**The Influence of Construct Scale on the Composition and Functional
Properties of Cartilaginous Tissues Engineered Using Bone-Marrow
Derived Mesenchymal Stem Cells**

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Abstract

Engineering cartilaginous tissue of a scale necessary to treat defects observed clinically is a well documented challenge in the field of cartilage tissue engineering. The objective of this study was to determine how the composition and mechanical properties of cartilaginous tissues engineered using bone marrow derived mesenchymal stem cells (MSCs) depend on the scale of the construct. Porcine bone marrow derived MSCs were encapsulated in agarose hydrogels and constructs of different cylindrical geometries ($\text{Ø}4 \times 1.5 \text{ mm}$; $\text{Ø}5 \times 3 \text{ mm}$; $\text{Ø}6 \times 4.5 \text{ mm}$; $\text{Ø}8 \times 4.5 \text{ mm}$) were fabricated and maintained in a chemically defined serum-free medium supplemented with transforming growth factor- $\beta 3$ (TGF- $\beta 3$) for 42 days. Total sulphated glycosaminoglycan (sGAG) accumulation by day 42 increased from 0.14 % w/w to 0.88 % w/w as the construct geometry increased from $\text{Ø}4 \times 1.5 \text{ mm}$ to $\text{Ø}8 \times 4.5 \text{ mm}$, with collagen accumulation increasing from 0.31 % w/w to 1.62 % w/w. This led to an increase in the dynamic modulus from 90.81 kPa to 327.51 kPa as the engineered tissue increased in scale from $\text{Ø}4 \times 1.5 \text{ mm}$ to $\text{Ø}8 \times 4.5 \text{ mm}$. By decreasing the external oxygen tension from 20% to 5%, it was possible to achieve these higher levels of mechanical functionality in the smaller engineered tissues. Constructs were then sectioned into smaller sub-regions in order to quantify the spatial accumulation of extracellular matrix components, and a model of oxygen diffusion and consumption was used to predict spatial gradients in oxygen concentration throughout the construct. sGAG accumulation was always highest in regions where oxygen concentration was predicted to be lowest. Furthermore, as the size of the engineered construct increased, different regions of the construct preferentially supported either sGAG or collagen accumulation, suggesting that gradients in regulatory factors other than oxygen were playing a role in determining levels of collagen synthesis. The identification of such factors and the means to control their spatial concentration within developing tissues represents a central challenge in engineering large cartilaginous grafts.

Introduction

Engineering cartilaginous tissues of a scale necessary to treat large defects remains a significant challenge. Overcoming such challenges is also central to engineering partial or whole synovial joints.¹ The problem of core degradation due to nutrient transfer limitations within developing cartilaginous tissues is well documented.² A number of different approaches have been proposed to overcome this problem, from the introduction of nutrient channels into cell seeded constructs,^{3, 4} to the use of bioreactors to improve mass transport.^{2, 5, 6} Perfusion bioreactors have been developed for scaffold cell seeding and culture and can be used to generate homogenous tissues of clinically relevant thicknesses.⁷ More recently the use of such bioreactor based systems has been extended in order to engineer large diameter cartilage grafts using human chondrocytes.⁸

There has been increased interest in using mesenchymal stem cells (MSCs) for functional cartilage tissue engineering.⁹ The potential advantages of using MSCs as an alternative to chondrocytes for cartilage repair strategies are well documented.¹⁰ Isolating chondrocytes requires retrieving a biopsy of articular cartilage that can produce additional damage to the joint surface.¹¹ In addition, chondrocytes have demonstrated a decreased capacity for chondrogenesis with extended *in vitro* passaging and with age.¹²⁻¹⁴ For these reasons, numerous studies report using progenitor cells isolated from tissues such as bone marrow,¹⁵⁻²¹ synovium,²²⁻²⁵ infrapatellar fat pad²⁶⁻³⁰ and adipose tissue,³¹⁻³³ among others, for cartilage tissue engineering. Such progenitor cells have been combined with a variety of scaffolds, from relatively inert marine-derived biomaterials such as agarose and alginates,³⁴⁻³⁹ to silk scaffolds⁴⁰⁻⁴² and electrospun meshes,⁴³ to more bioactive scaffolds derived from native articular cartilage extracellular matrix.^{44, 45} Whether such approaches can be used to engineer cartilaginous grafts of sufficient scale to treat large defects has yet to be addressed.

A feature of MSCs in regenerative events such as endochondral ossification during fracture healing is their ability to synthesize large volumes of cartilaginous tissue in avascular regions of the regenerating tissue. In a tissue engineering context, a similar low oxygen environment may be created within cell-seeded constructs due to cellular utilization of oxygen in the peripheral regions of the developing tissue.⁴⁶ Low oxygen tension has been shown in numerous studies to enhance chondrogenesis of MSCs.^{21, 26, 47-50} The objective of this study is to determine how the properties of cartilaginous tissues engineered using bone marrow derived MSCs will change both spatially and temporally as the scale of the construct increases. It is hypothesised that the functional properties of such tissues will improve as the geometrical volume of the engineered construct is increased due to the development of a lower oxygen environment within larger volume constructs. Computational modelling will be used to predict the oxygen tension within such cell seeded constructs. It is further hypothesized that the more chondrogenic microenvironment generated in such large MSC-seeded constructs can be recapitulated in smaller construct geometries by lowering the surrounding oxygen tension, thereby enhancing the functional properties of the resulting tissue.

Methods

Cell isolation and construct assembly

Bone marrow was aseptically harvested from the femoral diaphysis of two four-month old pigs (~50 kg). Marrow was aspirated repeatedly using a 16 gauge needle to break up large aggregates prior to centrifuging at 650g for 5 min. The separated floating adipose layer was discarded and the cell pellet was resuspended in expansion media (EM) consisting of high-glucose Dulbeccos Modified Eagles Medium (hgDMEM) GlutaMAX supplemented with 10% foetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (all GIBCO,

Biosciences, Dublin, Ireland). Cells were triturated through a 20 gauge needle to create a single cell suspension and filtered through a 40 μm nylon cell strainer (B.D. Falcon, Unitech, Dublin, Ireland). An aliquot of this suspension was treated with 4% acetic acid to lyse the red blood cells and viable mononuclear cells were counted using a haemocytometer in the presence of 0.4% trypan blue. Mononuclear cells were initially seeded at a density of 50,000 cells/ cm^2 in T-75 cm^2 flasks. Cells were subcultured at a density of 5,000 cells/ cm^2 following colony formation and expanded to passage two (P2). We have previously demonstrated that porcine bone marrow derived MSCs expanded in this manner can differentiate down an osteogenic, adipogenic or chondrogenic pathway.²⁷

Cells were mixed with 4% agarose (Type VII, Sigma-Aldrich, Arklow, Ireland) at a ratio of 1:1 at $\sim 40^\circ\text{C}$ to yield a final gel concentration of 2% at a density of 15 million cells/ml. The agarose-cell suspension was cast in stainless steel moulds and allowed to set for 20mins before coring with sterile biopsy punches to produce cylindrical constructs of the following dimensions (diameter x height): $\text{Ø}4 \times 1.5\text{mm}$; $\text{Ø}5 \times 3\text{mm}$; $\text{Ø}6 \times 4.5\text{mm}$; $\text{Ø}8 \times 4.5\text{mm}$. During the fabrication stage a hatched pattern was moulded onto the top surface of constructs to permit tracking of construct orientation for histology purposes and also to ensure constructs were not turned over during the culture period. Constructs did not float during the culture period, and rested on the bottom surface of wells of each culture plate. Details of media volumes and well plates are provided in Fig 1A.

Constructs were maintained in a chemically defined chondrogenic medium consisting of hgDMEM supplemented with 100 U/mL penicillin/streptomycin (both GIBCO), 100 $\mu\text{g/ml}$ sodium pyruvate, 40 $\mu\text{g/ml}$ L-proline, 50 $\mu\text{g/ml}$ L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, $1 \times$ insulin-transferrin-selenium, 100 nM dexamethasone (all Sigma-Aldrich) and 10ng/ml of transforming growth factor $\beta 3$ (TGF- $\beta 3$, ProSpec-Tany TechnoGene Ltd., Israel). Medium was changed twice per week for six weeks. In parallel, constructs of

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3 dimensions Ø5x3mm and Ø6x4.5mm were cultured at either 20% or 5% oxygen tension at
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5 37°C and 5% CO₂. For culturing at 5% oxygen tension a hypoxia incubator was used
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7 (Brunswick Galaxy 48R incubator with 1–19% O₂ control) which is maintained with nitrogen
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9 gas to create this low oxygen environment. All samples were assessed at day 42.
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11 12 13 14 ***Histology and immunohistochemistry*** 15

16 At each time point, two additional samples per group were fixed in 4% paraformaldehyde
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18 overnight, dehydrated, and embedded in paraffin. The constructs were sectioned (6 µm
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20 thickness) perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX
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22 (Sigma-Aldrich) in 0.1 M HCl for sulphated glycosaminoglycans (sGAG), and picro-sirius
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24 red for collagen. The deposition of collagen types I and II were identified through
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26 immunohistochemistry. Briefly sections were rinsed with PBS and quenched of peroxidase
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28 activity for 20 mins and treated with chondroitinase ABC (Sigma, 0.25 units/ml) in a
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30 humidified environment at 37°C for 1 hr to enhance permeability of the extracellular matrix
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32 by removal of chondroitin sulphate. Slides were again rinsed with PBS and blocked with 10%
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34 goat serum for 30 mins. Sections were incubated with mouse monoclonal collagen type I
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36 diluted 1:400 (Abcam, UK) (concentration 5.4 mg/ml) or mouse monoclonal anti-collagen
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38 type II diluted 1:100 (Abcam, United Kingdom) (concentration 1 mg/ml) for 1 hr at room
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40 temperature. After washing in PBS, the secondary antibody for type I and type II collagen
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42 (Anti-Mouse IgG Biotin antibody produced in goat) (concentration 1 g/L) binding was
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44 applied for 1 hr. Colour was developed using the Vectastain ABC reagent (Vectastain ABC
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46 kit, Vector Laboratories, United Kingdom) for 45 min and 5 min exposure to peroxidase
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48 DAB substrate kit (Vector laboratories, United Kingdom). Slides were dehydrated through
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50 ethanol and xylene and mounted with Vectamount medium (Vector Laboratories, United
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Kingdom). Positive and negative controls (porcine cartilage and ligament) were included in the immunohistochemistry staining protocol for each batch.

Quantitative biochemical analysis

To quantify the spatial accumulation of biochemical constituents, all but the Ø4x1.5mm constructs were sectioned into specific sub-regions and analysed separately (Fig. 1A). Samples were digested in screw cap microtubes with 500 µl of 125 µg/mL papain (Papain from papaya latex, P3125, Batch Num: 010M7013) in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich) at 60°C under constant rotation (Stuart® Rotator SB3; Lennox Laboratory Supplies, Dublin, Ireland) for 18 hrs. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay as described previously⁵¹ with a calf thymus DNA standard. sGAG content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110°C for 18 hrs in 38% HCl and assayed using a chloramine-T assay⁵² with a hydroxyproline:collagen ratio of 1:7.69.⁵³ Each biochemical constituent was normalised to the tissue wet weight.

Mechanical analysis

Constructs from each group were mechanically tested in unconfined compression between impermeable platens using a standard material testing machine with a 5N load cell (Zwick Z005, Roell, Germany) as previously described.⁵⁴ Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 µm/s until 10% strain was obtained and maintained until equilibrium was reached (30 min). Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain amplitude of 1%

superimposed upon the 10% strain was applied for 10 cycles at 1 Hz. The compressive equilibrium modulus and dynamic modulus were determined from these tests.

Statistical analysis

Statistical analysis was performed using MINITAB 15.1 software package (Minitab Ltd., Coventry, UK) with 3-4 samples analysed for each group at each time point. Groups were analyzed for significant differences using a general linear model for analysis of variance with factors, culture time, construct size, construct region and culturing regime (normal or low oxygen) and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p < 0.05$. Numerical and graphical results are displayed as mean $\pm$ standard deviation. The entire experiment was replicated with cells from the second donor which confirmed the findings presented in this manuscript.

Finite element modeling of oxygen concentration profiles

A numerical method was developed to predict the oxygen gradients within cylindrical constructs using a commercial finite element package (COMSOL Multiphysics™ v.3.2) in a similar fashion as previously described.⁵⁵⁻⁵⁷ An axisymmetric model was employed with symmetry boundary conditions imposed at the construct centerline. The concentrations at the edge and top surfaces of the construct in contact with the culture medium were assumed to be equivalent. The oxygen concentration C_{ox} as a function of time t within a cylindrical construct can be described as:

$$\frac{\partial C_{ox}(z,r,t)}{\partial t} = D_{construct} \left(\frac{\partial^2 C_{ox}(z,r,t)}{\partial x^2} + \frac{\partial^2 C_{ox}(z,r,t)}{\partial r^2} + \frac{2}{r} \frac{\partial C_{ox}(z,r,t)}{\partial r} \right) - R(z,r,t) \quad \text{Eqn (1)}$$

where oxygen is assumed to diffuse with a constant diffusion coefficient, $D_{construct}$ (m^2s^{-1}). The oxygen concentration is denoted by C_{ox} ($mol\ m^{-3}$). The depth into the construct sample is z (m). The reaction rate, R ($mol\cdot m^{-3}s^{-1}$), is the rate at which oxygen is consumed by the cells within the hydrogel system. The reaction rate R ($mol\cdot m^{-3}s^{-1}$) is dependent on the local number of cells (n_{cell}) and on the maximal rate of consumption of oxygen of the cells V_{max} ($mol\cdot cell^{-1}\cdot s^{-1}$). As oxygen concentration, c , becomes depleted the oxygen consumption rate of the cells alters according to Michaelis-Menten kinetics, with K_m ($mol\cdot m^{-3}$) being the oxygen concentration at half maximal oxygen consumption, yielding an equation for R :

$$R(z, r, t) = n_{cell} \left(\frac{V_{max} C_{ox}(z, r, t)}{C_{ox}(z, r, t) + K_m} \right) \quad \text{Eqn (2)}$$

The diffusion coefficient $D_{construct}$ was estimated using the Mackie and Meares relation,^{58, 59} defined as the ratio of diffusivity in the construct ($D_{construct}$) to diffusivity in free solution (D_0) which is a function of the volume fraction of water in the construct:

$$\frac{D_{construct}}{D_0} = \frac{(\phi^w)^2}{(2 - \phi^w)^2} \quad \text{Eqn (3)}$$

where D_0 is the diffusion coefficient of oxygen in medium ($3.0 \times 10^{-9} m^2 s^{-1}$) at $37^\circ C$,⁶⁰ and ϕ^w is = 0.98 for 2% agarose hydrogel yielding a value for $D_{construct} = 2.77 \times 10^{-9} m^2 s^{-1}$. Based on the average reported oxygen consumption rate for bone marrow MSCs undergoing chondrogenesis in a pellet culture system over 21 days⁶¹ V_{max} was estimated to be $18.7\ fmol\ hr^{-1}\ cell^{-1}$. As this parameter is known to vary during chondrogenesis,⁶¹ in a second simulation

we predicted how the spatial concentration of oxygen would change after doubling this rate ($V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$). The Michaelis-Menten constant, K_m , was set to $5 \times 10^{-3} \text{ mol m}^{-3}$.⁶⁰ The cell density, n_{cell} , was set to $15 \times 10^6 \text{ cells/ml}$. Results for the oxygen concentrations as a function of depth (centerline) and along the central radial direction were predicted as depicted in Fig 1B.

Results

Model predictions of oxygen concentration within MSC seeded constructs

Oxygen concentration profiles within the MSC seeded hydrogels were predicted to depend on construct size (Fig. 2A). The oxygen concentration was predicted to decrease with increasing distance from both the top (Fig. 2C) and side (Fig. 2D) surfaces of the construct. The predicted oxygen concentration within the bottom core of the construct decreased as the size of the construct increased from Ø4x1.5mm to Ø8x4.5mm. Assuming a peripheral oxygen tension of 20%, an oxygen concentration of approximately 10.8% was predicted within the core of the largest Ø8x4.5mm construct, compared to a predicted concentration of approximately 18.2% in the core of the smallest Ø4x1.5mm construct. As expected, doubling the oxygen consumption rate decreased the predicted oxygen concentration within constructs (Fig 2B, E & F). For example, the minimum oxygen tension in the core of the largest Ø8x4.5mm construct was predicted to reduce from 10.8% to 2.6% when $V_{\max}$ was increased by a factor of two. Reducing the oxygen concentration at the edge and top surfaces from 20% to 5% (Fig. 3) was also predicted to reduce core oxygen concentration values from approximately 16.3% to 1.9% in the Ø5x3mm constructs, and from approximately 14.3% to 0.7% in the Ø6x4.5mm construct ($V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$).

Composition and distribution of extracellular matrix within constructs of different scales

Less intense staining for sGAGs and collagen was observed for smaller sized constructs (Fig. 4). The bottom core of constructs cultured in a normoxic environment (20% O₂) generally stained intensely for type II collagen, with cells appearing to be slightly larger and/or more clustered in this region. This spatial difference in cell shape was particularly evident in intermediate sized constructs, where cells appeared larger in the core of the construct compared to the periphery (see Fig. 4). In a normoxic environment a peripheral region staining weakly for sGAGs and type II collagen was also observed, particularly in the smaller and intermediately sized constructs. A more homogenous staining for sGAGs in the Ø5x3mm and Ø6x4.5mm constructs was observed when maintained in a low oxygen environment (5% O₂). For larger constructs (Ø8x4.5mm), it was observed that the very core region stained less intensely for cartilaginous matrix by day 42. For the constructs cultured in a low oxygen environment the central core regions of the constructs also stained slightly less intensely for collagen compared to the surrounding tissue.

A slight decrease in DNA content was observed in the largest constructs (Ø8x4.5mm) compared to all other construct sizes (Fig. 5A). Total sGAG and collagen accumulation, measured as a percentage of wet weight or normalised to DNA, increased as the construct geometry increased from Ø4x1.5mm to Ø8x4.5mm (Fig.5 B&C). Total sGAG accumulation increased from a value of 0.14 ± 0.0 % w/w for the Ø4x1.5mm construct to 0.88 ± 0.04 % w/w for the Ø8x4.5mm construct at day 42. Total collagen accumulation increased from a value of 0.31 ± 0.01 % w/w for the Ø4x1.5mm construct to 1.62 ± 0.17 % w/w for the Ø8x4.5mm construct. Reducing the external oxygen tension to 5% O₂ resulted in a decrease in DNA content for Ø6x4.5mm constructs (Fig 5D). In addition, reducing the external oxygen tension led to an increase in sGAG accumulation in both Ø5x3mm and Ø6x4.5mm

constructs (Fig. 5E&F). Higher collagen accumulation was also observed in the Ø5x3mm constructs when cultured at 5% O₂, but not in the Ø6x4.5mm constructs (Fig 5E&F).

The entire experiment was replicated using MSCs isolated from a second donor, with identical findings observed in relation to the influence of both construct scale and external oxygen tension, albeit with higher overall levels of matrix accumulation observed in the replicate study (see supplementary Fig. S1).

All but the smallest Ø4x1.5mm constructs were sectioned into smaller sub-regions in order to quantify the spatial changes in both DNA content and the accumulation of extracellular matrix components (Fig. 6 & Table 1). DNA content was generally lower in the bottom core of all construct sizes, with no evidence of enhanced cell viability observed in constructs maintained at lower oxygen tensions (Fig. 6A & Table 1). sGAG accumulation was generally highest in the bottom core, decreasing towards the construct periphery. These spatial differences were maintained, or even accentuated, when sGAG levels were normalised to DNA content. sGAG accumulation was greater in the bottom core compared to the top core for the Ø5x3mm and Ø6x4.5mm constructs ($p < 0.0001$), with near comparable levels in the top and bottom cores of the larger Ø8x4.5mm constructs (Fig. 6B & Table 1). There was less spatial variation in sGAG accumulation in constructs maintained at 5% O₂ compared to 20% O₂, although sGAG accumulation was still higher in the core compared to the annulus.

Collagen accumulation was also higher in the core of the intermediate sized constructs, however it was lower in the core for the larger Ø8x4.5mm constructs (Fig. 6C & Table 1). In fact, collagen accumulation was highest in the outer annular regions of the Ø8x4.5mm constructs compared to both the inner annulus and core regions. These differences were less dramatic, although still present, when collagen levels were normalised to DNA content (Table 1). There was less spatial variation in collagen accumulation in the Ø5x3mm constructs cultured at 5% O₂ compared to 20% O₂, with collagen accumulation

higher in the annulus compared to the core. Collagen accumulation was reduced in all regions of the Ø6x4.5mm constructs when cultured at 5% O₂.

Structure-function relations

The equilibrium modulus of the Ø8x4.5mm constructs (36.52 ± 2.12 kPa) was significantly ($p<0.05$) higher than all other sized constructs (Fig. 7A). The dynamic modulus was observed to increase with increasing construct size, reaching a maximum value of 327.5 ± 30.3 kPa for the Ø8x4.5mm constructs (Fig. 7A). Culture in a low (5%) oxygen environment was also observed to increase both the equilibrium and dynamic mechanical properties of the engineered constructs (Fig 7B&C), with a 1.9 fold increase in the dynamic modulus for the Ø5x3mm constructs when maintained at 5% oxygen tension (Fig. 7C).

Structure-function plots (Fig. 7E-H) relating both the equilibrium and dynamic modulus of the engineered tissues to biochemical constituents revealed strong correlations. This analysis considered the effects of including all constructs (those maintained at both 20% and 5% O₂) or the 20% O₂ constructs only in the regression analysis. For equilibrium modulus, when all constructs are considered (20% and 5% O₂), a correlation existed for sGAG content ($r^2=0.68$) but not collagen content ($r^2=0.40$). These correlations became stronger when only constructs maintained at 20% O₂ were considered in the analysis (sGAG: $r^2=0.82$, collagen: $r^2=0.82$). This suggests that for the same total amount of matrix components, constructs cultured at 5% O₂ appear stiffer. Similar trends were seen for the dynamic modulus correlations.

Discussion

Engineering cartilaginous tissue of a scale necessary to treat defects observed clinically is a significant challenge in the field of cartilage tissue engineering. The results of this study

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2
3 suggest that the inherent capacity of bone marrow derived MSCs to form large cartilaginous
4 templates in low oxygen environments can be manipulated to engineer tissues of the scale
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6 necessary to treat large cartilaginous defects. The mechanical properties of the engineered
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8 tissues were observed to increase with increasing construct scale, reaching a maximum value
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10 for the Ø8x4.5mm engineered tissues. Comparable values of equilibrium and dynamic
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12 modulus were obtained for smaller constructs maintained at lower oxygen tensions,
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14 demonstrating that it is possible to recapitulate the chondrogenic oxygen microenvironment
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16 that develops within larger constructs in smaller constructs by altering the external oxygen
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18 tension.
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23 A simplified model of oxygen diffusion and consumption was used to predict spatial
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25 gradients in oxygen concentration throughout the construct. The validity of this model
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27 strongly depends on the value of the reaction rate, R , inputted into the model. This term was
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29 based on the average reported oxygen consumption rate for bone marrow MSCs undergoing
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31 chondrogenesis in a pellet culture system over 21 days,⁶¹ however this term will depend
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33 upon multiple factors, including the material in which the cells are encapsulated⁶² and the
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35 local levels of glucose and lactic acid.⁶³⁻⁶⁵ For higher oxygen consumption rates, the model
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37 predicts lower oxygen concentrations in the cores of MSC seeded constructs. For example, if
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39 the oxygen consumption rate ($V_{\max}$) is increased by a factor of two, the oxygen concentration
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41 in the bottom core of the larger Ø8x4.5mm constructs reduces to nearly 2.6%, however for
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43 this consumption rate the oxygen concentration is predicted to reduce to 0.26% in the smaller
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45 Ø5x3mm constructs maintained in low oxygen conditions. Therefore the model utilized in
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47 this study will always predict a lower oxygen tension in the core of the smaller Ø5x3mm
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49 constructs maintained in low oxygen conditions compared to the larger Ø8x4.5mm
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51 constructs maintained in normoxic conditions for reasonable values of R . (The potential
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53 implications of this are discussed in the following paragraph). Direct measurements are
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needed to confirm these predictions as oxygen consumption rates are known to increase with low glucose availability,⁶⁴ which may exist in the core of the larger constructs. A further assumption of the model is that the diffusivity of the construct to oxygen is constant. Clearly as large amounts of extracellular matrix are laid down the diffusivity of the tissue to oxygen will reduce, further reducing core oxygen levels.

sGAG accumulation was always highest in regions of the construct where oxygen concentration was predicted to be lowest. Reducing the ambient oxygen tension to 5% O₂ increased sGAG accumulation in nearly all regions of the Ø5x3mm and Ø6x4.5mm constructs. Therefore sGAG synthesis would appear to be inversely correlated with oxygen tension (see supplementary Fig.S2), although this may not be the only determinant of synthetic activity. A more complex relationship exists between collagen synthesis and spatial levels of oxygen concentration (see supplementary Fig. S2). Collagen accumulation is greater in the core of intermediately sized constructs, where oxygen concentration is clearly lower; however for larger constructs (Ø8x4.5mm) collagen accumulation was greater in the annular region. Furthermore, maintaining the Ø6x4.5mm constructs at 5% O₂ was observed to suppress collagen synthesis. The disparity in the spatial accumulation of sGAG and collagen is particularly evident in the larger Ø8x4.5mm constructs. While an oxygen tension below 20% O₂ is clearly beneficial for collagen synthesis, the fact that a lower oxygen microenvironment in the larger Ø8x4.5mm enhances sGAG but not collagen accumulation suggests that environmental conditions that promote sGAG synthesis are not necessarily optimal for collagen synthesis, and that gradients in other regulatory factors are playing an equally important role in determining rates of collagen synthesis. For example, collagen synthesis could be more sensitive to nutrient availability than sGAG synthesis, which may explain why collagen accumulation is lower in the core of the larger Ø8x4.5mm constructs compared to the core of the smaller constructs maintained at 5% O₂, where predicted oxygen

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3 concentrations are lower but where nutrient availability is presumably greater. An additional
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5 factor to consider may be the consumption rates of nutrients such as glucose, which have
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7 been shown to increase in low oxygen environments.⁶⁵ In addition it has been shown that
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9 cellular oxygen consumption increases at low glucose concentrations,^{59, 63, 64} thereby
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11 increasing the likelihood of anoxic, nutrient deficient regions developing within the cores of
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13 large tissue engineered constructs under certain conditions. Further studies are required to
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15 quantify gradients in oxygen and nutrient concentrations in large MSC seeded constructs.
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17 Finally it should be noted that spatial differences in collagen accumulation are not as
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19 dramatic when normalised to DNA content, suggesting that the lower levels of collagen
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21 within the cores of larger constructs can be partially attributed to lower cell viability in these
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23 regions.
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27 As expected, both the equilibrium and dynamic modulus of the engineered tissues
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29 were strongly correlated to the sGAG content. Interestingly, for comparable levels of
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31 extracellular matrix accumulation, constructs maintained at 5% O₂ appeared stiffer than those
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33 maintained at 20% O₂. This can potentially be explained by the fact that matrix is more
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35 homogenously accumulated in constructs maintained at 5% O₂, while a greater proportion of
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37 sGAG accumulates in the core when maintained at 20% O₂. Inhomogeneous accumulation of
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39 extracellular matrix can negatively impact upon the magnitude of material constants derived
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41 from mechanical tests that assume a homogenous tissue.⁶⁶ Alternatively, a lower oxygen
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43 tension could potentially alter the ratios or amounts of different types of collagen being
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45 generated by MSCs, or collagen cross-linking, all of which are known to impact upon the
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47 mechanical properties of tissue engineered cartilage.^{67, 68}
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51 There are numerous studies reported in the literature comparing the chondrogenic
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53 capacity of stem cells isolated from different tissue sources^{22, 24, 25, 69-72} and/or comparing
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55 stem cells to chondrocytes for cartilage tissue engineering.^{19, 26, 27, 73, 74} We have previously
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3 shown that stem cells and chondrocytes seeded into agarose hydrogels respond differentially
4 to variations in oxygen tension.²⁶ Coupled with the results of this study, this suggests that
5 comparisons between different cell types must be made with caution, as for a given scaffold
6 type and cell seeding density, chondrogenesis will be dependent on the geometry of the
7 construct. For example, it has been shown that the functional properties of cartilaginous
8 tissues engineered using chondrocyte seeded hydrogels or scaffolds are superior to that
9 generated using MSCs for small constructs,^{19, 38} but whether this result would translate to
10 larger constructs is unclear. Ideally comparisons would be made using a range of scaffold
11 geometries.
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23 The oxygen microenvironment within MSC seeded constructs will depend, among
24 other factors, on the scaffold geometry and architecture, the local concentrations of glucose
25 and lactic acid, cell-seeding density and the external oxygen tension. In this study we have
26 demonstrated that cartilaginous extracellular matrix accumulation within MSC seeded
27 agarose hydrogels increases with increasing construct size, and we have provided indirect
28 support for the hypothesis that this is due to a reduced oxygen concentration within these
29 larger engineered tissues. This led to increases in the mechanical functionality of the
30 engineered tissue, with both the equilibrium and dynamic modulus shown to be strongly
31 correlated with the sGAG content within the engineered tissue. As the size of the engineered
32 construct increased, different regions of the construct preferentially supported either sGAG or
33 collagen accumulation, suggesting that gradients in regulatory factors other than oxygen were
34 playing a role in determining levels of matrix synthesis. The results of this study demonstrate
35 that all of these factors must be tailored to the geometry of the tissue being engineered in
36 order to enhance chondrogenesis within the developing construct.
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Disclosure Statement

No competing financial interests exist.

Figure Legends & Tables

FIG. 1: (A) Schematic representation of the different construct sizes, Ø=diameter (mm) x height (mm), and experimental details of media volume and culture dishes used throughout. Each well contained a single construct. Illustration represents the regions of sectioning for biochemical assays with each region being analysed separately, with the exception of the Ø4x1.5mm constructs which were analysed in total. TA: Top annulus, BA: Bottom annulus, TIA: Top inner annulus, BIA: Bottom inner annulus, TC: Top core and BC: Bottom core. (B) Illustration of the partial 3D hydrogel geometry from which the axisymmetric finite element model was developed to predict oxygen concentration profiles.

FIG. 2: Predicted oxygen concentration profiles for hydrogel constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) cultured at 20% oxygen tension as illustrated in **FIG. 1**. (A) Contour plots using an oxygen consumption rate, $V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (B) Contour plots using an oxygen consumption rate, $V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) Profile along vertical centreline ($V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (D) Profile along central radial line ($V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) (E) Profile along vertical centreline ($V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (F) Profile along central radial line ($V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$).

FIG. 3: Predicted oxygen concentration profiles for hydrogel constructs (Ø5x3mm; Ø6x4.5mm) cultured at 5% oxygen tension (A) Contour plots using an oxygen consumption rate, $V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (B) Contour plots using an oxygen consumption rate, $V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) Profile along vertical centreline ($V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (D) Profile along central radial line ($V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) (E) Profile along vertical centreline ($V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (F) Profile along central radial line ($V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$).

FIG. 4: Histological evaluation (sGAG, collagen, collagen type II and light microscopic surface image) of MSC seeded constructs of differing geometries at day 42. Constructs of dimension Ø5x3mm and Ø6x4.5mm were also cultured at a lower oxygen tension of 5%. Dashed line indicates the centerline of the engineered tissue. Scale bar for low magnification= 1mm. Higher magnification images in collagen type II stained sections are from core and annulus regions showing the difference in cell shape, scale bar = 50µm.

FIG. 5: Biochemical accumulation of engineered cartilaginous constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) at day 42 cultured under normoxic (20%) and low oxygen (5%) conditions. (A) Total DNA (ng/mg w/w) at 20% O₂ (B) Total sGAG and collagen (%w/w) at 20% O₂, (C) Total sGAG/DNA and collagen/DNA at 20% O₂. (D) Total DNA (ng/mg w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂ (E) Total sGAG and collagen (%w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂, (F) Total sGAG/DNA and collagen/DNA for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂. (&) $p < 0.05$, compared to all other constructs. (*, !) $p < 0.05$ indicates significance compared to preceding sized construct, (\$) $p < 0.05$ compared to same sized constructs cultured at 20% O₂. n=3 constructs per group.

FIG. 6: Spatial accumulation of (A) DNA (ng/mg w/w), (B) sGAG (% w/w) and (C) collagen (% w/w) for specific regions of engineered constructs at day 42. (!) $p < 0.05$, (&) $p < 0.001$, (*) $p < 0.0001$ indicates significance, n =3 for all groups. Mean data ± standard deviations are provided in Table 1.

FIG. 7: (A) Equilibrium and dynamic modulus of constructs cultured under 20% O₂ conditions. (B) Equilibrium & (C) Dynamic modulus of constructs (Ø5x3mm and Ø6x4.5mm) cultured at 20% and 5% O₂. (D) & (E) Structure-function correlation plots relating measured equilibrium modulus to biochemical constituents (F) & (G) Structure-function correlation plots relating measured dynamic modulus to biochemical constituents. This analysis considered the effects of including all constructs (20% and 5% O₂) or the 20% O₂ constructs only in the regression analysis. (*, &) p < 0.05 indicates significance compared to preceding sized construct. (!) p < 0.0001 indicates significance compared to same sized construct cultured at 20% O₂. (%) p < 0.001 indicates significance compared to other construct size at the same oxygen tension. n=3 constructs per group.

SUPPLEMENTARY FIG. S1: Comparison of engineered cartilaginous constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) from two different donors at day 42 cultured under normoxic (20%) and low oxygen (5%) conditions. (A) Total sGAG and (B) collagen (%w/w) at 20% O₂ (C) Total sGAG and (D) collagen (%w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂, n=3 constructs per group.

SUPPLEMENTARY FIG. S2: Correlation plots of mean oxygen concentration (%) with biochemical constituents normalised by DNA content. This analysis was performed using the averaged oxygen concentrations determined in the finite element models for specific regions as illustrated in FIG 1, for two specific values of oxygen consumption ($V_{\max}$). (A) & (B) $V_{\max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) & (D) $V_{\max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$.

TABLE 1: Mean $\pm$ standard deviation data corresponding to spatial distribution of matrix components as illustrated in Fig 7, n= 3 constructs per group analysed.

References

1. Williams, G.M., Chan, E.F., Temple-Wong, M.M., Bae, W.C., Masuda, K., Bugbee, W.D. and Sah, R.L. Shape, loading, and motion in the bioengineering design, fabrication, and testing of personalized synovial joints. *Journal of Biomechanics* **43**, 156, 2010.

2. Martin, I., Wendt, D. and Heberer, M. The role of bioreactors in tissue engineering. *Trends in Biotechnology* **22**, 80, 2004.

3. Buckley, C.T., Thorpe, S.D. and Kelly, D.J. Engineering of large cartilaginous tissues through the use of microchanneled hydrogels and rotational culture. *Tissue Eng Part A* **15**, 3213, 2009.

4. Bian, L., Angione, S.L., Ng, K.W., Lima, E.G., Williams, D.Y., Mao, D.Q., Ateshian, G.A. and Hung, C.T. Influence of decreasing nutrient path length on the development of engineered cartilage. *Osteoarthritis Cartilage* **17**, 677, 2009.

5. Mauck, R.L., Hung, C.T. and Ateshian, G.A. Modeling of Neutral Solute Transport in a Dynamically Loaded Porous Permeable Gel: Implications for Articular Cartilage Biosynthesis and Tissue Engineering. *Journal of Biomechanical Engineering* **125**, 602, 2003.

6. Chahine, N.O., Albro, M.B., Lima, E.G., Wei, V.I., Dubois, C.R., Hung, C.T. and Ateshian, G.A. Effect of dynamic loading on the transport of solutes into agarose hydrogels. *Biophysical Journal* **97**, 968, 2009.

7. Wendt, D., Stroebel, S., Jakob, M., John, G.T. and Martin, I. Uniform tissues engineered by seeding and culturing cells in 3D scaffolds under perfusion at defined oxygen tensions. *Biorheology* **43**, 481, 2006.

8. Santoro, R., Olivares, A.L., Brans, G., Wirz, D., Longinotti, C., Lacroix, D., Martin, I. and Wendt, D. Bioreactor based engineering of large-scale human cartilage grafts for joint resurfacing. *Biomaterials* **31**, 8946, 2010.
9. Huang, A.H., Farrell, M.J. and Mauck, R.L. Mechanics and mechanobiology of mesenchymal stem cell-based engineered cartilage. *Journal of Biomechanics* **43**, 128, 2010.
10. Pelttari, K., Steck, E. and Richter, W. The use of mesenchymal stem cells for chondrogenesis. *Injury* **39**, 58, 2008.
11. Lee, C.R., Grodzinsky, A.J., Hsu, H.P., Martin, S.D. and Spector, M. Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. *Journal of Orthopaedic Research* **18**, 790, 2000.
12. Barbero, A., Grogan, S., Schäfer, D., Heberer, M., Mainil-Varlet, P. and Martin, I. Age related changes in human articular chondrocyte yield, proliferation and post-expansion chondrogenic capacity. *Osteoarthritis and Cartilage* **12**, 476, 2004.
13. Luyten, F.P., Dell'Accio, F. and De Bari, C. Skeletal tissue engineering: Opportunities and challenges. *Best Practice and Research: Clinical Rheumatology* **15**, 759, 2001.
14. Darling, E.M. and Athanasiou, K.A. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *Journal of Orthopaedic Research* **23**, 425, 2005.
15. Kopesky, P.W., Lee, H.Y., Vanderploeg, E.J., Kisiday, J.D., Frisbie, D.D., Plaas, A.H.K., Ortiz, C. and Grodzinsky, A.J. Adult equine bone marrow stromal cells produce a cartilage-like ECM mechanically superior to animal-matched adult chondrocytes. *Matrix Biology* **29**, 427, 2010.
16. Li, Z., Yao, S.J., Alini, M. and Stoddart, M.J. Chondrogenesis of Human Bone Marrow Mesenchymal Stem Cells in Fibrin-Polyurethane Composites is Modulated

by Frequency and Amplitude of Dynamic Compression and Shear Stress. *Tissue Eng Part A* **16**, 575, 2009.

17. Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A. and Kelly, D.J. The response of bone marrow-derived mesenchymal stem cells to dynamic compression following TGF-beta3 induced chondrogenic differentiation. *Ann Biomed Eng* **38**, 2896, 2010.

18. Williams, C.G., Kim, T.K., Taboas, A., Malik, A., Manson, P. and Elisseeff, J. In vitro chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. *Tissue Eng* **9**, 679, 2003.

19. Mauck, R.L., Yuan, X. and Tuan, R.S. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. *Osteoarthritis Cartilage* **14**, 179, 2006.

20. Thorpe, S.D., Buckley, C.T., Vinardell, T., O'Brien, F.J., Campbell, V.A. and Kelly, D.J. Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells. *Biochem Biophys Res Commun* **377**, 458, 2008.

21. Meyer, E.G., Buckley, C.T., Thorpe, S.D. and Kelly, D.J. Low oxygen tension is a more potent promoter of chondrogenic differentiation than dynamic compression. *J Biomech* **43**, 2516, 2010.

22. Fan, J., Varshney, R.R., Ren, L., Cai, D. and Wang, D.A. Synovium-Derived Mesenchymal Stem Cells: A New Cell Source for Musculoskeletal Regeneration. *Tissue Eng Part B Rev* **15**, 75, 2009.

23. Pei, M., He, F., Boyce, B.M. and Kish, V.L. Repair of full-thickness femoral condyle cartilage defects using allogeneic synovial cell-engineered tissue constructs. *Osteoarthritis Cartilage* **17**, 714, 2009.

24. Sakaguchi, Y., Sekiya, I., Yagishita, K. and Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**, 2521, 2005.
25. Yokoyama, A., Sekiya, I., Miyazaki, K., Ichinose, S., Hata, Y. and Muneta, T. In vitro cartilage formation of composites of synovium-derived mesenchymal stem cells with collagen gel. *Cell Tissue Res* **322**, 289, 2005.
26. Buckley, C.T., Vinardell, T. and Kelly, D.J. Oxygen tension differentially regulates the functional properties of cartilaginous tissues engineered from infrapatellar fat pad derived MSCs and articular chondrocytes. *Osteoarthritis Cartilage* **18**, 1345, 2010.
27. Vinardell, T., Buckley, C.T., Thorpe, S.D. and Kelly, D.J. Composition-function relations of cartilaginous tissues engineered from chondrocytes and mesenchymal stem cells isolated from bone marrow and infrapatellar fat pad. *J Tissue Eng Regen Med* (In Press), 2010.
28. Dragoo, J.L., Samimi, B., Zhu, M., Hame, S.L., Thomas, B.J., Lieberman, J.R., Hedrick, M.H. and Benhaim, P. Tissue-engineered cartilage and bone using stem cells from human infrapatellar fat pads. *J Bone Joint Surg Br* **85**, 740, 2003.
29. Jurgens, W.J.F.M., Van Dijk, A., Doulabi, B.Z., Niessen, F.B., Ritt, M.J.P.F., Van Milligen, F.J. and Helder, M.N. Freshly isolated stromal cells from the infrapatellar fat pad are suitable for a one-step surgical procedure to regenerate cartilage tissue. *Cytotherapy* **11**, 1052, 2009.
30. Buckley, C.T., Vinardell, T., Thorpe, S.D., Haugh, M.G., Jones, E., McGonagle, D. and Kelly, D.J. Functional properties of cartilaginous tissues engineered from infrapatellar fat pad-derived mesenchymal stem cells. *J Biomech* **43**, 920, 2010.

31. Estes, B.T., Wu, A.W. and Guilak, F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis Rheum* **54**, 1222, 2006.

32. Kisiday, J.D., Kopesky, P.W., Evans, C.H., Grodzinsky, A.J., McIlwraith, C.W. and Frisbie, D.D. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *J Orthop Res* **26**, 322, 2008.

33. Guilak, F., Estes, B.T., Diekman, B.O., Moutos, F.T. and Gimple, J.M. 2010 Nicolas Andry Award: Multipotent adult stem cells from adipose tissue for musculoskeletal tissue engineering. *Clinical Orthopaedics and Related Research* **468**, 2530, 2010.

34. Erickson, I.E., Huang, A.H., Chung, C., Li, R.T., Burdick, J.A. and Mauck, R.L. Differential maturation and structure-function relationships in mesenchymal stem cell- and chondrocyte-seeded hydrogels. *Tissue Eng Part A* **15**, 1041, 2009.

35. Awad, H.A., Wickham, M.Q., Leddy, H.A., Gimple, J.M. and Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **25**, 3211, 2004.

36. Buschmann, M.D., Gluzband, Y.A., Grodzinsky, A.J., Kimura, J.H. and Hunziker, E.B. Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. *J Orthop Res* **10**, 745, 1992.

37. Buschmann, M.D., Gluzband, Y.A., Grodzinsky, A.J. and Hunziker, E.B. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. *J Cell Sci* **108 (Pt 4)**, 1497, 1995.

38. Vinardell, T., Thorpe, S.D., Buckley, C.T. and Kelly, D.J. Chondrogenesis and integration of mesenchymal stem cells within an in vitro cartilage defect repair model. *Ann Biomed Eng* **37**, 2556, 2009.

- 1
2
3 39. Meyer, E.G., Buckley, C.T., Steward, A.J. and Kelly, D.J. The effect of cyclic
4 hydrostatic pressure on the functional development of cartilaginous tissues engineered
5 using bone marrow derived mesenchymal stem cells. *J Mech Behav Biomed Mater* **4**,
6 1257, 2011.
7
8
9
10
11 40. Hofmann, S., Knecht, S., Langer, R., Kaplan, D.L., Vunjak-Novakovic, G., Merkle,
12 H.P. and Meinel, L. Cartilage-like tissue engineering using silk scaffolds and
13 mesenchymal stem cells. *Tissue Engineering* **12**, 2729, 2006.
14
15
16
17
18 41. Meinel, L., Hofmann, S., Karageorgiou, V., Zichner, L., Langer, R., Kaplan, D. and
19 Vunjak-Novakovic, G. Engineering cartilage-like tissue using human mesenchymal
20 stem cells and silk protein scaffolds. *Biotechnology and Bioengineering* **88**, 379,
21 2004.
22
23
24
25
26
27 42. Wang, Y., Kim, U.J., Blasioli, D.J., Kim, H.J. and Kaplan, D.L. In vitro cartilage
28 tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal
29 stem cells. *Biomaterials* **26**, 7082, 2005.
30
31
32
33
34 43. Li, W.J., Tuli, R., Okafor, C., Derfoul, A., Danielson, K.G., Hall, D.J. and Tuan, R.S.
35 A three-dimensional nanofibrous scaffold for cartilage tissue engineering using
36 human mesenchymal stem cells. *Biomaterials* **26**, 599, 2005.
37
38
39
40
41 44. Cheng, N.C., Estes, B.T., Awad, H.A. and Guilak, F. Chondrogenic differentiation of
42 adipose-derived adult stem cells by a porous scaffold derived from native articular
43 cartilage extracellular matrix. *Tissue Engineering - Part A* **15**, 231, 2009.
44
45
46
47 45. Diekman, B.O., Rowland, C.R., Lennon, D.P., Caplan, A.I. and Guilak, F.
48 Chondrogenesis of adult stem cells from adipose tissue and bone marrow: Induction
49 by growth factors and cartilage-derived matrix. *Tissue Engineering - Part A* **16**, 523,
50 2010.
51
52
53
54
55
56
57
58
59
60

1
2
3 46. Demol, J., Lambrechts, D., Geris, L., Schrooten, J. and Van Oosterwyck, H. Towards
4 a quantitative understanding of oxygen tension and cell density evolution in fibrin
5 hydrogels. *Biomaterials* **32**, 107, 2011.
6
7
8
9
10 47. Khan, W.S., Adesida, A.B. and Hardingham, T.E. Hypoxic conditions increase
11 hypoxia-inducible transcription factor 2alpha and enhance chondrogenesis in stem
12 cells from the infrapatellar fat pad of osteoarthritis patients. *Arthritis Res Ther* **9**, R55,
13 2007.
14
15
16
17
18 48. Wang, D.W., Fermor, B., Gimble, J.M., Awad, H.A. and Guilak, F. Influence of
19 oxygen on the proliferation and metabolism of adipose derived adult stem cells.
20 *Journal of Cellular Physiology* **204**, 184, 2005.
21
22
23
24
25 49. Kanichai, M., Ferguson, D., Prendergast, P.J. and Campbell, V.A. Hypoxia promotes
26 chondrogenesis in rat mesenchymal stem cells: A role for AKT and hypoxia-inducible
27 factor (HIF)-1 α . *Journal of Cellular Physiology* **216**, 708, 2008.
28
29
30
31
32 50. Robins, J.C., Akeno, N., Mukherjee, A., Dalal, R.R., Aronow, B.J., Koopman, P. and
33 Clemens, T.L. Hypoxia induces chondrocyte-specific gene expression in
34 mesenchymal cells in association with transcriptional activation of Sox9. *Bone* **37**,
35 313, 2005.
36
37
38
39
40 51. Kim, Y.J., Sah, R.L., Doong, J.Y. and Grodzinsky, A.J. Fluorometric assay of DNA
41 in cartilage explants using Hoechst 33258. *Anal Biochem* **174**, 168, 1988.
42
43
44
45 52. Kafienah, W. and Sims, T.J. Biochemical methods for the analysis of tissue-
46 engineered cartilage. *Methods Mol Biol* **238**, 217, 2004.
47
48
49
50 53. Ignat'eva, N.Y., Danilov, N.A., Averkiev, S.V., Obrezkova, M.V., Lunin, V.V. and
51 Sobol, E.N. Determination of hydroxyproline in tissues and the evaluation of the
52 collagen content of the tissues. *J Anal Chem* **62**, 51, 2007.
53
54
55
56
57
58
59
60

54. Buckley, C.T., Thorpe, S.D., O'Brien, F.J., Robinson, A.J. and Kelly, D.J. The effect of concentration, thermal history and cell seeding density on the initial mechanical properties of agarose hydrogels. *J Mech Behav Biomed Mater* **2**, 512, 2009.
55. Buckley, C.T. and O'Kelly, K.U. Fabrication and characterization of a porous multidomain hydroxyapatite scaffold for bone tissue engineering investigations. *J Biomed Mater Res B Appl Biomater* **93**, 459, 2010.
56. Radisic, M., Malda, J., Epping, E., Geng, W., Langer, R. and Vunjak-Novakovic, G. Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue. *Biotechnol Bioeng* **93**, 332, 2006.
57. Malda, J., Rouwkema, J., Martens, D.E., Le Comte, E.P., Kooy, F.K., Tramper, J., van Blitterswijk, C.A. and Riesle, J. Oxygen gradients in tissue-engineered PEGT/PBT cartilaginous constructs: measurement and modeling. *Biotechnol Bioeng* **86**, 9, 2004.
58. Gu, W.Y., Yao, H., Vega, A.L. and Flagler, D. Diffusivity of ions in agarose gels and intervertebral disc: effect of porosity. *Ann Biomed Eng* **32**, 1710, 2004.
59. Sengers, B.G., Heywood, H.K., Lee, D.A., Oomens, C.W.J. and Bader, D.L. Nutrient utilization by bovine articular chondrocytes: A combined experimental and theoretical approach. *Journal of Biomechanical Engineering* **127**, 758, 2005.
60. Haselgrove, J.C., Shapiro, I.M. and Silverton, S.F. Computer modeling of the oxygen supply and demand of cells of the avian growth cartilage. *Am J Physiol* **265**, C497, 1993.
61. Pattappa, G., Heywood, H.K., de Bruijn, J.D. and Lee, D.A. The metabolism of human mesenchymal stem cells during proliferation and differentiation. *J Cell Physiol* **226**, 2562, 2011.

62. Guaccio, A., Borselli, C., Oliviero, O. and Netti, P.A. Oxygen consumption of chondrocytes in agarose and collagen gels: a comparative analysis. *Biomaterials* **29**, 1484, 2008.

63. Zhou, S., Cui, Z. and Urban, J.P. Nutrient gradients in engineered cartilage: metabolic kinetics measurement and mass transfer modeling. *Biotechnol Bioeng* **101**, 408, 2008.

64. Heywood, H.K., Bader, D.L. and Lee, D.A. Rate of oxygen consumption by isolated articular chondrocytes is sensitive to medium glucose concentration. *J Cell Physiol* **206**, 402, 2006.

65. Peansukmanee, S., Vaughan-Thomas, A., Carter, S.D., Clegg, P.D., Taylor, S., Redmond, C. and Mobasheri, A. Effects of hypoxia on glucose transport in primary equine chondrocytes in vitro and evidence of reduced GLUT1 gene expression in pathologic cartilage in vivo. *J Orthop Res* **27**, 529, 2009.

66. Kelly, D.J. and Prendergast, P.J. Effect of a degraded core on the mechanical behaviour of tissue-engineered cartilage constructs: a poro-elastic finite element analysis. *Med Biol Eng Comput* **42**, 9, 2004.

67. Yan, D., Zhou, G., Zhou, X., Liu, W., Zhang, W.J., Luo, X., Zhang, L., Jiang, T., Cui, L. and Cao, Y. The impact of low levels of collagen IX and pyridinoline on the mechanical properties of in vitro engineered cartilage. *Biomaterials* **30**, 814, 2009.

68. Kelly, D.J., Crawford, A., Dickinson, S.C., Sims, T.J., Mundy, J., Hollander, A.P., Prendergast, P.J. and Hatton, P.V. Biochemical markers of the mechanical quality of engineered hyaline cartilage. *J Mater Sci Mater Med* **18**, 273, 2007.

69. Koga, H., Muneta, T., Nagase, T., Nimura, A., Ju, Y.J., Mochizuki, T. and Sekiya, I. Comparison of mesenchymal tissues-derived stem cells for in vivo chondrogenesis: suitable conditions for cell therapy of cartilage defects in rabbit. *Cell Tissue Res* **333**, 207, 2008.

- 1
2
3 70. Mochizuki, T., Muneta, T., Sakaguchi, Y., Nimura, A., Yokoyama, A., Koga, H. and
4
5 Sekiya, I. Higher chondrogenic potential of fibrous synovium- and adipose synovium-
6
7 derived cells compared with subcutaneous fat-derived cells: distinguishing properties
8
9 of mesenchymal stem cells in humans. *Arthritis Rheum* **54**, 843, 2006.
10
11
12 71. Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H. and Sekiya, I.
13
14 Comparison of rat mesenchymal stem cells derived from bone marrow, synovium,
15
16 periosteum, adipose tissue, and muscle. *Cell Tissue Res* **327**, 449, 2007.
17
18
19 72. Pei, M., He, F. and Vunjak-Novakovic, G. Synovium-derived stem cell-based
20
21 chondrogenesis. *Differentiation* **76**, 1044, 2008.
22
23
24 73. Mauck, R.L., Byers, B.A., Yuan, X. and Tuan, R.S. Regulation of cartilaginous ECM
25
26 gene transcription by chondrocytes and MSCs in 3D culture in response to dynamic
27
28 loading. *Biomech Model Mechanobiol* **6**, 113, 2007.
29
30
31 74. English, A., Jones, E.A., Corscadden, D., Henshaw, K., Chapman, T., Emery, P. and
32
33 McGonagle, D. A comparative assessment of cartilage and joint fat pad as a potential
34
35 source of cells for autologous therapy development in knee osteoarthritis.
36
37 *Rheumatology* **46**, 1676, 2007.
38
39
40
41
42
43
44
45
46
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FIG. 1: (A) Schematic representation of the different construct sizes, Ø=diameter (mm) x height (mm), and experimental details of media volume and culture dishes used throughout. Each well contained a single construct. Illustration represents the regions of sectioning for biochemical assays with each region being analysed separately, with the exception of the Ø4x1.5mm constructs which were analysed in total. TA: Top annulus, BA: Bottom annulus, TIA: Top inner annulus, BIA: Bottom inner annulus, TC: Top core and BC: Bottom core. (B) Illustration of the partial 3D hydrogel geometry from which the axisymmetric finite element model was developed to predict oxygen concentration profiles.

FIG. 2: Predicted oxygen concentration profiles for hydrogel constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) cultured at 20% oxygen tension as illustrated in FIG. 1. (A) Contour plots using an oxygen consumption rate, $V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (B) Contour plots using an oxygen consumption rate, $V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) Profile along vertical centreline ($V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (D) Profile along central radial line ($V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) (E) Profile along vertical centreline ($V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (F) Profile along central radial line ($V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$). 197x291mm (150 x 150 DPI)

FIG. 3: Predicted oxygen concentration profiles for hydrogel constructs (Ø5x3mm; Ø6x4.5mm) cultured at 5% oxygen tension (A) Contour plots using an oxygen consumption rate, $V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (B) Contour plots using an oxygen consumption rate, $V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) Profile along vertical centreline ($V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (D) Profile along central radial line ($V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) (E) Profile along vertical centreline ($V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$) and (F) Profile along central radial line ($V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$).
175x291mm (150 x 150 DPI)

FIG. 4: Histological evaluation (sGAG, collagen, collagen type II and light microscopic surface image) of MSC seeded constructs of differing geometries at day 42. Constructs of dimension Ø5x3mm and Ø6x4.5mm were also cultured at a lower oxygen tension of 5%. Dashed line indicates the centerline of the engineered tissue. Scale bar for low magnification= 1mm. Higher magnification images in collagen type II stained sections are from core and annulus regions showing the difference in cell shape, scale bar = 50µm.

205x230mm (150 x 150 DPI)

FIG. 5: Biochemical accumulation of engineered cartilaginous constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) at day 42 cultured under normoxic (20%) and low oxygen (5%) conditions. (A) Total DNA (ng/mg w/w) at 20% O₂ (B) Total sGAG and collagen (%w/w) at 20% O₂, (C) Total sGAG/DNA and collagen/DNA at 20% O₂. (D) Total DNA (ng/mg w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂ (E) Total sGAG and collagen (%w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂, (F) Total sGAG/DNA and collagen/DNA for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂. (*, !) p < 0.05 indicates significance compared to preceding sized construct, (\$) p < 0.05 compared to same sized constructs cultured at 20% O₂. n=3 constructs per group. 190x269mm (150 x 150 DPI)

FIG. 6B
181x123mm (150 x 150 DPI)

FIG. 6: Spatial accumulation of (A) DNA (ng/mg w/w), (B) sGAG (% w/w) and (C) collagen (% w/w) for specific regions of engineered constructs at day 42. (!) $p < 0.05$, (&) $p < 0.001$, (*) $p < 0.0001$ indicates significance, $n = 3$ for all groups. Mean data $\pm$ standard deviations are provided in Table 1.

FIG. 7: (A) Equilibrium and dynamic modulus of constructs cultured under 20% O2 conditions. (B) Equilibrium & (C) Dynamic modulus of constructs (Ø5x3mm and Ø6x4.5mm) cultured at 20% and 5% O2. (D) & (E) Structure-function correlation plots relating measured equilibrium modulus to biochemical constituents (F) & (G) Structure-function correlation plots relating measured dynamic modulus to biochemical constituents. This analysis considered the effects of including all constructs (20% and 5% O2) or the 20% O2 constructs only in the regression analysis. (*, &) $p < 0.05$ indicates significance compared to preceding sized construct. (!) $p < 0.0001$ indicates significance compared to same sized construct cultured at 20% O2. (%) $p < 0.001$ indicates significance compared to other construct size at the same oxygen tension. $n=3$ constructs per group.

203x278mm (150 x 150 DPI)

Construct Size	Region	O ₂ (%)	DNA (ng/mg w/w)	sGAG (% w/w)	sGAG/DNA	Collagen (% w/w)	Collagen/DNA
Ø4x1.5mm	Total	20	75.39 ± 7.19	0.14 ± 0.00	18.72 ± 2.03	0.31 ± 0.01	40.80 ± 4.98
Ø5x3mm	Top Annulus	20	73.04 ± 10.67	0.21 ± 0.01	28.92 ± 3.39	0.23 ± 0.07	31.76 ± 5.67
	Top Core	20	57.87 ± 4.01	0.58 ± 0.03	100.33 ± 10.48	0.77 ± 0.08	132.62 ± 7.91
	Bottom Annulus	20	71.76 ± 8.26	0.31 ± 0.02	43.62 ± 3.27	0.43 ± 0.03	61.10 ± 7.93
	Bottom Core	20	48.53 ± 5.19	1.07 ± 0.13	223.56 ± 39.06	1.00 ± 0.08	208.30 ± 7.50
	Top Annulus	5	67.50 ± 3.32	0.98 ± 0.10	144.13 ± 7.62	2.73 ± 0.41	406.72 ± 72.02
	Top Core	5	69.06 ± 9.82	1.31 ± 0.15	190.05 ± 7.23	1.92 ± 0.68	276.27 ± 86.47
	Bottom Annulus	5	63.51 ± 9.24	1.06 ± 0.09	168.69 ± 21.36	2.11 ± 0.27	337.42 ± 63.62
	Bottom Core	5	43.91 ± 14.06	1.36 ± 0.16	322.75 ± 63.92	1.00 ± 0.37	466.25 ± 182.04
Ø6x4.5mm	Top Annulus	20	61.48 ± 3.93	0.32 ± 0.02	52.25 ± 2.41	0.60 ± 0.16	97.51 ± 22.07
	Top Core	20	71.67 ± 5.63	0.97 ± 0.03	204.69 ± 13.72	1.43 ± 0.02	300.42 ± 18.94
	Bottom Annulus	20	68.53 ± 6.12	0.44 ± 0.02	64.96 ± 8.25	0.89 ± 0.50	127.74 ± 70.76
	Bottom Core	20	47.49 ± 3.12	1.34 ± 0.07	188.56 ± 19.62	1.17 ± 0.19	163.32 ± 14.24
	Top Annulus	5	45.61 ± 4.11	1.06 ± 0.05	232.36 ± 22.09	0.41 ± 0.14	92.36 ± 38.12
	Top Core	5	65.52 ± 8.35	1.40 ± 0.05	217.04 ± 32.82	0.88 ± 0.12	136.46 ± 27.43
	Bottom Annulus	5	57.71 ± 6.08	1.06 ± 0.02	185.97 ± 18.73	0.60 ± 0.03	103.78 ± 6.07
	Bottom Core	5	36.79 ± 12.86	1.35 ± 0.04	400.44 ± 138.74	0.99 ± 0.17	285.60 ± 88.53
Ø8x4.5mm	Top Annulus	20	83.24 ± 6.65	0.62 ± 0.01	74.15 ± 6.91	2.79 ± 0.74	332.04 ± 62.58
	Top Inner Annulus	20	45.46 ± 13.80	0.76 ± 0.09	173.04 ± 33.80	0.71 ± 0.23	156.95 ± 24.87
	Top Core	20	53.17 ± 18.09	1.18 ± 0.06	236.63 ± 65.68	0.74 ± 0.13	146.00 ± 42.41
	Bottom Annulus	20	64.28 ± 21.05	0.88 ± 0.11	72.27 ± 18.77	2.89 ± 0.71	487.72 ± 191.07
	Bottom Inner Annulus	20	21.87 ± 0.63	0.86 ± 0.03	391.80 ± 2.68	0.44 ± 0.10	202.45 ± 48.47
	Bottom Core	20	33.26 ± 10.78	1.19 ± 0.01	389.07 ± 150.81	0.46 ± 0.18	190.39 ± 72.11

TABLE 1: Mean ± standard deviation data corresponding to spatial distribution of matrix components as illustrated in Fig 7, n= 3 constructs per group analysed.
200x172mm (300 x 300 DPI)

SUPPLEMENTARY FIG. S1: Comparison of engineered cartilaginous constructs (Ø4x1.5mm; Ø5x3mm; Ø6x3mm; Ø8x4.5mm) from two different donors at day 42 cultured under normoxic (20%) and low oxygen (5%) conditions. (A) Total sGAG and (B) collagen (%w/w) at 20% O₂ (C) Total sGAG and (D) collagen (%w/w) for Ø5x3mm and Ø6x4.5mm constructs at 20% and 5% O₂, n=3 constructs per group. 189x142mm (150 x 150 DPI)

SUPPLEMENTARY FIG. S2: Correlation plots of mean oxygen concentration (%) with biochemical constituents normalised by DNA content. This analysis was performed using the averaged oxygen concentrations determined in the finite element models for specific regions as illustrated in FIG 1, for two specific values of oxygen consumption (V_{max}). (A) & (B) $V_{max} = 18.7 \text{ fmol hr}^{-1} \text{ cell}^{-1}$ (C) & (D) $V_{max} = 37.4 \text{ fmol hr}^{-1} \text{ cell}^{-1}$.
194x153mm (150 x 150 DPI)