). NO has been widely linked to arthritis, particularly the upregulation of MMPs, matrix degradation and the suppression of proteoglycan and collagen synthesis (Abramson, 2008). Nitric oxide also has a negative effect on TGF-β expression which has leads to a reduction in GAG production as a result in the reduction of TGF-β (Studer *et al.*, 1999). Previous work in to this area has shown that the suppression of nitric oxide synthase (NOS) can reduce the severity of symptoms in a murine model of arthritis (McCartney-Francis *et al.*, 1993). Therefore, reducing the local presence of NO in joints during post-operative rehabilitation *e.g.* after microfracture, could improve the outcome of cartilage repair by reducing the effects of NO on cartilage matrix synthesis, cartilage degradation and TGF-β signalling. However, further work is required to identify the role of NO in response to load and the effect that it has on MSC chondrogenesis, as NO may play a direct role in mechanotransduction and, therefore, the generation of cartilage-like repair material in response to joint loading *in vitro*. A clear next step is to establish whether the NO plays a role in mechanotransduction, and whether it has an inhibitory effect on cartilage tissue development under these conditions and the extent to which NO levels can be modulated to improve chondrogenic outcomes.

Conclusions

The results of secretome analysis presented here show that there are clear similarities and differences between the secretomes of MSCs stimulated with multiaxial mechanical load or TGF-β1. Nineteen factors were identified by secretome analysis as being significantly different in two or more groups. Of particular interest are the four factors (angiopoietin 2, GROα, MMP13 and osteoprotegerin) whose changes in secretome profile between the groups were mirrored by their changes in gene expression as determined by real-time PCR. Alongside these factors, this work identifies for the first time an increase in NO production in response to joint stimulating mechanical load. These data demonstrate that the chondrogenic effects of multiaxial mechanical load and stimulation with exogenous TGF-β1 are not analogous. The factors identified by this work, and their potential for manipulation, presents a multitude of novel and interesting avenues for further investigation with regards to mesenchymal stem cells and cartilage tissue engineering.

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Discussion with Reviewers

Reviewer I: The discussion about NO is a strong section of the paper. However, some of the data indicate that NO could be good (inhibiting T-cell response?). Could the NO being produced by load be a good not bad thing? This was sort of alluded to in mentioning that it could play a role in mechanotransduction.

Authors: Further work is required to identify the role of NO in response to load and the effect that it has on MSC chondrogenesis, as NO may play a direct role in mechanotransduction and therefore for the generation of cartilage-like repair material in response to joint loading *in vitro*. A clear next step is to establish whether the NO plays a role in mechanotransduction, and whether it has an inhibitory effect on cartilage tissue development under

these conditions and the extent to which NO levels can be modulated to improve chondrogenic outcomes.

Reviewer I: One limitation of the study is that the secretome is only analysed at a single time point. Clearly chondrogenesis is a dynamic process and we would anticipate the secretome would change. Furthermore, as noted there were instances where protein and gene data do not correlate, which (as discussed by the authors) is not unreasonable given this dynamic process and other reasons for a non-linear correlation between gene and protein. Looking forward, how could future experiments address the dynamic process of chondrogenesis and hypertrophic maturation?
Authors: The question of timing and the change in the secretome profile over time is a really important one. This study aimed to identify candidate molecules. The follow up would be to repeat this experiment with several time points (*e.g.* 3, 7, 14 and 21 days). The candidate molecules could then be investigated in more detail with assays such as ELISA. Alongside this, follow up studies based on the work presented here will allow for the characterisation of some of the markers identified in this work. These novel markers may also be useful for looking at the process of chondrogenesis over time alongside the 'classic' markers that are commonly used in the field..

Reviewer II: Please explain why factors were selected for RT-PCR on the basis that they are soluble proteins.
Authors: This decision was made in order to remove possible differences in the data as a result of changes to turnover (including internalisation) and the shedding of membrane bound proteins (in particular in response to load) which may further cloud the differences between the media protein content and mRNA levels as determined by RT-PCR.

Reviewer II: What further experiments do you suggest to investigate the role of NO in load-induced chondrogenesis?
Authors: This is something we are really excited about. The first thing to establish is how load and NO are related. In order to do that, we would load in the presence of an NOS blocker. If NO is involved in mechano-transduction then the block may disrupt chondrogenesis. If blocking NO does not affect chondrogenesis and NO is being produced as part of a separate process, the benefit of blocking NO production on chondrogenesis could be investigated, to see if you can get a better chondrogenic outcome.

Editor's Note: Scientific Editor in charge of the paper: Chris Evans.