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Tissue Engineering Auricular Cartilage Using Late Passage Human Auricular Chondrocytes

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Abstract

Purpose—The significant shortcomings associated with current autologous reconstructive options for auricular deformities have inspired great interest in a tissue engineering solution. A major obstacle in the engineering of human auricular cartilage is the availability of sufficient autologous human chondrocytes. A clinically obtainable amount of auricular cartilage tissue (i.e. one gram) only yields approximately ten million cells, where 25 times this amount is needed for the fabrication of a full-scale pediatric ear. It is thought that repeated passaging of chondrocytes leads to de-differentiation and loss of the chondrogenic potential. However, little to no data exists regarding the ideal number of times that human auricular chondrocytes can be passaged in a manner that maximizes the cellular expansion while minimizing dedifferentiation.

Methods—Human auricular chondrocytes (HAuCs) were isolated from discarded otoplasty specimens. The HAuCs were then expanded and cells from passage 3, 4, and 5 were encapsulated into 8mm diameter discs made from type I collagen hydrogels with a cell density of 25 million cells/mL. The constructs were implanted subcutaneously in the dorsa of nude mice, and harvested after 1 and 3 months for analysis.

Results—Constructs containing passage 3, 4, and 5 chondrocytes all maintained their original cylindrical geometry. After 3 months *in vivo*, the diameters of the P3, P4, and P5 discs were 69±9%, 67±10%, and 73±15% of their initial diameter, respectively. Regardless of the passage number, all constructs developed a glossy white cartilaginous appearance, similar to native auricular cartilage. Histologic analysis demonstrated development of an organized perichondrium composed of collagen, a rich proteoglycan matrix, cellular lacunae, and a dense elastin fibrin network by Safranin-O and Verhoeff's stain. Biochemical analysis confirmed similar amounts of proteoglycan and hydroxyproline content in late passage constructs when compared to native auricular cartilage.

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Disclosures/Conflicts of Interest

Lawrence Bonassar is a co-founder and equity holder in 3D BioCorp. No competing financial interests exist for other authors.

Conclusion—These data indicate that late passage human auricular chondrocytes (up to passage 5) form elastic cartilage that is histologically, biochemically, and biomechanically similar to native human elastic cartilage and have the potential to be used for auricular cartilage engineering.

Introduction

Microtia is the most common congenital auricular anomaly, occurring in as many as 17 per 10,000 births [1 2]. If left uncorrected, auricular disfigurement can cause significant psychological distress and impact psychosocial function as a child matures [2 3 4 5 6 7]. In addition to congenital absence or malformation of the ear, acquired auricular deformities secondary to trauma or oncologic resection occur in 1 per 500 people annually [2 8 9].

The current gold standard for reconstruction of auricular defects is the use of autologous costal cartilage harvested from the abdomen, meticulously sculpted to recapitulate the native auricle, and implanted under the peri-auricular skin [10 11]. The benefits of this approach include the lack of antigenicity of the autologous construct and therefore long-term stability [1 10 12 13 14]. Despite these advantages, significant drawbacks remain and have been tolerated for lack of a better alternative. First, harvesting of costal cartilage is a painful procedure for a young child to endure and frequently leaves the patient with a visible chest deformity. [15 16]. Further, fibrous costal cartilage poorly mimics the mechanical properties of elastic auricular cartilage, resulting in inflexible and rigid tissue. Finally, this procedure requires tremendous technical expertise, as accurately sculpting a patient specific auricular facsimile is tremendously difficult [12 17 18].

Given the difficulties associated with autologous ear reconstruction, surgeons and researchers have long sought a tissue engineering solution for auricular reconstruction. Such a strategy would entail several components, including an appropriate cell source (i.e. patient derived auricular chondrocytes), the means to render the patient's normal auricle in three dimensions with a high resolution, as well as the capability to then transform that image into a three-dimensional biocompatible scaffold. The scaffold, comprised of naturally derived and/or synthetic biocompatible and biodegradable materials, provides a supportive environment for the deposition of elastic cartilage matrix by chondrocytes which results in the transformation of the scaffold into auricular neocartilage while maintaining the original geometry.

The ideal cell source for this strategy would be autologous auricular chondrocytes (AuCs) derived from the patient's microtic ear or, if necessary, from a small biopsy of the patient's unaffected, contralateral conchal bowl [19]. However, the use of patient specific AuCs is limited by their availability. A biopsy of ~1 g of auricular cartilage provides ~ 10 million cells [19], significantly less than the 200–250 million required to form a full-scale pediatric auricle [20 21]. In our previous work, we have found that 1 gram of elastic cartilage can be expanded to generate ~138 million AuCs after three passages. Whether human AuCs may be expanded further to the requisite number needed to fabricate a full scale ear scaffold, remains unknown [20]. Most previous studies reported that chondrocytes rapidly de-differentiate into fibroblast-like cells when expanded beyond the second passage, ultimately losing chondrogenic capacity [22 23 22 24]. These studies, however, used articular

chondrocytes isolated either from non-human animals or from discarded arthroplasty procedures [23 25 26 27]. Relatively few studies have investigated the use of late passage auricular chondrocytes to engineer elastic neocartilage and none have used only late passage chondrocytes as their sole cell source but instead mixed passaged chondrocytes with cryopreserved P0 cells or the addition of various types of supplemented media *in vitro* [13 28 29 30]. Herein, we examine the capacity of using late passage (P3–P5) human auricular chondrocytes (HAuCs) in standard cell culture conditions to form elastic cartilage *in vivo*.

Material and Methods

Ethics Statement

All animal care and experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals [31] and were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee (protocol # 2011-0036). Ear cartilage remnants were obtained by surgeons within their private practices with informed consent, and thus exempt from IRB approval.

Isolation of human auricular chondrocytes

Human auricular chondrocytes (HAuCs) were isolated as previously described [21 20 32]. Briefly, ear cartilage was obtained from otoplasty procedures (a 14-year old female and a 12-year old male). Under sterile conditions, auricular cartilage was dissected from the surrounding perichondrium, diced into 1 mm³ pieces, and digested overnight in 0.2% collagenase (Worthington Biochemicals Corp., Lakewood, NJ), Dulbecco's modified Eagle's medium (DMEM) (MediaTech Inc., Manassas, VA), 100 µg ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin. The following day, HAuCs were filtered, washed with 1x phosphate buffered saline (PBS) (MediaTech) with 100 µg ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, and counted.

Passaging of human auricular chondrocytes

HAuCs were plated at ~10,000 cells/cm² and cultured in DMEM with 10% fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 100 µg ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, and 0.1 mM non-essential amino acids under 5% pCO₂ and at 37 °C. HAuCs were expanded and split 3:1 at each passage using 0.25% trypsin (MediaTech) to release. At passages three, four, and five (P3, P4, P5 respectively), a portion of the HAuCs were encapsulated into hydrogel discs, as outlined in the following section.

Hydrogel disc construct fabrication

Type I collagen stock was extracted from rat tails, lyophilized, and reconstituted as previously described [33 34]. At the time of construct fabrication, collagen stock solution was neutralized to a pH of 7.0 and maintained at 300 mOsm by mixing with the appropriate volumes of 1N NaOH (Sigma-Aldrich, St. Louis, MO), 10x Phosphate Buffered Saline (PBS), and 1x PBS as previously described [35]. Cells from P3, P4, or P5 were mixed with neutralized collagen and formed into discs as previously described [32]. Briefly, HAuCs were resuspended in PBS and homogeneously mixed with neutralized collagen for a final cell density of 25 million cells ml⁻¹ and collagen density of 10 mg ml⁻¹. The cellularized

collagen was then extruded between two glass plates to form 2 mm thick sheet gels and allowed to undergo thermal gelation for 1 hour at 37 °C. Subsequently, an 8 mm dermal biopsy punch was used to create uniform diameter hydrogel discs, which were placed in the same media used for cell expansion and implanted within 48 hours.

Construct implantation and explantation

Hydrogel constructs were implanted as previously described [32]. Briefly, 10-week old male athymic nude mice (NU/NU; Charles River, Wilmington, MA) were anesthetized and prepped. Four medial vertical incisions, 2 on each side, were made on the dorsum of each mouse, followed by minimal dissection of the subcutaneous tissue to create pockets just large enough to accommodate 1 disc. Four discs from a single passage (P3, P4, or P5) were implanted into each mouse, with one disc per subcutaneous pocket. Incisions were closed with interrupted nylon sutures, and an overlying sterile, occlusive dressing was placed prior to reversal from anesthesia.

At 1 and 3 months, animals were sacrificed using carbon dioxide asphyxiation with cervical dislocation. Discs were harvested, weighed, measured in height and diameter, and imaged. One disc per mouse was fixed in 10% neutral buffered formalin for 48 hours and then transferred to 70% ethanol for histologic analyses. The remaining discs were flash frozen in liquid nitrogen for biochemical and biomechanical analyses. A total of 94 discs were explanted for analysis, 32 from P3, 32 from P4, and 30 from P5.

Histological analyses

Formalin-fixed samples were dehydrated via sequential washes in ethanol, embedded in paraffin, and cut into 5 µm thick sections. Sections were stained with Safranin O/Fast green to assess proteoglycan distribution, Picrosirius red to assess collagen organization, and Verhoeff's/Van Gieson to assess presence of elastin fibers. Images were taken in bright-field at 100×, 200×, and 400× using a Nikon Exclipse TE2000-S microscope (Nikon Instruments, Melville, NY) fitted with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI).

Biochemical analyses

Biochemical analyses were performed as previously described [36]. Briefly, frozen disc constructs were thawed, weighed, frozen, lyophilized, and re-weighed. Samples were digested with 1.25 mg ml⁻¹ papain (Sigma-Aldrich) solution at 60°C overnight, and analyzed for DNA content via the Hoechst DNA assay [37], sulfated glycosaminoglycan (GAG) content via a modified 1,9-dimethylmethylene blue (DMMB) assay [38] and collagen and elastin via a hydroxyproline assay [39]. Biochemical properties were reported normalized to sample wet weight (WW).

Mechanical analysis

Three mm diameter by one mm height cylinders were cut from the center of each frozen disc construct. Confined compression testing was performed as previously described [32 42]. Briefly, samples were thawed in PBS containing protease inhibitors (Roche Diagnostics, Indianapolis, IN) and placed in a cylindrical confining chamber mounted in an ELF 3200

test frame (Enduratec, Eden Prairie, MN). Samples were compressed to 50% of their original height in 10 steps of 50 μm , with 5 minutes between steps to allow for full stress relaxation. Resultant stresses were recorded at 1 Hz and the temporal profiles of stress were fit to a poroelastic model of tissue behavior using custom MATLAB (MathWorks, Natick, MA) code to calculate the equilibrium modulus and hydraulic permeability [36].

Statistical analysis

Statistical analysis was performed using SigmaPlot 11.0 (Systat Software, Inc, San Jose, CA). Data were analyzed by 2-way ANOVA with Tukey's post hoc analysis. A value of $P < 0.05$ was used as a threshold for statistical significance. All data are expressed as means plus one standard deviation.

Results

Gross Analysis

Disc constructs from all passages (P3, P4, and P5) maintained a cylindrical geometry following both 1 and 3 months of implantation. Collagen hydrogel discs were translucent and ranged from colorless to pink prior to implantation (Figure 1A), whereas after 1 and 3 months *in vivo* all constructs developed a white, shiny auricular cartilage like appearance (Figure 1B). Prior to implantation, the disc constructs were soft and required delicate manipulation, but after explantation, all discs featured gross flexibility similar to native auricular cartilage when handled.

Although all disc constructs experienced some contraction after 3 months *in vivo*, the contraction was consistent between the various cell passages and all passages generated nearly equivalent amounts of auricular cartilage. The diameters of the P3, P4, and P5 discs were $69 \pm 9\%$, $67 \pm 10\%$, and $73 \pm 15\%$ of their initial diameter, respectively. Although the diameters at 3 months were consistently significantly higher than the diameters at 1 month, no differences existed between the various passages disc diameter at 3 months ($P > 0.05$, Figure 2A), likely reflecting the different patient cell sources used for the 1 and 3 month time points. P3, P4, and P5 discs retained $55 \pm 6\%$, $59 \pm 11\%$, and $47 \pm 9\%$ of initial construct height, respectively (Figure 2B). Three-month discs with P5 cells displayed significantly less height retention compared to discs at 1 month or discs with P4 cells, but importantly were not different from P3, 3 month discs ($P > 0.05$). In addition to maintaining geometry, all constructs generated a final explant mass between 16.4 and 24.5 mg, with no significant differences between the various cell passage discs or time points ($P > 0.05$, Figure 2C)

Histologic Analyses

Implanted disc constructs generated auricular cartilage-specific extracellular matrix and microstructure following 3 months *in vivo*. Picrosirius red staining showed the formation of bundled collagen fibers along the construct surface in discs from all passages, resembling the perichondrial layer characteristic of native ear cartilage (Figure 3A–C). The bundling of collagen was also apparent after only 1 month *in vivo* (Figure S1A–C). Safranin-O staining showed the formation of rich proteoglycan matrix within the interior bulk of the disc tissue after 3 months for all passages (Figure 3D–F). In addition, all discs displayed development

of cellular lacunae within proteoglycan-dense tissue, while cells within the perichondrium were smaller, bearing a resemblance to fibroblasts. Verhoeff's staining showed the development of a nascent elastin fiber network in the interior of the tissue in disc constructs from all passages at 3 months (Figure 3G–I). In contrast, after only one month *in vivo*, P3, P4, and P5 discs displayed less dense staining for proteoglycans, with no evidence of elastin fiber formation at this time point (Figure S1D–I).

Biochemical Analyses

Implanted disc constructs demonstrated maturation towards auricular cartilage biochemical composition, in terms of water, cellular, proteoglycan, collagen, and elastin content. Discs containing P5 HAuCs were $92\pm2\%$ and $92\pm2\%$ water at 1 month and 3 months, respectively, significantly higher than those with P3 ($86\pm3\%$ and $86\pm4\%$) or P4 ($84\pm6\%$ and $85\pm6\%$) cells (Figure 4A, $P < 0.05$). DNA normalized to construct wet weight (WW) was analyzed to represent cellular content. After 3 months, discs displayed 1.7 ± 1.2 , 3.4 ± 1.7 , and 1.7 ± 2.4 μg DNA per mg WW for P3, P4, and P5, respectively (Figure 4B). In total, the DNA content of constructs after 3 months was significantly higher than at 1 month ($P < 0.05$).

Disc constructs developed key auricular cartilage extracellular matrix components during *in vivo* maturation. All constructs displayed development of proteoglycan content, measured by sulfated glycosaminoglycans (GAG), after 1 and 3 months of implantation. GAG content normalized to WW was significantly higher in discs after 3 months compared to 1 month, with P3 discs containing 7.8 ± 5.5 $\mu\text{g}/\text{mg}$, P4 containing 8.9 ± 5.6 $\mu\text{g}/\text{mg}$, and P5 containing 9.9 ± 2.6 $\mu\text{g}/\text{mg}$ (Figure 4C, $P < 0.05$). Collagen and elastin composition, measured by hydroxyproline content, was present in all implanted discs, with no statistical difference observed between time points or cell passage number (Figure 4D, $P < 0.05$).

Mechanical Analysis

Disc constructs displayed increased mechanical stiffness following implantation. Through confined compression testing, auricular cartilage formed from all cell passages displayed equilibrium moduli, indicative of stiffness, on the order of hundreds of kPa, showing cartilage maturation (Figure 5). Constructs at 3 months displayed significantly higher equilibrium moduli compared to 1 month discs ($P < 0.05$) while no differences were observed between cell passages.

Discussion

In this study, we set out to determine whether late passage (P3–P5) auricular chondrocytes cultured under standard conditions could be used to engineer auricular cartilage *in vivo*. These data indicate that human AuCs expanded as far as the fifth passage successfully generate robust auricular cartilage as demonstrated by the elaboration of gross cartilage-like appearance with retention of overall construct shape and the development of elastic cartilage with the microstructure, biochemical, and mechanical properties similar to human elastic cartilage. After three months, all disc constructs developed key auricular cartilage matrix structures, including a perichondrial layer, dense proteoglycan deposition, cellular lacunae,

and elastin fibers, similar to those observed in native human auricular cartilage [13 28] and in previous tissue engineered auricular cartilage containing cells of bovine origin [21 32].

We have previously demonstrated successful generation of auricular cartilage by combining collagen hydrogels with bovine cells, utilizing both AuCs alone [20 21] and in combination with mesenchymal stem cells [32]. However, clinical translation of auricular cartilage tissue engineering requires cells of human origin. Autologous human AuCs can be acquired from a patient, either from a microtic ear remnant [43] or the healthy contralateral conchal bowl [19]. This small amount (~1g) of cartilage tissue would only yield ~10 million HAuCs [19], meaning that a significant challenge remains in generating a full-size human ear, which requires over 200 million cells to produce [20 21].

It has been commonly accepted that chondrocyte expansion beyond second passage results in “de-differentiation”, marked by decreased deposition of chondrogenic matrix materials (i.e. type II collagen) and increased fibroblastic behavior (i.e. type I collagen deposition) [24 22]. This change in phenotype may result from chondrocytes being removed from their physiologic three-dimensional cartilage matrix and placed in a two-dimensional monolayer, which abrogates normal extrusion of extracellular matrix components [23 44]. However, with minimal exceptions, most of these data were obtained from studies of articular chondrocytes.

Not surprisingly, the few studies that did utilize late passage human auricular chondrocytes, were no more optimistic in their assessment of their utility for cartilage engineering. Mandl *et al* found that HAuCs expanded through P4 had significantly decreased GAG content and type II collagen expression with passaging, as less than 20% of cells stained positive for type II collagen by P4 [19]. Rodriguez *et al* explored the de-differentiation of extensively expanded HAuCs, finding that cells incubated *in vitro* for greater than 6 weeks were significantly less likely to generate healthy cartilage tissue following implantation [44]. However, because this study did not report passage number, and the cartilage tissue generated was characterized only by gross appearance and deposition of elastin fibers, without quantitative measurement of biochemical and biomechanical maturation, it is difficult to assess the true effect of late passaging on the cell phenotype. [44].

Perhaps because of established dogma regarding the use of late passaged chondrocytes, several other studies that have explored AuC expansion did not analyze chondrocyte behavior beyond the third passage. Expansion of human ear, nasal, and rib chondrocytes through the second passage found significant decreases in collagen type II mRNA levels between P1 and P2, and did not attempt further expansion [30]. Another study used both sheep and human AuCs, in which the cells were isolated and expanded through P3, then combined with primary (P0) AuCs and implanted in mice [28]. While the sheep P0–P3 combination generated auricular cartilage, the human cell implants generated fibrous tissue containing no type II collagen, proteoglycans, or elastin. Similarly, other groups have investigated the chondrogenic capacity of AuCs by combining of passaged chondrocytes with primary cells, however all were still limited to only two passages, which will not produce sufficient yield to generate large engineered tissue [25 26].

Alternatively, several investigators have accepted the hypothesized de-differentiation process and have attempted to salvage 2D expanded AuCs by returning them to three-dimensional culture conditions in hopes of coaxing chondrocytes into a more differentiated phenotype, with varying degrees of success. [22 23 25 26 27]. Others have used growth factors [27 45] to prevent de-differentiation. Tseng *et al* found that expanding human AuCs in basic fibroblast growth factor (bFGF) conditioned media failed to prevent dedifferentiation by P3 [28].

To the best of our knowledge, the current study provides the first evidence that human auricular chondrocytes can be passaged up to 5 times in monolayer culture and still retain the ability to produce robust human auricular cartilage within a three-dimensional construct *in vivo*. The use of late passage auricular chondrocytes may be crucial for successful translation of auricular tissue engineering strategies, as it allows for expansion of patient specific donor chondrocytes to the requisite number needed to engineer a full-scale ear construct. The successful generation of auricular cartilage from extensively expanded HAuCs, in contrast to previous published literature, may reside in both our abundant experience working with primary human tissue (a factor which involves a steep learning curve and is not available to all laboratories) and the use of human tissue samples from healthy pediatric patients.

Despite this success, certain limitations must still be overcome. Although the disc constructs retained cylindrical geometry following implantation, there was significant loss of volume. In addition, time points beyond 3 months are necessary to demonstrate long-term stability of the generated tissue.

In summary, contrary to previously published findings, we have demonstrated that late passage human auricular chondrocytes can produce robust elastic cartilage that is equivalent to native tissue. As a result of the geometrically increased number of HAuCs available after each passage, a sufficient number of autologous auricular chondrocytes may now be available from each patient to allow for fabrication of a full sized auricular scaffold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Gross images of tissue engineered disc constructs prior to (A) and following (B) implantation. Implanted constructs with AuCs from all passages displayed gross shape retention after 1 and 3 months *in vivo*, with all constructs generating shiny, white, cartilage-like appearances. All scale bars = 4 mm. Pre-implant disc containing P3 AuCs.

Figure 2.

Analysis of disc construct geometry (**A,B**) and amount of cartilage tissue generation (**C**). All constructs contracted from initial dimensions. No significant differences observed between passage groups or time points for final tissue mass. * indicates significant difference from P3 within time point. % indicates significant difference from P4 within time point. # indicates significant difference from 1 Month within passage number. Bar indicates significant difference for all constructs from 1 Month. $P < 0.05$, $n = 10-16$.

Figure 3. Histological evaluation of auricular cartilage microstructure in disc constructs following 3-month implantation. Constructs from P3, P4, and P5 all displayed similar microstructural components and organization after 3 months. Picrosirius Red staining (A–C) displayed formation of bundled collagen fibers at the tissue surface, resembling a perichondrial layer (PC). Safranin-O staining (D–F) revealed deposition of rich proteoglycan matrix within discs. Verhoeff staining (G–I) showed initial formation of an elastic fiber (EF) network within the bulk of the cartilage tissue. All scale bars = 100 μ m. All images taken of 3-month constructs.

Figure 4.

Biochemical composition of generated auricular cartilage tissue in implanted disc constructs. Constructs maintained water content (A) from 1 to 3 months, featured increased DNA (B) and glycosaminoglycan (GAG) (C) content between time groups, and displayed no significant differences in hydroxyproline content (D), representative of collagen and elastin, between time or passage groups. * indicates significant difference from P3 within time point. % indicates significant difference from P4 within time point. # indicates significant difference from 1 Month within passage number. Bar indicates significant difference for all constructs from 1 Month. DNA, GAG, and hydroxyproline content normalized to construct wet weight (WW). $P < 0.05$, $n = 10-12$.

Figure 5.

Mechanical analysis of tissue engineered disc constructs following implantation. The equilibrium modulus, representative of tissue compressive stiffness, increased significantly between 1- and 3-month implantation of disc constructs. # indicates significant difference from 1 Month within passage number. Bar indicates significant difference for all constructs from 1 Month. $P < 0.05$, $n = 8-12$.