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7 **Composition-function relations of cartilaginous tissues engineered**
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9 **from chondrocytes and mesenchymal stem cells isolated from bone**
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11 **marrow and infrapatellar fat pad**
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33 **Running title:**
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35 Engineered cartilage from different MSC sources
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56 **Key Words:** Infrapatellar fat pad; Cartilage; TGF- β 3; Mesenchymal stem cells;
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58 Chondrocytes; Hydrogel.
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Abstract

The objective of this study was to determine the functional properties of cartilaginous tissues generated by porcine MSCs isolated from different tissue sources, and to compare these properties to those derived from chondrocytes (CC). MSCs were isolated from bone marrow (BM) and infrapatellar fat pad (FP), while CC were harvested from the articular surface of the femoro-patellar joint. Culture-expanded CC and MSCs were encapsulated in agarose hydrogels and cultured in the presence of TGF- β 3. Samples were analysed biomechanically, biochemically and histologically at day 0, day 21 and day 42. After 42 days in free swelling culture, mean GAG content was 1.50 % w/w in CC seeded constructs, compared to 0.95 % w/w in FP and 0.43 % w/w in BM seeded constructs. Total collagen accumulation was highest in FP constructs. DNA content increased with time for all the groups. The mechanical functionality of cartilaginous tissues engineered using CCs was superior to that generated from either source of MSCs. Differences were also observed in the spatial distribution of matrix components in tissues engineered using CC and MSCs, which appears to have a strong influence on the apparent mechanical properties of the constructs. Therefore while functional cartilaginous tissues can be engineered using MSCs isolated from different sources, the spatial composition of these tissues is unlike that generated using chondrocytes, suggesting that MSCs and chondrocytes respond differently to the regulatory factors present within developing cartilaginous constructs.

1. Introduction

Due to its limited capacity for regeneration and self repair, as well as a paucity of therapeutic options, degeneration of articular cartilage can have severe consequences (Pelttari *et al.*, 2008). Clinical procedures for cartilage defect repair such as subchondral abrasion arthroplasty, microfracture or autologous chondrocyte implantation often result in only a temporary partial repair, producing a biomechanically inferior tissue that can ultimately degenerate leading to osteoarthritis (Schumann *et al.*, 2006). Engineering functional cartilage tissue using mesenchymal stem cells (MSCs) seeded into scaffolds or hydrogels represents a promising alternative treatment for articular cartilage defects. Many adult tissues maintain populations of MSCs that are not terminally differentiated and which could potentially be used for tissue regeneration following trauma, disease or aging (Pittenger *et al.*, 1999; Fuchs and Segre 2000; Hadjantonakis and Papaioannou 2001; Wickham *et al.*, 2003). Such cells possess the ability to proliferate extensively *ex vivo* while maintaining their differentiation capabilities (Caplan 1991; Pittenger *et al.*, 1999).

Bone marrow (BM) derived MSCs have been studied extensively *in vitro* for their capacity to differentiate and synthesize markers associated with adipocytes, chondrocytes (CC) and osteoblasts (Maniatopoulos *et al.*, 1988; Caplan 1991; Johnstone *et al.*, 1998; Pittenger *et al.*, 1999), and for their ability to generate a mechanically functional cartilaginous tissue (Mauck *et al.*, 2006; Mauck *et al.*, 2007; Thorpe *et al.*, 2008; Thorpe *et al.*, 2008; Erickson *et al.*, 2009; Huang *et al.*, 2009). However traditional bone marrow procurement procedures may be painful, time consuming, expensive and risk cell contamination and loss (Pelttari *et al.*, 2008; Khan *et al.*, 2009). An ideal source of autologous cells would be both easy to obtain and

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3 would result in minimal patient discomfort during harvesting (Zuk et al., 2001).
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5 Recent studies have shown that MSCs isolated from other connective tissues such as
6
7 fat pad (FP), synovial membrane and subcutaneous fat may possess significant
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9 plasticity in their multi-lineage capabilities (De Bari et al., 2001; Zuk et al., 2001; Zuk
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11 et al., 2002; Dragoo et al., 2003; Wickham et al., 2003; Huang et al., 2005; English et
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13 al., 2007; Pei et al., 2008). These tissues represent attractive cell sources for tissue
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15 engineering because they are generally accessible with minimal donor site morbidity.
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20 Comparisons of the chondrogenic potential of different sources of MSCs
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22 (synovium, bone marrow, subcutaneous fat and muscle) in a pellet culture system
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24 suggest that synovial membrane derived MSCs possess a superior chondrogenic
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26 capability to other sources (Koga *et al.*, 2008). It has also been reported that human
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28 osteoarthritic fat pad (FP) tissue contains highly clonogenic and multipotent MSCs
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30 with stable chondrogenic potential *in vitro* (English et al., 2007), which express
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32 markers common with other sources of MSCs (Khan et al., 2008). We have recently
33
34 demonstrated that functional cartilaginous tissue can be engineered using infrapatellar
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36 FP derived MSCs embedded in agarose hydrogel (Buckley et al., 2009). While it has
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38 been demonstrated that cartilaginous tissues engineered using BM derived MSCs
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40 embedded in agarose hydrogel possess inferior mechanical properties to those
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42 engineered using CC (Mauck et al., 2007; Erickson et al., 2009), it is unclear if this
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44 finding extends to MSCs isolated from sources other than the bone marrow.
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51 The objective of this study is to compare the biomechanical and biochemical
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53 properties of tissues engineered using porcine MSCs isolated from the infrapatellar fat
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55 pad and bone marrow to those derived from CC. A porcine model was chosen as they
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57 are similar to humans in terms of their genetics, anatomy and physiology (Vacanti et
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59 al., 2005). The tripotentiality of porcine MSCs from different tissue sources was first
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3 assessed (chondrogenic, adipogenic and osteogenic potential). Next CC and MSCs
4 from each source were encapsulated in agarose hydrogels and cultured in the presence
5 of TGF- β 3. Our hypothesis was that MSCs isolated from within the synovial joint
6 would possess a potential to generate functional cartilaginous tissues comparable to
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8 CC isolated from articular cartilage.
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17 **2. Materials and methods**

18 **2.1. Cell isolation and expansion**

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21 Four month old porcine MSCs were aseptically harvested from the bone marrow
22 (BM) of the femur and from the whole infrapatellar fat pad (FP) (the outer and the
23 inner layers of the FP were not separated, but any fibrous tissue attached to the FP
24 was removed). Chondrocytes (CC) were harvested from the articular surface of the
25 femoro-patellar joint. Cells from each tissue source were harvested from 2 animals
26 and pooled. BM MSCs were isolated and expanded according to a modified method
27 developed for human MSCs (Lennon and Caplan 2006). Muscle and fascia was
28 removed and bones were sawn close to the femoral head. BM was removed from the
29 medullary canal and transferred to a 50mL tube containing expansion medium (EM)
30 consisting of high-glucose Dulbecco's Modified Eagle Medium (hgDMEM,
31 GlutaMAX) containing 10% foetal bovine serum and 1% penicillin (100 U/mL)-
32 streptomycin (100 μ g/mL) (all from GIBCO, Biosciences, Dublin, Ireland). Marrow
33 was aspirated repeatedly to break up large aggregates prior to centrifugation at 650g
34 for 5 min. The separated fatty layer was discarded and the cell pellet was re-
35 suspended in EM, triturated through a 16 gauge needle and filtered through a 40 μ m
36 nylon cell strainer (B.D. Falcon, Unitech, Dublin, Ireland). Red blood cells were lysed
37 using 4% acetic acid and mononuclear cells were counted using a haemocytometer.
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Cartilage slices were rinsed with phosphate buffered saline containing penicillin/streptomycin (200U/ml) and amphotericin B (2.5µg/ml). Cartilage and FP pieces were incubated with DMEM/F12 containing collagenase type II (125 U/mg) (all from Sigma–Aldrich, Dublin, Ireland) for 16-18 hours under constant rotation at 37°C. The resulting cell suspension was then filtered through a 40µm pore-size cell sieve (Falcon Ltd, Sarstedt, Ireland) and the filtrate centrifuged and rinsed with PBS twice.

CC were seeded at a density of 50,000 cells/cm² in 175 cm² T flasks and expanded to passage one (P1). Viable cells were counted using a hemacytometer and 0.4% trypan blue staining. Isolated CC from all joints were pooled and maintained in DMEM/F-12 (Sigma–Aldrich, Arklow, Ireland) supplemented with 10% v/v foetal bovine serum (FBS) and 100U/ml penicillin/streptomycin (GIBCO, Biosciences, Dublin, Ireland) during the expansion phase. MSCs from the BM and FP were seeded at a density of 5,000 cells/cm² in 175 cm² T flasks and expanded to passage three (P3).

2.2. Adipogenesis and Osteogenesis

Expanded MSCs from the BM and FP were plated in 9.5cm² six well plates at a density of 10³cells/cm² and cultured for 7 days in complete medium (DMEM GlutaMAX supplemented with 10% v/v foetal bovine serum (FBS) and 100U/ml penicillin/streptomycin) and changed to osteogenic or adipogenic medium for 21 days. Osteogenic medium consisted of complete medium supplemented with 100nM dexamethasone, 10mM β-glycerolphosphate and 0.05mM ascorbic acid, while adipogenic medium consisted of complete medium supplemented with 100nM dexamethasone, 0.5mM isobutylmethylxanthine and 50µM indomethacin (all from

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3 Sigma-Aldrich, Ireland). After 21 days cells were washed twice with PBS and fixed
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5 with ethanol for ten minutes. The wells were then stained for two minutes as follows:
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7 1% oil red solution, which stains lipid deposits, was used as a marker of adipogenesis;
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9 while 1% alizarin red solution, which stains calcium deposits, was used as a marker of
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11 osteogenesis.
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17 **2.3. Chondrogenesis**

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19 A pellet culture system was used to assess the chondrogenic capacity of MSCs.
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21 500,000 cells were placed in 1.5 ml conical microtubes and centrifuged at 650G for 5
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23 minutes. The pellets were cultured in a chemically defined chondrogenic medium
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25 (CM): DMEM GlutaMAX supplemented with penicillin (100 U/ml)-streptomycin
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27 (100 µg/ml) (both GIBCO, Biosciences, Ireland), 100 µg/ml sodium pyruvate, 40
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29 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 4.7 µg/ml linoleic acid, 1.5
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31 mg/ml BSA, 1× insulin–transferrin–selenium, 100 nM dexamethasone (all from
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33 Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human transforming growth
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35 factor-β3 (TGF-β3; Prospec, Israel). For histological evaluation the pellets were
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37 embedded in paraffin, cut into 5µm thick sections, and stained with 1% alcian blue
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39 8GX (Sigma–Aldrich, Ireland) in 0.1M HCl to assess glycosaminoglycan content.
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46 Immunohistochemistry was used to detect type II collagen.
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50 **2.4. Cell-agarose constructs**

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52 Culture-expanded CC and MSCs were encapsulated in agarose (Type VII) at a final
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54 gel concentration of 2% and a cell density of 15×10^6 cells/ml. The agarose-cell
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56 suspension was cast in a stainless steel mould to produce cylindrical constructs (Ø
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58 5mm×3mm thickness). Agarose hydrogel constructs were maintained in the CM
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3 described above for 6 weeks. There were 2 samples per well with 2.5 ml of medium
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5 per construct. Medium was changed every 2-3 days. The study was designed with 5
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7 gels per cell source at each time point; samples were analyzed at day 0, day 21 and
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9 day 42.
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12 13 14 15 **2.5. Mechanical testing**

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17 Constructs were mechanically tested between impermeable platens using a standard
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19 materials testing machine (Zwick Roell Z005, Herefordshire, UK) with a 5N load cell,
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21 as previously described (Buckley et al., 2009). Agarose constructs were kept hydrated
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23 through immersion in a PBS bath maintained at room temperature. To assess construct
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25 functionality, an unconfined stress relaxation test was performed. Contact between the
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27 loading platen and the construct was first visually confirmed following the application
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29 of a small pre-load (0.01N), and then a ramp compression to 10% strain followed by a
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31 hold period until equilibrium of the sample was achieved in a period of 30 minutes.
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33 The equilibrium Young's modulus was determined from the equilibrium force. This
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35 was followed by a 1Hz cyclic test at 1% strain amplitude for 10 cycles in unconfined
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37 compression to determine the dynamic modulus. Construct dimensions were
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39 measured using a toolmaker's microscope (Mitutoyo UK Ltd., Andover, UK).
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51 Constructs were fixed in 4% paraformaldehyde overnight, rinsed in PBS, embedded in
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53 paraffin and sectioned to 8µm thickness. The histological sections were stained with
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55 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1M HCl to assess
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57 glycosaminoglycan content and picosirius red to detect collagen. Collagen type I, II
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59 and X contents were evaluated with a standard immunohistochemical technique as
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3 previously described (Thorpe et al., 2010). Positive and negative controls were
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5 included in the immunohistochemistry staining protocol for each batch.
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10 **2.7. Biochemical analysis**

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12 The 5mm diameter constructs were cored using a 3mm biopsy punch. All samples
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14 were digested in papain (125µg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl,
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16 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Dublin, Ireland) at 60°C under
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18 constant rotation for 18 hours. Aliquots of the digested samples were assayed
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20 separately for DNA, collagen and sulphated glycosaminoglycan (GAG) content. DNA
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22 content was quantified using the Quant-it Picogreen DNA assay (Invitrogen, UK).
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24 The proteoglycan content was estimated by quantifying the amount of sulphated
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26 glycosaminoglycan (GAG) in constructs using the dimethylmethylene blue dye-
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28 binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate
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30 standard. Total collagen content was determined by measuring the hydroxyproline
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32 content, using a hydroxyproline-to-collagen ratio of 1:7.69 (Kafienah and Sims 2004;
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34 Ignat'eva et al., 2007). Matrix accumulation (total collagen and GAG) was normalised
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36 to the tissue wet weight and to DNA.
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46 **2.8. Statistical analysis**

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48 Mechanical and biochemical properties of engineered constructs are expressed in the
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50 form of mean ± standard deviation (SD). Five or six samples were analysed per group
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52 at each time point; 3 or 4 samples were used for mechanical and biochemical analyses
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54 and 2 samples were used for histology. Differences in mechanical and biochemical
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56 properties with cell type and/or time-in-culture were determined by a general linear
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58 model for analysis of variance with groups as factors. Statistics were performed with
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3 MINITAB 15.1 (Minitab Ltd., Coventry, UK) software package. Tukey's test for
4 multiple comparisons was used to compare conditions. Commercially available
5 software (GraphPadPrism 4, San Diego, USA) was also used for a correlation
6 analyses and to compare correlation slopes between conditions. A level of $p < 0.05$ was
7 considered significant. Each experimental arm (CC, FP and BM) was replicated at
8 least once using MSCs isolated from 2 different porcine donors to the original donors,
9 with the same trends observed in replicate experiments. The results of the first
10 replicate are presented in the manuscript. The data from the second replicate is
11 included in the correlation analysis of mechanical properties with biochemical
12 composition (see below).
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32 **3. Results**

33 **3.1. MSC Tripotentiality**

34 After 21 days in culture, pellets in chondrogenic conditions became spherical and
35 increased in size, in particular the FP pellets. Alcian blue staining for GAG was
36 stronger for FP pellets compared to BM pellets (Fig. 1). Pellets were also stained for
37 type II collagen; the immunoreaction revealed strong staining for FP pellets in
38 comparison with weaker staining for BM pellets (data not shown). After 3 weeks in
39 culture with osteogenic medium BM cells deposited calcium, as evident by staining
40 with alizarin red. In comparison, weak staining for alizarin red was observed for the
41 FP group (Fig. 1). All the cell sources demonstrated deposition of fat generating oil
42 droplets that stained with oil red. MSCs cultured in control conditions did not stain
43 with either alizarin red or oil red.
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3.2. Agarose hydrogel culture

Histological evaluation and immunohistochemical analysis (Fig. 2) revealed CC, FP and BM groups stained positively for sulphated proteoglycan and collagen accumulation. Immunohistochemical analysis also revealed the presence of type II collagen in CC, FP and BM constructs. Only weak staining for type I and type X collagen was observed (Fig. 2). For CC seeded hydrogels, the annular region exhibited stronger type II collagen staining compared to the core, with an opposite effect observed for the MSC groups. Matrix staining appeared more diffuse for CC and FP constructs, while a more pericellular staining was observed for BM constructs. FP constructs stained strongly for alcian blue with evidence of cell clustering and development of lacunae.

Significantly greater total GAG accumulation was observed in CC seeded hydrogels in comparison to both MSC groups, while the highest collagen accumulation was found in FP constructs (Fig. 3). Overall analysis of variance results showed that GAG, total collagen and DNA deposition for each cell seeded agarose hydrogel was dependent on time in culture ($p < 0.0001$), cell type ($p < 0.0001$) and region of construct (annulus or core) ($p < 0.0001$). At day 42, GAG accumulation for CC constructs reached 1.50% w/w (± 0.05) compared to 0.95% w/w (± 0.04) for FP constructs and 0.43% w/w (± 0.03) for BM constructs. At day 42, collagen accumulation reached 0.88% w/w (± 0.02) for FP, 0.54% w/w (± 0.01) for CC and 0.48% w/w (± 0.02) for BM. DNA content generally increased with time for all the groups, where a reduction in DNA content was observed between day 0 and day 42. Normalisation of the GAG content to DNA generally revealed a similar trend as the normalisation to wet weight.

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GAG accumulation was higher in the core region of the construct for the two MSC groups, compared to greater GAG accumulation in the annular region for CC constructs (Fig. 4). By day 42, in terms of percentage of wet weight the core regions of BM and FP scaffolds showed significantly greater GAG accumulation compared to their corresponding annular region (BM annulus = 0.27 ± 0.04 %w/w, BM core = 0.79 ± 0.06 %w/w, FP annulus = 0.61 ± 0.06 %w/w and FP core = 1.48 ± 0.11 %w/w). Similar differences were observed in core-annulus accumulation when normalised by DNA content, but with higher GAG/DNA in the core regions of CC constructs compared to their annulus (Fig. 4). FP cores contained similar amounts of GAG per DNA ($112.85 \mu\text{g}/\mu\text{g} \pm 8.58$) as the core ($127.71 \mu\text{g}/\mu\text{g} \pm 4.45$) and annular ($110.32 \mu\text{g}/\mu\text{g} \pm 6.16$) region of CC constructs.

Total collagen accumulation in MSC constructs was greater in the core compared to the annulus (BM annulus = 0.43 ± 0.01 %w/w, BM core = 0.66 ± 0.02 %w/w, FP annulus = 0.56 ± 0.02 %w/w and FP core = 1.383 ± 0.1 %w/w). CC collagen content was lower in the core (0.38 ± 0.04 %w/w) in comparison with the annulus (0.66 ± 0.09 %w/w). When normalised by DNA, collagen values were higher in the core of the FP constructs compared to other groups.

For BM constructs, no significant differences were observed in the DNA content between annular or core regions. DNA content was higher within core regions ($131.87 \text{ ng}/\text{mg w/w} \pm 7.12$) for FP constructs in comparison with the annulus ($109.50 \text{ ng}/\text{mg w/w} \pm 1.17$). The opposite trend was observed in CC constructs, with DNA content of $107.68 \text{ ng}/\text{mg w/w} (\pm 9.14)$ for the core region and $131.87 \text{ ng}/\text{mg w/w} (\pm 7.12)$ for the annulus.

Time and cell type were significant factors in both mechanical measures ($p < 0.05$). After 42 days of culture CC constructs exhibited higher equilibrium

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3 modulus (39.4kPa \pm 0.3) compared to MSC constructs ($p < 0.001$) (Fig. 5). Within the
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5 MSCs groups, FP gels demonstrated comparable equilibrium modulus (16.9kPa \pm 1.6)
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7 to that of BM constructs (15.6kPa \pm 0.61). The dynamic modulus significantly
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9 increased ($p < 0.05$) with time in culture for all groups. In the FP group the modulus
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11 increased from (29.0kPa \pm 0.62) at day 0 to (133.7kPa \pm 8.4) at week 6, in the BM
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13 group from (33.6kPa \pm 1.62) to (112.6kPa \pm 13.7) and in the CC group from (38.1kPa
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15 \pm 0.19) to (256.8kPa \pm 9.9). The dynamic modulus of FP constructs was significantly
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17 higher than BM constructs after 6 weeks of culture ($p < 0.05$).
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25 **3.3. Structure-function correlation analysis**

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27 The equilibrium modulus and dynamic modulus at day 42 were plotted against the
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29 percentage of wet weight of total collagen and GAG content for CC, FP and BM
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31 constructs (Fig. 6). All correlations were found to be statistically significant (Table 1),
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33 except the relation between total collagen content and the equilibrium modulus in the
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35 FP group. Comparisons between groups were not statistically significant.
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44 **4. Discussion**

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46 In this study we evaluated the potential of porcine BM and infrapatellar FP MSCs
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48 embedded in agarose hydrogels to generate functional cartilaginous tissue, and
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50 compared the properties of these tissues to those generated using CC isolated from
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52 articular cartilage. Our objective was not to identify the optimal isolation, expansion
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54 and chondrogenic differentiation conditions for different MSC sources, but rather to
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56 compare composition-function relations of cartilaginous tissues generated using a
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58 number of different cell sources under near identical culture conditions.
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3 Understanding how the functional properties of cartilaginous tissue evolve is an
4 essential pre-requisite to the development of any MSC-based therapy regardless of the
5 cell source. While porcine BM and FP derived MSCs underwent robust
6 chondrogenesis in agarose culture, construct properties were overall inferior to CC
7 seeded constructs. This agrees in part with a previous study using a hyaluronan-based
8 scaffold that demonstrated that cartilaginous constructs generated using human CC
9 contained higher fractions of GAG compared to FP derived cells and other cell
10 sources, and only constructs generated using CC stained positive for type II collagen
11 (Marsano *et al.*, 2007). Interestingly, in the present study core levels of total GAG and
12 collagen accumulation in the FP group were comparable to that found in the CC
13 group, suggesting that under the environmental conditions created in the core of these
14 constructs, the chondrogenic capability of FP MSCs is comparable to CC. Therefore
15 while we were unable to fully corroborate our initial hypothesis that FP derived MSCs
16 possess a potential to generate functional cartilaginous tissues comparable to CC,
17 these are a source of chondro-progenitor cells worthy of further investigation for
18 cartilage regeneration therapies.

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41 The infrapatellar FP is composed of adipocytes and adipose connective tissue
42 containing collagen and glycosaminoglycans, covered by a layer of synovial
43 membrane which articulates with the trochlear cartilage of the distal femur
44 (Mochizuki *et al.*, 2006). MSCs from the FP are presumably derived either from the
45 perivascular region of vessels and capillaries invading the tissue (Khan *et al.*, 2008),
46 and/or from the synovial layer (De Bari *et al.*, 2001) that covers the tissue. It has
47 recently been demonstrated that MSCs isolated from intra-articular tissues and CC
48 share similar gene expression profiles distinct from other mesenchymal tissue-derived
49 cells such as bone marrow and adipose derived MSCs (Segawa *et al.*, 2009).
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3 Differences in gene expression profiles, possibly due to differences in their respective
4 stem cell niches, provide one possible explanation for the apparent superior functional
5 properties of FP constructs compared to BM constructs in this study. However, direct
6 comparisons of different MSC sources must, for a number of reasons, be made with
7 caution. Firstly, the initial fraction of MSCs within the different tissues has not been
8 measured, and therefore the exact number of population doublings that have occurred
9 in each group during the expansion phase cannot be accurately quantified. This may
10 be important as the chondrogenic capacity of porcine BM derived MSCs is known to
11 diminish during prolonged passaging (Vacanti *et al.*, 2005). It should also be noted
12 that BM derived MSCs cultured in our laboratory under similar conditions to that
13 described here have generated cartilaginous tissues with comparable ECM content
14 and mechanical properties to that measured here for FP derived MSCs (Thorpe *et al.*,
15 2008). Variability in the chondrogenic capability of BM MSCs has been documented,
16 compared to reports of relatively consistent chondrogenesis of synovium derived
17 MSCs (Sakaguchi *et al.*, 2005). Therefore, before concluding that any one source of
18 MSCs is superior for functional cartilage tissue engineering, it will be necessary to
19 first optimise the isolation, expansion and differentiation conditions for each cell type.
20 For example, it has been demonstrated that the initial monolayer culture conditions
21 used to expand adipose derived stem cells determine their subsequent chondrogenic
22 capacity in agarose hydrogel culture (Estes *et al.*, 2008). Future studies will
23 investigate how variables associated with MSC expansion protocols such as plating
24 density and growth factor supplementation (e.g. FGF-2, TGF- β) will influence the
25 subsequent functional properties of engineered cartilaginous tissues, as these factors
26 have been shown to regulate proliferation and subsequent chondrogenic
27 differentiation of MSCs isolated from different sources (Colter *et al.*, 2000; Erickson

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3 et al., 2002; Bianchi et al., 2003; Solchaga et al., 2005; Estes et al., 2006; Yanada et
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5 al., 2006; Marsano et al., 2007; Stewart et al., 2007; Khan et al., 2008; Khan et al.,
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7 2008).

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10 GAG and collagen accumulation in MSC groups was greater in the core, while
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12 CC constructs had a higher concentration of extracellular matrix in the annular region.
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14 A similar inhomogeneous distribution of the extracellular matrix synthesised by
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16 MSCs embedded in agarose hydrogels was also observed in our previous studies
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18 (Thorpe et al., 2008; Buckley et al., 2009; Thorpe et al., 2010). The development of
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20 an inhomogeneous cartilaginous tissue following encapsulation of CC in agarose has
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22 also previously been reported (Kelly et al., 2006; Buckley et al., 2009; Kelly et al.,
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24 2009). This distribution seems to be specific to CC in agarose gels and may be due to
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26 spatial gradients of nutrients, oxygen and growth factors. The reason for the specific
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28 matrix distributions has not yet been elucidated, but it does suggest that CC and
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30 MSCs respond differently to the gradients in regulating factors present within the
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32 developing tissue.
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39 The mechanical properties of MSC seeded hydrogels were lower than those of
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41 CC seeded hydrogels after 21 and 42 days in culture under identical conditions. It has
42
43 previously been reported that immature MSCs may possess a more limited
44
45 chondrogenic capacity compared to CC in an agarose hydrogel model (Mauck et al.,
46
47 2006; Vinardell *et al.*, 2009). The results of this study extend this finding to other
48
49 sources of MSCs. Composition-function relations (Figure 6) revealed that even in
50
51 cases where total GAG and collagen content was comparable or higher in FP
52
53 hydrogels, the equilibrium modulus of the engineered tissue was higher for CC seeded
54
55 constructs. This result is unexpected, as the equilibrium modulus is strongly
56
57 dependant on the GAG and collagen content. Differences in the basic structure and
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3 properties of proteoglycans synthesised by MSCs and CC may partially explain these
4 results, although it has been suggested that the bone marrow MSC-produce aggrecan
5 with a phenotype more characteristic of young tissue than chondrocyte-produced
6 aggrecan (Kopesky et al., 2010). The types of proteoglycan synthesised by engineered
7 tissues from different cell sources, and their ability to assemble, aggregate and form a
8 stable tissue, may also play a key role (Connelly et al., 2008; Babalola and Bonassar
9 2010).

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20 The spatial composition and organization of engineered cartilage tissues will
21 also influence the apparent mechanical properties of the construct (Kelly and
22 Prendergast 2004). Mechanical tests such as that utilized in this study to quantify
23 construct properties make the assumption of tissue homogeneity. The spatial
24 distribution of GAG within CC seeded constructs is typically more homogenous than
25 MSC groups, where the majority of GAG is accumulated within the construct core
26 (Figure 5). Such an inhomogeneous distribution of matrix components will lower the
27 apparent equilibrium modulus of the tissue compared to a more homogenous
28 construct, even if the total matrix accumulation is identical. This spatial distribution of
29 matrix would also appear to influence the correlation between the dynamic modulus
30 of the engineered tissue and its composition. The relationship between the total GAG
31 content and the dynamic modulus of CC and MSC seeded constructs is quite similar,
32 despite the spatial differences in tissue composition. However the collagen content of
33 an engineered cartilaginous construct will also have a strong bearing on the dynamic
34 properties of a tissue. For example, comparing the relationship between the dynamic
35 modulus and the biochemical content in CC and FP MSC constructs, it appears that
36 greater amounts of collagen are required in FP MSC constructs to achieve comparable
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3 values of dynamic modulus to CC constructs, which again may be due in part to the
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5 greater heterogeneity of the tissues engineered using MSCs.
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8 It has been demonstrated that bovine CC in agarose hydrogels produce a more
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10 mechanically functional tissue when TGF- β is withdrawn from the media after two
11
12 weeks of culture (Byers *et al.*, 2008), while we have demonstrated that porcine FP
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14 MSCs respond more favourably to continued growth factor supplementation (Buckley
15
16 *et al.*, 2009). In this study, all cell types received continued TGF- β 3 supplementation,
17
18 so it is possible that the results of this study may be underestimating the superior
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20 functional cartilage tissue forming ability of CC compared to MSCs. Clearly more
21
22 optimal expansion and differentiation conditions exist for all cell types.
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27 While not reported in this study, we have observed that porcine subcutaneous
28
29 fat derived MSCs undergo poor chondrogenesis following encapsulation in agarose
30
31 hydrogels, with significant cell death occurring. Other authors have also observed
32
33 decreases in cell viability following encapsulation of adipose derived MSCs in
34
35 agarose hydrogels (Estes *et al.*, 2008), and poorer chondrogenesis of adipose derived
36
37 MSCs in agarose hydrogels compared to BM MSCs (Kisiday *et al.*, 2008). A number
38
39 of other studies also suggest a poorer chondrogenic capacity for adipose derived
40
41 MSCs compared to other sources (Winter *et al.*, 2003; Awad *et al.*, 2004; Huang *et al.*,
42
43 2005; Im *et al.*, 2005; Afizah *et al.*, 2007; Koga *et al.*, 2008; Peng *et al.*, 2008; Vidal *et*
44
45 *al.*, 2008; Danišovič *et al.*, 2009; Diekman *et al.*, 2009; Hildner *et al.*, 2009; Jakobsen
46
47 *et al.*, 2009). Future studies will investigate whether the mixed population of cells
48
49 from the synovial layer and adipose tissue of FP biopsies has a positive effect on
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51 chondrogenesis, or whether they should be used in isolation for cartilage tissue
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53 engineering.
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4 The results of this study demonstrate that differences in the chondrogenic
5
6 capacity of different MSC sources in pellet culture reported here and in previous
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8 studies translate into differences in the functional properties of cartilage engineered
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10 using these different cell populations. Given its anatomical location, a large number of
11
12 MSCs can be obtained from a FP biopsy during arthroscopy for tissue engineering
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14 therapies. There have been reports stating that removing the entire FP during total
15
16 knee replacement procedures can produce postoperative pain for the patient
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18 (Meneghini *et al.*, 2007). On the other hand, removal of the FP leads to significant
19
20 improvement in symptoms and function after the surgery in cases of impingement
21
22 (Ogilvie-Harris and Giddens 1994). In general, there is little clinical evidence to
23
24 suggest that taking biopsies of FP could be seriously detrimental to the patient.
25
26 Therefore harvesting a biopsy of FP tissue has a lower degree of invasiveness
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28 compared to bone marrow aspiration, and causes minimal complications during joint
29
30 arthroscopy, especially if the goal is to treat a chondral or osteochondral defect in the
31
32 joint. In conclusion, functional cartilaginous tissues can be engineered using MSCs
33
34 isolated from different sources. The development of these tissues is different to those
35
36 generated using CCs, suggesting that MSCs and CCs respond differently to the
37
38 regulatory factors present within developing cartilaginous constructs. Using the same
39
40 expansion and differentiation conditions, MSCs isolated from the FP would appear to
41
42 possess a potential to generate functional cartilaginous tissues at least comparable to
43
44 BM MSCs, although CC still represent a standard to which all MSC-based cartilage
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46 tissue engineering strategies should be compared.
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For Peer Review

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8 **List of figures**
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12 **Figure 1:**
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15 Osteogenic potential of mesenchymal stem cells: colonies staining positive for
16 alizarin red staining (left column). Adipogenic potential of mesenchymal stem cells:
17 colonies staining positive for oil red staining (right column). Chondrogenic potential
18 of mesenchymal derived stem cells: pellet staining positive for glycosaminoglycan
19 with alcian blue (middle column).
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29 **Figure 2:**
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32 Agarose gels seeded with either chondrocytes or mesenchymal stem cells at day 42
33 stained for type II collagen, alcian blue (stains glycosaminoglycans) picosirius red
34 (stains collagen), type X collagen and type I collagen. Original magnification x 100.
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41 **Figure 3:**
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44 DNA, GAG and total collagen content in engineered tissues at day 0, 21 and 42
45 expressed in percentages of wet weight and per μg of DNA. Data represent the mean
46 \pm SD of three samples. **a** significant vs bone marrow, **b** vs fat pad, # vs day 21 and *
47 vs day 42 . $p < 0.05$.
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55 **Figure 4:**
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58 DNA, GAG and total collagen content in engineered tissues at day 42 expressed in
59 percentages of wet weight and per μg of DNA in construct annulus (A) and core (C).
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3 Data represent the mean \pm SD of three samples. **a** significant vs bone marrow, **b** vs fat
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5 pad, # vs day 21 and * vs day 42 . $p < 0.05$.
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10 **Figure 5:**

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12 Equilibrium and 1Hz dynamic modulus at day 0, day 21 and day 42. Data represent
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14 the mean \pm SD of three samples. **a** significant vs bone marrow, **b** vs fat pad, # vs day
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16 21 and * vs day 42 . $p < 0.05$.
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22 **Figure 6:**

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24 Correlation plots relating measured mechanical properties to biochemical constituents.
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26 Note that replicate data from separate experiments is included in this analysis.
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32 **Table 1:**

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34 Correlation of mechanical properties and biochemical content for different sources of
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36 progenitor cells and chondrocyte constructs. Correlation coefficients relating
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38 measured mechanical properties with concentration of GAG and total collagen. NS:
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40 not significant. Note that replicate data from separate experiments is included in this
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42 analysis.
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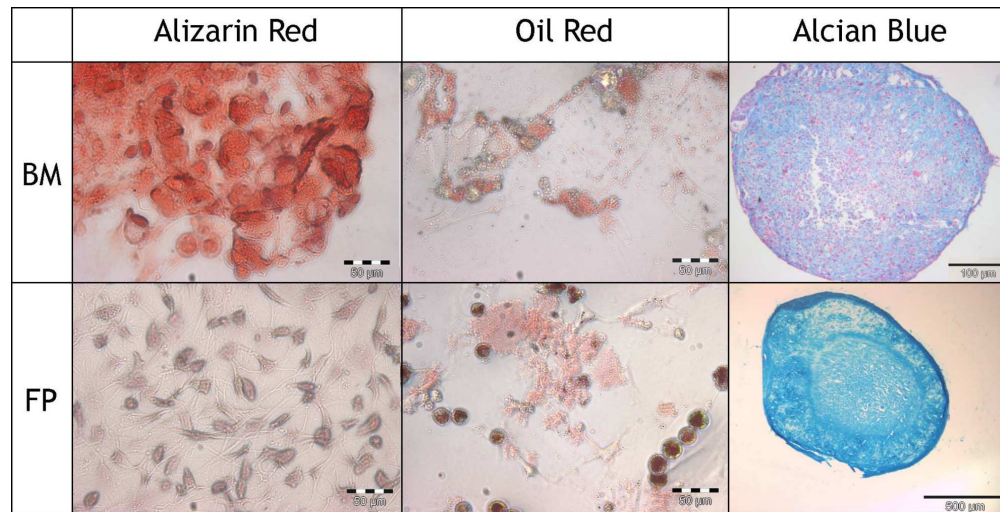


Figure 1. Osteogenic potential of mesenchymal stem cells: colonies staining positive for alizarin red staining (left column). Adipogenic potential of mesenchymal stem cells: colonies staining positive for oil red staining (right column). Chondrogenic potential of mesenchymal derived stem cells: pellet staining positive for glycosaminoglycan with alcian blue (middle column).
83x42mm (600 x 600 DPI)

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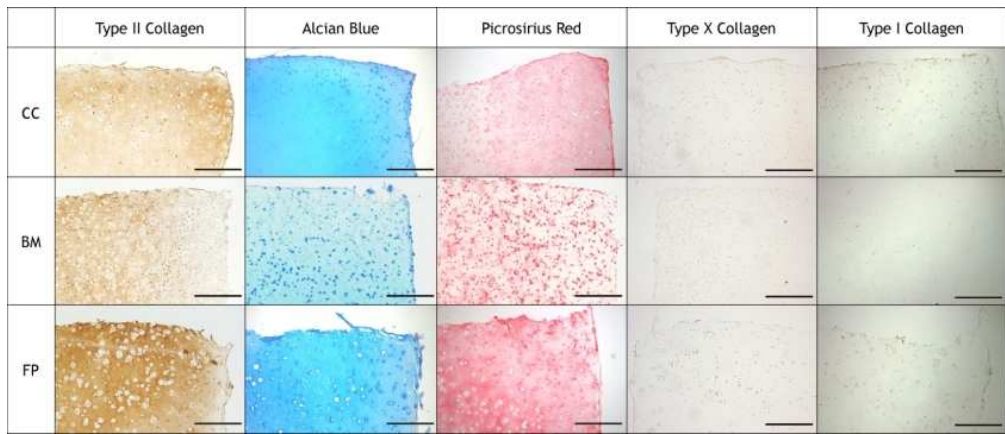


Figure 2: Agarose gels seeded with either chondrocytes or mesenchymal stem cells at day 42 stained for type II collagen, alcian blue (stains glycosaminoglycans) picrosirius red (stains collagen), type X collagen and type I collagen. Original magnification x 100.

71x30mm (300 x 300 DPI)

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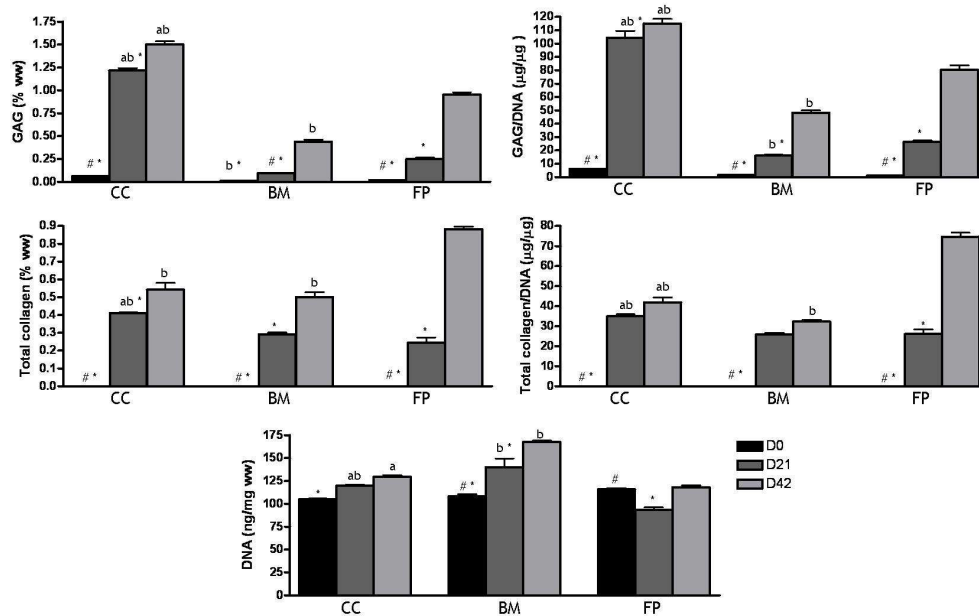


Figure 3. DNA, GAG and total collagen content in engineered tissues at day 0, 21 and 42 expressed in percentages of wet weight and per μg of DNA. Data represent the mean + SD of three samples. a significant vs bone marrow, b vs fat pad, # vs day 21 and * vs day 42. $p < 0.05$.
71x45mm (600 x 600 DPI)

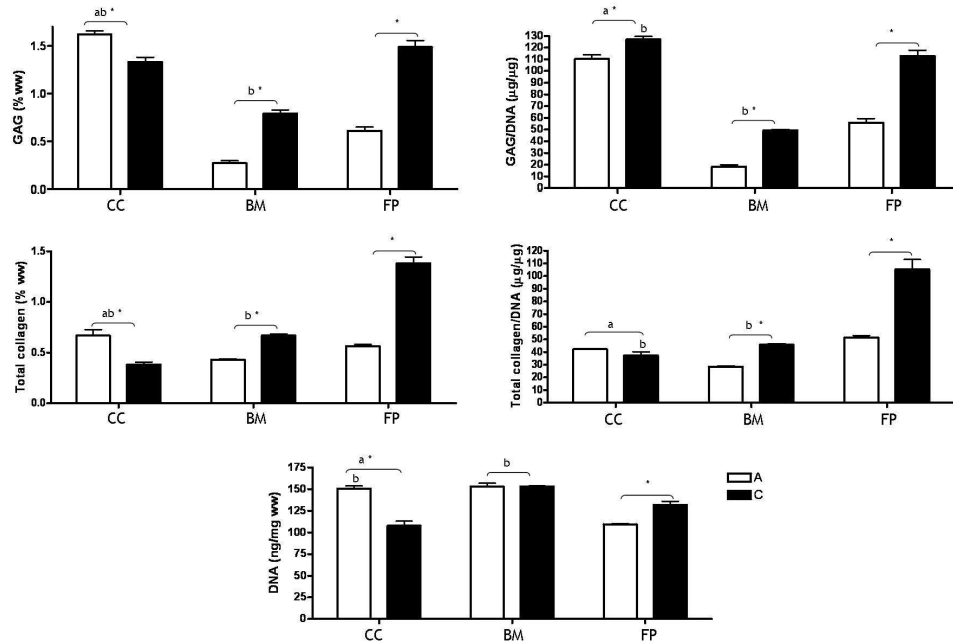


Figure 4. DNA, GAG and total collagen content in engineered tissues at day 42 expressed in percentages of wet weight and per µg of DNA in construct annulus and core. Data represent the mean + SD of three samples. a significant vs bone marrow, b vs fat pad, # vs day 21 and * vs day 42. $p < 0.05$.

78x54mm (600 x 600 DPI)

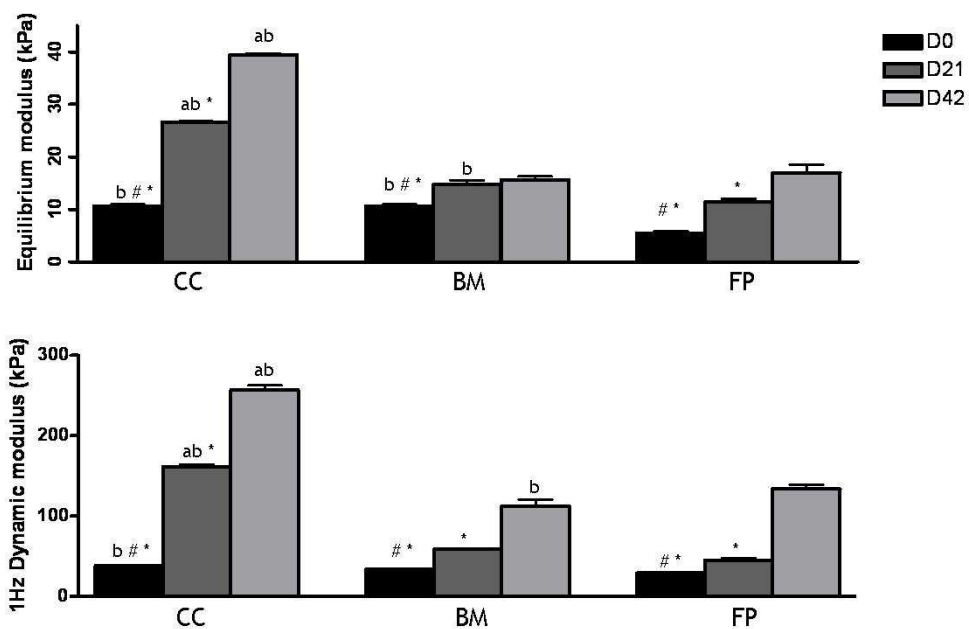


Figure 5. Equilibrium and 1Hz dynamic modulus at day 0, day 21 and day 42. Data represent the mean + SD of three samples. a significant vs bone marrow, b vs fat pad, # vs day 21 and * vs day 42. $p < 0.05$.

48x33mm (600 x 600 DPI)

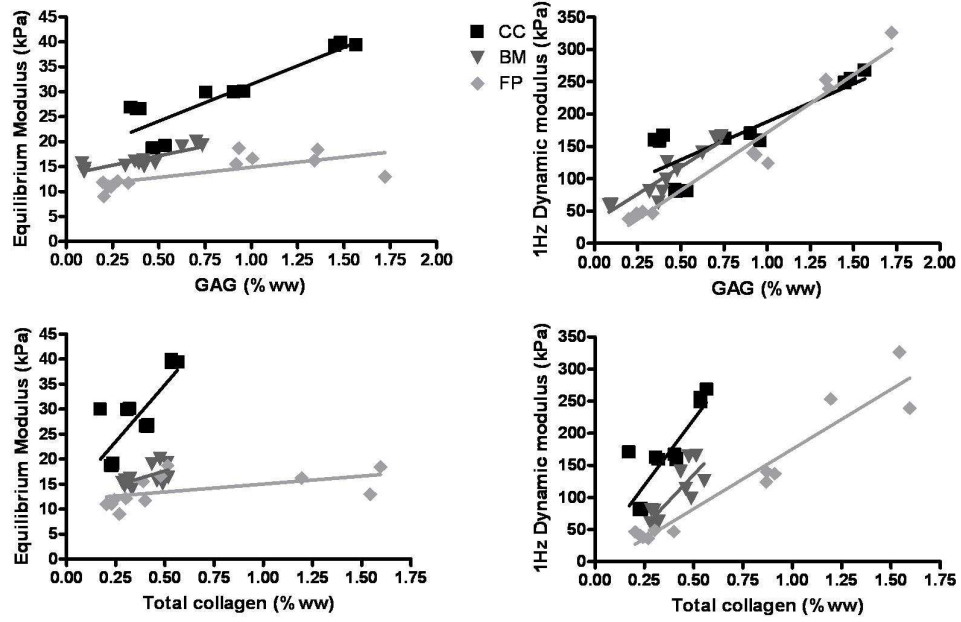


Figure 6. Correlation plots relating measured mechanical properties to biochemical constituents. Note that replicate data from separate experiments is included in this analysis. 70x45mm (600 x 600 DPI)

Review

Table 1: Structure-Function Correlation

<i>Cell source</i>	<i>Comparison</i>	<i>Slope</i>	<i>R²</i>	<i>P</i>	<i>vs CC</i>	<i>vs BM</i>
CC	Eq Modulus vs. GAG	14.7	0.7797	P=0.0001		
	Eq Modulus vs. Total collagen	45.5	0.6346	P<0.01		
	Dyn Modulus vs. GAG	117.7	0.6939	P<0.001		
	Dyn Modulus vs. Total collagen	412.2	0.7289	P<0.001		
BM	Eq Modulus vs. GAG	7.6	0.7535	P<0.001	NS	
	Eq Modulus vs. Total collagen	11.8	0.3293	P=0.05	NS	
	Dyn Modulus vs. GAG	168.2	0.862	P<0.0001	NS	
	Dyn Modulus vs. Total collagen	327.3	0.7003	P<0.001	NS	
FP	Eq Modulus vs. GAG	4.0	0.4911	P=0.01	NS	NS
	Eq Modulus vs. Total collagen	3.1	0.2693	NS	NS	NS
	Dyn Modulus vs. GAG	179.2	0.9584	P<0.0001	NS	NS
	Dyn Modulus vs. Total collagen	185.9	0.9232	P<0.0001	NS	NS

Table 1: Correlation of mechanical properties and biochemical content for different sources of progenitor cells and chondrocyte constructs. Correlation coefficients relating measured mechanical properties with concentration of GAG and total collagen. NS: not significant. Note that replicate data from separate experiments is included in this analysis.

203x138mm (150 x 150 DPI)