HYDROSTATIC PRESSURE ACTS TO STABILISE A CHONDROGENIC PHENOTYPE IN PORCINE JOINT TISSUE DERIVED STEM CELLS

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Abstract

Hydrostatic pressure (HP) is a key component of the *in vivo* joint environment and has been shown to enhance chondrogenesis of stem cells. The objective of this study was to investigate the interaction between HP and TGF-β3 on both the initiation and maintenance of a chondrogenic phenotype for joint tissue derived stem cells. Pellets generated from porcine chondrocytes (CCs), synovial membrane derived stem cells (SDSCs) and infrapatellar fat pad derived stem cells (FPSCs) were subjected to 10 MPa of cyclic HP (4 h/day) and different concentrations of TGF-β3 (0, 1 and 10 ng/mL) for 14 days. CCs and stem cells were observed to respond differentially to both HP and TGF-β3 stimulation. HP in the absence of TGF-β3 did not induce robust chondrogenic differentiation of stem cells. At low concentrations of TGF-β3 (1 ng/mL), HP acted to enhance chondrogenesis of both SDSCs and FPSCs, as evident by a 3-fold increase in *Sox9* expression and a significant increase in glycosaminoglycan accumulation. In contrast, HP had no effect on cartilage-specific matrix synthesis at higher concentrations of TGF-β3 (10 ng/mL). Critically, HP appears to play a key role in the maintenance of a chondrogenic phenotype, as evident by a down-regulation of the hypertrophic markers *type X collagen* and *Indian hedgehog* in SDSCs irrespective of the cytokine concentration. In the context of stem cell based therapies for cartilage repair, this study demonstrates the importance of considering how joint specific environmental factors interact to regulate not only the initiation of chondrogenesis, but also the development of a stable hyaline-like repair tissue.

Keywords: Hydrostatic pressure, cartilage, synovial membrane, infrapatellar fat pad, transforming growth factor (TGF)-β3, stem cells, chondrocytes.

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Introduction

Articular cartilage lesions do not heal adequately, leading to intense effort towards developing new therapies to repair and regenerate articular cartilage (Buckwalter, 1998; Elder and Athanasiou, 2009). The variety of treatment options available implies that there is no single, ideal, reliable and predictable therapy (Hildner *et al.*, 2011). The use of mesenchymal stem cells (MSCs) as part of a cell based therapy for cartilage repair is very appealing. MSCs have a high potential for proliferation and differentiation (Pittenger *et al.*, 1999) and overcome many of the drawbacks associated with the use of autologous chondrocytes (CCs), including the creation of a new defect in the articular surface. Stem cells isolated from synovial joint tissues such as synovial membrane (SM) (De Bari *et al.*, 2001; Pei *et al.*, 2008a; Pei *et al.*, 2008b) and infrapatellar fat pad (FP) (Dragoo *et al.*, 2003; Wickham *et al.*, 2003; English *et al.*, 2007; Khan *et al.*, 2007; Khan *et al.*, 2008) are a particularly promising alternative to CCs as they are phenotypically similar to CCs and accessible during arthroscopy (Segawa *et al.*, 2009). This has led to increased interest in using joint tissue derived stem cells for engineering functional cartilaginous grafts (Buckley *et al.*, 2010a; Buckley *et al.*, 2010b; Vinardell *et al.*, 2010). To fully realise their potential, in this regard, requires a more thorough understanding of how biochemical and biophysical factors will influence their phenotypic stability and biosynthetic activities during *in vitro* cultivation and following implantation into the joint environment.

The pellet culture model is commonly used to explore chondrogenic differentiation of MSCs (Johnstone *et al.*, 1998). In this system, cell-cell interactions within the MSC condensation coupled with the extrinsic signal provided by the addition of transforming growth factor beta 1 (TGF-β1) has been shown to induce chondrogenesis of MSCs. Chondrogenesis was found to be dependent on the concentration of TGF-β1 within the culture media, with more robust differentiation observed at higher concentrations of TGF-β1 (Johnstone *et al.*, 1998). Chondrogenesis of synovial membrane derived stem cells (SDSCs) and infrapatellar fat pad derived stem cells (FPSCs) has also been demonstrated using the pellet culture system (Mochizuki *et al.*, 2006; Marsano *et al.*, 2007; Vinardell *et al.*, 2010). Additional growth factors have been used in combination with TGF-β stimulation with synergistic effects observed (Lee *et al.*, 2008; Pei *et al.*, 2008b). While cellular condensation coupled with endogenous TGF-β stimulation have been shown to induce chondrogenic differentiation *in vitro*, other environmental

signals, including biophysical stimuli, will play a key role in determining stem cell fate.

Biophysical stimuli such as tension, compression, fluid flow and hydrostatic pressure (HP) are thought to regulate chondrogenesis of stem cells (Stoddart *et al.*, 2009; Kelly and Jacobs, 2010). Finite element models have been used to support the hypothesis that mechanical loading can regulate tissue differentiation and that HP can promote cartilage formation and potentially suppress endochondral ossification (Carter *et al.*, 1998; Loboa *et al.*, 2001). *In vitro* studies have demonstrated that physiological frequencies and magnitudes of HP (3 to 10 MPa at frequencies of 1 Hz) increase CCs matrix synthesis (Parkkinen *et al.*, 1993; Toyoda *et al.*, 2003a; Toyoda *et al.*, 2003b; Elder *et al.*, 2006; Elder and Athanasiou, 2008; Elder and Athanasiou, 2009). In either the absence or presence of TGF, cyclic HP also seems to enhance chondrogenesis of bone marrow MSCs at the gene and/or protein level (Angele *et al.*, 2003; Miyanishi *et al.*, 2006b; Finger *et al.*, 2007; Elder and Athanasiou, 2008; Wagner *et al.*, 2008; Meyer *et al.*, 2011). HP has also been shown to enhance chondrogenesis of SDSCs (Sakao *et al.*, 2008; Wu *et al.*, 2008), however the interaction between HP and members of the TGF-β family of growth factors in regulating the initiation and maintenance of a chondrogenic phenotype in joint tissue derived stem cells remains poorly understood.

MSCs offer tremendous potential for the treatment of damaged and diseased articular cartilage. The success of new therapies utilising joint tissue derived stem cells will require a fundamental understanding of how local environmental factors regulate their phenotype and subsequent synthetic activities. While both TGF-β stimulation and hydrostatic pressure have been identified as key determinants of stem cell differentiation, little is known about how these signals interact to regulate their fate. Given that both stimuli are key components of the regenerative environment, the goal of this study was to investigate the interaction between HP and TGF-β3 in regulating the induction and maintenance of a chondrogenic phenotype in joint tissue derived stem cells. To this end, this study explored the combined effects of HP and different concentrations of TGF-β3 (0, 1 and 10 ng/mL) on chondrogenic gene expression and matrix synthesis for SDSCs and FPSCs and compared this response to CCs isolated from articular cartilage from the same joint. Given that retrieving stem cells from multiple tissues in healthy humans is challenging, a porcine model was chosen to address this research question. Pigs are similar to humans in terms of their genetics, anatomy and physiology (Vacanti *et al.*, 2005), and the tripotentiality of porcine MSCs from FPSCs and SDSCs has been demonstrated in previous studies (Pei *et al.*, 2008b; Vinardell *et al.*, 2010).

Materials and Methods

Cell isolation and expansion

CCs and joint tissue derived stem cells were aseptically harvested from the femoropatellar joint of four month old pigs (two donors). Cartilage tissue harvested from the tibial, patellar and femoral surface were pooled for each pig in order to obtain sufficient numbers of CCs to perform the study. Briefly, full depth articular cartilage slices were rinsed with phosphate buffered saline (PBS) containing penicillin/streptomycin (200 U/mL) (GIBCO, Biosciences, Dublin, Ireland). The cartilage slices were then combined and digested with DMEM GlutaMAX (GIBCO) containing collagenase type II (315 U/mg) (Worthington, Langanbach Services, Bray, Ireland) and amphotericin B (2.5 μg/mL) (Sigma-Aldrich, Dublin, Ireland) for 16-18 h under constant rotation at 37 °C. The resulting cell suspension was then filtered through a 40 μm pore-size cell sieve (Falcon, Sarstedt, Wexford, Ireland) and the filtrate centrifuged and rinsed with PBS twice. SM and FP tissues were harvested, sliced and rinsed with PBS (Sigma-Aldrich) containing penicillin/streptomycin (200 U/mL) and amphotericin B (2.5 μg/mL). SM and FP pieces were incubated with DMEM GlutaMAX containing collagenase type II (315 U/mg) for 3-4 h under constant rotation at 37 °C. The resulting cell suspension was then filtered through a 40 μm pore-size cell sieve and the filtrate centrifuged and rinsed with PBS twice. CCs, SDSCs and FPSCs were seeded at a density of 5×10^3 cells/cm^2 in 175 cm^2 T-flasks in a humidified atmosphere at 37 °C and 5 % CO_2 and expanded to passage one for CCs (approximately 7 population doublings) and to passage two for the stem cells (approximately 14-15 population doublings). Cells were also cryopreserved for replicate studies (hgDMEM supplemented with 10 % v/v foetal bovine serum (Gibco; FBS) and 10 % dimethylsulphoxide (Sigma-Aldrich; DMSO) in a liquid nitrogen tank). Viable cells were counted using a haemocytometer and 0.4 % trypan blue staining. Isolated cells from different tissues were maintained in DMEM GlutaMAX supplemented with 10 % v/v foetal bovine serum (FBS) (Gibco) and 100 U/mL penicillin/streptomycin during the monolayer expansion phase with 5 ng/mL of human fibroblast growth factor-2 (FGF-2) (Prospec, Ness-Ziona, Israel).

Pellet formation

250 000 cells were placed in 1.5 mL conical microtubes and centrifuged at 650 G for 5 min. The pellets were cultured in a chemically defined chondrogenic medium: DMEM GlutaMAX supplemented with 100 U/mL penicillin/streptomycin, 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid-2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin (BSA), 1× insulin-transferrin-selenium, 100 nM dexamethasone (all from Sigma-Aldrich). The pellets were cultured with different concentrations of human TGF-β3 (Prospec) 0, 1 and 10 ng/mL. Medium was exchanged every 3 or 4 days with 1 mL samples of media taken for biochemical analysis.

Application of hydrostatic pressure

Pellets were subjected to HP after an initial 24 h of free swelling culture HP (10-12 per group). Pellets were sealed in sterile plastic bags with 4 mL of chondrogenic medium with different concentrations of TGF-β3 and assigned to receive either mechanical stimulation (HP group), or were left unloaded (Free swelling: FS group). The loaded group was exposed to 10 MPa of HP at a frequency of 1 Hz for 4 h per day, for 14 days, while the unloaded controls were kept

in bags in an incubator immersed in water at 37 °C during the loading period. After loading, all pellets were returned to culture dishes. A stainless steel pressure chamber (Parr Instruments Company, Moline, IL, USA), connected to a piston (phd Inc., Irish Pneumatics, Kildare, Ireland), driven by an Instron 8801 materials testing machine was used to generate the required levels of HP. Further details are available elsewhere (Meyer *et al.*, 2011).

Histology and immunohistochemistry

For histological evaluation pellets were fixed in 4 % paraformaldehyde and embedded in paraffin, cut into 5 µm thick sections, and stained with 1 % alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl to assess glycosaminoglycan content and picrosirius red to detect collagen. Type I, II and X collagen content were evaluated with a standard immunohistochemical technique; briefly, sections were treated with peroxidase (between steps, slides were washed in PBS) followed by treatment with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37 ºC to enhance permeability of the extracellular matrix. After incubation with goat serum to block non-specific sites, the collagen type I (ab6308, 1:400; 5.7 mg/mL), collagen type II (ab3092,1:100; 1 mg/mL) or collagen type X (ab49945,1:200; 1.4 mg/mL) primary antibody (mouse monoclonal, Abcam, Cambridge, UK) was applied on the sections for 1 h at room temperature. Next, the secondary antibody (Anti–Mouse IgG biotin conjugate (1:200; 2.1 mg/mL), Sigma-Aldrich) was added for 1 h, followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs, Peterborough, UK) for 45 min. Finally, the slides were developed with DAB peroxidase (Vector Labs) for 5 min. Positive and negative controls were included in the immunohistochemistry staining protocol for each batch.

Biochemical analysis

All samples were digested in papain (125 µg/mL) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich) at 60 °C under constant rotation for 18 h. Aliquots of the digested samples were assayed separately for DNA, collagen and sulphated glycosaminoglycan (sGAG) content. DNA content was quantified using the Quant-it Picogreen DNA assay (Invitrogen, Paisley, UK). The proteoglycan content was estimated by quantifying the amount of sGAG in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor, Carrickfergus, Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline-to-collagen ratio of 1:7.69 (Kafienah and Sims, 2004; Ignat'eva *et al.*, 2007). Alkaline phosphatase activity was determined using SensoLyte® pNPP Alkaline Phosphatase Assay Colorimetric kit (AnaSpec, San Jose, CA, USA) and the absorbance was measured at 405 nm. At each media exchange, samples of media were taken for analysis of alkaline phosphatase activity, sGAG and total collagen secreted into the media. The total release to the media over the 14 day culture period for all pellets in each experimental group was measured and from this the average release per pellet was determined. The concentration of TGF-β1 in the culture media was quantified using a TGF-β1 ELISA kit (Booster Immunoleader, Wuhan, China) following the manufacturer's instructions. The concentration of TGF-β3 in the media was quantified using a DuoSet ELISA Development System (R&D Systems, Abingdon, UK) following the manufacturer's protocol.

RNA isolation and quantitative real-time polymerase chain reaction

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression changes in chondrogenic specific genes over time and subsequent to the application of loading. Total RNA was extracted from SDSCs and FPSCs pellets following 14 days of culture. Day zero groups were harvested following centrifugation and formation of cellular pellets. Loaded and unloaded groups were harvested directly after the application of loading at day 14. Pellet construct pooling was performed for all groups, with a total of 5 pellets pooled per group. Total cellular RNA was extracted from each pellet group by physical homogenisation using a motor driven pestle and mortar system (Sigma-Aldrich) in 1 mL of TRIZOL reagent (Invitrogen), followed by a chloroform (Sigma-Aldrich) extraction with PureLink™ RNA Mini kit (Invitrogen) as per manufacturer's instructions. RNA was re-suspended in RNase free water prior to reverse transcription.

Total RNA concentrations were determined using the Quant-iT™ RNA assay and a Qubit® fluorometer (Invitrogen) and adjusted to a standard concentration prior to cDNA synthesis. To quantify mRNA expression, 100 ng of total RNA was reverse transcribed into cDNA using iScript™ cDNA systhesis kit (BioRad, Hemel Hempstead, UK) as per manufacturer's instructions. TaqMan® gene expression Assays (Applied Biosystems/ Life Technologies, Paisley, UK) which contain forward and reverse primers, and a FAM-labelled TaqMan probe for porcine (*Sus scrofa*) *Sox9* (Ss03392406m1), *aggrecan* (Ss03374822m1), *collagen type I alpha I* (Ss03373340m1), *collagen type II alpha 1* (Ss03373344g1), *collagen type X alpha 1* (Ss03391766m1), *TGF-β receptor 3* (Ss03394536m1), *Indian Hedgehog* (Ss03373541m1) and *Glyceraldehyde-three-phosphate dehydrogenase (GAPDH)* (Ss03373286u1) were used in this study. Each assay was chosen based on the amplicon spanning an exon junction. qRT-PCR was performed using an ABI 7500 Sequence Detection system (Applied Biosystems). A 20 µL volume was added to each well (5 µL of cDNA preparation diluted 1:5 with RNase free water, 1 µL of gene assay, 10 µL of TaqMan Universal PCR Master mix (Applied Biosystems) and 4 µL RNase free water). Samples were assayed in triplicate in one run (40 cycles). qRT-PCR data were analysed using relative quantification and the Delta C^t-method as described previously (Livak and Schmittgen, 2001) with the FS day zero samples used as the calibrator and *GAPDH* as the endogenous control gene. Relative quantification values are presented as fold changes in gene expression plus/minus the standard error of the mean relative to the control group, which was normalised to one.

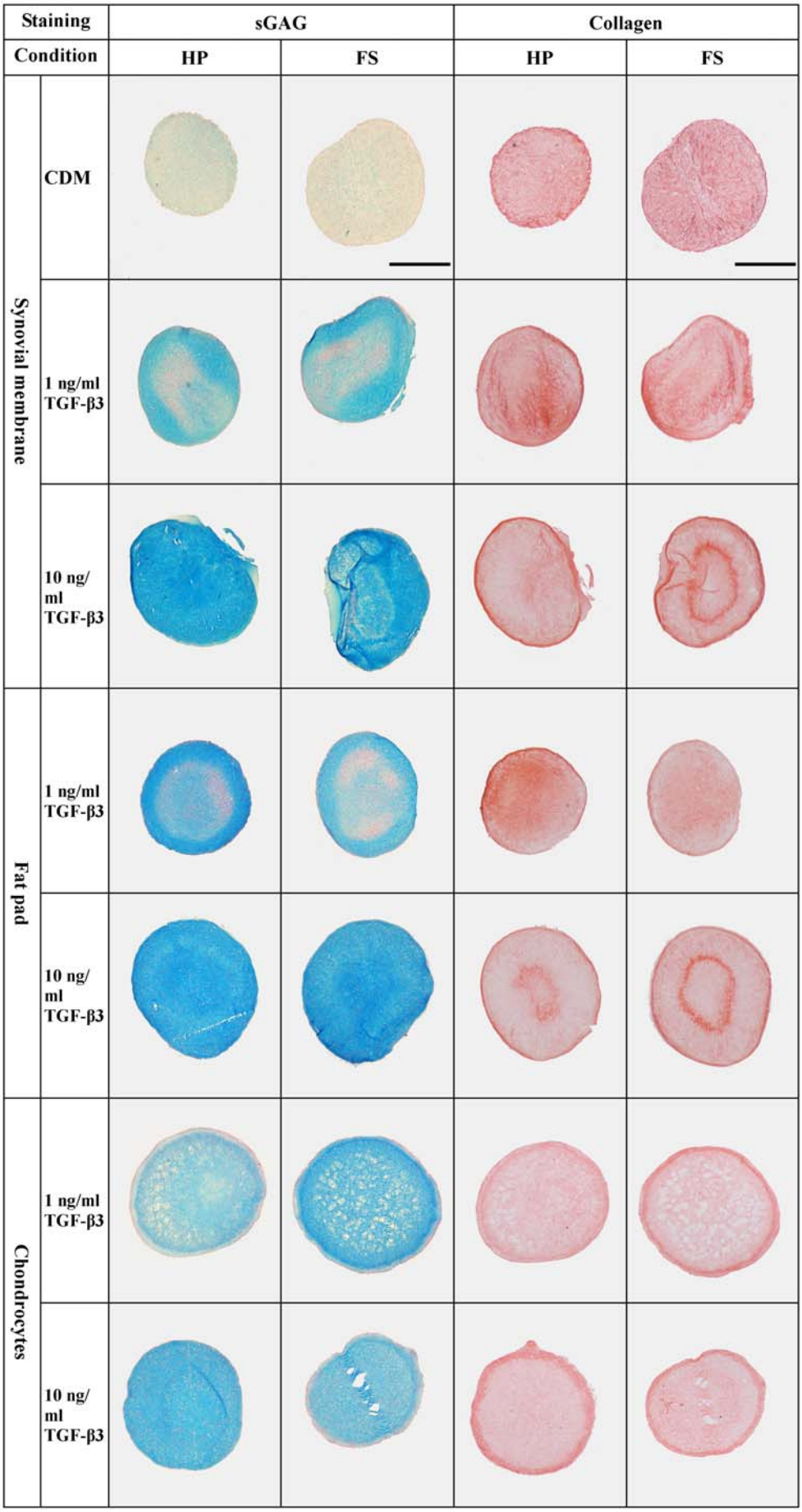

Fig. 1. Histology sections of synovial membrane, fat pad and chondrocytes pellets kept in free swelling conditions (FS) or exposed to hydrostatic pressure (HP) following 14 days of culture. Pellets were stained with alcian blue (stains sulphated glycosaminoglycan, left columns) and picro-sirius red (stains collagen, right columns); scale bar 500 µm.

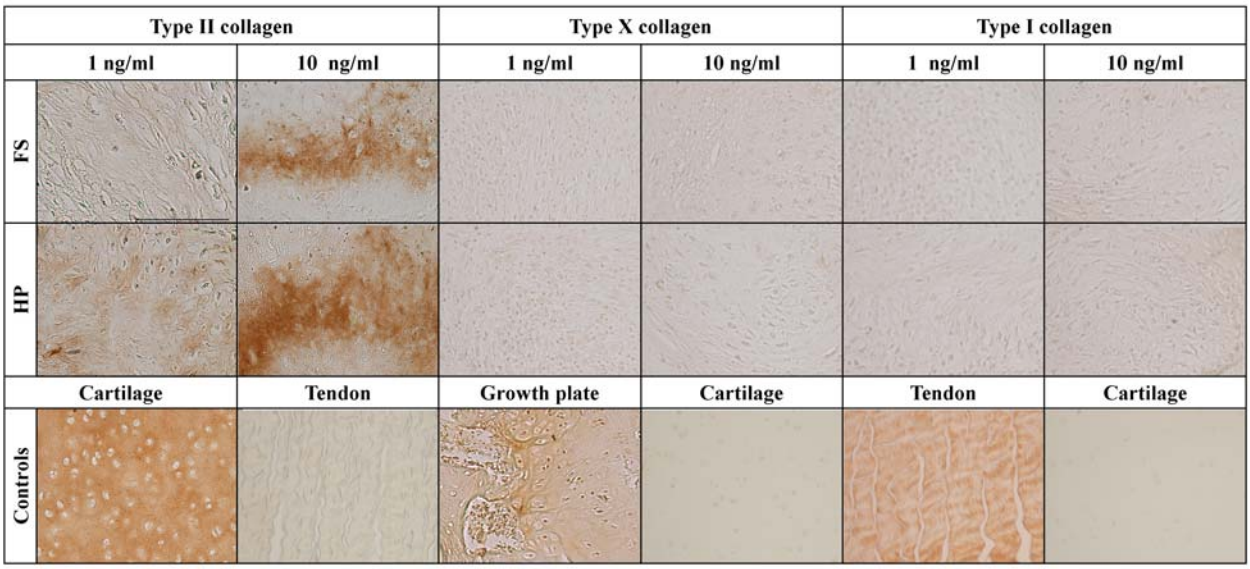

Fig. 2. Synovial membrane pellets immunostained for type II, X and I collagen with subsequent controls (Type II collagen, positive control: cartilage, negative control: ligament; type X collagen, positive control: growth plate, negative control: cartilage; type I collagen positive control: ligament, negative control: cartilage). Images are taken from regions of the pellets that stained positive for picro-sirius red. Scale bar 100 µm.

Statistical analysis

For each cell source, at least two replicates of the entire study were performed using cells from the same donor. For each of these replicates, twelve samples were analysed per group at each time point, with 5 samples used for biochemical analysis of matrix components, 5 pooled samples for qRT-PCR and 2 samples were used for histology. In addition, the average sGAG and collagen release to the media per pellet in each experimental group, as well as the concentration of TGF-β1 and TGF-β3 in the media, was determined for each replicate of the experiment. The data from all replicates was then pooled for subsequent statistical analysis. Differences in gene expression, mechanical and biochemical properties with cell type and/or time-in-culture were determined by *t*-tests and by analysis of variance with time or loading as independent factors, followed by Tukey's *post-hoc* test (95 % confidence Interval). Statistics were preformed with MINITAB 15.1 (Minitab, Coventry, UK) and the GraphPadPrism 4 software package (San Diego, CA, USA). Data are expressed in the form of mean ± standard error of the mean (SEM). Statistical significance was declared at $p \leq 0.05$. Given the unexpected response of chondrocytes to different concentrations of TGF-β3 (see data below) we replicated this arm of the study using cells derived from a different donor and observed the same response.

Results

Effect of HP on pellet morphology

After 14 days in culture, pellets were mostly spherical and stained positive for proteoglycan and collagen deposition when supplemented with TGF-β3, while minimum staining was observed in both the non-supplemented FS and HP groups (Fig. 1). In general, an increase in matrix staining was observed for FPSCs and SDSCs when supplemented with higher concentrations of TGF-β3. Moreover, HP appeared to influence the organisation of the neo-tissue, with a more obvious core region in the FS SDSC pellets supplemented with 10 ng/mL of TGF-β3 compared to a more homogeneous distribution of extracellular matrix in the HP group (Fig. 1). CCs pellets supplemented with 1 ng/mL of TGF-β3 presented some evidence of cell clustering, with more intense staining observed in the FS group in comparison with pellets subjected to HP. Immunohistochemistry confirmed the presence of type II collagen in the SDSCs pellets (Fig. 2). The same pattern of staining was observed in FPSCS and CCs pellets (data not shown). Weak staining for type I and X collagen was observed.

Effect of HP on matrix production

HP had no effect on DNA content for any cell type at 0, 1 and 10 ng/mL of TGF-β3 (data not shown). HP significantly increased sGAG accumulation for both FPSCs and SDSCs supplemented with 1 ng/mL of TGF-β3 (FPSCs HP: 10.5 µg (± 1.1) *vs.* FPSCs FS: 7.01 µg (± 0.6); SDSCs HP: 8.35 µg (± 1.3) *vs.* SDSCs FS: 5.5 µg (± 0.5)), but did not affect collagen synthesis (Fig. 3a). In contrast, HP had no effect on sGAG synthesis for both FPSCs and SDSCs when supplemented with 10 ng/mL of TGF-β3 (Fig. 3b). For the CCs pellets, HP decreased sGAG accumulation when supplemented with 1 ng/mL of TGF-β3 (FS: 27.7 µg (± 2.3) *vs.* HP: 14.3 µg (± 1.7)) (Fig. 3a). A similar result was observed for the total amount of sGAG synthesised (addition of sGAG retained in the pellet and the amount of sGAG secreted to the media). HP was also observed to inhibit collagen accumulation for CCs when supplemented

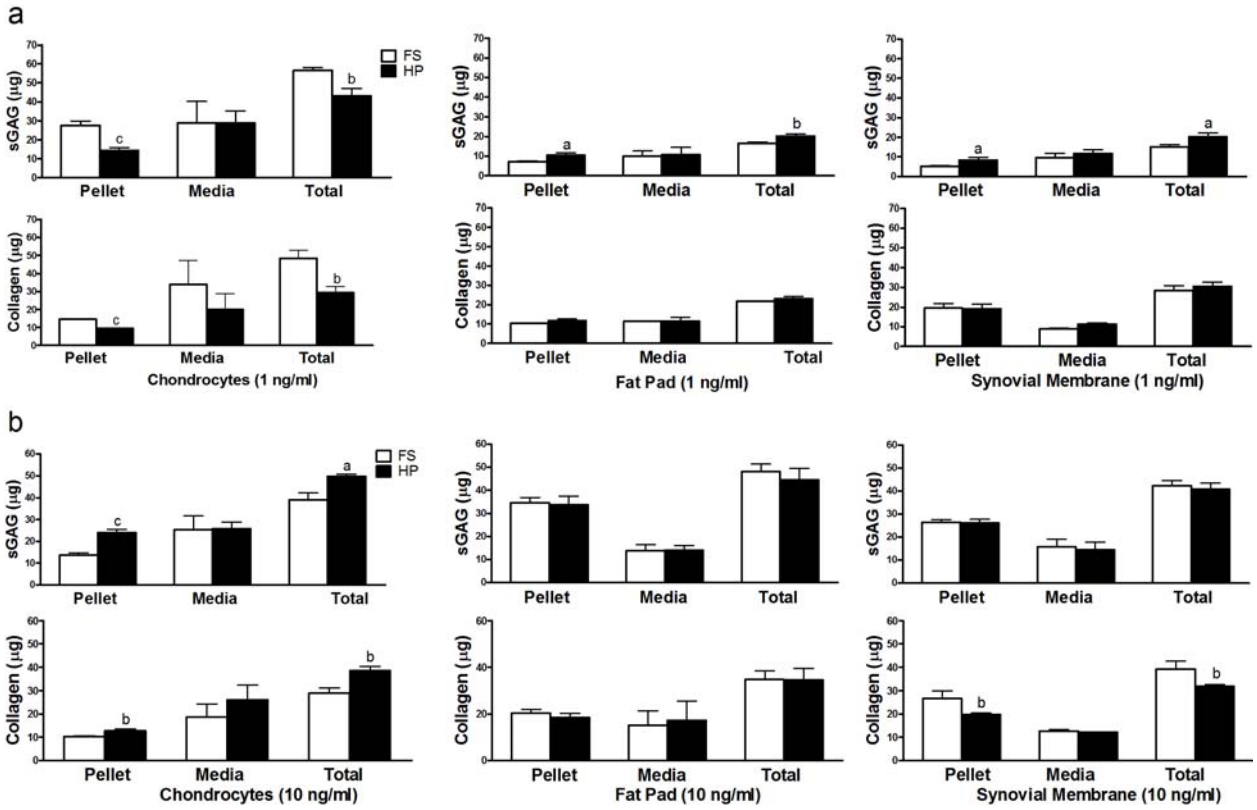

Fig. 3. Sulphated glycosaminoglycan (sGAG) and collagen content measured in the pellets and culture media of chondrocytes and joint tissue derived stem cells with (**a**) 1 ng/mL of TGF-β3 or (**b**) 10 ng/mL of TGF-β3. Free swelling (FS); hydrostatic pressure (HP). Media values represent the total amount of matrix components released per pellet over the entire culture period. The total content represents the sum of matrix components being retained in each pellet and released to the media. a: $p < 0.05$; b: $p < 0.001$; c: $p < 0.0001$.

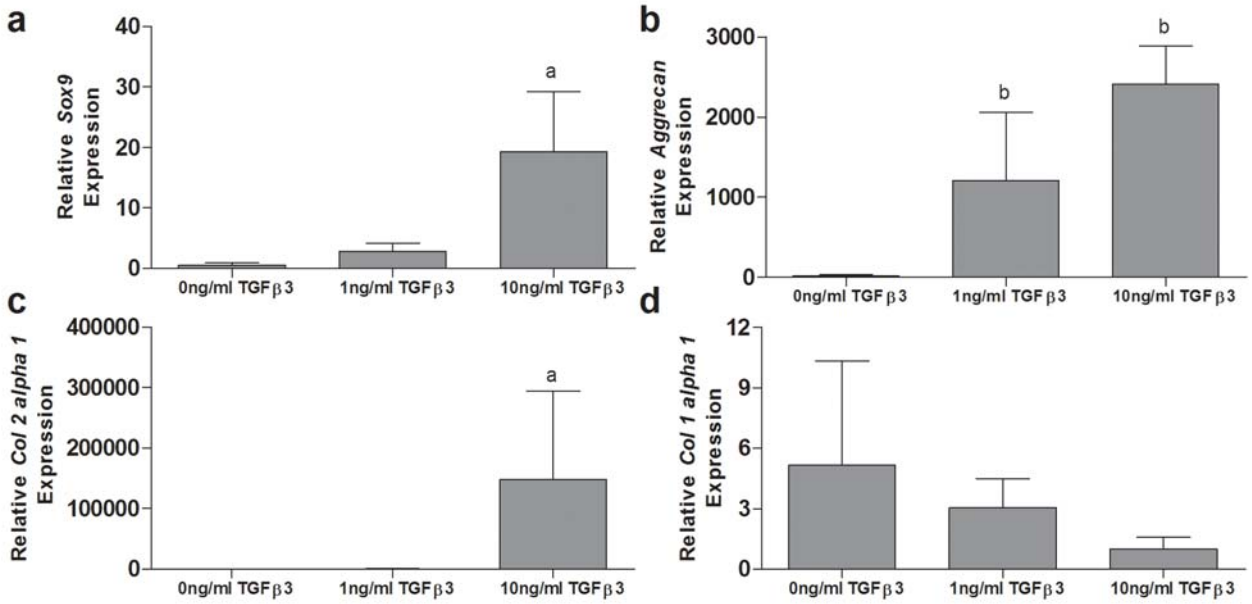

Fig. 4. Chondrogenic and extracellular matrix-specific relative gene expression in synovial membrane pellets cultured in free swelling conditions with 0, 1 or 10 ng/mL TGF-β3 over 14 days. Calibration performed according to expression of the endogenous control gene *GAPDH* and normalised to day 0 free swelling controls. *Sox9* (**a**), *Aggrecan* (**b**), *Collagen type II alpha 1* (**c**) and *Collagen type I alpha 1* (**d**) expression. Data is expressed as relative expression per group and represented as mean ±SEM. Pellets were analysed from 3 independent experimental replicates, using 5 pellets pooled from each replicate. a: $p < 0.05$, b: $p < 0.0001$ *vs.* gene expression at day 0.

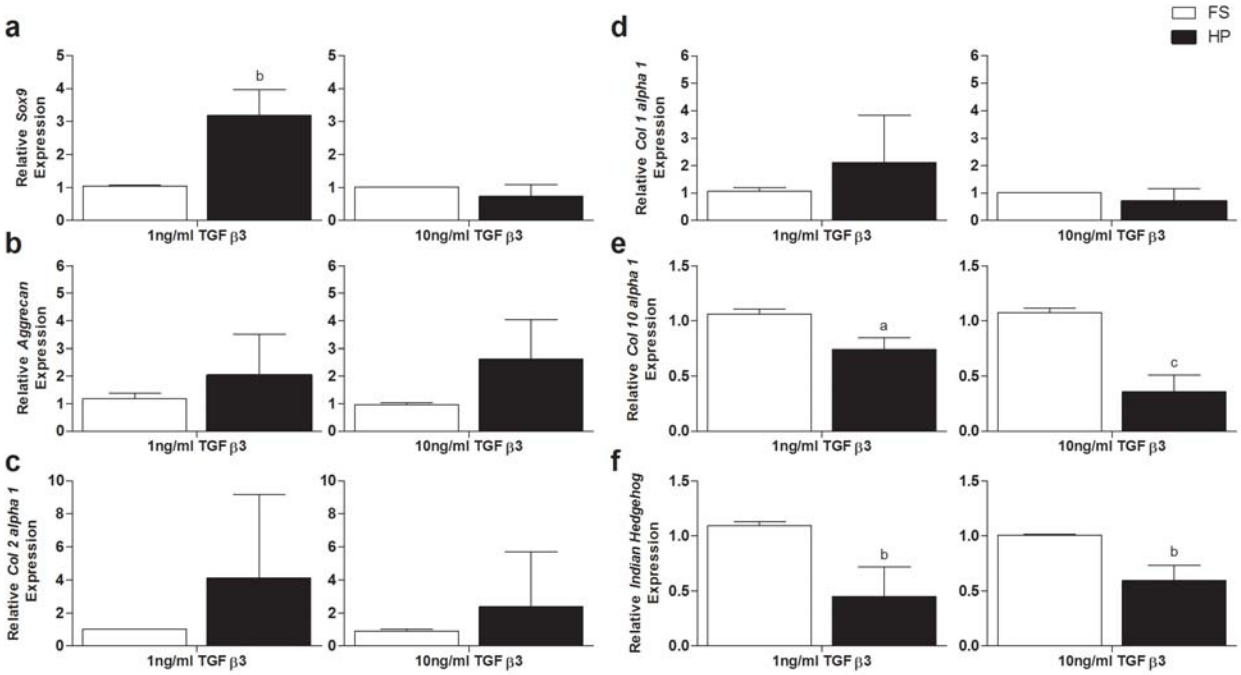

Fig. 5. The influence of hydrostatic pressure on gene expression in synovial membrane pellets at day 14 cultured in free swelling (FS) and hydrostatic pressure (HP) conditions with 1 or 10 ng/mL TGF-β3. Calibration of each gene to *GAPDH* expression levels and normalised to free swelling controls. *Sox9* (**a**), *Aggrecan* (**b**), *Collagen type II alpha 1* (**c**), *Collagen type I alpha 1* (**d**), *Collagen type X alpha 1* (**e**) and *Indian Hedgehog* (*Ihh*) (**f**) expression. Data is expressed as relative expression per group and represent mean ±SEM. Pellets were analysed from 3 independent experimental replicates, using 5 pellets pooled from each replicate. a: $p < 0.1$, b: $p < 0.05$, c: $p < 0.01$. *vs.* gene expression at day 14 for FS control.

with 1 ng/mL of TGF-β3 (FS: 14.8 µg (± 0.25) *vs.* HP: 9.34 µg (± 0.05)) (Fig. 3a). When CCs were supplemented with 10 ng/mL of TGF-β3, HP increased both sGAG (FS: 13.7 µg (± 1.1) *vs.* HP: 23.9 µg (± 1.6)) and collagen (FS: 10.1 µg (± 0.4) *vs.* HP: 12.8 µg (± 0.7)) accumulation. Analysis of the media from all groups revealed negligible levels of alkaline phosphatase activity (data not shown).

Influence of exogenous TGF-β3 and HP on gene expression and TGF-β protein synthesis

SDSCs responded to the addition of 10 ng/mL of TGF-β3 with a greater than 19-fold increase in *Sox9* expression, 2,400-fold increase in *Aggrecan* expression and a 140,000-fold increase in *type II collagen* expression following 14 days in FS conditions compared to day 0 control levels (Fig. 4a, b, c). The addition of 1 ng/mL of TGF-β3 resulted in a 1,200-fold (D14 FS: 1202.7 (± 858.6) *vs.* D0 FS: 0.92 (± 0.04), $p \leq 0.0001$) increase in *Aggrecan* relative gene expression compared to day 0 control levels (Fig. 4b). No difference was observed for *type I collagen* with and without TGF-β3 supplementation following 14 days in FS culture (Fig. 4d). *Type X collagen* and *Indian Hedgehog* were not expressed at day 0. By day 14, the expression of *type X collagen and Indian Hedgehog* was 100 fold and 5 fold higher respectively in pellets supplemented with 10 ng/mL compared to 1 ng/mL of TGF-β3 (data not shown).

The application of HP had no significant effect on the expression of *Sox9, Aggrecan* and *type II collagen* in the absence of growth factor supplementation at day 14, but did lead to significant decrease in *type I collagen* gene expression (HP: 0.13 (± 0.07) *vs.* FS: 1.02 (± 0.002), $p \leq 0.01$) (data not shown). Applying HP in the presence of 1 ng/mL of TGF-β3 resulted in a 3-fold increase in the mRNA levels of *Sox9* (HP: 3.19 (± 0.45) *vs.* FS: 1.21 (± 0.09), $p \leq 0.05$) in comparison to FS controls (Fig. 5a). A similar trend was observed in the FPSCs group following the application of HP and 1 ng/mL of TGF-β3 stimulation with a 3.5-fold increase in *Sox9* (HP: 3.53 (± 0.45) *vs.* FS: 1.05 (± 0.03), $p \leq 0.1$) relative gene expression in comparison to FS controls (data not shown). No significant difference was observed for the extracellular matrix specific genes *aggrecan*, *type II collagen* and *type I collagen* following the application of HP combined with either 1 or 10 ng/mL TGF-β3 (Fig. 5b, c, d). Applying HP to SDSCs pellets in the presence of TGF-β3 at 1 and 10 ng/mL also lead to decreases in the mRNA levels of the hypertrophic marker *type X collagen* and the developmental regulatory gene *Indian Hedgehog* (Fig. 5e, f).

Finally, in an attempt to explain the mechanism by which HP enhances chondrogenesis in SDSCs supplemented with 1 ng/mL of TGF-β3, we explored if HP regulated the endogenous production of TGF-β by measuring the concentration of TGF-β1 and TGF-β3 in the media of FS and HP pellets. The application of HP did not lead to additional endogenous production of TGF-β3 by SDSCs already exogenously stimulated with TGF-β3 (Fig. 6a). Exogenous stimulation of SDSCs with TGF-β3 did result in endogenous production of TGF-β1, but this

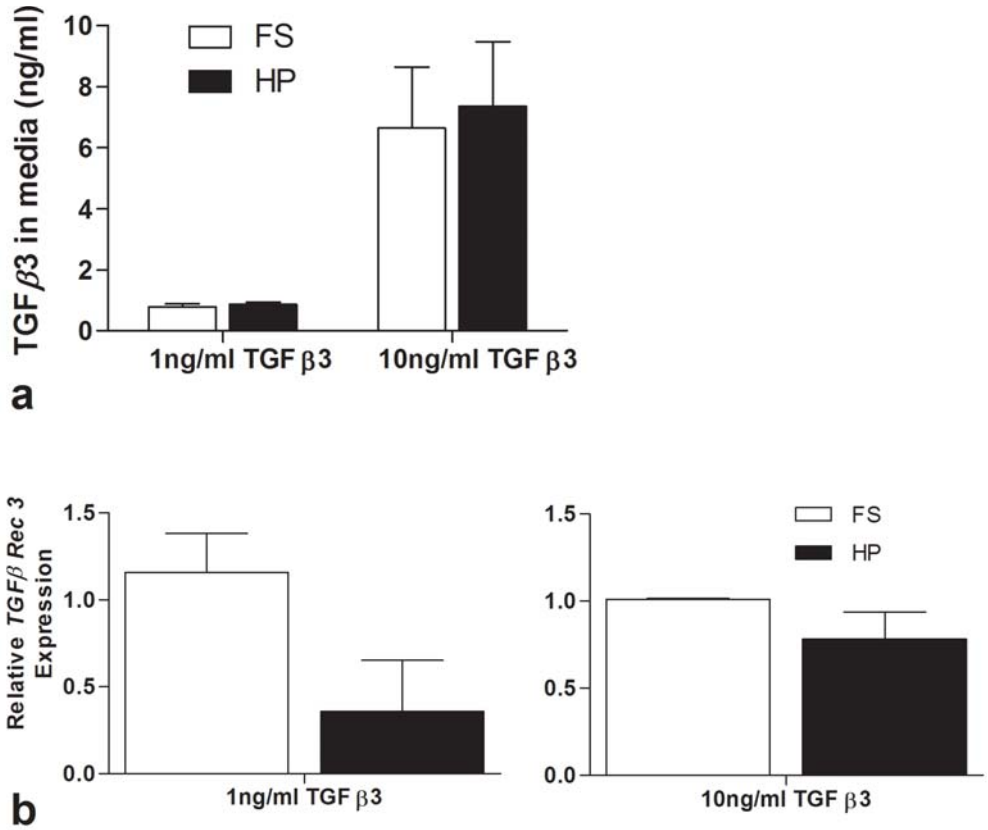

Fig. 6. (**a**) The influence of hydrostatic pressure on TGF-β3 synthesis, as determined by measuring the concentration of TGF-β3 in the media of pellets exogenously supplemented with either 1 or 10 ng/mL of TGF-β3. (**b**) The influence of hydrostatic pressure on the expression of TGF-β receptor 3.

was not influenced by the application of HP (FS: 0.152 ± 0.028 ng/mL TGF-β1; HP: 0.156 ± 0.037 ng/mL TGF-β1; both groups supplemented with 1 ng/mL of TGF-β3). Furthermore, HP did not significantly affect the mRNA levels of TGF-β receptor 3 (Fig. 6b).

Discussion

Understanding how various environmental cues interact to regulate chondrogenesis of stem cells will be critical to the development of new regenerative medicine therapies for treating damaged and diseased articular cartilage. The objective of this study was to investigate the interaction between HP and TGF-β3 in regulating the induction and maintenance of a chondrogenic phenotype in joint tissue derived stem cells. HP alone did not induce robust chondrogenesis of joint derived stem cells, however when applied with low concentrations of TGF-β3, it acted synergistically to increase *Sox-9* gene expression, promote sGAG synthesis and inhibit *Indian Hedgehog* and *type X collagen* gene expression, the latter two being markers of pre-hypertrophy and terminal differentiation. At high magnitudes of TGF-β3, HP had no additional synergistic effect on matrix synthesis, suggesting an upper limit on the stimulatory effects these two chondrogenic cues can have on matrix production, although it continued to act to suppress *Indian Hedgehog* and *type X collagen* gene expression. Interestingly, CCs responded differently to both TGF-β3 stimulation and HP than joint tissue derived stem cells, with synthesis of sGAG and collagen inhibited at higher concentrations of TGF-β3.

HP is one of the main mechanical stimuli exerted on articular cartilage and understanding its effects on stem cells and CCs is a central challenge in the field of articular cartilage tissue engineering and regeneration (Elder and Athanasiou, 2009). The loading regime chosen for this study was based on previous reports demonstrating that 10 MPa of HP applied for 4 h daily at a frequency of 1 Hz enhanced chondrogenesis (Angele *et al.*, 2003; Ikenoue *et al.*, 2003; Miyanishi *et al.*, 2006b). Exposure to 10 MPa of HP influenced the organisation of the tissue, resulting in the formation of a more homogenous pellet. A similar observation has been reported using human bone marrow MSCs, where pellets exposed to 10 MPa of HP exhibited a more compact and uniform structure than FS controls (Miyanishi *et al.*, 2006a; Miyanishi *et al.*, 2006b). Such alterations in the distribution of extracellular matrix components might be expected to lead to improvements in apparent construct mechanical functionality (Kelly and Prendergast, 2004) should the use of HP be extended to engineering cartilaginous grafts using scaffolds seeded with joint tissue derived MSCs (Meyer *et al*., 2011).

Chondrocytes and joint tissue derived stem cells were found to differentially respond to altered TGF-β3 concentrations. Johnstone *et al*. evaluated the effect of different concentrations of TGF-β (0.5 to 10 ng/mL) on bone marrow derived MSCs, concluding that lowering the TGF-β concentration decreased their chondrogenic potential (Johnstone *et al*., 1998). A similar response to TGF-β3 stimulation was observed in this study for joint tissue derived stem cells. FPSCs and SDSCs responded similarly to TGF-β3 stimulation, corroborating previous reports where these two stem cell sources demonstrate a comparable level of chondrogenesis in response to cytokine stimulation (Mochizuki *et al*., 2006; Marsano *et al*., 2007). Interestingly when CCs were supplemented with higher concentrations of TGF-β3, lower levels of sGAG and collagen accumulation were observed, which has also been reported for bovine CCs where supplementation with 10 ng/mL of TGF-β3 was observed to suppress chondrogenesis (Erickson *et al*., 2011). This suppression of CCs chondrogenesis at high concentrations of TGF-β3 was partially overcome when cells were subjected to HP. It is possible that HP is activating an alternative pathway to that of TGF-β3 stimulation, one that acts independently or perhaps cross-talks intracellularly, or alternatively that HP is directly influencing TGF-β signalling, although the results of this study do not provide any support for the hypothesis that HP regulates TGF-β signalling in SDSCs. It has been suggested that HP can directly affect the conformation of transmembrane proteins (Kornblatt and Kornblatt, 2002; Elder and Athanasiou, 2009), raising the possibility that such extrinsic mechanical loading can physically alter TGF-β receptors on the cell surface, and hence their binding affinity for TGF-β3.

HP, in the absence of TGF-β3, did not appear to induce robust chondrogenesis of joint derived stem cells. In the case of bone marrow derived MSCs, some uncertainty exists in the literature as to the ability of HP to induce chondrogenesis in the absence of growth factors. Some report that HP has little or no effect on chondrogenic gene expression and matrix production (Zeiter *et al*., 2009), while others report increases in *Sox-9*, *type II collagen* and *aggrecan* gene expression but no increase in pellet wet weight due to loading (Miyanishi *et al*., 2006b). HP has also been shown to promote *Sox-9* gene expression and protein production in SDSCs (Sakao *et al*., 2008; Wu *et al*., 2008), although such increases in the expression of regulatory genes due to HP does not necessarily lead to changes in the expression of *aggrecan* and *type II collagen* (Finger *et al*., 2007). In the present study we found that HP acts synergistically with low concentrations of TGF-β3 to enhance chondrogenesis of joint tissue derived MSCs, as evidenced by a 3-fold increase in *Sox9* expression and enhanced sGAG synthesis. As both biochemical and biophysical stimuli are present within the *in vivo* joint environment, further work is necessary to fully understand how these various cues interact to regulate stem cell fate.

In agreement with previous studies (Zeiter *et al*., 2009), we also observed that HP had no beneficial effect on cartilage-specific matrix production when stem cells were supplemented with 10 ng/mL of TGF-β3. In contrast, a number of other studies have reported a synergistic effect for bone marrow MSCs supplemented with 10 ng/mL of TGF-β3 (Miyanishi *et al*., 2006b; Wagner *et al*., 2008). Similar results have been observed for adipose derived stem cells (Ogawa *et al*., 2009). Given that synovial fibroblasts have been shown to produce TGF-β when subjected to HP (Wu *et al*., 2008), we initially hypothesised that HP would exert its effects on joint tissue derived stem cells, at least in part, *via* this pathway. If this were the case, it might explain why HP only enhances chondrogenesis at lower concentrations of TGF-β, with the additional signal provided by HP being lost when saturation levels of TGF-β are present. Previous studies have demonstrated that mechanical loading can enhance chondrogenesis of human bone marrow derived MSCs, which was promoted through the TGF pathway by up-regulating TGF gene expression and protein synthesis (Li *et al*., 2010). However, the results of this study do not provide evidence to support the hypothesis that HP influences TGF-β synthesis in SDSCs, suggesting instead that it may act to regulate other signalling pathways involved in chondrogenesis of SDSCs. It has recently been demonstrated that the primary cilium is involved in signal transduction in response to hydrostatic compressive loading of growth plate chondrocytes (Shao *et al*., 2011). Given that the primary cilium is known to act as a mechanosensory organelle in numerous orthopaedic cells (Malone *et al*., 2007; Kwon *et al*., 2010; Hoey *et al*., 2011; Shao *et al*., 2011; Hoey *et al*., 2012), it is possible that HP exerts its effects on joint tissue derived stem cells through any one of numerous signalling pathways (e.g. *Hedgehog*, *Wnt*) that acts through the primary cilia.

It has been demonstrated that cartilaginous tissues engineered using SDSCs and FPSCs do not form stable cartilage following subcutaneous implantation (De Bari *et al*., 2004; Marsano *et al*., 2007; Dickhut *et al*., 2009). A major difference between the ectopic and orthotopic environment is the absence of mechanical signals such as HP, which may play a key role in achieving a type X collagen negative permanent cartilage phenotype (Steck *et al*., 2009). Joint loading has been shown to be an essential requirement for the homeostasis of normal articular cartilage (Palmoski *et al*., 1980; Kiviranta *et al*., 1987) and definition of articular tissues during development (Nowlan *et al*., 2010; Roddy *et al*., 2011). As chondrogenically primed bone marrow MSCs proceed to terminal differentiation, they express *type X collagen* (Winter *et al*., 2003) and *alkaline phosphatase* (Pelttari *et al*., 2006), indicative of a hypertrophic phenotype (Dickhut *et al*., 2009). SDSCs have been shown to deposit similar levels of type X collagen to bone marrow and adipose derived stem cells after 5 weeks of chondrogenic induction *in vitro* (Dickhut *et al*., 2009), although they have a reduced tendency to undergo ectopic calcification, instead undergoing fibrous degeneration or complete degeneration. The hypertrophic markers analysed in this study: *Indian hedgehog*, which is expressed in the pre-hypertrophic zone during bone development (Kronenberg, 2003; Day and Yang, 2008) and *type X collagen*, a marker of the terminally differentiated (hypertrophic) chondrocyte phenotype, were both down-regulated due to the application of HP; suggesting that mechanical signals play a key role in the formation of a permanent or stable chondrocyte phenotype.

Further support for such a hypothesis can be found in the results of previous studies demonstrating that HP down-regulates the expression of *MMP-13*, a proteolytic enzyme in hypertrophic cartilage required for vascularisation of calcified cartilage (Wong *et al.*, 2003). Given that our finding that HP, in the absence of TGF-β stimulation, had little effect on cartilage-specific matrix accumulation, but that it did suppress the expression of genes associated with hypertrophy, regardless of the concentration of TGF-β3, provides support for the idea that HP may play a more important role in the maintenance rather than the induction of a chondrogenic phenotype in joint derived stem cells. Mechanobiological models have previously suggested that hydrostatic pressure could inhibit endochondral ossification during skeletal regeneration (Carter *et al.*, 1998), and here we provide experimental data in support of this hypothesis. *In vivo*, hydrostatic pressure may interact with the local oxygen environment (Sheehy *et al.*, 2011) to regulate endochondral ossification.

A possible limitation of the study is the use of stem cells and chondrocytes isolated from skeletally immature pigs. Pigs share an underlying genetic and physiological similarity to humans (Vacanti *et al.*, 2005), and it has been suggested that they may provide a useful animal model system to evaluate tissue engineering strategies (Ringe *et al.*, 2002). Another limitation of the study is that we did not directly explore if joint tissue derived stem cells display a donor dependant response to HP or if the passage number of the cells makes a difference to their response to mechanical stimulation. We accept that the number of passages (Wiseman *et al.*, 2004; Das *et al.*, 2008) and some donor dependant response of joint tissue derived stem cells to biochemical and biophysical stimuli is highly likely, although this was not the primary question being addressed as part of this study

In conclusion, this study demonstrates that HP and TGF-β3 interact to regulate chondrogenesis of joint tissue derived stem cells. The data demonstrates that HP can act synergistically with low concentrations of TGF-β3 to up-regulate *Sox9* expression and the synthesis of cartilage-specific matrix molecules. Furthermore, HP acts to down-regulate the expression of genes associated with pre-hypertrophy and terminal differentiation (*Indian hedgehog* and *type X collagen*). In the context of stem cell based therapies for articular cartilage repair, the results of this study demonstrate the importance of considering how both biochemical and biophysical joint specific environmental factors might interact to regulate not only the initiation of chondrogenesis, but also the development of a stable cartilage repair tissue.

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Discussion with Reviewers

Reviewer I: You state in Material and Methods that "at each media exchange samples of media were taken for analysis of ALP, sGAG and total collagen …." To what did you normalise the values measured in the media samples taken at each time point or media exchange – as digestions of the samples was only done at day 0 and day 14?
Authors: A sample of media was taken at each media exchange and then the amount GAG and collagen and ALP activity was analysed separately and normalised by the number of pellets, i.e., the average release per pellet was determined.

Reviewer I: Please comment on the differences in cellular composition of synovial membrane and fat tissue in comparison to cartilage.
Authors: The synovial membrane is a vascular connective tissue that lines the inner surface of the joint capsule and articulates with itself or with the margins of the articular cartilage (Radin, 2001). The synovium is composed of an inner layer (intima) and a deep layer (subintima). Within the inner layer are 4 types of synovial lining cells that overlap one in another in 2 or 3 layers: macrophages, fibroblasts, endothelial cells and mastocysts. The deep layer is composed of adipose and synovial cells (Dewire and Einhorn, 2001).

Intra-articular fat pads are a feature of some joints. They are composed of adipocytes and adipose connective tissue containing collagen and glycosaminoglycans. Its surface is covered with synovial membrane and its role is to facilitate the distribution of synovial fluid within the joint and act as a cushion protecting exposed articular surfaces (Vahlensieck *et al*., 2002; Nakano *et al*., 2004).

Further commentary on the differences between SDSCs and FPSCs can be found in Mochizuki *et al*. (2006) (text reference).

Reviewer I: What is the percentage of cells that can really be considered as stem cells in these tissues?
Authors: A minimal amount of synovial tissue is required to extract the SMSCs successfully. Based on the CFU-F data obtained in our laboratory from 9 different porcine donors, approximately 2-4 % of cells from these joint tissues are colony-forming, although it must be stated that this in itself cannot be used to state that this percentage of the population are true stem cells.

Reviewer I: How did the cell yield and expansion period differ for the three investigated tissues and did this influence differentiation capacity of the cells?
Authors: CCs were expanded to passage one (7 population doubling) and stem cells to passage two (14-15 population doublings for each stem cell source). Because CCs are known to de-dedifferentiate in monolayer culture (Benya *et al*., 1978), we aimed to minimise the number of population doublings for the CC group. As is well reported in the literature, expanding joint derived stem cells to passage 14 doesn't seem to affect their chondrogenic potential (De Bari *et al*., 2001; Mochizuki *el al*., 2006, text references; Sakaguchi *et al*., 2005, additional reference).

Reviewer I: Does this influence the responsivity of the cells to mechanical loading?
Authors: Das *et al.* (2008) (text reference) have shown that expansion alters the response of human chondrocytes to stretch. Therefore, the answer to the reviewer's question is yes, but in order to have the sufficient number of cells to perform the study we had to expand them.

Reviewer II: Would it be possible to investigate the isoforms TGF-β1, TGF-β2 and TGF-β3? Does HP affect their expression differentially?

This is even more pertinent when considering of the studies referenced Miyanishi *et al.* (2006a,b) (text references) used TGF-β3 (as in this study) whereas Zeiter *et al*. (2009) (text reference) used TGF-β1. There are few papers investigating the differences of the various isoforms, and the fact that isoforms exist indicates they will have different effects. Is the response to HP one of them?
Authors: To address the reviewers comment, we analysed both TGF-β1 and TGF-β3 production in SMSCs in response to HP for the 1 ng/mL of TGF-β3 arm of the study where this mechanical stimulus has been shown to enhance cartilage specific matrix synthesis. We found that while measurable levels of TGF-β1 were found in the media, these values did not increase with the application of HP. Furthermore, HP did not influence the synthesis of TGF-β3 or the expression of TGF-β receptor 3. Therefore, we were unable to provide evidence to support the hypothesis that HP exerts its influence on stem cell chondrogenesis by altering TGF-β signalling.

Reviewer II: One interesting observation is that IHH is down-regulated. Does this translate into a change in protein levels? As can be seen from the authors' own data, the 2-3,000 fold increase in aggrecan mRNA only did not lead to the same level of sulphated GAG.
Authors: We did not measure IHH at the protein level. While HP led to decreases in type X gene expression, immunohistochemistry did not reveal any dramatic difference in type X collagen production. Therefore, our feeling is that while HP is impacting on the expression of these proteins at the gene level by day 14, noticeable differences in protein production will not be observed until later time points as stem cells proceed towards terminal differentiation.

Reviewer III: What was the baseline value from cells without exposure to TGFβ?
Authors: Negligible amounts of sGAG were produced by stem cells not supplemented with TGF-β3. It was therefore concluded that they did not undergo robust chondrogenesis and were not considered for further analysis.

Reviewer III: The authors conclude in their last sentence of the paper that their study has important results relevant to stem cell based therapies for articular cartilage. One assumes that they are meaning, in the main, for treating humans who will be at least skeletally mature, if not of a more advanced age. How do they think that their results, which have been carried out only on 4-month old pigs, might have been different if performed on skeletally

mature individuals (with pigs attaining skeletal maturity at approximately 18 months of age)?

Authors: It has been demonstrated that cartilage matrix formation by MSCs in three-dimensional culture in response to TGF-β stimulation is age-dependent (Erickson *et al*., 2011). Given that the response of MSCs to biochemical cues is age-dependent, it is reasonable to believe that the response of MSCs to mechanical signals will also be age-dependent. Further studies are required to explore this hypothesis.

Additional References

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