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RESEARCH ARTICLE

Engineering biomechanically functional neocartilage derived from expanded articular chondrocytes through the manipulation of cell-seeding density and dexamethasone concentration

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Abstract

Recent work has established methods to engineer self-assembled, scaffold-free neocartilage from an expanded articular chondrocyte (AC) cell source. In continuing such work, the objective of the present study was to investigate the effects of cell-seeding density and dexamethasone concentration on these neocartilage constructs. Neocartilage discs (5 mm diameter) were formed by self-assembling passaged leporine articular chondrocytes into non-adherent agarose moulds. The cell-seeding densities (2, 3, 4, 5 and 6 million cells/construct) and dexamethasone concentrations (10 and 100 nM) in the culture medium were varied in a full-factorial study. After 4 weeks, the neocartilage constructs were assessed for morphological, biochemical and biomechanical properties. The cell-seeding density profoundly affected neocartilage properties. The two dexamethasone concentrations explored did not induce overall significant differences. Constructs formed using lower cell-seeding densities possessed much higher biochemical and biomechanical properties than constructs seeded with higher cell densities. Notably, the 2 million cells/construct group formed hyaline-like neocartilage with a collagen wet weight (WW) content of ~7% and a Young's modulus of ~4 MPa, representing the high end of values achieved in self-assembled neocartilage. Excitingly, the mechanical properties of these constructs were on a par with that of native cartilage tissues tested under similar conditions. Through optimization of cell-seeding density, this study shows for the first time the use of expanded ACs to form homogeneous self-assembled neocartilage with exceptionally high tensile strength. With such functional properties, these engineered neocartilage constructs provide a promising alternative for treating articular lesions. Copyright © 2016 John Wiley & Sons, Ltd.

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1. Introduction

Tissue-engineered articular cartilage has the potential to overcome several shortcomings of current cartilage repair options. It is known that microfracture results in the formation of fibrocartilage that is biomechanical properties inferior to those of native tissue (Krych *et al.*, 2012). Autologous chondrocyte implantation (ACI) and its matrix-assisted variant, MACI, can result in inconsistent repair tissue, with several studies showing that only 15–29% of patients develop hyaline-like repair tissue, while the majority develop a fibrocartilaginous or fibrous fill (Bartlett *et al.*, 2005; Ringe *et al.*, 2012; Shekkeris *et al.*, 2012). On the other hand, scaffold-free neocartilage

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constructs formed by the self-assembly process, using passaged articular chondrocytes (ACs) as a cell source, have been shown to have a high type II:type I collagen ratio indicative of predominantly hyaline-like articular cartilage (Huey and Athanasiou, 2013). In addition, the tissue quality of self-assembled neocartilage constructs may be less inconsistent than that of ACI or other matrix-assisted variants, as the constructs are cultured under controlled *in vitro* conditions for weeks prior to implantation. Therefore, self-assembled neocartilage may represent a suitable treatment option for cartilage lesions that result from trauma or diseases, such as osteoarthritis.

Recent studies have established methods to generate self-assembled neocartilage using passaged ACs as a cell source (Huey and Athanasiou, 2013), as opposed to the primary ACs used in previous studies (Elder and Athanasiou, 2009; Hu and Athanasiou, 2006b). Briefly, the methods first entailed expanding primary ACs under chondrogenically-tuned conditions, which involves the use of serum-free, FGF-2-supplemented medium and prolonged culture past cell confluence (Huey and Athanasiou, 2013). Cells were then cultured in aggregate suspensions in the presence of TGFβ1 to induce redifferentiation of the dedifferentiated chondrocytes (Huey and Athanasiou, 2013). Cells isolated from these aggregates were then formed into scaffold-free, 3 mm diameter discs using the self-assembly process (Hu and Athanasiou, 2006b). After 4 weeks of culture, hyaline-like neocartilage constructs were formed.

To enhance the functional properties of tissue-engineered neocartilage, i.e. type II collagen content and biomechanical properties, various biomimetic stimuli and strategies can be used. These include optimizing cell expansion conditions (Grogan *et al.*, 2014; Mandl *et al.*, 2004), the addition of growth factors and other chemical agents (Elder and Athanasiou, 2009; Hsieh-Bonassera *et al.*, 2009) and the application of mechanical stimuli (Hu and Athanasiou, 2006a; Raizman *et al.*, 2009). This study aimed to evaluate the effects of cell-seeding density and dexamethasone concentration on the properties of self-assembled neocartilage formed with passaged ACs.

Cell-seeding density has been previously shown to influence the morphological, biochemical and biomechanical properties of self-assembled neocartilage (Murphy *et al.*, 2013; Revell *et al.*, 2008). In a study using primary ACs, higher cell-seeding densities yielded neocartilage constructs with higher glycosaminoglycan (GAG) content and aggregate modulus, while lower cell-seeding densities yielded constructs with higher collagen content. A range of 3.75–5.5 million cells/5 mm diameter construct was found to be optimal (Revell *et al.*, 2008). However, when ACs were expanded to the third (P3) or fourth (P4) passage and used to form 3 mm diameter constructs at a density proportional to 5.5 million cells/5 mm diameter construct, a fluid-filled, acellular central region developed (Huey and Athanasiou, 2013). In another study, which utilized expanded costochondrocytes (CCs) as a cell source, 2 million cells/5 mm construct was found to be optimal compared to 3 and 4 million cells/construct (Murphy *et al.*, 2013). These studies demonstrate that the seeding density used to form self-assembled neocartilage is a critical factor that can significantly affect neocartilage properties. The optimal density seems to differ between primary and passaged cells and potentially between different cell types. This is the first study to investigate the effects of cell-seeding density on self-assembled neocartilage derived from passaged ACs.

Dexamethasone can also have a potentially significant effect on the properties of self-assembled neocartilage. Dexamethasone is a potent agonist of the glucocorticoid receptor and is often a supplement in chondrogenic culture medium formulations, typically at a concentration of 100 nM, to induce chondrogenesis of stem cells (Diekman *et al.*, 2012; Johnstone *et al.*, 1998); 100 nM dexamethasone has also been used in the culture medium of chondrocytes and chondrocyte-derived neotissues with the notion that it will help maintain or enhance the chondrogenic phenotype. Indeed, some studies have shown that application of dexamethasone to primary or passaged chondrocytes can upregulate chondrogenic gene expression (James *et al.*, 2007; Sekiya *et al.*, 2001) and matrix accumulation (Giovannini *et al.*, 2010; Lu *et al.*, 2011; Sadowski and Steinmeyer, 2001). However, many studies have also shown an adverse effect of dexamethasone on chondrocyte survival, proliferation and matrix synthesis (Fujita *et al.*, 2004; Miyazaki *et al.*, 2000). Furthermore, glucocorticoid therapy is known to stunt long bone development (Mazziotti and Giustina, 2013). The results of one study optimizing chondrocyte culture conditions suggested reducing the standard concentration of 100 nM by 50% (Enochson *et al.*, 2012). Therefore, a lower concentration of dexamethasone (10 nM) was investigated alongside the current standard concentration (100 nM) to determine more beneficial conditions for culturing self-assembled neocartilage.

In this study, the effects of cell-seeding density (2, 3, 4, 5 and 6 million cells/construct) and dexamethasone concentration (10 and 100 nM) on neocartilage properties were investigated in a full-factorial design. At the end of 4 weeks of culture, the constructs were evaluated for their matrix content (GAGs, total collagen and collagens I and II) and biomechanical properties (compressive and tensile). A lower seeding density than the previously used density of 5.5 million cells/5 mm diameter construct was hypothesized to prevent the development of fluid-filled central regions and to form homogeneous, solid neotissues with significantly improved biochemical and biomechanical properties. Assessment of these properties would also determine whether 10 or 100 nM dexamethasone concentration was more suitable for the culture of self-assembled neocartilage.

2. Materials and methods

2.1. Chondrogenic medium formulation and dexamethasone concentration

The chondrogenic medium formulation used throughout the study consisted of Dulbecco's modified eagle's

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medium (DMEM; 25 mM glucose/GlutaMAX™; Life Technologies, Carlsbad, CA, USA), 1% penicillin-streptomycin-fungizone (PSF; Lonza, Basel, Switzerland), 1% insulin-transferrin-selenium (ITS; BD Biosciences, San Jose, CA, USA), 1% non-essential amino acids (NEAA; Life Technologies), 100 μg/ml sodium pyruvate (Thermo Fischer Scientific, Waltham, MA, USA), 50 μg/ml ascorbate-2-phosphate (Sigma, St. Louis, MO, USA) and 40 μg/ml L-proline (Sigma). Dexamethasone at a concentration of 10 or 100 nM (Sigma) was added, using a 10 mM stock in ethanol. These dexamethasone concentrations were maintained throughout the entire experiment, which includes the cell passaging, aggregate redifferentiation and neocartilage culture periods.

2.2. Isolation of juvenile rabbit articular chondrocytes

ACs were isolated from full-thickness cartilage of the femoral condyle, trochlear groove and tibial plateau from 6–8 week-old New Zealand White rabbits (Jones Rabbit Farm, Santa Rosa, CA, USA). Cells were pooled from six animals of mixed genders. The tissues were minced, washed with phosphate-buffered saline (PBS) and digested with 500 U/ml collagenase type 2 (Worthington Biochemical, Lakewood, NJ, USA) in chondrogenic medium + 3% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA) for 18 h at 37°C/10% CO_2. The digested tissue solution was strained through a 70 μm filter and the cells washed three times by centrifugation/resuspension steps. The cells were then counted and cryopreserved in chondrogenic medium + 20% FBS + 10% dimethyl sulphoxide (DMSO; Sigma). Cell viability, as observed by trypan blue staining, was $>$ 95%. Each knee yielded $\sim 3 \times 10^6$ ACs.

2.3. Chondrocyte passaging in monolayers

Primary ACs were passaged in monolayers in chondrogenic medium + 5 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA) in a chondrogenically-tuned process, as previously described (Huey *et al.*, 2012), until passage 3 (P3) ACs were obtained. Briefly, P0 ACs were thawed and seeded into culture flasks at 25 000 cells/cm^2. For the first 24 h, 10% FBS was supplemented to promote cell adhesion. The medium was changed every 3–4 days. All cultures took place at 37°C/10% CO_2. When cells reached 95% confluence, the monolayers were cultured for an additional 4 days, as conducted in the previous study (Huey *et al.*, 2012). Cell sheets were then lifted with 20 min incubation in 0.25% trypsin–EDTA (Invitrogen) and the sheets digested with 500 U/ml collagenase in chondrogenic medium + 3% FBS for 30 min. The cells were filtered through a 70 μm cell strainer, washed three times, counted and either passaged, as described above, or redifferentiated, as described next.

2.4. Chondrocyte redifferentiation in aggregate cultures

P3 ACs were redifferentiated in aggregate 3D cultures, as previously described (Huey and Athanasiou, 2013). Briefly, 10 cm Petri dishes were first coated with a thin layer of 2% agarose to prevent cell adhesion; 15×10^6 P3 ACs were seeded into each dish with 15 ml chondrogenic medium + 10 ng/ml TGFβ1 (Peprotech). The dishes were put onto an orbital shaker (Model 3500, VWR, Radnor, PA, USA) at $\sim$1 Hz for 24 h and then cultured statically for 6 days. The medium was changed every 2 days. At day 7, the cell aggregates were digested with 0.25% trypsin–EDTA for 20 min after which the trypsin was removed, and then digested in 500 U/ml collagenase in chondrogenic medium +3% FBS for 30–40 min. The cells were filtered through a 70 μm cell strainer, washed three times, counted and used for the self-assembly of neocartilage constructs.

2.5. Self-assembly of tissue-engineered cartilage

Disk-shaped, 5 mm diameter neocartilage constructs were grown via the self-assembly process previously described (Hu and Athanasiou, 2006b); 5 mm diameter wells made of 2% agarose were formed in 24-well plates, using custom-machined stainless steel pegs as a negative cast. Several changes of medium were added to the agarose wells over 3 days to saturate the agarose with medium. Empty wells were seeded with 100 μl medium containing 2, 3, 4, 5 or 6 million P3 ACs that had been passaged and redifferentiated. After 4 h of incubation, a loose construct was formed and 500 μl medium was carefully added to each well ($t = 0$ days); 500 μl medium was changed every 24 h until day 14. At day 14, the constructs were gently unconfined (Elder and Athanasiou, 2008) from the wells and placed into six-well plates (five constructs/10 ml medium/well). During unconfinement, some constructs with fluid-filled cavities were broken, as described in Results. Part of the medium (10 ml) was changed every 48 h. After 4 weeks, the constructs were removed from culture, their wet weights (WWs) measured and photographed. Each construct was then sectioned into appropriately sized pieces for histology and biochemical and biomechanical testing.

2.6. Histology

Tissue samples, approximately $1 \times 1 \times 1$ mm, were cryoembedded in HistoPrep (Thermo Fisher Scientific) and sectioned at 16 μm thickness. After fixation in formalin, the sections were stained for GAGs with safranin O, fast green and Weigert's haematoxylin. Picrosirius red was used to stain for all collagens.

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2.7. Biochemical analysis

Tissue samples, 3 × 1 × 1 mm, from the solid tissue regions of each construct were weighed to obtain WWs, lyophilized for 3 days and then reweighed to obtain dry weights (DWs). Water content (%) was calculated as (WW – DW)/WW × 100%. Each sample was digested in 900 μl 1.1 mg/ml pepsin (Sigma)/0.05 M acetic acid/0.44 M NaCl for 7 days at 4°C on a rocker. Undigested tissues were then homogenized with an ultrasonicator (Misonix XL-2000, Qsonica, Newtown, CT, USA). Samples were then neutralized with 100 μl 10× TBS and treated with elastase (Sigma) at a concentration of 0.09 mg/ml for 2 days at 4°C on a rocker.

Total collagen content was determined by measuring hydroxyproline content, using a modified chloramine-T colorimetric assay (Woessner, 1961); 100 μl digested sample solution was diluted in 100 μl 1× TBS, hydrolysed with 200 μl 4 N NaOH at 120°C for 15 min in an autoclave and neutralized with 200 μl 4 N HCl. Hydroxyproline oxidation was achieved with the addition of 1.25 ml 0.062 M chloramine T (Sigma) in an acetate–citrate buffer (0.45 M NaOH, 0.45 M sodium acetate, 0.14 M citric acid and 0.11 M acetic acid) and incubation for 20 min at room temperature. The oxidation reaction was stopped and colour developed with the addition of 1.25 ml 1.2 M Ehrlich's reagent (Sigma) in 30% perchloric acid/70% isopropanol and 20 min incubation at 65°C. Samples were plated in duplicate and absorbance measured at 550 nm using a microplate reader. Equal amounts of digest solution had been added to the standards (bovine collagen from the Sircol Collagen Assay, Biocolor, Carrickfergus, UK) and samples to ensure consistency.

Total sulphated GAG content was measured using the Blyscan GAG Assay kit (Biocolor), following the manufacturer's instructions. Briefly, 10 μl digested sample was incubated with 500 μl Dye Reagent for 30 min with intermittent vortexing, the precipitate centrifuged and the pellet dissolved in 500 μl Dissociated Reagent for absorbance measurement at 650 nm.

Collagens type I and II content was measured using an ELISA sandwich assay, as previously described (Huey *et al.*, 2012). Chondrex reagents (Chondrex, Redmond, WA, USA) and protocols were used. For collagen I ELISA, a similar protocol was employed, using antibodies from US Biological (Swampscott, MA, USA).

2.8. Biomechanical analysis

For tensile testing, dog bone-shaped samples were created with a scalpel and a 2 mm biopsy punch. The samples were photographed, glued to paper tabs (Natoli *et al.*, 2010) and subjected to uniaxial tension, using an Instron model 5565 with a 50 N load cell (Instron, Canton, MA, USA). A strain rate of 1% of the gauge length/s was used. The gauge length of 1.27 mm was set as the distance between the paper tabs. Cross-sectional areas were calculated from front- and side-view images of the dog bone uploaded to ImageJ. The Young's modulus was obtained from the linear region of the stress–strain curve. The ultimate tensile strength (UTS) was chosen to be the maximum stress reached.

For compressive testing, a 3 mm diameter tissue sample was taken from the middle of the construct, using a biopsy punch. If the construct contained a fluid-filled cavity, the thicker of the two pieces was used to maintain a degree of consistency. The sample was placed in a PBS bath at room temperature and subjected to an unconfined, incremental stress–relaxation test, as described previously (Allen and Athanasiou, 2006). Briefly, sample heights were determined by lowering the platen until a load change of 0.02 N was detected. Samples were compressed to 10% strain at a rate of 1% of the sample height/s, held for 200 s, compressed to 20% strain and held for 450 s. The instantaneous modulus and relaxation modulus of the 20% strain curve were determined by curve-fitting parameters into the standard linear solid (SLS), finite deformation (FD) model (Allen and Athanasiou, 2006), using MatLab software.

2.9. Statistics

All datasets (n = 6–8/group) were analysed with two-factor ANOVA and Tukey's *post hoc* test ($p < 0.05$), using JMP 10 software (SAS Institute). Errors bars represent the standard deviation (SD) around the mean. Significant differences exist between groups not sharing the same letters.

3. Results

3.1. Chondrocyte passaging, aggregate suspension culture and self-assembly

Juvenile rabbit ACs expanded from P0 to P3 in monolayers underwent an average of 202-fold expansion (7.7 doublings). Passaged cells developed a progressively fibroblastic phenotype, indicative of dedifferentiation (Figure 1a). Dexamethasone concentration (10 and 100 nM) did not significantly affect proliferation rate or cell yields at the end of each passage, as indicated by paired *t*-test (data not shown). After 24 h of rotational culture, as part of the first phase of the aggregate suspension culture step, P3 ACs formed 10–50 cell aggregates of relatively uniform size (Figure 1b). In the second phase, these aggregates were statically cultured for 6 more days and formed a contiguous tissue mass, due to the aggregates adhering to one another. Cells isolated from these aggregates, which showed >90% viability, were then used for the self-assembly of 5 mm diameter constructs.

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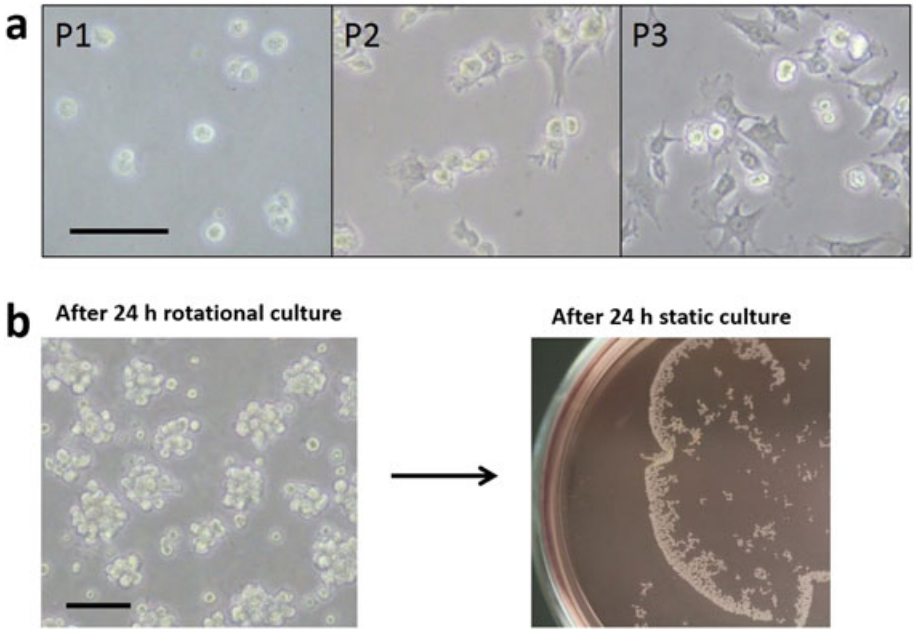

Figure 1. (a) Morphology of juvenile rabbit ACs in monolayer culture at 24 h post-seeding during passage (P)1, P2 and P3; the ACs developed a progressively fibroblastic phenotype over the passages, typical of chondrocyte dedifferentiation; scale bar = 50 μm. (b) To redifferentiate passaged ACs, the cells underwent 24 h of 3D rotational culture, after which they formed cell aggregates consisting of 10–50 cells; this was followed by 6 days of static 3D culture, during which the majority of aggregates further coalesced into a contiguous mass within 24 h; scale bar = 50 μm

3.2. Neocartilage morphology and histology

After 4 weeks of culture the neocartilage constructs showed a variety of morphologies, highly dependent on the initial cell-seeding density. Constructs seeded with 2 million chondrocytes formed solid, homogeneous neotissues but had slightly bent- or bowl-shaped geometries (Figure 2a). Constructs seeded with 3–5 million cells developed large fluid-filled cavities (Figure 3a). In the 4 and 5 million cells/construct group, the fluid-filled cavities had broken when the constructs were unconfined from their wells at day 14, thus forming constructs with an empty cavity at the end of the 28 day culture period. Constructs seeded with 6 million cells formed small constructs with outlying, unaggregated cells scattered at the periphery of the well. Overall, the 2 million cells/construct group formed the most homogeneous constructs.

Histological analysis revealed a fairly homogeneous distribution of GAGs and collagen throughout the solid tissue regions of all the neocartilage constructs (Figure 2b). Differences in staining intensity and staining distribution were not observed among any of the groups. In the higher seeding-density groups where a fluid-filled cavity developed, histological stains revealed a region devoid of cells and rich in matrix components (Figure 3 b), whereas the 2 million cells/construct group showed a homogeneous tissue with even staining throughout the construct. Histological characteristics qualitatively support the formation of hyaline-like cartilage in all constructs.

3.3. Neocartilage biochemical properties

The water content, used as an indicator of the density of neotissue components, and the matrix content (GAGs, total collagen and collagens I and II) of neocartilage constructs were assessed (Figure 4a–d). Samples for biochemical analysis were acquired from regions of the constructs that did not contain components of the fluid-filled cavities. Constructs seeded with 2 million cells had the lowest water content (79.3 ± 0.8%), followed by constructs seeded with 6 million cells (Figure 4a). For several seeding densities (4 and 5 million cells/construct), culture with 10 nM dexamethasone yielded constructs with lower water content, although this trend was not consistent among the other seeding densities.

Total collagen WW was highest in the 2 million cells/construct group (6.7 ± 1.2%) (Figure 4b). Increasing the seeding density to 3 million cells/construct significantly lowered collagen WW in the constructs. Although the 5 and 6 million cells/construct groups had similar collagen WW values to the 2 million cells/construct group, this was believed to be an artifact, due to the loss of cells or significant breakage of the constructs during the unconfinement process at day 14. This artifact may have been less significant in the 4 million cells/construct group, which had less construct breakage. GAG/WW was highest in the 2 million cells/construct group (7.9 ± 0.5%) (Figure 4c). Increasing seeding densities above 2 million cells decreased the GAG content. Collagen II WW was found to follow a trend similar to that of total collagen

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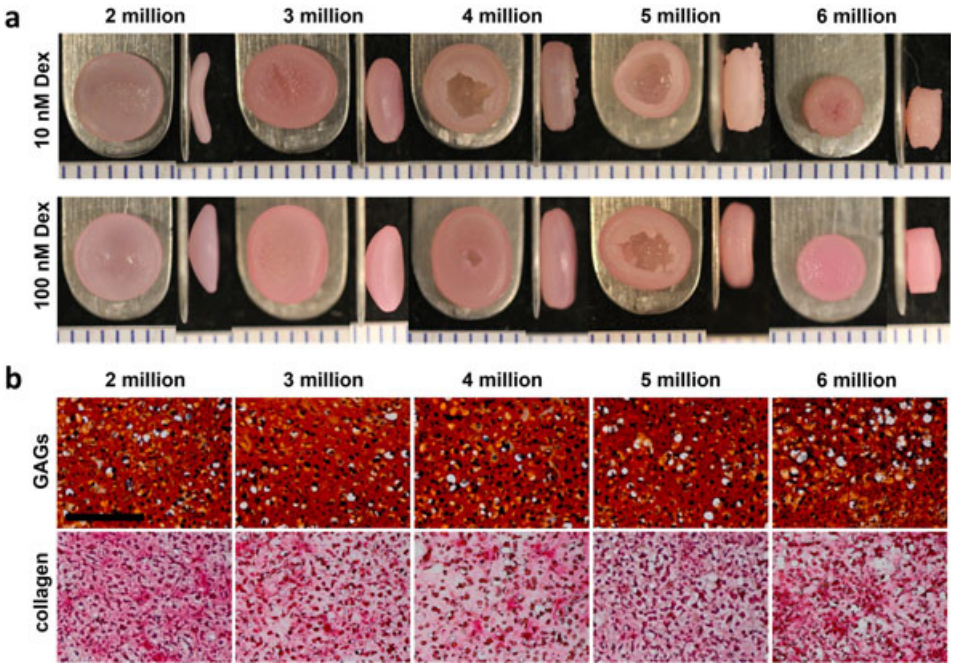

Figure 2. (a) Gross morphology of 5 mm diameter self-assembled neocartilage discs seeded with 2, 3, 4, 5 and 6 million cells and cultured with 10 or 100 nM dexamethasone (Dex) at day 28: the 4 and 5 million cells groups had broken upon unconfinement at day 14, resulting in abnormal morphology; the 6 million cells group did not fully self-assemble and many cells were lost upon unconfinement at day 14; ruler at the bottom of each image show 1 mm spacings. (b) Histology of the solid tissue regions of the constructs show homogeneous distribution of GAGs and collagens: constructs cultured in 10 nM dexamethasone appeared similar and are not shown; scale bar = 100 μm

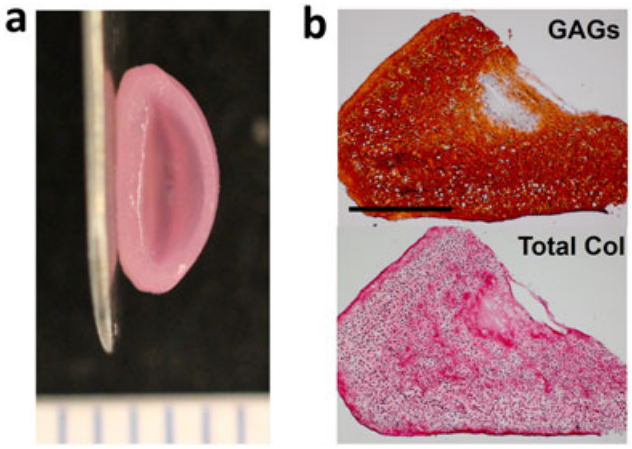

Figure 3. At high cell-seeding densities, the neocartilage constructs developed a fluid-filled cavity devoid of cells and rich in matrix components, as shown in (a) the cross-sectional gross morphological and (b) histological representative images of the 3 million cells/construct 100 nM dexamethasone group; scale bar =1 mm

WW (Figure 4d); specifically, the 2 million cells/construct group, taken as a factor in a two-way ANOVA statistical test, was found to be significantly higher than the other seeding-density groups. Dexamethasone concentration did not have a significant effect on GAG and total collagen content. Collagen I could not be detected in any of the samples, indicating the formation of only hyaline-like neocartilage in all the groups. The results indicate that 2 million cells/construct formed hyaline cartilage with the highest overall matrix content.

3.4. Biomechanical properties of neocartilage

The tensile and compressive properties of the neocartilage constructs were also assessed (Figure 5a–d). The tensile properties of the 2 million cells/construct group were substantially higher than those of the other seeding-density groups, reaching a Young's modulus of 4.15 ± 1.19 MPa and a UTS of 1.14 ± 0.37 MPa. The compressive properties of the 2 million cells/construct group were also significantly higher than those of the other seeding-density groups, reaching an instantaneous modulus of 375 ± 157 kPa and a relaxation modulus of 162 ± 54 kPa (at 20% strain). Dexamethasone concentration did not affect the biomechanical properties. The results indicated superior biomechanical properties of the 2 million cells/construct group.

4. Discussion

This study established key processing methods to generate functionally robust, self-assembled neocartilage using passaged ACs as a cell source. Cell-seeding density was discovered to be a critical factor in forming homogeneous tissues with abundant matrix and high biochemical properties. The hypothesis was formed that

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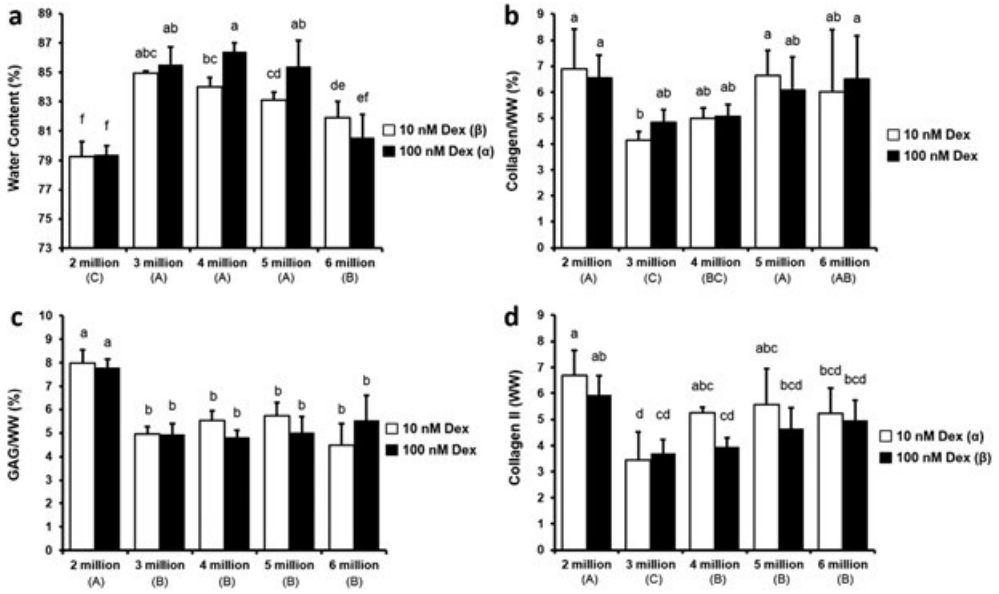

Figure 4. Neocartilage biochemical properties at the end of 4 weeks of culture: (a) water, (b) total collagen, (c) GAGs and (d) type II collagen contents normalized by weight

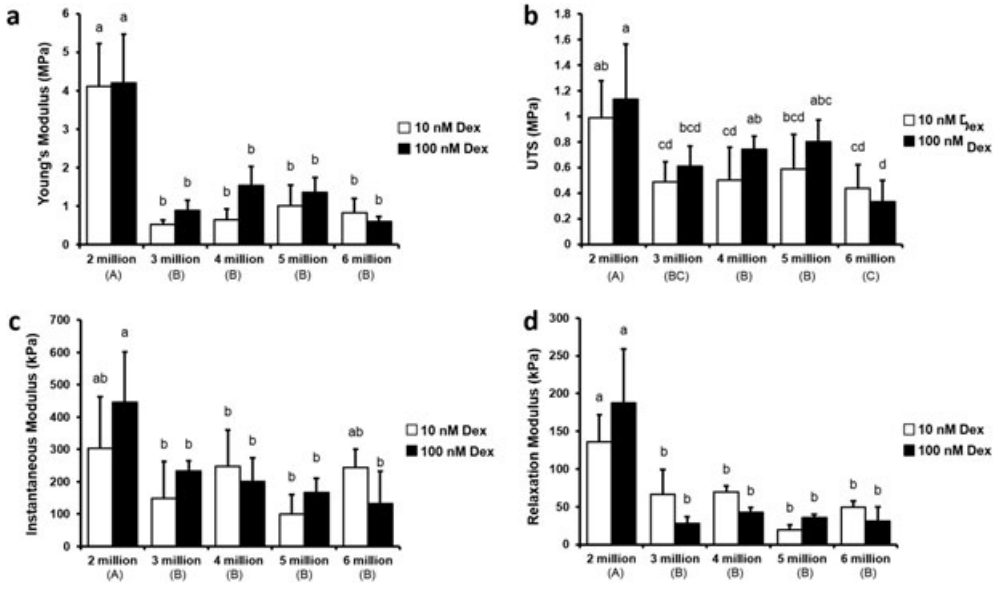

Figure 5. Neocartilage tensile and compressive properties at the end of 4 weeks of culture: (a) Young's modulus and (b) UTS were used to assess tissue tensile properties, while (c) instantaneous modulus and (d) relaxation modulus were used to assess the compressive properties of the tissue

a lower seeding density (e.g. 2 million cells/5 mm construct) could prevent the formation of fluid-filled cavities and help form neocartilage with superior properties. A dexamethasone concentration of 10 or 100 nM in the culture medium did not broadly effect construct properties. Of the most significant note, neocartilage constructs formed in this study reached a Young's modulus of ~4 MPa, representing the high end of values achieved in self-assembled neocartilage (Huey and Athanasiou, 2013; Murphy *et al.*, 2013; Responte *et al.*, 2012) and also values approaching those of native juvenile cartilage tissues tested under similar conditions (Eleswarapu *et al.*, 2011; Paschos *et al.*, 2014). The combination of a lower seeding density and use of a juvenile cell source are two likely factors contributing to the observed five-fold increase of tensile properties compared to previous work that had used passaged ACs to form self-assembled neocartilage (Huey and Athanasiou, 2013). Also noteworthy was that the ACs used in this study had undergone substantial *in vitro* expansion (202× cell expansion, or 7.7 doublings), which typically induces significant AC dedifferentiation in human cell lines (Giovannini *et al.*, 2010). However, in this study and in previous work using leporine ACs (Huey and Athanasiou, 2013), the resulting neocartilage constructs contained undetectable amounts of type I

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collagen and were rich in type II collagen, indicating the formation of hyaline-like cartilage. This study represents important first steps towards developing functional neocartilage constructs for future preclinical studies and for the end goal of treating lesions of articular cartilage.

Cell-seeding density was found to be a profound factor influencing neocartilage morphology, matrix content and biomechanical properties. Constructs seeded with 2 million cells formed homogeneous tissues with superior functional properties, while higher cell densities formed constructs with inferior functional properties and morphological abnormalities, such as fluid-filled cavities (3, 4 and 5 million cells/construct) or incomplete self-assembly of cells (6 million cells/construct). In terms of biochemical properties, the 2 million cells/construct group had significantly higher GAG WW, higher collagen II WW and higher or the same total collagen WW contents than in all other groups. In a previous study where passaged CCs were used to form self-assembled neocartilage, a lower seeding density was also observed to contribute to higher GAG and collagen contents of the constructs' solid tissue regions (Murphy *et al.*, 2013). The lower water content of the 2 million cells/construct group also indicates the formation of a denser tissue. The superior properties of the low seeding-density group could be a result of better access to nutrients in these thinner neotissues, thus allowing for unimpeded, energy-intensive, anabolic processes such as matrix synthesis.

In terms of biomechanical properties, the 2 million cells/construct group had significantly higher compressive and tensile properties than the higher cell-seeding density groups. In cartilage tissue, the GAG and collagen contents have been generally correlated with compressive (Sanchez-Adams *et al.*, 2011) and tensile (Eleswarapu *et al.*, 2011) properties, respectively. In this study, the significantly higher GAG content of the 2 million cells/construct group resulted in higher compressive properties. Likewise, the higher type II collagen content of this group was associated with a higher Young's modulus and UTS. Total collagen values showed some correlation with tensile property values, but some disparities exist. As one study shows, total collagen, as measured by hydroxyproline content, may not be a suitable biochemical indicator for tensile properties (Kelly *et al.*, 2007). Overall, the 2 million cells/construct group possessed robust morphological, biochemical and biomechanical properties suitable for future development as an engineered cartilage graft.

Dexamethasone at 10 or 100 nM did not have an appreciable or consistent effect on the constructs' biochemical and biomechanical properties. As a group, constructs cultured in 10 nM dexamethasone had significantly lower water content and higher type II collagen WW contents than those cultured in 100 nM dexamethasone. However, the beneficial effects of the 10 nM dose were not seen across any biomechanical measures. Furthermore, when comparing the 10 and 100 nM concentrations within the 2 million cells/construct group, the only relevant cell density group for future studies, no difference in any parameters was observed. The small changes observed in construct properties between the two groups indicate the possibility of some degree of receptor saturation at 10 nM dexamethasone. Further investigation could explore a wider range of dexamethasone concentrations (0–1000 nM) to determine their effects on self-assembled neocartilage in detail.

It is envisioned that the self-assembled neocartilage generated in this study could be developed to meet the clinical requirements necessary for translation. Specifically, one important clinical criterion is to generate constructs of a practical size and thickness to adequately fill various lesions that can occur in diseased or injured joints. In the human knee, cartilage lesions occur predominantly in the medium femoral condyle (58% of cases) (Hjelle *et al.*, 2002), and the cartilage thickness of this region is on average 1.5–2.5 mm (Coleman *et al.*, 2013; Li *et al.*, 2005). When treating cartilage lesions with mature cartilage grafts, such as with auto/allografts, the grafts are often implanted flush to the surrounding native tissue. On the other hand, the self-assembled neocartilage constructs described in this study, which were ~0.8 mm thick, are envisioned to be implanted slightly depressed relative to the surrounding native tissues. Because these neocartilage constructs have been shown to secrete matrix and grow in size (i.e. thickness and diameter) beyond the 4-week endpoint that was used in this study (Ofek *et al.*, 2008), they have the potential to completely fill the defect site over time *in vivo*. Previous preclinical and clinical studies have also demonstrated the ability of relatively small neocartilage constructs to fill large cartilage defects *in vivo* (Fickert *et al.*, 2012; Lewis *et al.*, 2009). To match the shape and area of the defect site, self-assembled neocartilage constructs can be formed using a system of moulds and dead weights (MacBarb *et al.*, 2013). Therefore, the neocartilage constructs formed in this study could potentially meet the dimensional requirements to fill cartilage defect sites of the medium femoral condyle or other areas.

In cases where thicker cartilage constructs are required, other methods need to be used to increase the thickness of the self-assembled neocartilage constructs. The addition of more cells to a fixed area may not be a viable strategy, as this study demonstrates that fluid-filled cavities form at seeding densities >2 million cells/5 mm disc. The formation of these cavities is not a well-understood process. Higher seeding densities may lead to diffusion limitations in the central region of the construct, potentially inducing cell death and a disconnected matrix of low quality. This central region may then eventually develop into a large fluid-filled cavity during construct growth. Previous studies have shown that thick, cell-dense scaffolds seeded with chondrocytes develop necrotic centres (Heywood *et al.*, 2004, 2006). To improve the initial and long-term accessibility of nutrients to cells, the employment of sequential seeding of cells or use of a perfusion bioreactor may be feasible strategies. Use of high-glucose medium or a larger medium volume for

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culture have also been shown to mitigate necrosis induced by nutrient deficiency (Heywood *et al.*, 2004, 2006). Diffusion limitations may not be the only explanation of the formation of fluid-filled cavities, as previous work has shown that self-assembled neocartilage formed using primary ACs can reach thicknesses of 2 mm (Hu and Athanasiou, 2006a). This observation supports the idea that cellular differences between primary ACs and the redifferentiated, passaged ACs used in this study could also be a contributing factor. Therefore, the use of biomimetic mechanical (Hu and Athanasiou, 2006a) or chemical stimuli (Natoli *et al.*, 2009) that can further redifferentiate passaged ACs towards the primary cell phenotype may also aid in the formation of thicker neocartilage constructs.

The use of juvenile ACs in this study, as opposed to adult ACs used in the previous study (Huey and Athanasiou, 2013), may provide several advantages in the manufacture and clinical efficacy of engineered cartilage. Juvenile ACs have been shown to significantly secrete more type II collagen and GAGs than adult ACs (Adkisson *et al.*, 2010b; Bonasia *et al.*, 2011). As an allogeneic source, use of juvenile ACs abolishes the need to obtain cartilage biopsies in a second surgery, which has many advantages related to medical costs, accessibility and patient welfare. Finally, juvenile allogeneic chondrocytes have been reported to show minimal immunogenicity in *in vitro* (Adkisson *et al.*, 2010a), animal (Lu *et al.*, 2005) and clinical (McCormick *et al.*, 2013) studies. Although this study utilized leporine cells, ongoing work is being conducted to replicate these results with human ACs. Juvenile ACs are potentially a highly clinically relevant cell source for the clinical repair of cartilage.

Most importantly, the neocartilage properties achieved in this study (collagen WW 6.7 ± 1.2%; Young's modulus 4.15 ± 1.19 MPa) represent the high end of values achieved in self-assembled articular neocartilage and biomechanical properties, near to or on a par with native cartilage tissues. Compared to self-assembled neocartilage from previous studies using primary, juvenile ACs as a cell source, the constructs in this study had ~three-fold higher Young's moduli (Elder and Athanasiou, 2008). Compared to self-assembled neocartilage using adult, passaged ACs as a cell source, the constructs in this study had ~five-fold higher Young's moduli (Huey and Athanasiou, 2013). Remarkably, these neocartilage constructs had tensile properties on a par with those of native, juvenile cartilage tissues tested under similar conditions (Eleswarapu *et al.*, 2011; Paschos *et al.*, 2014). When compared to native adult human cartilage, these constructs had ~one third of the collagen content and ~one quarter of the tensile modulus (Temple *et al.*, 2007). However, with only 4 weeks of culture, the self-assembled neocartilage in this study was still in much in an immature state. Studies have shown that self-assembled neocartilage can increase in biomechanical properties over longer *in vitro* (Ofek *et al.*, 2008) and *in vivo* (Responte *et al.*, 2012) culture. The possibility of these constructs gaining full biomechanical functionality is potentially achievable.

In conclusion, this study demonstrates that homogeneous, hyaline-like neocartilage constructs can be formed from passaged ACs, even after extensive monolayer expansion. Most excitingly, these constructs possessed exceptional tensile properties and collagen content that was near to, or on a par with, those of native tissue. Such constructs provide a promising tissue-engineering solution for the long-term repair of articular cartilage lesions.

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Conflict of Interest

The authors have declared no conflict of interest.

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