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Passaged Goat Costal Chondrocytes Provide a Feasible Cell Source for Temporomandibular Joint Tissue Engineering

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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) and collagens, makes them an appealing cell source for fibrocartilage 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. It was hypothesized that earlier passage constructs would have more cartilaginous proteins and less fibrocartilaginous proteins. 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 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 groups. These results suggest not only feasibility but potential superiority of passaged CCs over primary CCs, which may lead to functional engineered fibrocartilage.

Keywords—Fibrochondrocytes, Fibrocartilage, Cartilage, Extracellular matrix, Mechanical properties.

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.[29] There are various treatment options depending on the level of degeneration as reviewed elsewhere.[12,51] 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.[8,13,14,27,30,32,35,38] Costal cartilage grafts are also used in ear, nose, and other craniofacial reconstructions.[8] By using autologous tissue, complications with patient rejection of non-biologic materials, as was seen with the Vitek Proplast implant, are eliminated.[45–47] The costal cartilage can be harvested easily, and while these surgeries are performed frequently, there was a risk of chest wall deformity that has largely been eliminated with current surgical techniques.[53] A common problem with this approach in the jaw is overgrowth of the costal cartilage in the TMJ, which can require further surgery.[5,33,37,49] 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 engineered construct *in vitro* with costal chondrocytes may yield a completely functional tissue without undesirable complications. Ease of use and practicality 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,[3,15,24,34,42] tracheal,[43,48] elastic,[20,43,44,52] and, most recently, fibrocartilage.[23] Previous work suggests that CCs produce considerable quantities of extracellular matrix (ECM) proteins and could provide a more clinically feasible cell source for TMJ disc tissue engineering.[23] However, the acellular nature of cartilage limits the amount of viable cells available from a piece of tissue.[41] 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

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appear to lose their proliferative ability, showing little expansion beyond passage 5.[36] 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.[10,43] Articular chondrocytes have exhibited a 10-fold decrease in collagen type II gene expression and a 20-fold increase in collagen type I gene expression from passage 0 to 4.[10] It is encouraging to note, however, that the resulting 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 numbers, difficult to harvest, and likely diseased in any patient interested in a TMJ replacement (data not shown), they will serve as a control in this experiment. It is hypothesized that constructs composed of higher passage CCs will contain less cartilaginous proteins and more fibrocartilaginous proteins. Specifically, there will be less collagen type II and glycosaminoglycans and more collagen type I and total collagen.

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.[22] 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% by volume fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1% by volume Penicillin–Streptomycin–Amphotericin B (PSF), 1% by volume 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 1× trypsin-EDTA (Gibco, Carlsbad, CA) and used in this experiment at passage 2, which was required to obtain the quantity of cells necessary to create the appropriate number of constructs for this study.

Costal cartilage tissue was scraped from non-floating ribs, minced, and digested with 0.2% type II collagenase (Worthington, Lakewood, NJ) for 18 h. 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% CO_2.

Construct Culture

At least 22 samples were created and examined at two time points. Constructs were created via a scaffoldless modified method used by Hu and Athanasiou.[19] In this technique the agarose is used as a non-adherent surface, around which the cells must form. 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 picrosirius 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% H_2O_2 in methanol and Vectastain protein block (Vector Laboratories, Burlingame, CA). Primary antibodies were incubated with the samples for 1 h. 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*-acetylcysteine (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.[25] Total collagen was measured with a modified colorimetric hydroxyproline assay, described previously.[50] 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 dimethylmethlylene 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.[9] Briefly, the sample digests were incubated on high-affinity plates for 18 h 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 bizidine (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 a minimum of 6 samples at the 6-wk time point. Compressive properties were determined by conducting creep tests under unconfined compression.[4] Costal chondrocyte samples were cut through the diameter of the samples to create an even testing surface, and were tested with the cut surface oriented toward the testing platen. 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 min 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 creeped for 1 h. The load was then removed, and the sample was again allowed to equilibrate to the specimen's recovery height. Samples remained intact after testing. Creep data were then analyzed with the curve fitting tool in Matlab (The Math Works, Inc.) using the viscoelastic model described previously.[26] 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.

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

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. CC samples were consistently oriented in the radial direction of the sample. The resulting size (less than 1 mm in diameter) of the TMJ disc constructs required nearly all of the constructs, with only a central region cut out to form the dog-bone shape. 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 two-way analysis of variance (ANOVA). The two factors, 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 morphology, ELISA, and biochemical data. Mechanical data were analyzed with a one-way ANOVA, where cell type was the only factor.

TABLE 1. Dimensions and weights of constructs at 6 wks.

	Week 3			Week 6		
	Diameter (mm)	Wet weight (mg)	Dry weight (mg)	Diameter (mm)*	Wet weight (mg)	Dry weight (mg)*
TMJ	0.99 ± 0.07^{d}	0.32 ± 0.07^{c}	0.09 ± 0.03^{d}	0.84 ± 0.12^{d}	0.22 ± 0.06^{c}	0.06 ± 0.02^{d}
P0	3.01 ± 0.15^{b}	21.56 ± 3.36^{a}	1.95 ± 0.27^{a}	3.08 ± 0.22^{b}	24.85 ± 1.95^{a}	3.38 ± 0.25^{a}
P1	3.37 ± 0.22^{a}	11.88 ± 0.95^{b}	1.52 ± 0.19^{b}	3.35 ± 0.07^{a}	16.80 ± 1.33^{b}	2.67 ± 0.15^{b}
P3	3.12 ± 0.16^{a}	15.84 ± 1.78^{b}	1.93 ± 0.30^{ab}	3.35 ± 0.13^{a}	19.09 ± 2.60^{b}	2.83 ± 0.39^{ab}
P5	2.54 ± 0.148^{c}	9.23 ± 1.56^{b}	1.21 ± 0.14^{c}	2.85 ± 0.08^{c}	11.99 ± 0.90^{b}	1.81 ± 0.10^{c}

Data are shown as mean ± SD. Groups separated by different superscripted letters are statistically significant ($p < 0.05$). The asterisk indicates that data at 6 wks were significantly greater for diameter and wet weight. TMJ constructs were significantly the smallest. 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.

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 ($p < 0.0001$). 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 more spherical 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 and 6 wks ($p = 0.0021$).

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

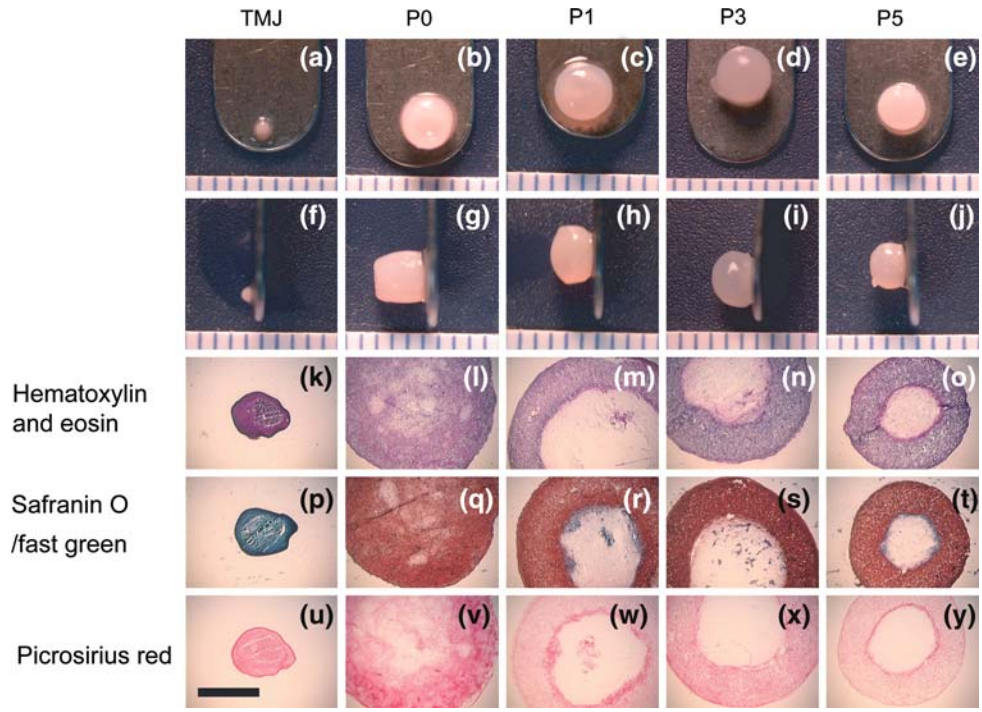

FIGURE 1. Images a–j show gross morphology of each construct from the top (a–e) and side (f–j) at 6 wks. Spaces below the constructs are 1 mm. H&E (k–o), safranin O/fast green for GAG (p–t), and picrosirius red for collagen (u–y) staining are also shown for each construct at 6 wks. The scale bar in the bottom left is 1 mm. TMJ constructs contracted into small spheres. 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.

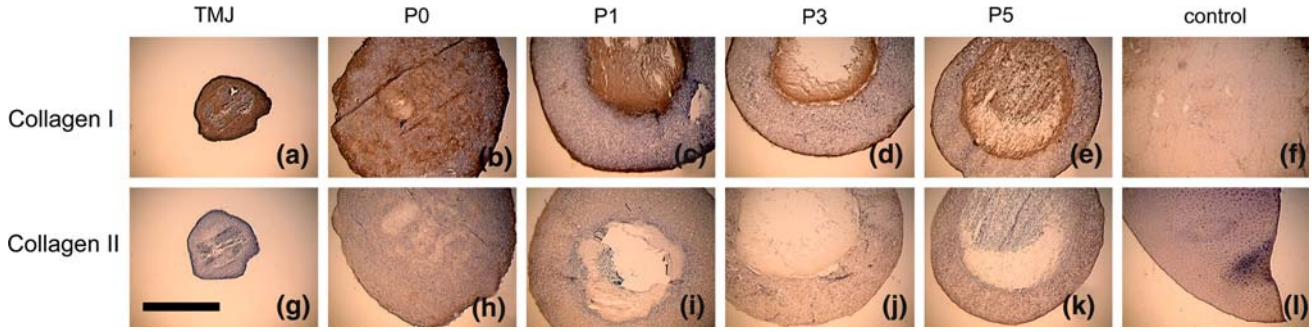

FIGURE 2. IHC staining for collagen type I (a–f) and type II (g–l) for all constructs at 6 wks (with hematoxylin for cells). Positive controls are seen in frames f (knee meniscus tissue) and l (articular cartilage tissue). Collagen I was seen throughout TMJ and P0 constructs as indicated by the brown staining. 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, which only stained purple with hematoxylin for cell nuclei. Scale bar = 1 mm.

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. There were not profound differences in cell morphology between the groups.

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. While the collagen type II stained less strongly than type I, there is no collagen II staining present in the TMJ constructs, while all the other groups stained positive.

Biochemistry

The number of cells per construct (Fig. 3a) was significantly higher in the P3, P0, and P5 groups with P1 being significantly lower and the TMJ group the lowest ($p < 0.0001$). For most groups, cells tended to increase ($p = 0.0540$) 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 ($p < 0.0001$), 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 ($p < 0.0001$). 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 ($p < 0.0001$).

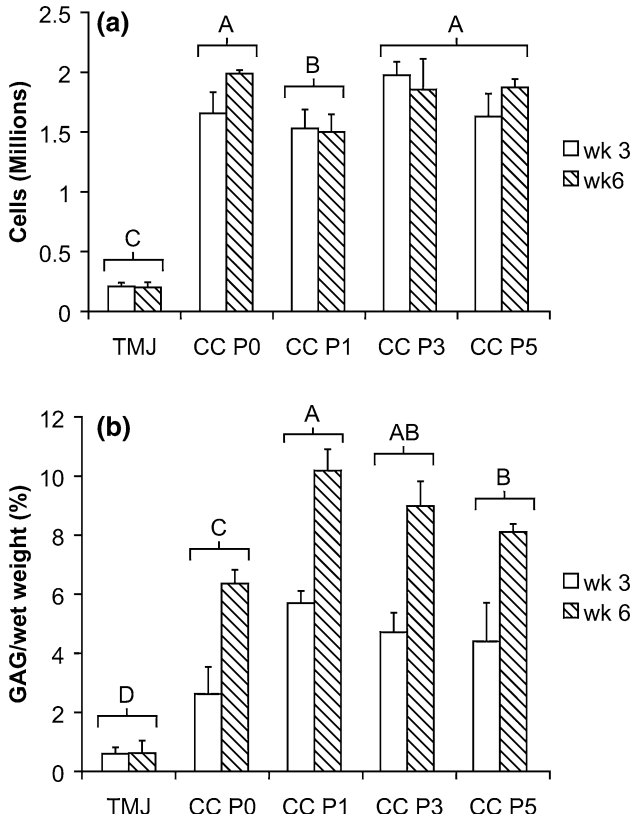

FIGURE 3. Cells per construct (a) and GAG per wet weight (b) quantities for all groups (mean + SD). Groups separated by different letters, above the data bars, are considered significantly different ($p < 0.05$). TMJ constructs were significantly lower for both cell and GAG content from all other groups. 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.

ELISA

Collagen I and II quantities are also illustrated in Fig. 4. As with total collagen, TMJ constructs

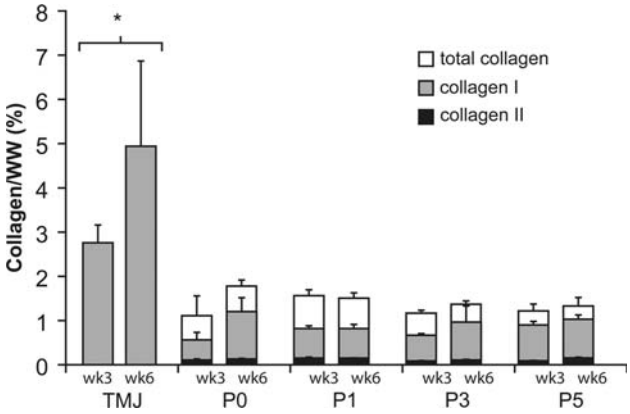

FIGURE 4. Collagen content normalized by wet weight (mean + SD) for all groups at 3 and 6 wks. Gray and black bars show the stacked quantities of collagen type I and collagen type II, respectively, from the ELISAs. The remaining white bars were the quantities measured with the hydroxyproline assay, which 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.

produced the most collagen I per weight wet ($p < 0.0001$). 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 ($p < 0.0001$).

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 ($p = 0.0004$) and a significantly larger viscosity than all other groups ($p < 0.0001$). The P5 group had a significantly higher E_∞ than P3 ($p = 0.0226$). In tension, the TMJ constructs had a significantly larger E than any other group ($p = 0.0010$) and a significantly larger UTS than all the groups except P1 ($p = 0.0004$).

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.[23] This previous work showed that the CCs were superior to dermal fibroblasts or a mixture of CCs and dermal fibroblasts. Costal cartilage is used frequently in various reconstructive surgeries, because it is an abundant source of cartilage that is easy to obtain.[7,8,13,14,27,30,32,35] 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 purpose 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 a greater capacity for creating a functional fibrocartilage tissue replacement than TMJ disc cells or primary CCs.

The hypothesis that increasing passage would decrease the chondrocytic proteins while increasing the fibrochondrocytic proteins in the constructs proved partially true. Higher passages showed a significant

TABLE 2. Mechanical properties at 6 wks.

	Compressive properties			Tensile properties	
	E_∞ (kPa)	E_0 (kPa)	Viscosity (s*kPa)	UTS (MPa)	E (MPa)
TMJ	44.5 ± 12.5^{ab}	190 ± 58.4^{a}	7200 ± 2810^{a}	1.11 ± 0.661^{a}	2.28 ± 1.67^{a}
P0	32.2 ± 24.5^{ab}	93.9 ± 72.2^{b}	868 ± 753^{b}	0.147 ± 0.0565^{b}	0.326 ± 0.189^{b}
P1	28.3 ± 10.3^{ab}	76.8 ± 32.7^{b}	261 ± 126^{b}	0.668 ± 0.427^{ab}	0.958 ± 0.641^{b}
P3	23.1 ± 6.06^{b}	55.0 ± 14.7^{b}	223 ± 91.1^{b}	0.178 ± 0.102^{b}	0.165 ± 0.0818^{b}
P5	50.0 ± 10.2^{a}	117 ± 22.1^{ab}	451 ± 511^{b}	0.320 ± 0.199^{b}	0.496 ± 0.557^{b}

Data are shown as mean $\pm$ SD. Groups separated by different superscripted letters are statistically significant ($p < 0.05$). In compression, statistical differences were observed in E_0 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.

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.[18] Alternatively, CC constructs grew in diameter over time (although P5 constructs contracted in diameter initially). The time-dependent growth in the P3 constructs was a 7% increase in diameter from 3 to 6 wks.

Dead cells were noted only in the center of passaged constructs. Previous research has shown that chondrocytes dedifferentiate with passage, and it is possible there are multiple cell subpopulations that form with slightly different phenotypes within the entire population of passaged condrocytes.[10,43] When these cells are seeded into a three-dimensional construct, the subpopulations may aggregate into distinct regions through their surface receptors.[40] The outer core of cells may limit nutrient diffusion into the center or waste transport removal causing the inner population of cells to die. Additionally or alternatively, the inner population of cells may have developed adhesion dependence, which may lead to their death upon being placed in this non-adherent culture. Experimentation with passaged CCs following this study suggests that the "cyst" forms within the first 48 h 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 as 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.[11] Total collagen/wet weight of the native TMJ disc is approximately 30%,[16] while GAG/wet weight is 2%.[39] The results obtained in this study showed that CCs had insufficient collagen production, but GAG concentration actually exceeded native values. 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,[43] collagen type III,[17] and collagen type X.[21] 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.

It has been established that mechanical properties are related 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 correspond directly 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. However, 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 tension[6] and 2–8 times less than the native

tissue in compression.[1] The lower mechanical properties observed in the CC constructs are likely due to a lack of ECM organization, which may be improved with *in vitro* mechanical stimulation.

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. Indeed, the TMJ disc cells were passaged for this study, and previous work has shown a substantial decrease in aggrecan, collagen I, and collagen II gene expression for TMJ disc cells with passaging.[2] While these decreases in expression occured at passage 2, there was not a complete loss of the original phenotype as was seen at the later passages, which suggests these early passage cells are the most feasible passage for a tissue engineering study.[2] Additionally, the cell number in the TMJ disc cell 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 (over 3 mm in diameter, about one-third the anteroposterior dimension of the TMJ disc) with relevant ECM (collagen and GAG). Furthermore, while there was not a clear trend in the effects of CC passage number on the resulting constructs, the passaged CCs consistently outperformed primary CCs, although the differences were not always significant. To better understand these cells after expansion, more characterization of the primary and passaged cells, perhaps relating to their gene expression, may be desirable. 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, particularly collagen type I, 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.

While this work has shown exciting data that dramatically improves upon previous work toward TMJ disc tissue engineering, there are several aspects of TMJ disc replacement that must also be considered before a tissue engineered replacement can be implemented. In addition to the functional and clinical aspects discussed previously, considerations of construct integration and surgical attachment are important ones. Successful methods used in articular cartilage or the knee meniscus may not be applicable to the TMJ disc, like periosteal flaps,[31] sutures, or polymer staples,[28] due to the large movement that occurs within the disc and its thinness. Care must also be taken not to introduce additional complications into the joint with the surgery, which is a common concern in oral and maxillofacial surgery.[51] Considerable future work is needed to develop methods for integrating and attaching a tissue engineered replacement.

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REFERENCES

[1]Allen, K. D., and K. A. Athanasiou. A surface-regional and freeze-thaw characterization of the porcine temporomandibular joint disc. *Ann. Biomed. Eng.* 33:951–962, 2005. doi:10.1007/s10439-005-3872-6.

[2]Allen, K. D., and K. A. Athanasiou. Effect of passage and topography on gene expression of temporomandibular joint disc cells. *Tissue Eng.* 13:101–110, 2007. doi:10.1089/ten.2006.0094.

[3]Amiel, D., R. D. Coutts, F. L. Harwood, K. K. Ishizue, and J. B. Kleiner. The chondrogenesis of rib perichondrial grafts for repair of full thickness articular cartilage defects in a rabbit model: a one year postoperative assessment. *Connect. Tissue Res.* 18:27–39, 1988. doi:10.3109/03008208809019070.

[4]Athanasiou, K. A., A. Agarwal, and F. J. Dzida. Comparative study of the intrinsic mechanical properties of the human acetabular and femoral head cartilage. *J. Orthop. Res.* 12:340–349, 1994. doi:10.1002/jor.1100120306.

[5]Baek, R. M., and Y. T. Song. Overgrowth of a costochondral graft in reconstruction of the temporomandibular joint. *Scand. J. Plast. Reconstr. Surg. Hand Surg.* 40:179–185, 2006. doi:10.1080/02844310600763725.

[6]Beatty, M. W., M. J. Bruno, L. R. Iwasaki, and J. C. Nickel. Strain rate dependent orthotropic properties of pristine and impulsively loaded porcine temporomandibular joint disk. *J. Biomed. Mater. Res.* 57:25–34, 2001. doi:10.1002/1097-4636(200110)57:1<25::AID-JBM1137>3.0.CO;2-H.

[7]Brent, B. Technical advances in ear reconstruction with autogenous rib cartilage grafts: personal experience with 1200 cases. *Plast. Reconstr. Surg.* 104:319–334, 1999; discussion 335–338.

[8]Caccamese, J. F., Jr., R. L. Ruiz, and B. J. Costello. Costochondral rib grafting. *Atlas Oral Maxillofac. Surg. Clin. North Am.* 13:139–149, 2005. doi:10.1016/j.cxom.2005.05.004.

[9]Darling, E. M., and K. A. Athanasiou. Growth factor impact on articular cartilage subpopulations. *Cell Tissue Res.* 322:463–473, 2005. doi:10.1007/s00441-005-0020-4.

[10]Darling, E. M., and K. A. Athanasiou. Rapid phenotypic changes in passaged articular chondrocyte subpopulations. *J. Orthop. Res.* 23:425–432, 2005. doi:10.1016/j.orthres.2004.08.008.

[11]Detamore, M. S., J. G. Orfanos, A. J. Almarza, M. M. French, M. E. Wong, and K. A. Athanasiou. Quantitative analysis and comparative regional investigation of the extracellular matrix of the porcine temporomandibular joint disc. *Matrix Biol.* 24:45–57, 2005.

[12]Dimitroulis, G. The role of surgery in the management of disorders of the temporomandibular joint: a critical review of the literature. Part 2. *Int. J. Oral Maxillofac. Surg.* 34:231–237, 2005.

[13]Fernandes, R., T. Fattahi, and B. Steinberg. Costochondral rib grafts in mandibular reconstruction. *Atlas Oral Maxillofac. Surg. Clin. North Am.* 14:179–183, 2006. doi:10.1016/j.cxom.2006.05.007.

[14]Figueroa, A. A., B. J. Gans, and S. Pruzansky. Long-term follow-up of a mandibular costochondral graft. *Oral Surg. Oral Med. Oral Pathol.* 58:257–268, 1984. doi:10.1016/0030-4220(84)90050-1.

[15]Freed, L. E., J. C. Marquis, A. Nohria, J. Emmanual, A. G. Mikos, and R. Langer. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. *J. Biomed. Mater. Res.* 27:11–23, 1993. doi:10.1002/jbm.820270104.

[16]Gage, J. P., R. M. Shaw, and F. B. Moloney. Collagen type in dysfunctional temporomandibular joint disks. *J. Prosthet. Dent.* 74:517–520, 1995. doi:10.1016/S0022-3913(05)80355-5.

[17]Goldring, M. B., J. Birkhead, L. J. Sandell, T. Kimura, and S. M. Krane. Interleukin 1 suppresses expression of cartilage-specific types II and IX collagens and increases types I and III collagens in human chondrocytes. *J. Clin. Invest.* 82:2026–2037, 1988. doi:10.1172/JCI113823.

[18]Hoben, G. M., J. C. Hu, R. A. James, and K. A. Athanasiou. Self-assembly of fibrochondrocytes and chondrocytes for tissue engineering of the knee meniscus. *Tissue Eng.* 13:939–946, 2007. doi:10.1089/ten.2006.0116.

[19]Hu, J. C., and K. A. Athanasiou. A self-assembling process in articular cartilage tissue engineering. *Tissue Eng.* 12:969–979, 2006. doi:10.1089/ten.2006.12.969.

[20]Isogai, N., H. Kusuhara, Y. Ikada, H. Ohtani, R. Jacquet, J. Hillyer, E. Lowder, and W. J. Landis. Comparison of different chondrocytes for use in tissue engineering of cartilage model structures. *Tissue Eng.* 12:691–703, 2006. doi:10.1089/ten.2006.12.691.

[21]Jikko, A., H. Murakami, W. Yan, K. Nakashima, Y. Ohya, H. Satakeda, M. Noshiro, T. Kawamoto, S. Nakamura, Y. Okada, F. Suzuki, and Y. Kato. Effects of cyclic adenosine 3′,5′-monophosphate on chondrocyte terminal differentiation and cartilage-matrix calcification. *Endocrinology* 137:122–128, 1996. doi:10.1210/en.137.1.122.

[22]Johns, D. E., and K. A. Athanasiou. Improving culture conditions for temporomandibular joint disc tissue engineering. *Cells Tissues Organs.* 185:246–257, 2007. doi:10.1159/000102173.

[23]Johns, D. E., and K. A. Athanasiou. Engineering the TMJ disc with clinically-relevant cell sources. *J. Dent. Res.* 87:548–552, 2008.

[24]Johnson, T. S., J. W. Xu, V. V. Zaporojan, J. M. Mesa, C. Weinand, M. A. Randolph, L. J. Bonassar, J. M. Winograd, and M. J. Yaremchuk. Integrative repair of cartilage with articular and nonarticular chondrocytes. *Tissue Eng.* 10:1308–1315, 2004.

[25]Kim, Y. J., R. L. Sah, J. Y. Doong, and A. J. Grodzinsky. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal. Biochem.* 174:168–176, 1988. doi:10.1016/0003-2697(88)90532-5.

[26]Leipzig, N. D., and K. A. Athanasiou. Unconfined creep compression of chondrocytes. *J. Biomech.* 38:77–85, 2005.

[27]Lindqvist, C., J. Jokinen, P. Paukku, and A. Tasanen. Adaptation of autogenous costochondral grafts used for temporomandibular joint reconstruction: a long-term clinical and radiologic follow-up. *J. Oral Maxillofac. Surg.* 46:465–470, 1988. doi:10.1016/0278-2391(88)90413-2.

[28]Lu, Y., S. Dhanaraj, Z. Wang, D. M. Bradley, S. M. Bowman, B. J. Cole, and F. Binette. Minced cartilage without cell culture serves as an effective intraoperative cell source for cartilage repair. *J. Orthop. Res.* 24:1261–1270, 2006. doi:10.1002/jor.20135.

[29]NIDCR, TMJ Disorders, 2006.

[30]Obeid, G., S. A. Guttenberg, and P. W. Connole. Costochondral grafting in condylar replacement and mandibular reconstruction. *J. Oral Maxillofac. Surg.* 46:177–182, 1988. doi:10.1016/0278-2391(88)90079-1.

[31]Ochi, M., Y. Uchio, K. Kawasaki, S. Wakitani, and J. Iwasa. Transplantation of cartilage-like tissue made by tissue engineering in the treatment of cartilage defects of the knee. *J. Bone Joint Surg. Br.* 84:571–578, 2002. doi:10.1302/0301-620X.84B4.11947.

[32]Perrott, D. H., H. Umeda, and L. B. Kaban. Costochondral graft construction/reconstruction of the ramus/condyle unit: long-term follow-up. *Int. J Oral. Maxillofac. Surg.* 23:321–328, 1994. doi:10.1016/S0901-5027(05)80046-3.

[33]Pietila, K., T. Kantomaa, P. Pirttiniemi, and A. Poikela. Comparison of amounts and properties of collagen and proteoglycans in condylar, costal and nasal cartilages. *Cells Tissues Organs.* 164:30–36, 1999. doi:10.1159/000016640.

[34]Popko, J., P. Szeparowicz, B. Sawicki, S. Wolczynski, and J. Wojnar. Rabbit articular cartilage defects treated with cultured costal chondrocytes (preliminary report). *Folia Morphol. (Warsz)* 62:107–112, 2003.

[35]Ross, R. B. Costochondral grafts replacing the mandibular condyle. *Cleft Palate Craniofac. J.* 36:334–339, 1999. doi:10.1597/1545-1569(1999)036<0334:CGRTMC>2.3.CO;2.

[36]Saadeh, P. B., B. Brent, B. J. Mehrara, D. S. Steinbrech, V. Ting, G. K. Gittes, and M. T. Longaker. Human cartilage engineering: chondrocyte extraction, proliferation, and characterization for construct development. *Ann. Plast. Surg.* 42:509–513, 1999. doi:10.1097/00000637-199905000-00008.

[37]Samman, N., L. K. Cheung, and H. Tideman. Overgrowth of a costochondral graft in an adult male. *Int. J. Oral Maxillofac. Surg.* 24:333–335, 1995. doi:10.1016/S0901-5027(05)80484-9.

[38]Samman, N., L. K. Cheung, and H. Tideman. Variations in costochondral grafting of the mandibular ramus. *Ann. R. Australas. Coll. Dent. Surg.* 13:144–153, 1996.

[39]Sindelar, B. J., S. P. Evanko, T. Alonzo, S. W. Herring, and T. Wight. Effects of intraoral splint wear on proteoglycans in the temporomandibular joint disc. *Arch. Biochem. Biophys.* 379:64–70, 2000. doi:10.1006/abbi.2000.1855.

[40]Steinberg, M. S., and M. Takeichi. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl. Acad. Sci. U.S.A.* 91:206–209, 1994. doi:10.1073/pnas.91.1.206.

[41]Stockwell, R. A. The cell density of human articular and costal cartilage. *J. Anat*. 101:753–763, 1967.
[42]Szeparowicz, P., J. Popko, B. Sawicki, S. Wolczynski, and M. Bierc. Comparison of cartilage self repairs and repairs with costal and articular chondrocyte transplantation in treatment of cartilage defects in rats. *Rocz. Akad. Med. Bialymst*. 49(Suppl. 1):28–30, 2004.
[43]Tay, A. G., J. Farhadi, R. Suetterlin, G. Pierer, M. Heberer, and I. Martin. Cell yield, proliferation, and postexpansion differentiation capacity of human ear, nasal, and rib chondrocytes. *Tissue Eng*. 10:762–770, 2004. doi:10.1089/1076327041348572.
[44]Ting, V., C. D. Sims, L. E. Brecht, J. G. McCarthy, A. K. Kasabian, P. R. Connelly, J. Elisseeff, G. K. Gittes, and M. T. Longaker. In vitro prefabrication of human cartilage shapes using fibrin glue and human chondrocytes. *Ann. Plast. Surg*. 40:413–420, 1998; discussion 420–421.
[45]Trumpy, I. G., and T. Lyberg. In vivo deterioration of proplast-teflon temporomandibular joint interpositional implants: a scanning electron microscopic and energy-dispersive X-ray analysis. *J. Oral Maxillofac. Surg*. 51:624–629, 1993.
[46]Trumpy, I. G., and T. Lyberg. Surgical treatment of internal derangement of the temporomandibular joint: long-term evaluation of three techniques. *J. Oral Maxillofac. Surg*. 53:740–746, 1995; discussion 746–747.
[47]Trumpy, I. G., B. Roald, and T. Lyberg. Morphologic and immunohistochemical observation of explanted Proplast-Teflon temporomandibular joint interpositional implants. *J. Oral Maxillofac. Surg*. 54:63–68, 1996; discussion 68–70.
[48]Walles, T., B. Giere, P. Macchiarini, and H. Mertsching. Expansion of chondrocytes in a three-dimensional matrix for tracheal tissue engineering. *Ann. Thorac. Surg*. 78:444–448, 2004; discussion 448–449.
[49]Ware, W. H., and S. L. Brown. Growth centre transplantation to replace mandibular condyles. *J. Maxillofac. Surg*. 9:50–58, 1981. doi:10.1016/S0301-0503(81)80012-4.
[50]Woessner, J. F. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch. Biochem. Biophys*. 93:440–447, 1961. doi:10.1016/0003-9861(61)90291-0.
[51]Wong, M. E., K. D. Allen, and K. A. Athanasiou. Tissue engineering of the temporomandibular joint. In: Biomedical Engineering Handbook Third Edition: Tissue Engineering and Artificial Organs, edited by J. D. Bronzino. CRC Press, 2006, pp. 51-1–52-22.
[52]Xu, J. W., V. Zaporojan, G. M. Peretti, R. E. Roses, K. B. Morse, A. K. Roy, J. M. Mesa, M. A. Randolph, L. J. Bonassar, and M. J. Yaremchuk. Injectable tissue-engineered cartilage with different chondrocyte sources. *Plast. Reconstr. Surg*. 113:1361–1371, 2004. doi:10.1097/01.PRS.0000111594.52661.29.
[53]Yotsuyanagi, T., M. Mikami, M. Yamauchi, Y. Higuma, S. Urushidate, and K. Ezoe. A new technique for harvesting costal cartilage with minimum sacrifice at the donor site. *J. Plast. Reconstr. Aesthet. Surg*. 59:352–359, 2006. doi:10.1016/j.bjps.2005.04.049.