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

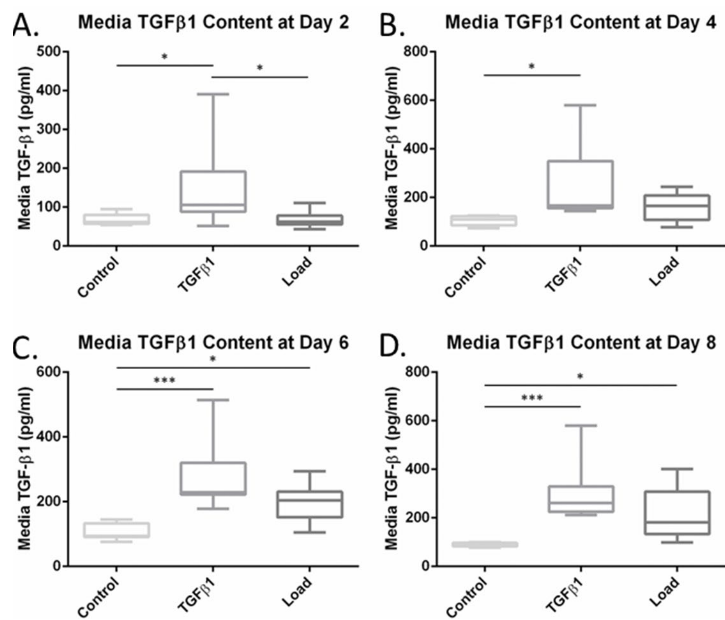
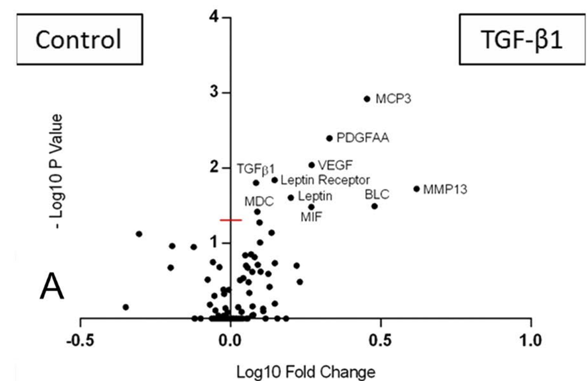
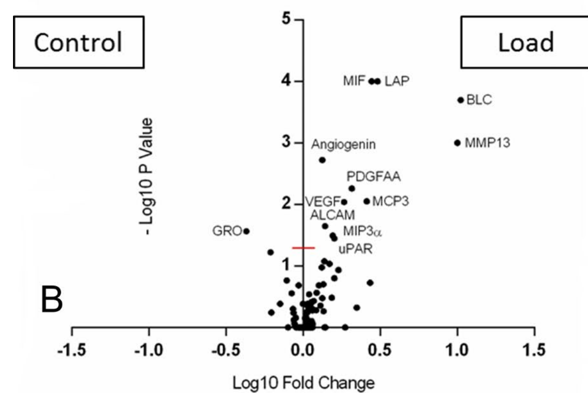


Fig. 1. Quantification of total TGF-β1 in the culture media of control, TGF-β1 stimulated and loaded scaffolds by week, over four weeks of culture. Statistical significance was defined as $p \leq 0.05$ and determined using the Kurskal-Wallis and Dunn's multiple comparison tests. * represents $p \leq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





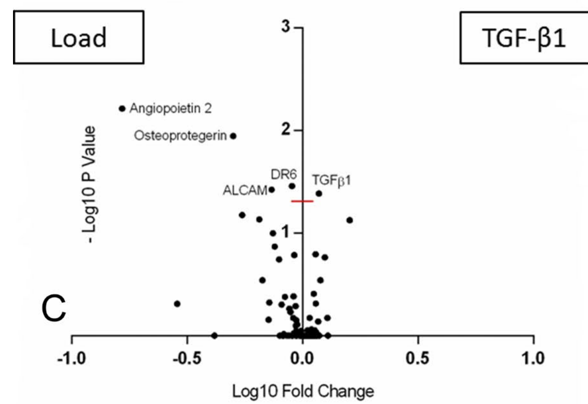


Fig. 2. Volcano plots showing the results of the three sets of statistical comparisons made between groups. These plots have −Log10 *p*-value of the comparison on the Y-axis and Log10 fold change of the comparison for each factor on the X-axis. As a result the greater the fold change the further a factor 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.