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Passaged costal chondrocytes provide a viable cell source for temporomandibular joint tissue engineering

D.E. Johns¹ and K.A. Athanasiou^{1,*}

¹ Department of Bioengineering, Rice University, Houston, TX

Abstract

Costal cartilage is commonly harvested for various types of facial reconstructive surgery. The ability of costal chondrocytes (CCs) to produce relevant extracellular matrix, including glycosaminoglycans (GAGs), collagen type I, and collagen type II, makes them an appealing cell source for fibrocartilage tissue engineering. In order to obtain enough cells for tissue engineering, however, cell expansion will likely be necessary. This study examined CCs at passages 0, 1, 3, and 5, as well as temporomandibular (TMJ) disc cells, in a scaffoldless tissue engineering approach. TMJ disc constructs had over twice the collagen content of any other group, as well as the largest tensile properties; however, the substantial contraction of the constructs and limited cell numbers make it a non-feasible cell source for tissue engineering. In general, statistical differences in mechanical properties or total collagen content of the various CC groups were not observed; however, significantly more GAG was produced in the passaged CCs than the primary CCs. More collagen type II was also observed in some of the passaged cell groups than in passage 0. These results suggest not only feasibility but potential superiority of passaged CCs over primary CCs, which may lead to a functional engineered fibrocartilage tissue.

Introduction

The temporomandibular joint (TMJ) has the essential function of allowing fluid jaw movement. Degeneration or injury of this joint leads to pain during everyday activities like eating or talking, which can become physically and emotionally painful. In the United States alone, there are over 10 million patients with TMJ disorders.¹ There are various treatment options depending on the level of degeneration as reviewed elsewhere.^{2, 3} In severe cases, treatment options have limited success, and there is no consensus as to a standard method of treatment.

When TMJ disorders are severe or traumatic injury occurs in the TMJ, total joint reconstruction may be necessary. A widely accepted approach for replacing the mandible of the jaw is to use a rib and costal cartilage graft.^{4–11} Costal cartilage grafts are also used in ear, nose, and other craniofacial reconstructions.⁴ By using autologous tissue, complications with patient rejection are eliminated. The costal cartilage can be harvested easily, and donor site morbidity is largely eliminated with current surgical techniques.¹² A common problem with this approach in the jaw is overgrowth of the costal cartilage in the TMJ, which can require further surgery.^{13–16} The tissue overgrowth suggests that using the costal cartilage directly may not be appropriate for reconstructing a soft tissue, like the TMJ disc; however, creation of a tissue engineering construct *in vitro* with costal chondrocytes may yield a completely functional tissue without undesirable complications. Ease of use and practicality

*Corresponding author: Kyriacos A. Athanasiou, Rice University, Department of Bioengineering: MS-142, P.O. Box 1892, Houston, TX, 77251, athanasiou@rice.edu, Tel.: 713-348-6385, Fax: 713-348-5877.

in a clinical setting motivates the exploration of costal chondrocytes (CCs) in a tissue engineering approach.

Indeed, CCs have been used in tissue engineering for various cartilage applications: articular,^{17–21} tracheal,^{22, 23} elastic,^{23–26} and, most recently, fibrocartilage.²⁷ Previous work suggests that CCs are quite productive and could provide a more clinically feasible cell source for TMJ disc tissue engineering.²⁷ However, the acellular nature of cartilage limits the amount of viable cells available from a piece of tissue.²⁸ For this reason, cell expansion and passaging appeals to tissue engineers as a way to obtain large numbers of cells, which are frequently needed for a tissue engineering approach. Previous work has shown that CCs have the ability to expand up to passage 5, at which point they appear to lose their proliferative ability, showing little expansion beyond passage 5.²⁹ Passaging, however, causes chondrocyte dedifferentiation to a more fibrochondrocyte-like cell type; just a single passage is capable of causing a significant drop in collagen type II and glycosaminoglycan (GAG) production.^{23, 30} It is encouraging to note, however, that the resultant dedifferentiation may prove beneficial for the purposes of tissue engineering fibrocartilages, like the TMJ disc, which contain less collagen type II, more collagen type I, and less GAG than articular cartilage.

This study examines the use of CCs at passage 0, 1, 3, and 5 and TMJ disc cells in a scaffoldless tissue engineering approach. While TMJ disc cells are not a feasible option for tissue engineering as they are extremely limited in cell number, difficult to harvest, and likely diseased in any patient interested in a TMJ replacement, they will serve as a control in this experiment. It is hypothesized that increasing passages decreases the cartilaginous proteins while increasing the fibrocartilaginous proteins in the constructs.

Materials and Methods

Cell isolation

Both cell types were taken from three skeletally mature goats, which were obtained from a local abattoir immediately after death. TMJ disc cells were removed and isolated, as described previously.³¹ These cells were expanded in Dulbecco's modified Eagle medium (DMEM) with L-glutamine and 4.5 g/L glucose (Biowhittaker, Walkersville, MS) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1% Penicillin-Streptomycin-Amphotericin B (PSF), 1% non-essential amino acids (NEAA) (Life Technologies, Carlsbad, CA), 25 µg/mL L-ascorbic acid (Sigma, St. Louis, MO), and 1 µg/mL insulin (Sigma). At 70–90% confluence, TMJ disc cells were passaged with 1X trypsin-EDTA (Gibco, Carlsbad, California) and used in this experiment at passage 2.

Costal cartilage tissue was scraped from non-floating ribs, minced, and digested with 0.2% type II collagenase (Worthington, Lakewood, NJ) for 18 hrs. A portion of the primary cells (P0s) were used for construct formation and the remainder were plated on tissue culture treated plastic and cultured as described above. CCs were expanded in DMEM (Gibco) with 10% FBS, 1% PSF, 1% NEAA, and 25 µg/mL L-ascorbic acid. CCs at passage 1 (P1), passage 3 (P3), and passage 5 (P5) were collected to form tissue engineered constructs. All cells were cultured in a standard incubator at 37°C and 5% CO₂.

Construct culture

Constructs were created via a modified method used by Hu and Athanasiou.³² Briefly, each sample group of cells was seeded into 3 mm, 2% agarose wells to form self-assembled constructs, containing 2×10^6 cells each. These constructs were cultured in the wells for 2 wks before being transferred into agarose coated 6-well plates. Partial media changes of DMEM with 1% PSF, 1% NEAA, 1% ITS+ premix (BD Biosciences, San Jose, CA), 0.1

μM dexamethasone, 40 μg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50 μg/mL ascorbate 2-phosphate (Sigma), and 100 μg/mL sodium pyruvate (Fisher) occurred everyday.

Histology

Two constructs were removed at both time points (3 and 6 wks), frozen in HistoPrep™ Frozen Tissue Embedding Media (Fisher), cut to 14 μm sections in a cryotome, and put on glass slides. Slides were placed on a 30°C warm plate overnight and formalin-fixed for histology. Sections were stained with picosirius red for collagen, safranin O/fast green for GAGs, and hematoxylin and eosin for cell visualization. Immunohistochemistry (IHC) for collagen types I and II was also performed on these samples. IHC slides were stored at -80°C, fixed in acetone, and stained with a Biogenex i6000 autostainer (San Ramon, CA). Samples were washed in phosphate buffer saline solution with Tween before every step except the primary antibody. Slides were blocked with 3% H₂O₂ in methanol and Vectastain protein block (Vector Laboratories, Burlingame, CA). Primary antibodies were incubated with the samples for 1 hr. Antibodies for collagen type I were mouse monoclonal (Accurate Chemical and Scientific, Westbury, NY), and rabbit polyclonal antibodies were used for collagen type II (Chemicon, Temecula, CA). Secondary antibodies for the appropriate species were provided by the Vectastain ABC kit. Staining was visualized with a DAB substrate kit (Vector Laboratories). IHC slides were counter stained with Harris's hematoxylin (Fisher).

Biochemistry

Six samples were taken at 3 and 6 wks for biochemical analysis. Samples were weighed before and after a 2 day lyophilization step to determine both wet and dry weights of the constructs. Once dried, samples were digested in 125 μg/mL papain (Sigma) in 50 mM phosphate buffer (pH=6.5) containing 2 mM N-acetyl-cysteine (Sigma) and EDTA for 7 days followed by 2 days of 1 mg/mL elastase (Sigma) digestion. The entire digest occurred at 4°C with constant mechanical agitation.

DNA was quantified with a PicoGreen® dsDNA reagent (Molecular probes), and cell numbers were calculated using a conversion factor of 7.7 pg DNA/cell, as determined previously.³³ Total collagen was measured with a modified colorimetric hydroxyproline assay, described previously.³⁴ Briefly, samples were hydrolyzed with NaOH and neutralized with HCl. Chloramine T and Ehrlich's solutions were added and incubated at 60°C. Sulfated GAGs were quantified with a dimethylmethyle blue Blyscan kit, according to the manufacturer's protocol (Biocolor, Newtownabbey, Ireland).

ELISA

Collagen type I was measured with an indirect ELISA, as described previously.³⁵ Briefly, the sample digests were incubated on high-affinity plates for 18 hrs at 4°C. Samples were then exposed to a primary mouse antibody (Accurate Chemical) followed by a HRP-conjugated secondary antibody (Chemicon). Samples were visualized by incubating wells with tetramethyl biazidine (Chemicon) and quantified by comparing them to an ELISA grade collagen I standard. Collagen type II was quantified with a Chondrex (Redmond, WA) collagen detection kit, according to the manufacturer's protocol.

Mechanical testing

Mechanical testing was performed on at least 6 samples at the 6 wk time point. Compressive properties were determined by conducting creep tests under unconfined compression.³⁶ Costal chondrocyte samples were cut in half through the diameter of the samples to create an

even testing surface. The initial heights of all the samples were measured with digital calipers. Samples were then placed in saline solution and positioned under the platen so that the sample surface and platen were parallel. Each specimen was loaded with a tare weight of 0.002 N until equilibrium was reached (deformation less than 10^{-6} mm/s) or 10 minutes elapsed. A step load was then applied to the sample with a creep test weight of 0.007 N until equilibrium was again reached or the sample crept for 1 hr. The load was then removed, and the sample was again allowed to equilibrate to the specimen's recovery height. Creep data were then analyzed with the curve fitting tool in Matlab (The Math Works, Inc) using the viscoelastic model described previously.³⁷ Equation 1 describes the behavior of the viscoelastic solid where u_z is the deformation, σ is the applied stress, E_∞ is the relaxed modulus, z is the creep distance, τ_ϵ is the stress relaxation time constant, τ_σ is the creep time constant, and $h(t)$ is the step function. Fitting the data gives solutions for the relaxed modulus and time constants, from which the viscosity and instantaneous modulus (E_0) can be calculated.

$$u_z(r, z(r, t), t) = \frac{2\sigma}{3E_\infty} z(r, 0) \left[1 + \left(\frac{\tau_\epsilon}{\tau_\sigma} - 1 \right) e^{-t/\tau_\sigma} \right] h(t) \quad \langle \text{Eq. 1} \rangle$$

An Instron 5565 (Norwood, MA) with a 50 N load cell was used for tensile testing. Samples were cut into a dog bone shape and measured with digital calipers across the smallest cross sectional area. Samples were secured with cyanoacrylate glue on a paper frame with a standard gauge length. The paper was attached to the Instron grips before cutting it—leaving only the sample to be tested. Samples were tested at a 10% strain per minute until failure. Displacement and load data were collected and converted into stress and strain. Ultimate tensile strength (UTS) and elastic modulus (E) were calculated for each data set.

Statistics

When applicable, data were analyzed for statistical significance with a 2-way analysis of variance (ANOVA). Factors of cell type and time had five and two levels, respectively. When a main effects test indicated significance ($p < 0.05$), a Tukey's *post hoc* test was used to determine differences among the levels. This statistical model was used for ELISA and biochemical data. Mechanical data were analyzed with a 1-way ANOVA, where cell type was the only factor.

Results

Morphology and histology

Quantitative size data are shown in Table 1 and illustrated in the first two rows of Fig. 1. TMJ and P5 constructs contracted early in culture resulting in statistically smaller diameters than the other constructs. TMJ constructs were also statistically smaller in both diameter and volume than P5 constructs. Contraction in the TMJ constructs resulted in a primarily spherical shape. Constructs composed of passaged CCs formed rounder shapes compared to the P0 constructs, which were cylindrical in appearance. CC constructs grew over time resulting in a significant difference in diameters between 3 wks and 6 wks.

Figure 1 illustrates the histological and gross morphological differences between the different cell types at 6 wks. In terms of staining localization and intensity, little difference was observed between 3 and 6 wks. TMJ constructs contracted into dense spheres of cells with little ECM, as seen by the H&E staining. These constructs did not stain for GAGs, but did stain for collagen throughout. P0 constructs stained for collagen, GAG, and cells

throughout the construct in a mostly uniform manner. P1, P3, and P5 constructs stained positive for GAG and collagen, but staining was only seen in an outer ring of the construct. Cells for these constructs were localized to the outer ring. While a few cells were located in the center, trypan blue staining of the fluid from this inner region indicated the cells were dead.

IHC staining is shown in Fig. 2. Collagen type I was seen throughout all constructs, but was denser on the outer surface and in the center of the passaged constructs. All but the TMJ constructs stained positive for collagen II.

Biochemistry

The number of cells (Fig. 3a) was significantly higher in the P3, P0, and P5 groups with P1 being significantly lower and the TMJ group the lowest. For most groups, cells tended to increase ($p = 0.05$) with increasing time. Quantitative sulfated GAG content normalized by construct wet weight is compared in Fig. 3b. GAG significantly increased from 3 to 6 wks, approximately doubling in all groups except the TMJ. The GAG content of the TMJ constructs were significantly lower than any other group, and P0 constructs contained significantly lower GAG than any of the passaged groups. P5 constructs also contained significantly less GAG than P1 or P3 constructs. Total collagen from the hydroxyproline assay normalized by wet weight (Fig. 4) was the greatest in the TMJ constructs, with no other statistical differences.

ELISA

Collagen I and II quantities are also illustrated in Fig. 4. As with total collagen, TMJ constructs produced the most collagen I per weight wet ($p < 0.05$). No collagen II was produced in the TMJ constructs at 3 wks, which supports the IHC results, and only trace amounts (at the limits of detection) were measured at 6 wks. P1 constructs had the most (significantly more than P0, P3, or TMJ constructs) collagen type II per wet weight, and P5 constructs had significantly more than P3 or TMJ constructs.

Mechanical properties

Mechanical properties are listed in Table 2. In compression, TMJ constructs had a significantly larger E_0 than P0, P1, and P3 constructs and a significantly larger viscosity than all other groups. The P5 group had a significantly higher E_∞ than P3. In tension, the TMJ constructs had a significantly larger E than any other group and a significantly larger UTS than all the groups except P1.

Discussion

With the limited treatment options available for patients with TMJ disorders, tissue engineering seeks to create a functional TMJ disc replacement from a patient's own cells. Previous work has shown the potential functionality of costal chondrocytes in a scaffoldless, fibrocartilage tissue engineering approach.²⁷ Costal cartilage is used frequently in various reconstructive surgeries, because it is an abundant source of cartilage that is easy to obtain.^{4–9, 11, 38} However, complications that have been seen when using the whole tissue for jaw replacement suggest that using the cells with an *in vitro* tissue engineering approach may provide greater control for creating a functional TMJ disc replacement. To achieve the desired cell numbers from this hypocellular tissue, cell passaging may be necessary and may provide a more functional construct for the purposes of engineering the TMJ disc. This study examined the biochemical and mechanical characteristics of constructs from P0, P1, P3, and P5 CCs, in addition to TMJ disc cells. The data presented here show that passaged CCs have the greatest capacity for creating a functional fibrocartilage tissue replacement.

The hypothesis that increasing passage would decrease the chondrocytic proteins while increasing the fibrochondrocytic proteins proved partially true. Higher passages showed a significant decrease in collagen type II with P3 containing less collagen II per wet weight than P1 constructs. However, the higher passages did not increase collagen type I quantities. GAG per wet weight was significantly decreased in P5 from other passage constructs but was also significantly less in P0 constructs over the passaged constructs. It was originally expected that higher passage constructs would most closely resemble TMJ disc cell constructs, but this was largely not the case. The TMJ constructs had the smallest diameter, highest percentage of collagen I and total collagen, and lowest amounts of GAG and cells. While P5 constructs also had the smallest average diameter of the CC groups, P0 constructs had the closest GAG quantity, P1 had the closest cell quantity, and P3 had the closest collagen II quantities to the TMJ constructs. None of the CC constructs approached the total collagen or collagen I quantities seen in the TMJ disc constructs.

The overall appearance of the constructs created in this study suggests that CCs are a promising cell source for tissue engineering. TMJ constructs contracted to a small sphere with a diameter one-third the size of the other groups; this contraction event is undesirable, because the decrease in diameter and tissue volume makes it more difficult to create a tissue replacement of clinically relevant dimensions. These results were also seen with fibrocartilage from the knee meniscus.³⁹ Alternatively, CC constructs grew in diameter over time (although P5 constructs contracted in diameter initially). The time-dependent growth was particularly prominent in the P3 constructs, which exhibited a 7% increase in diameter from 3 wks to 6 wks.

Dead cells were noted only in the center of passaged constructs. Previous research has shown that cartilage dedifferentiates with passage, and it is possible there are multiple cell subpopulations that form with slightly different phenotypes.^{23, 30} When these cells are seeded into a three-dimensional construct, the subpopulations may aggregate into distinct regions through their surface receptors.⁴⁰ Additionally or alternatively, the outer core of cells may limit nutrient diffusion into the center or waste transport removal causing the inner population of cells to die. Experimentation with passaged CCs following this study suggests that the “cyst” forms within the first 48 hrs after seeding, but using a lower cell seeding density (cells per area) may eliminate this phenomenon (unpublished data). Altering the media composition or delivery, for example, by adding growth factors or using a perfusion bioreactor, may also prevent cell death in the core. Eliminating this unique structural characteristic may improve the cellular communication and/or overall biochemical and mechanical properties of the constructs such that passaged cells may create more functional constructs.

Biochemical assessment showed that passaged CC constructs produced almost twice as much GAG and equivalent amounts of collagen type I and total collagen to primary CCs. This ECM production is critical to the functionality of a tissue engineered construct. Collagen type II was also greater for passaged constructs with the exception of passage 3; however, this collagen type is uncharacteristic of a TMJ disc and may be detrimental to its functionality.⁴¹

The total collagen measured with hydroxyproline was not fully accounted for by the collagen types I and II ELISAs. This is likely due to other ECM molecules that would be detected with the hydroxyproline assay including other types of collagen and elastin. CCs have been shown to produce elastin,²³ collagen type III,⁴² and collagen type X.⁴³ Additional collagen types could also be present like types IX and XII, which are associated with types II and I, respectively.

CC constructs were able to retain the initial cell seeding density of 2×10^6 cells. These groups had between 1.5×10^6 to 2×10^6 cells per construct, while TMJ constructs lost 7/8 of this initial seeding density within the first 3 wks of culture. These cells either did not initially assemble into the construct, or the cells died and sloughed off within the first 3 wks. Also, the cell number did not increase over time in this group, suggesting a lack of proliferation, or proliferation was equilibrated with cell death. In any case, the loss of cells again suggests that even more cells would be needed to produce a TMJ disc cell construct of relevant dimensions. This illustrates once again the lack of feasibility in using TMJ disc cells in this tissue engineering approach.

Mechanical properties can frequently be linked to ECM content. While the GAG content of the constructs was not well correlated to the trend in compressive properties, the significant differences between the groups for UTS and E almost exactly correspond to the differences in total collagen and collagen type I. TMJ constructs had 2–10 times greater tensile properties than the other groups and about a 5 times greater total collagen content. Unfortunately, the TMJ disc cell constructs, in addition to being made of a non-feasible cell source and contracting significantly, still lack the mechanical integrity to function *in vivo*. Even the best mechanical data are still 4–84 times (depending on the direction tested) less than the native TMJ disc in tension⁴⁴ and 2–8 times less than the native tissue in compression.⁴⁵

Overall, this study illustrates the potential for passaged CCs in tissue engineering and also suggests the need for future work with these cells. Despite the larger tensile properties, collagen content, and in some cases compressive properties, TMJ disc cells are not a feasible option for tissue engineering. They are difficult to harvest, likely diseased in a patient considering a TMJ disc replacement, and extremely limited in number. Additionally, the cell number in the constructs decreased from initial seeding and contracted to become prohibitively small. On the other hand, CCs can be easily obtained through a minimally invasive procedure and produce constructs of reasonable size with relevant ECM. Furthermore, while there was not a clear trend in the effects of passaging on CCs, the passaged CCs consistently outperformed primary CCs. Passaging increases time between tissue harvest and implantation but yields more cells, which may be essential to create a construct of relevant size. Mechanical properties and collagen content also need to be improved, and employing some of the strategies mentioned previously, like adding growth factors or a bioreactor, will be important future work. Incorporating these strategies could improve this tissue engineering approach such that it becomes a feasible option for sufferers of TMJ disorders.

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References

1. NIDCR. TMJ Disorders. 2006.
2. Dimitroulis G. The role of surgery in the management of disorders of the temporomandibular joint: a critical review of the literature. Part 2. International journal of oral and maxillofacial surgery 2005;34(3):231–237. [PubMed: 15741028]
3. Wong, ME.; Allen, KD.; Athanasiou, KA. Tissue engineering of the temporomandibular joint. In: Bronzino, JD., editor. Biomedical Engineering Handbook, Third Edition: Tissue Engineering and Artificial Organs. CRC Press; 2006. p. 51-51-52-22.
4. Caccamese JF Jr, Ruiz RL, Costello BJ. Costochondral rib grafting. Atlas of the oral and maxillofacial surgery clinics of North America 2005;13(2):139–149. [PubMed: 16139760]

5. Fernandes R, Fattahi T, Steinberg B. Costochondral rib grafts in mandibular reconstruction. *Atlas of the oral and maxillofacial surgery clinics of North America* 2006;14(2):179–183. [PubMed: 16959605]
6. Figueroa AA, Gans BJ, Pruzansky S. Long-term follow-up of a mandibular costochondral graft. *Oral surgery, oral medicine, and oral pathology* 1984;58(3):257–268.
7. Lindqvist C, Jokinen J, Pauku P, Tasanen A. Adaptation of autogenous costochondral grafts used for temporomandibular joint reconstruction: a long-term clinical and radiologic follow-up. *J Oral Maxillofac Surg* 1988;46(6):465–470. [PubMed: 3164050]
8. Obeid G, Guttenberg SA, Connoles PW. Costochondral grafting in condylar replacement and mandibular reconstruction. *J Oral Maxillofac Surg* 1988;46(3):177–182. [PubMed: 3280758]
9. Ross RB. Costochondral grafts replacing the mandibular condyle. *Cleft Palate Craniofac J* 1999;36(4):334–339. [PubMed: 10426600]
10. Samman N, Cheung LK, Tideman H. Variations in costochondral grafting of the mandibular ramus. *Annals of the Royal Australasian College of Dental Surgeons* 1996;13:144–153. [PubMed: 9178987]
11. Perrott DH, Umeda H, Kaban LB. Costochondral graft construction/reconstruction of the ramus/condyle unit: long-term follow-up. *International journal of oral and maxillofacial surgery* 1994;23(6 Pt 1):321–328. [PubMed: 7699266]
12. Yotsuyanagi T, Mikami M, Yamauchi M, Higuma Y, Urushidate S, Ezoe K. A new technique for harvesting costal cartilage with minimum sacrifice at the donor site. *J Plast Reconstr Aesthet Surg* 2006;59(4):352–359. [PubMed: 16756249]
13. Baek RM, Song YT. Overgrowth of a costochondral graft in reconstruction of the temporomandibular joint. *Scandinavian journal of plastic and reconstructive surgery and hand surgery/Nordisk plastikkirurgisk forening [and] Nordisk klubb for handkirurgi* 2006;40(3):179–185.
14. Pietila K, Kantomaa T, Pirtiniemi P, Poikela A. Comparison of amounts and properties of collagen and proteoglycans in condylar, costal and nasal cartilages. *Cells, tissues, organs* 1999;164(1):30–36. [PubMed: 10940671]
15. Samman N, Cheung LK, Tideman H. Overgrowth of a costochondral graft in an adult male. *International journal of oral and maxillofacial surgery* 1995;24(5):333–335. [PubMed: 8627095]
16. Ware WH, Brown SL. Growth centre transplantation to replace mandibular condyles. *Journal of maxillofacial surgery* 1981;9(1):50–58. [PubMed: 6939773]
17. Amiel D, Coutts RD, Harwood FL, Ishizue KK, Kleiner JB. The chondrogenesis of rib perichondrial grafts for repair of full thickness articular cartilage defects in a rabbit model: a one year postoperative assessment. *Connective tissue research* 1988;18(1):27–39. [PubMed: 3180813]
18. Popko J, Szeparowicz P, Sawicki B, Wolczynski S, Wojnar J. Rabbit articular cartilage defects treated with cultured costal chondrocytes (preliminary report). *Folia morphologica* 2003;62(2):107–112. [PubMed: 12866669]
19. Szeparowicz P, Popko J, Sawicki B, Wolczynski S, Biernacki M. Comparison of cartilage self repairs and repairs with costal and articular chondrocyte transplantation in treatment of cartilage defects in rats. *Roczniki Akademii Medycznej w Białymstoku* (1995) 2004;49 (Suppl 1):28–30. [PubMed: 15638365]
20. Johnson TS, Xu JW, Zaporozjan VV, Mesa JM, Weinand C, Randolph MA, et al. Integrative repair of cartilage with articular and nonarticular chondrocytes. *Tissue engineering* 2004;10(9–10):1308–1315. [PubMed: 15588391]
21. Freed LE, Marquis JC, Nohria A, Emmanuel J, Mikos AG, Langer R. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. *J Biomed Mater Res* 1993;27(1):11–23. [PubMed: 8380593]
22. Walles T, Giere B, Macchiarini P, Mertsching H. Expansion of chondrocytes in a three-dimensional matrix for tracheal tissue engineering. *The Annals of thoracic surgery* 2004;78(2):444–448. discussion 448–449. [PubMed: 15276493]
23. Tay AG, Farhadi J, Suetterlin R, Pierer G, Heberer M, Martin I. Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes. *Tissue engineering* 2004;10(5–6):762–770. [PubMed: 15265293]

24. Ting V, Sims CD, Brecht LE, McCarthy JG, Kasabian AK, Connelly PR, et al. In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes. *Annals of plastic surgery* 1998;40(4):413–420. discussion 420–411. [PubMed: 9555998]
25. Xu JW, Zaporozhan V, Peretti GM, Roses RE, Morse KB, Roy AK, et al. Injectable tissue-engineered cartilage with different chondrocyte sources. *Plastic and reconstructive surgery* 2004;113(5):1361–1371. [PubMed: 15060348]
26. Isogai N, Kusuhara H, Ikada Y, Ohtani H, Jacquet R, Hillyer J, et al. Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. *Tissue engineering* 2006;12(4):691–703. [PubMed: 16674284]
27. Johns DE, Athanasiou KA. Engineering the TMJ disc with clinically-relevant cell sources. *J Dent Res*. 2008 Accepted.
28. Stockwell RA. The cell density of human articular and costal cartilage. *Journal of anatomy* 1967;101(Pt 4):753–763. [PubMed: 6059823]
29. Saadeh PB, Brent B, Mehrara BJ, Steinbrech DS, Ting V, Gittes GK, et al. Human cartilage engineering: chondrocyte extraction, proliferation, and characterization for construct development. *Annals of plastic surgery* 1999;42(5):509–513. [PubMed: 10340859]
30. Darling EM, Athanasiou KA. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J Orthop Res* 2005;23(2):425–432. [PubMed: 15734258]
31. Johns DE, Athanasiou KA. Improving culture conditions for temporomandibular joint disc tissue engineering. *Cells, tissues, organs* 2007;185(4):246–257. [PubMed: 17587799]
32. Hu JC, Athanasiou KA. A self-assembling process in articular cartilage tissue engineering. *Tissue engineering* 2006;12(4):969–979. [PubMed: 16674308]
33. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Analytical biochemistry* 1988;174(1):168–176. [PubMed: 2464289]
34. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961;93:440–447. [PubMed: 13786180]
35. Darling EM, Athanasiou KA. Growth factor impact on articular cartilage subpopulations. *Cell and tissue research* 2005;322(3):463–473. [PubMed: 16047167]
36. Athanasiou KA, Agarwal A, Dzida FJ. Comparative study of the intrinsic mechanical properties of the human acetabular and femoral head cartilage. *J Orthop Res* 1994;12(3):340–349. [PubMed: 8207587]
37. Leipzig ND, Athanasiou KA. Unconfined creep compression of chondrocytes. *Journal of biomechanics* 2005;38(1):77–85. [PubMed: 15519342]
38. Brent B. Technical advances in ear reconstruction with autogenous rib cartilage grafts: personal experience with 1200 cases. *Plastic and reconstructive surgery* 1999;104(2):319–334. discussion 335–318. [PubMed: 10654675]
39. Hoben GM, Hu JC, James RA, Athanasiou KA. Self-assembly of fibrochondrocytes and chondrocytes for tissue engineering of the knee meniscus. *Tissue engineering* 2007;13(5):939–946. [PubMed: 17484700]
40. Steinberg MS, Takeichi M. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proceedings of the National Academy of Sciences of the United States of America* 1994;91(1):206–209. [PubMed: 8278366]
41. Detamore MS, Orfanos JG, Almaraz AJ, French MM, Wong ME, Athanasiou KA. Quantitative analysis and comparative regional investigation of the extracellular matrix of the porcine temporomandibular joint disc. *Matrix Biol* 2005;24(1):45–57. [PubMed: 15749001]
42. Goldring MB, Birkhead J, Sandell LJ, Kimura T, Krane SM. Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. *The Journal of clinical investigation* 1988;82(6):2026–2037. [PubMed: 3264290]
43. Jikko A, Murakami H, Yan W, Nakashima K, Ohya Y, Satake H, et al. Effects of cyclic adenosine 3',5'-monophosphate on chondrocyte terminal differentiation and cartilage-matrix calcification. *Endocrinology* 1996;137(1):122–128. [PubMed: 8536602]

44. Beatty MW, Bruno MJ, Iwasaki LR, Nickel JC. Strain rate dependent orthotropic properties of pristine and impulsively loaded porcine temporomandibular joint disk. *J Biomed Mater Res* 2001;57(1):25–34. [PubMed: 11416845]
45. Allen KD, Athanasiou KA. A surface-regional and freeze-thaw characterization of the porcine temporomandibular joint disc. *Ann Biomed Eng* 2005;33(7):951–962. [PubMed: 16060536]

Figure 1.

Images a-j show gross morphology of each construct from the top (a-e) and side (f-j) at 6 wks. Spaces below the constructs are 1mm. H&E (k-o), safranin O/fast green for GAG (p-t), and picrosirius red for collagen (u-y) staining are also shown for each group. The scale bar in the bottom left is 1mm. TMJ constructs contracted into small spheres, primarily consisting of cells, as indicated by the H&E staining. They did not stain for GAG and stained uniformly for collagen. P0 constructs were tall cylinders while passaged constructs also rounded into spheres. P0 constructs stained mostly uniform for collagen, GAG, and cells. Passaged cells, however, formed fluid-filled spheres that did not stain for cells, GAG, or collagen. Only an outer ring of tissue stained for cells and ECM.

Figure 2. IHC staining for collagen type I (a-f) and type II (g-l) for all constructs at 6 wks. Positive controls are seen in frames f and l. Collagen I was seen throughout TMJ and P0 constructs. Passaged constructs showed the most intense collagen I staining around the outside of the constructs and within the fluid-filled center. Collagen type II was seen throughout all the tissue-like areas of the CC constructs, but was not seen in TMJ constructs. Scale bar = 1mm.

Figure 3.

Cell (a) and GAG per wet weight (b) quantities for all groups (mean + SD). Groups separated by different letters are considered significantly different ($p < 0.05$). TMJ constructs were significantly lower for both cell and GAG content from all other groups. P1 had significantly lower cell numbers than other CC constructs. P0 had the lowest GAG content of all the CC constructs, and P5 had significantly lower GAG quantities than P1 constructs. Time was a significant factor for GAG with 6 wks being greater than 3 wks as was very apparent in CC constructs.

Figure 4.

Collagen content normalized by wet weight (mean + SD) for all groups at 3 and 6 wks. Gray and black bars show the stacked quantities of collagen type I and collagen type II, respectively, from the ELISAs. The remaining white bars were the quantities measured with the hydroxyproline assay, which were unaccounted for by the ELISAs. All results indicated a significant increase in the time factor from 3 to 6 wks. The TMJ group had the most ($p < 0.05$) collagen type I and total collagen, as indicated by the asterisk. Collagen II results (statistics not represented in the figure) were greatest in P1 constructs, which had significantly more collagen type II per wet weight than P0, P3, or TMJ constructs, followed by P5 constructs, which were also significantly greater than P3 or TMJ constructs.

Table 1

Data are shown as mean $\pm$ SD. Groups separated by different letters are statistically significant ($p < 0.05$). TMJ constructs were significantly smaller by all metrics when compared to the other groups. P5s were significantly smaller than other CCs in diameter and dry weight. P1 and P3 constructs had the largest diameters. P0 constructs had the largest wet weight and a significantly greater dry weight than the P1 group.

	Diameter (mm)	Thickness (mm)	Wet weight (mg)	Dry weight (mg)
TMJ	0.837 $\pm$ 0.119 ^d	0.893 $\pm$ 0.211 ^b	0.218 $\pm$ 0.0608 ^c	0.0617 $\pm$ 0.0204 ^d
P0	3.08 $\pm$ 0.224 ^b	2.64 $\pm$ 0.936 ^a	24.9 $\pm$ 1.95 ^a	3.38 $\pm$ 0.245 ^a
P1	3.35 $\pm$ 0.0727 ^a	2.32 $\pm$ 0.133 ^a	16.8 $\pm$ 1.33 ^b	2.67 $\pm$ 0.151 ^b
P3	3.35 $\pm$ 0.127 ^a	2.76 $\pm$ 0.176 ^a	19.1 $\pm$ 2.60 ^b	2.829 $\pm$ 0.391 ^{ab}
P5	2.85 $\pm$ 0.0841 ^c	2.51 $\pm$ 0.0952 ^a	12.0 $\pm$ 0.904 ^b	1.81 $\pm$ 0.0974 ^c

Table 2

Data are shown as mean $\pm$ SD. Groups separated by different letters are statistically significant ($p < 0.05$). In compression, statistical differences were observed in E_o and viscosity with TMJ being significantly larger than all except P5s and all groups, respectively. The P5 group had a significantly larger E_{∞} than the P3 group. In tension, the UTS for TMJ constructs was significantly larger than P0, P3, and P5 constructs. TMJ constructs also had a significantly larger elastic modulus than all other constructs.

	Compressive Properties			Tensile Properties	
	E_{∞} (kPa)	E_o (kPa)	Viscosity (s*kPa)	UTS (MPa)	E (MPa)
TMJ	44.5 $\pm$ 12.5 ^{ab}	190 $\pm$ 58.4 ^a	7200 $\pm$ 2810 ^a	1.11 $\pm$ 0.661 ^a	2.28 $\pm$ 1.67 ^a
P0	32.2 $\pm$ 24.5 ^{ab}	93.9 $\pm$ 72.2 ^b	868 $\pm$ 753 ^b	0.147 $\pm$ 0.0565 ^b	0.326 $\pm$ 0.189 ^b
P1	28.3 $\pm$ 10.3 ^{ab}	76.8 $\pm$ 32.7 ^b	261 $\pm$ 126 ^b	0.668 $\pm$ 0.427 ^{ab}	0.958 $\pm$ 0.641 ^b
P3	23.1 $\pm$ 6.06 ^b	55.0 $\pm$ 14.7 ^b	223 $\pm$ 91.1 ^b	0.178 $\pm$ 0.102 ^b	0.165 $\pm$ 0.0818 ^b
P5	50.0 $\pm$ 10.2 ^a	117 $\pm$ 22.1 ^{ab}	451 $\pm$ 511 ^b	0.320 $\pm$ 0.199 ^b	0.496 $\pm$ 0.557 ^b