Development of Scaffold-Free Elastic Cartilaginous Constructs with Structural Similarities to Auricular Cartilage

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External ear reconstruction with autologous cartilage still remains one of the most difficult problems in the fields of plastic and reconstructive surgery. As the absence of tissue vascularization limits the ability to stimulate new tissue growth, relatively few surgical approaches are currently available (alloplastic implants or sculpted autologous cartilage grafts) to repair or reconstruct the auricle (or pinna) as a result of traumatic loss or congenital absence (e.g., microtia). Alternatively, tissue engineering can offer the potential to grow autogenous cartilage suitable for implantation. While tissue-engineered auricle cartilage constructs can be created, a substantial number of cells are required to generate sufficient quantities of tissue for reconstruction. Similarly, as routine cell expansion can elicit negative effects on chondrocyte function, we have developed an approach to generate large-sized engineered auricle constructs ($\geq 3 \text{ cm}^2$) directly from a small population of donor cells (20,000–40,000 cells/construct). Using rabbit donor cells, the developed bioreactor-cultivated constructs adopted structural-like characteristics similar to native auricular cartilage, including the development of distinct cartilaginous and perichondrium-like regions. Both alterations in media composition and seeding density had profound effects on the formation of engineered elastic tissue constructs in terms of cellularity, extracellular matrix accumulation, and tissue structure. Higher seeding densities and media containing sodium bicarbonate produced tissue constructs that were closer to the native tissue in terms of structure and composition. Future studies will be aimed at improving the accumulation of specific tissue constituents and determining the clinical effectiveness of this approach using a reconstructive animal model.

Introduction

RECONSTRUCTION OF THE EXTERNAL EAR (total or partial) remains one of the most difficult challenges in reconstructive and plastic surgery.^{1–4} However, there are a few approaches currently available for external ear reconstruction, such as alloplastic implants and sculpted autologous cartilage grafts—both of which have their advantages and disadvantages. While the availability of alloplastic implants in the shape of the ear is typically not an issue, these implants are susceptible to both infection and immunogenic issues and, as such, their long-term durability is uncertain.^{3,5} Sculpted autologous cartilage grafts, alternatively, have good long-term durability as well as the potential to grow with the patient.^{2,3,5} In contrast to alloplastic implants, sculpted autologous cartilage grafts require shaping at the time of surgery, can result in donor-site morbidity,^{2,5} and patients must have a sufficient supply of donor cartilage to be candidate for this approach.^{2,3} Alternatively, tissue engi-

neering can offer the potential to overcome the limitations with these conventional surgical approaches as autologous cartilaginous tissue can be grown *in vitro*.^{2,6} In addition, several studies have demonstrated that tissue-engineered cartilage constructs can precisely be developed in the predetermined shape of the external ear using cell seeded-polymer constructs.^{2,7} Despite this potential, recapitulation of the native structure of auricle cartilage and cell sourcing remain as technical hurdles toward the development of functional tissue-engineered implants.^{1,6}

Auricle cartilage is an elastic cartilaginous tissue with a distinct structure. The central cartilaginous region is similar to hyaline cartilage as it is rich in collagen type II fibers and large aggregating proteoglycans while also possessing abundant elastic fibers (cross-linked elastin and associated microfibrils). The sparsely populated chondrocytes that reside in this region are quite large in size (compared to chondrocytes in hyaline cartilage) and are responsible for the maintenance and remodeling of this tissue.^{8,9} This

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central cartilaginous region is surrounded by two layers of dense connective tissue on both sides and referred to as the perichondrium.¹⁰ The perichondrium, believed to be essential for the growth and maintenance of elastic cartilage,^{2,9–11} is rich in collagen type I fibers, small nonaggregating proteoglycans, and contains numerous fibroblastic-like cells. However, the cells in the inner layer of the perichondrium (adjacent to the cartilaginous region), while resembling fibroblasts, are chondroblasts and can easily differentiate into chondrocytes.^{10–15}

Although the use of autologous cells in tissue engineering applications is preferable,^{3,16} only a reasonable number of cells can be harvested from an individual. To overcome this limitation, a common approach has been to include a cell expansion phase before seeding the cells in, or on, a suitable scaffolding material.^{11,17–19} Although successful in producing large quantities of cells, routine cell expansion of chondrocytes tends to elicit negative side effects, such as dedifferentiation and loss of potency,^{20–22} thereby requiring a vast number of cells to synthesize sufficient quantities of engineered tissue. Alternatively, other sources of chondrocytes (e.g., costal) have been explored for auricular cartilage tissue engineering^{3,23–27}; however, the cytological differences between these cells and elastic chondrocytes may make it difficult to recapitulate a functional elastic cartilaginous extracellular matrix (ECM). Choosing the appropriate *in vitro* model and creating a microenvironment that sustains elastin production may be important to tissue engineer elastic cartilage, but the prerequisites for elastin formation are still largely unknown.¹⁰ To overcome these limitations, we have recently developed an approach to generate hyaline cartilaginous tissue directly from limited population of donor cells without a separate expansion phase.²⁸ Long-term bioreactor cultivation of articular chondrocytes resulted in the formation of large, scaffold-free cartilaginous tissue constructs (as little as 20,000 cells to create 3 cm² of engineered tissue). Upon implantation, the constructs were able to repair critical-sized defects and also adopted the structure of native articular cartilage.²⁹ Due to these promising results, the purpose of this study was to investigate whether this approach could be used to create scaffold-free auricular cartilaginous constructs that possess the distinct structure of elastic cartilage from a limited population of extracted cells.

Materials and Methods

Rabbit auricular chondrocyte harvest and isolation

This study was performed with the approval from the University Animal Care Committee (UACC) at the Queen's University. Full thickness auricular cartilage was harvested from the ear of adolescent female New Zealand white rabbits (2 ± 0.5 kg; ~12 weeks old) (Charles River Laboratories, Wilmington, MA) to reflect the general age of patients undergoing auricular reconstruction. Ears were completely dissected free of skin with the inner chondrogenic layer and the outer perichondrial layer were kept entirely intact. The tissue was cut in small fragments and digested with 0.5% protease (w/v) (Sigma-Aldrich, Oakville, Canada) followed by 0.15% collagenase A (w/v) (Roche Diagnostics Canada, Laval, QC, Canada) in Ham's F12 media (Hyclone, Logan, UT) overnight at 37°C with 95% relative humidity and 5% CO₂. Auricular chondrocytes

were isolated by centrifugation (700 g for 7 min) with the resultant cell pellet washed three times with Ham's F12 media. Viable cells, determined by Trypan Blue dye (Sigma-Aldrich) exclusion,³⁰ were then seeded in low-density monolayers in a continuous flow bioreactor in Ham's F12 media (Hyclone) supplemented with 25 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (Sigma-Aldrich) and an antibiotic solution containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Sigma-Aldrich).^{21,22,31} To minimize inter-animal variability, tissue was obtained from several ears (up to three per experiment) and pooled together.

Continuous flow bioreactor

A continuous flow bioreactor system was used to maintain a constant medium supply to the developing construct in a low-shear environment.^{28,32,33} Briefly, the reactor consisted of multichannel vented polypropylene chambers to house single constructs (3 cm² containing a maximum media volume of 4 mL). A constant flow of fresh media (from aerated reservoirs) at 10 µL/min was provided by a peristaltic pump (Ismatec; Cole Parmer Canada, Anjou, Canada) with waste media collected in a vented reservoir. The bioreactor was housed in an incubator maintained at 37°C with 95% relative humidity and 5% CO₂.

Effect of different seeding densities and media compositions

To determine the most effective method to synthesize large elastic cartilaginous constructs in reactor culture (≥ 3 cm²), two different seeding densities and media formulations were investigated. Cultures were seeded at either 6500 or 13,000 isolated cells/cm² (i.e., 20,000 or 40,000 cells/well) directly on the bottom surface of the reactor wells. These seeding densities reflect multiples of a routine cell passaging density (6500 cells/cm²). Similarly, constructs (from both seeding densities) were cultured in the media (Ham's F12 supplemented with 20 mM HEPES, 20% FBS, 100 µg/mL ascorbic acid, and antibiotics) in the presence or absence of 14 mM of sodium bicarbonate (NaHCO₃) to stimulate synthesis and accumulation of cartilaginous ECM.²⁸ After seeding, all preparations were maintained under no-flow conditions for 48 h. Preparations were then cultured under a constant media flow rate of 10 µL/min for a period of 4 weeks. Media reservoirs were changed every 2–3 days and supplemented with fresh ascorbic acid and antibiotics. After the 4-week culture period, developed constructs were harvested, weighed (wet weight), and then divided into three parts and processed for histological/immunohistochemical evaluation, biochemical analyses, and transmission electron microscopy (TEM), respectively. Four separate trials of this study were conducted (*n* = 8 samples/group).

Histological evaluation

Engineered constructs as well as native auricular cartilage tissue samples were fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.2) for 24 h, dehydrated in graded ethanol solutions, and embedded in paraffin at 65°C. Thin (5 µm thick) sections were cut and mounted on Superfrost slides (Fisher Scientific, Mississauga, Canada) and dried for

FIG. 1. Macroscopic appearance of the tissue generated from the monolayer cell preparations after 4 weeks of bioreactor culture. Scale bar: 1 cm. Color images available online at www.liebertpub.com/tea

24 h at 37°C. Sections were stained with either hematoxylin and eosin (H&E, a general connective tissue stain), safranin-O (proteoglycan stain), and Weigert's resorcin-fuchsin (elastin/elastic fiber stain).³⁴ Stained sections were examined by light microscopy using a Zeiss Axio-Image M1 microscope (Göttingen, Germany).

Tissue thickness measurements and mechanical testing

The thickness of the tissue constructs and native tissue samples were determined using the bright-field images of the histology sections. Bright-field images (at 20 $\times$) were digitally captured and calibrated using a calibration slide from which the thicknesses of the tissue samples could be determined using ImageJ software (Open Source; National Institutes of Health). Thickness measurements were then taken at 10 random locations throughout each section and the average value recorded. Mechanical testing of native auricular cartilage and engineered cartilage constructs was

FIG. 2. General architectural organization of the tissue generated from the monolayer cell preparations after 4 weeks in bioreactor culture compared to native auricular cartilage. (A) Native auricular cartilage. (B) Engineered construct generated from 6500 cells/cm² without sodium bicarbonate (NaHCO₃). (C) Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. (D) Engineered construct generated from 13,000 cells/cm² without NaHCO₃. (E) Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Hematoxylin and eosin stain; scale bar: 50 μ m. Color images available online at www.liebertpub.com/tea

performed using a Mach-1 Micromechanical Testing system (Biomomentum, Laval, QC, Canada) equipped with a 1 kg load cell (0.05 g resolution). Tissue mechanical properties (elastic modulus and Poisson's ratio at 37°C in Ham's F12 media) were determined using a double compressive indentation method^{35,36} using two plane-ended indentors (2 and 6 mm diameter). Compressive indentations were conducted at a ramp rate of 10% strain/s to a maximum of 10% strain. Samples were then allowed to equilibrate in media for ~20 min between indentations.

Immunochemical localization of elastin and collagen types

Immunofluorescence localization of elastin and collagen X in the engineered constructs and native auricular cartilage tissues samples was performed as previously described.²⁸

Briefly, after deparaffinization and dehydration, sections were enzymatically treated with 0.25 units/mL chondroitinase ABC (Sigma-Aldrich) in Tris-acetate buffer (40 mM Tris acetate with 1 mM EDTA), pH 8.5, for 1 h at 37°C followed by 0.25 units/mL Keratinase (Sigma-Aldrich) Tris-acetate buffer (40 mM Tris acetate with 1 mM EDTA), pH 8.5, for 30 min at 37°C to facilitate antibody binding. To reduce nonspecific protein binding, the sections were blocked with phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) for 30 min at room temperature. Sections were then incubated with mouse monoclonal antibodies against elastin (BA4 at 1:100 dilution; Abcam, Cambridge, MA) or with rabbit polyclonal antibodies against collagen type X (ab58632 at 1:150 dilution; Abcam), all diluted in phosphate buffer (pH 7.4) containing 1% BSA overnight at 4°C. Following primary antibody incubation, sections were rinsed in PBS (pH 7.4) and incubated with

FIG. 3. Proteoglycan organization of the tissue generated from the monolayer cell preparations after 4 weeks in bioreactor culture compared to native auricular cartilage. (A) Native auricular cartilage. (B) Engineered construct generated from 6500 cells/cm² without NaHCO₃. (C) Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. (D) Engineered construct generated from 13,000 cells/cm² without NaHCO₃. (E) Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Safranin-O stain; scale bar: 50 µm. Color images available online at www.liebertpub.com/tea

Texas Red (elastin) or FITC-labeled (collagen type X) anti-mouse or anti-rabbit secondary antibodies (1:200 dilution; Abcam) for 2 h at room temperature. The sections were counterstained with DAPI (Vector Laboratories, Inc., Burlingame, CA) and examined by fluorescent microscopy using a Zeiss Axio-Image M1 microscope with Axiovision software (Carl Zeiss, Oberkochen, Germany).

Immunohistochemical localization of collagen types I and II in the engineered constructs and native auricular cartilage tissues samples was performed as previously described.³⁷ Briefly, after deparaffinization and dehydration, sections were enzymatically treated with 0.05% of trypsin (pH 7.8) for 30 min at 37°C to facilitate antibody binding. Endogenous peroxidase activity was blocked with 1% H₂O₂ and 1% BSA in phosphate buffer for 30 min. Sections were then incubated with mouse monoclonal antibodies against collagen type I (I-8H5 at 40 µg/mL; Daiichi Fine Chemicals Co. Ltd.) or collagen type II (II-II6B3 at

187 µg/mL; Developmental Studies Hybridoma Bank, Iowa, IA), all diluted in phosphate buffer (pH 7.4) containing 1% BSA overnight at 4°C. Following primary antibody incubation, sections were rinsed in PBS (pH 7.4) and incubated with biotinylated anti-mouse secondary antibodies (Vector Laboratories) using the Vectastain Elite ABC kit (Vector Laboratories) for 2 h at room temperature, followed by incubation with diaminobenzidine for 6 min at room temperature. The sections were counterstained with Harris' hematoxylin and mounted in the permanent mounting medium. Stained sections were examined by light microscopy using a Zeiss Axio-Image M1 microscope (Göttingen, Germany).

For all immunohistochemical studies, nonspecific staining was assessed by the replacement of the primary antibody with nonimmune serum (data not shown). All the experiments were completed at least three times with no positive staining detected in the negative controls.

FIG. 4. Elastic fiber organization of the tissue generated from the monolayer cell preparations after 4 weeks in bioreactor culture compared to native auricular cartilage. (A) Native auricular cartilage. (B) Engineered construct generated from 6500 cells/cm² without NaHCO₃. (C) Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. (D) Engineered construct generated from 13,000 cells/cm² without NaHCO₃; (E) Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Arrows denote the presence of thin elastic fibers. Resorcin-fuchsin elastic fibers stain; scale bars: (A) 50 µm; (B–E) 20 µm. Color images available online at www.liebertpub.com/tea

Transmission electron microscopy

Engineered constructs and native auricular cartilage tissue samples were fixed in a Karnovsky solution for 2 h at room temperature. Samples were then postfixed in 1% osmium tetroxide for 1 h at 4°C, dehydrated in graded ethanol solutions, and embedded in epoxy resin Epon 812 (Electron Microscope Science, Hatfield, PA). Ultrathin sections (70 nm) were cut and collected on copper grids, stained with uranyl acetate and lead citrate, and examined using a LEO 906 transmission electron microscope (LEO Elektronenmikroskopie GmbH, Oberkochen, Germany).³⁸

Biochemical quantification of matrix constituents

Engineered construct samples were weighed again to determine the percent sample mass of the entire developed construct. The dry weight of the engineered construct as

well as native auricular cartilage tissue samples was then determined after overnight lyophilization. Samples were then digested by papain (bioreactor samples: 40 µg/mL; native tissue samples: 80 µg/mL in 20 mM ammonium acetate, 1 mM EDTA, and 2 mM dithiothreitol) for 72 h at 65°C and stored at -20°C until analysis. Aliquots of the digest were assayed separately for DNA, proteoglycan, and collagen content. The DNA assay was estimated using Hoechst 33258 dye (Sigma-Aldrich)³⁹ with calf thymus DNA as the standard (Sigma-Aldrich). The proteoglycan content was estimated by quantifying the amount of sulfated glycosaminoglycans using 1,9-dimethylmethylene blue dye binding assay^{40,41} with chondroitin sulfate sodium salt from bovine cartilage as the standard (Sigma-Aldrich). Total collagen content (combined content of all collagen types) was determined by the determination of hydroxyproline content. Briefly, aliquots of the papain digest were hydrolyzed in 6 N HCl for 18 h at 110°C, and the hydroxyproline

FIG. 5. Immunohistochemical localization of elastin in the engineered elastic cartilaginous constructs generated from monolayer preparations after 4 weeks of bioreactor culture compared to native auricular cartilage. (A, B) Native auricular cartilage. (C cartilaginous region; C' perichondrium-like region) Engineered construct generated from 6500 cells/cm² without NaHCO₃. (D cartilaginous region; D' perichondrium-like region) Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. (E cartilaginous region; E' perichondrium-like region) Engineered construct generated from 13,000 cells/cm² without NaHCO₃. (F cartilaginous region; F' perichondrium-like region) Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Binucleated cells were observed in the native tissue (circled in B) and also on the constructs (circled in E and F). Scale bars: (A) 50 µm; (B) 20 µm; (C-F') 10 µm. Color images available online at www.liebertpub.com/tea

content was determined in the hydrolyzate using chloramine-T/Ehrlich's reagent assay.⁴² Total collagen content was estimated assuming hydroxyproline accounts for 10% of the total collagen mass in cartilage.⁴³

Statistical analyses

All results were expressed as the mean $\pm$ standard error of the mean. Biochemical quantification data were analyzed using two-way analysis of variance (ANOVA) to determine the effect of cell seeding density and media formulation on elastic cartilaginous tissue growth. In addition, pair-wise comparisons between individual groups was conducted using a one-way ANOVA and Tukey's *post hoc* testing. Data were checked before performing statistical tests for both normality and equal variance. Statistical tests were conducted using statistical software (SPSS version 16; SPSS, Inc., Chicago, IL), and the significance was associated with *p*-values less than 0.05.

Results

Structure of engineered elastic cartilage constructs

Using a small population of isolated cells, scaffold-free elastic cartilage constructs were generated from long-term bioreactor culture of different monolayer preparations (6500 or 13,000 cells/cm²) with or without media supplemented with 14 mM NaHCO₃. Each of the preparations resulted in the formation of large tissue constructs (~ 3 cm²) (Fig. 1). Histological evaluation of the engineered constructs revealed that the generated neotissue possessed a similar morphology to that of native auricular cartilage with the appearance of defined cartilaginous and perichondrium-like regions. The neocartilage was composed of round-to-oval lacunae cells homogeneously distributed in a basophilic matrix (Fig. 2) with larger cells arranged in cartilaginous region and smaller cells in the perichondrium-like region. The ECM of the cartilaginous region stained positive for sulfated proteoglycans (Fig. 3) as well as displaying the

FIG. 6. Immunohistochemical localization of collagen X in the engineered elastic cartilaginous constructs generated from monolayer preparations after 4 weeks of bioreactor culture compared to native auricular cartilage. (A, B) Native auricular cartilage. (C: cartilaginous region; C': perichondrium-like region) Engineered construct generated from 6500 cells/cm² without NaHCO₃. (D: cartilaginous region; D': perichondrium-like region) Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. (E: cartilaginous region; E': perichondrium-like region) Engineered construct generated from 13,000 cells/cm² without NaHCO₃. (F: cartilaginous region; F': perichondrium-like region) Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Binucleated cells were observed in the native tissue (circled in B) and also on the constructs (circled in panel F). Scale bars: (A) 50 μ m; (B) 20 μ m; (C–F') 10 μ m. Color images available online at www.liebertpub.com/tea

presence of thin elastin fibers (Fig. 4). Immunohistochemical assessment confirmed the presence of elastin (Fig. 5). In the native tissue, elastin was evident in the matrix as well as inside the cells. For the engineered constructs, however, elastin was only observed intracellularly. In addition, the presence of binucleated cells was observed in both the native tissue and the engineered constructs (Fig. 5B, E, F). The perichondrium-like region was predominantly fibrous and displayed little positive proteoglycan staining. In the native tissue, the perichondrium was negative for elastin (Fig. 5A, B); however, elastin was present in the perichondrium-like region of the engineered constructs, which appeared to decrease with higher seeding densities (Fig. 5C–F).

Immunohistochemical assessment for collagen types showed that collagen X was present in the native tissue on the cartilaginous region, intra- and extracellularly but not within the perichondrium (Fig. 6A, B). In the engineered

constructs, collagen X was only detected intracellularly within the cartilaginous and perichondrium-like regions (Fig. 6C–F). Binucleated cells were also detected in these images in both the native tissue and the engineered constructs (Fig. 6B, F). Collagen type I was exclusively located in the perichondrium-like region (Fig. 7A, C, E), with more intense staining the boundary between this region and elastic cartilaginous matrix (Fig. 7C, E). Interestingly, collagen type I was not detected in the any of the constructs grown in the absence of NaHCO_3 (Fig. 7B, D). The distribution of collagen type II was more homogenous with staining throughout the construct (elastic cartilaginous matrix and perichondrium-like regions), with more intense staining in the elastic cartilaginous matrix region (Fig. 8). Among the different preparations investigated, tissues generated from the higher seeding density (13,000 cells/ cm^2) with NaHCO_3 supplemented media appeared to possess a structure more similar to the native tissue in

FIG. 7. Immunohistochemical localization of collagen I in the engineered elastic cartilaginous constructs generated from monolayer preparations after 4 weeks of bioreactor culture compared to native auricular cartilage. (A) Native auricular cartilage. (B) Engineered construct generated from 6500 cells/ cm^2 without NaHCO_3 . (C) Engineered construct generated from 6500 cells/ cm^2 with 14 mM NaHCO_3 . (D) Engineered construct generated from 13,000 cells/ cm^2 without NaHCO_3 . (E) Engineered construct generated from 13,000 cells/ cm^2 with 14 mM NaHCO_3 . Arrows indicate positive detection of collagen I in the perichondrium-like regions of the construct. Scale bar: 50 μm . Color images available online at www.liebertpub.com/tea

FIG. 8. Immunohistochemical localization of collagen II in the engineered elastic cartilaginous constructs generated from monolayer preparations after 4 weeks of bioreactor culture compared to native auricular cartilage. **(A)** Native auricular cartilage. **(B)** Engineered construct generated from 6500 cells/cm² without NaHCO₃. **(C)** Engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. **(D)** Engineered construct generated from 13,000 cells/cm² without NaHCO₃. **(E)** Engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. Scale bar: 50 μ m. Color images available online at www.liebertpub.com/tea

terms of cellular morphology and having a more defined perichondrium-like region.

Tissue ultrastructure

Ultrastructure of cells from the engineered elastic tissue constructs, compared to native elastic cartilage, was examined by TEM. In the native elastic cartilage, chondrocytes in the central region were observed with single, large cytoplasmic lipid droplets (Fig. 9A). These droplets were surrounded by cytofilaments primarily arranged in parallel (Fig. 9A, inset). These elements were more readily observed in cells that were less elongated (typical chondrocyte morphology). Similar morphologies were observed in the engineered tissues after 4 weeks of bioreactor cultivation (Fig. 9B, D). Cells present in the ECM of the engineered constructs were generally oval in shape and presented many indentations and deep recesses. These irregularly shaped margins represent a peculiar structural feature of young

chondroblasts of all cartilage tissues, and it was also observed on the native tissue. Similar morphology was also observed in the constructs grown in the absence of NaHCO₃ (data not shown). All groups possessed the same key elements of elastic tissue in terms of cytoskeleton, lipid droplets, and defined territorial and interterritorial matrices. It was possible to observe the presence of elastic fibers in the constructs grown in the presence of NaHCO₃. These appeared as electron-dense amorphous aggregates delineated by a thin fibrillary meshwork at the border of interterritorial matrix and not detected in the territorial matrix (Fig. 9C, E). Slight differences in ultrastructure were observed between the different preparations with the presence of NaHCO₃ appearing to have a greater effect than seeding density. Constructs grown in the absence of NaHCO₃ (data not shown) displayed less preserved cells and contained more thin/delicate fibrillar meshes compared to the construct grown in the presence of NaHCO₃. Similar to the histological and immunohistochemical evaluations, constructs

FIG. 9. Transmission electron micrographs of the middle of the engineered elastic cartilaginous constructs and native auricular cartilage. **(A)** Native auricular cartilage with inset showing the detail of the extracellular matrix. **(B)** Cellular ultrastructure within the engineered construct generated from 6500 cells/cm² with NaHCO₃. **(C)** Presence of elastic fiber formation (circled) within the engineered construct generated from 6500 cells/cm² with 14 mM NaHCO₃. **(D)** Cellular ultrastructure within the engineered construct generated from 13,000 cells/cm² with NaHCO₃. **(E)** Presence of elastic fiber formation (circled) within the engineered construct generated from 13,000 cells/cm² with 14 mM NaHCO₃. LD, lipid droplets; N, nucleus; IM, interterritorial matrix; TM, territorial matrix. Scale bars: **(A, B)** 5 µm; **(A inset)** 1 µm; **(D)** 2 µm; **(C, E)** 1 µm.

TABLE 1. BIOCHEMICAL AND PHYSICAL PROPERTIES OF ENGINEERED ELASTIC CARTILAGINOUS TISSUE

	<i>Seeding density and NaHCO₃ concentration</i>			
	<i>6500 (cells/cm²)</i>		<i>13,000 (cells/cm²)</i>	
	<i>0 (mM)</i>	<i>14 (mM)</i>	<i>0 (mM)</i>	<i>14 (mM)</i>
DNA (µg/mg dry wt.)	1.0 ± 0.04 ^a	1.1 ± 0.1	1.13 ± 0.06 ^{a,b}	1.0 ± 0.1 ^b
PG (µg/mg dry wt.)	1.0 ± 0.05	1.5 ± 0.5	1.3 ± 0.1 ^b	2.2 ± 0.9 ^b
Collagen (µg/mg dry wt.)	1.0 ± 0.1	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.1
PG/DNA (µg/µg)	1.0 ± 0.1	1.2 ± 0.3 ^a	1.0 ± 0.1 ^b	2.1 ± 0.8 ^{a,b}
Collagen/DNA (µg/µg)	1.0 ± 0.1	1.3 ± 0.2 ^a	0.89 ± 0.09 ^b	1.6 ± 0.2 ^{a,b}
Collagen/PG (µg/µg)	1.0 ± 0.2 ^b	1.6 ± 0.6 ^{a,b}	0.76 ± 0.07	0.9 ± 0.3 ^a
Thickness (mm)	1.0 ± 0.1 ^b	1.5 ± 0.3 ^b	1.2 ± 0.1 ^b	1.5 ± 0.2 ^b

Four separate experiments were performed, data expressed relative to control (6500 cells/cm²; 0 mM NaHCO₃), presented as mean ± SEM (*n* = 6–8 samples/group).

^aSignificant effect of seeding density (*p* < 0.05).

^bSignificant effect of sodium bicarbonate (*p* < 0.05).

PG, proteoglycans; SEM, standard error of the mean; NaHCO₃, sodium bicarbonate.

grown at the higher seeding density and in the presence of NaHCO_3 displayed the greatest similarity to the ultrastructure of native elastic cartilage.

Physical and biochemical properties of accumulated ECM

Both the presence of NaHCO_3 in the media and seeding density had a profound effect on the formation of engineered elastic tissue constructs in terms of tissue thickness, cellularity, and ECM accumulation. Tissue thickness was significantly affected by the presence of NaHCO_3 (50% increase; $p < 0.01$), with no observable effect of seeding density (Table 1). Tissue cellularity was also only significantly affected by the presence of NaHCO_3 (72–99% increase in DNA content; $p < 0.01$) (Fig. 10A). Cartilaginous ECM accumulation was significantly affected by both NaHCO_3 (83–139% increase in proteoglycan content, 129–221% increase in collagen content; $p < 0.01$) and seeding density (30% increase in proteoglycan content, 40% increase in collagen content; $p < 0.01$) (Fig. 10B, C). When normalized to tissue dry weight or cellularity, similar effects of NaHCO_3 and seeding density were observed (Table 1). However, when compared to native auricular cartilage, the obtained thickness, ECM accumulation, and cellularity in the engineered elastic constructs were lower with the exception of proteoglycan accumulation, which appeared to approach the values of native tissue (Table 2). Similar to the previous results, constructs grown at the higher seeding density and in the presence of NaHCO_3 displayed the greatest similarity to native elastic cartilage in terms of tissue thickness and biochemical composition (Table 2). In addition, while the elastic modulus of the engineered elastic constructs were significantly lower ($p < 0.01$) than native auricular cartilage (13,000 cells/ cm^2 with NaHCO_3 : 13 ± 4 kPa, $n = 8$; native auricular cartilage: 100 ± 19 kPa, $n = 7$); both tissues appeared to possess a similar Poisson's ratio (13,000 cells/ cm^2 with NaHCO_3 : 0.305 ± 0.003 , $n = 8$; native auricular cartilage: 0.281 ± 0.019 , $n = 7$).

Discussion

As there are relatively few surgical approaches currently available for total or partial ear reconstruction (alloplastic implants or sculpted autologous cartilage grafts),^{2,3,6,44} tissue engineering methods have been explored as a means to grow autologous cartilage suitable for implantation. However, as routine cell expansion can elicit negative effects on chondrocyte function,^{20–22} we have developed an approach to generate large-sized,^{28,45} scaffold-free engineered auricle constructs without the use of a separate cell expansion phase. After 4 weeks of bioreactor cultivation, the developed scaffold-free tissue constructs approached the thickness of native auricular cartilage (38–65% of native tissue thickness). Interestingly, the engineered constructs appeared to adopt a structural appearance similar to that of native auricular cartilage. The top portion of the constructs resembled the perichondrium of native auricular cartilage and contained smaller spindle-shaped cells in a fibrous ECM that stained positive for the presence of collagen types I and II with little staining for sulfated proteoglycans. However, the presence of collagen type I was primarily located at the

FIG. 10. Quantification of biochemical constituents in the engineered elastic cartilaginous constructs generated from monolayer preparations after 4 weeks of bioreactor culture. (A) DNA content; (B) proteoglycan content; (C) total collagen content (combined content of all collagen types). Data represented as mean $\pm$ SEM ($n = 8$ samples/group) normalized to the constructs generated from 6500 cells/ cm^2 without NaHCO_3 . ^aStatistical effect of NaHCO_3 ($p < 0.01$). ^bStatistical effect of seeding density ($p < 0.01$).

boundary between the cartilaginous and perichondrium-like regions of the developed constructs and there was some evidence of both collagen type X and elastin, which appeared to be modulated by changes in seeding density and the presence of NaHCO_3 . Interestingly, while the formation of a cartilaginous region in the developed constructs were observed among all the investigated conditions (seeding density and NaHCO_3 supplementation), the cultures supplemented with NaHCO_3 developed more distinct perichondrium-like regions. Although the development of perichondrium-like regions in neocartilage constructs have been observed in

TABLE 2. REPRESENTATIVE DATA OF THE BIOCHEMICAL AND PHYSICAL PROPERTIES OF ENGINEERED ELASTIC CARTILAGINOUS TISSUE AND NATIVE AURICULAR TISSUE FROM A SINGLE EXPERIMENT

	Seeding density and NaHCO ₃ concentration				Native tissue
	6500 (cells/cm ²)		13,000 (cells/cm ²)		
	0 (mM)	14 (mM)	0 (mM)	14 (mM)	
DNA (μg/mg dry wt.)	3.44±0.05	3.3±0.1	4.1±0.7	3.3±0.4	2.3±0.2
PG (μg/mg dry wt.)	202±41	263±2	315±64	234±15	231±26
Collagen (μg/mg dry wt.)	130±18	185±48	144±31	152±35	496±35
PG/DNA (μg/μg)	59±11	79±2	76±2	71±5	133±14
Collagen/DNA (μg/μg)	38±5	26±15	35±1	45±5	230±30
Collagen/PG (μg/μg)	0.65±0.04	0.3±0.1	0.46±0.01	0.6±0.1	2.2±0.2
Thickness (mm)	0.10±0.01	0.162±0.03	0.13±0.01	0.17±0.03	0.26±0.03

Bioreactor: $n = 2-8/\text{group}$; native tissue: $n = 6/\text{group}$.

relatively few previous studies—most notably by Yanaga and coworkers who also showed a spontaneous recapitulation of a perichondrium-like region after implantation,⁴⁶ the majority of elastic cartilage tissue engineering approaches tend to only utilize cells isolated from the cartilaginous region.^{10,11,47-51} The creation of engineered constructs possessing both cartilaginous and perichondrium regions, similar to that observed in native auricular cartilage, is of benefit toward the development of functional engineered constructs suitable for implantation.^{2,10,44}

The cartilaginous portion of the engineered constructs also appeared to be similar to that of native auricular cartilage with the presence of elastin, collagen types II and X, and sulfated proteoglycans. The presence of elastin expression and elastic fiber formation was confirmed by histology, immunohistochemistry, and TEM—which appeared as electron-dense aggregates delineated by a thin fibrillary meshwork located at the border of the interterritorial matrix. The reduced elastin expression in the engineered constructs (with little to no observable extracellular staining) may be due to mature chondrocytes being less capable of elastin synthesis or that *in vitro* culture models do not necessarily support elastin production by these cells.^{10,48} Thus, the upregulation of elastin synthesis and elastic fiber formation in the developed constructs needs to be improved for the development of functional elastic cartilage constructs.

While collagen type II appeared to be widely expressed, collagen type X was also expressed in the engineered constructs. Although collagen type X is typically associated as a marker of hypertrophic and mineralized cartilage,⁵²⁻⁵⁵ it is also present in nonmineralizing tissues, including auricular and neoseptal cartilages,⁵⁵ and on the surface of articular cartilage⁵⁶ and the meniscus.⁵⁷ In addition, a small population of binucleated cells was observed in the cartilaginous region of the native tissue and bioreactor-cultivated constructs. According to Moskalewski *et al.*,⁴⁷ the presence of binucleated cells is one of the hallmark observations in rabbit auricular cartilage, which appeared to be maintained after bioreactor cultivation. Although smaller populations of binucleated cells were observed in the present study (native tissue and engineered constructs) compared to the observations of Moskalewski (~25% of the cell population),⁴⁹ the engineered constructs were capable of maintaining the characteristic binucleated cell phenotype of elastic cartilage.^{47,49}

Although the developed cartilage constructs appeared to adopt structural-like characteristics of native auricular cartilage, the accumulation of ECM constituents and resultant mechanical properties were lower than that of the native tissue. Although the accumulation of proteoglycans in the developed constructs was similar to native auricular cartilage, the engineered constructs only accumulated between 26% and 37% of the collagen content of the native tissue. This effect resulted in lower collagen-to-proteoglycan ratios with the constructs achieving similar levels compared to that of native auricular cartilage. Previous studies have also noted reduced ECM accumulation and mechanical properties within engineered elastic cartilage constructs.^{3,6,9} However, the biochemical and mechanical properties of the developed constructs could potentially be affected by the culture conditions. Both seeding density and the use of medium supplemented with NaHCO₃ were sufficient to increase the accumulation of both proteoglycans (twofold increase) and collagen (threefold increase) relative to the control cultures (lower seeding density without NaHCO₃ supplementation). Although increasing maturation of the engineered constructs was observed through the modulation of the culture conditions, the development of constructs with improved biochemical and mechanical properties will most likely be required for the development of functional constructs that are suitable for implantation. Engineered constructs also appeared to be more cellular than native auricular cartilage (43–78% increase in tissue cellularity); an effect that has also been observed by others.⁵⁸ Interestingly, no significant differences in construct cellularity were observed with increased seeding densities (as opposed to the presence of NaHCO₃) suggesting that higher seeding densities could potentially be used to accelerate tissue formation within the bioreactor without deleterious effects on the cellularity of the developed constructs. Ultrastructural characterization of the constructs confirmed the observations of the similarities between the engineered constructs and native auricular cartilage. Key elements of the native tissue could also be observed in the constructs after 4 weeks of cultivation, including defined territorial and interterritorial matrices, cytoskeleton, and presence of lipid droplets. The cells in the engineered constructs were generally oval in shape and had cytoplasmic margins with many indentations and recesses similar to that observed in the native tissue.

These irregularly shaped margins represent a peculiar structural feature of young chondroblasts of all cartilaginous tissues.⁵⁹ The intercellular matrix within the constructs did not appear to differ morphologically from native auricular cartilage. However, the microfibrillar component of the elastic fibers was more prominent in the native tissue. Interestingly, due to the presence of a large cytoplasmic lipid droplets surrounded by cytofilaments and sparse ECM, some authors have suggested that tissue with these features resemble more of an adipose-like tissue, referred to as a secondary cartilage (a primitive chondroid tissue of lower vertebrates).^{60–62} In the present study, while we did observe some of these characteristics in both the engineered constructs and native auricular cartilage samples, further analyses are required to determine whether these tissues can be classified as secondary cartilages since these lipid droplets were also observed in the chondrogenic perichondrium of the native tissue (data not shown).

In this proof-of-principle study, isolated chondrocytes from adolescent animals were used to reflect the general age of patients undergoing auricular reconstruction for microtia. However, as surgical reconstruction is also performed in cases of ear injury (e.g., burns, bites), older and potentially less synthetically active autologous cells are available, which may affect the amount and quality of the tissue that can be generated through this approach. This is especially relevant in terms of elastogenesis, which is known to be adversely affected with increasing age.^{8,10,47,50} This could potentially be overcome through the maturation of the constructs *in vivo*⁴⁴ or the use of hormone or growth factor supplementation (insulin, IGF-1, or TGF- β 1), which have been shown to stimulate the elastogenesis.^{63–68} Another hurdle for the development of functional auricular tissue implants is the need to develop anatomically shaped constructs. To address this issue, patient-specific rapid-prototyped tissue culture molds could be used, which have previously been explored for the creation of engineered articular cartilage constructs tailored to the anatomy of a particular defect.²⁸ Finally, the translation toward the development of human engineered auricular constructs need to be undertaken before this approach could used clinically.

In this study, we have described an approach to generate large-size auricular cartilage constructs from a small population of isolated cells. The cells were cultivated in a continuous flow bioreactor to stimulate the production of cartilaginous tissue. Developed scaffold-free tissue constructs appeared to adopt structural-like characteristics similar to native auricular cartilage with the development of distinct cartilaginous and perichondrium-like regions. Alterations in media composition and seeding density had profound effects on the formation of engineered auricular tissue constructs in terms of cellularity, ECM accumulation, thickness, and tissue structure. Future studies will be aimed at improving the accumulation of specific tissue constituents, especially elastin and elastic fibers, and determining the clinical effectiveness of this approach using a reconstructive animal model.

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Disclosure Statement

No competing financial interests exist.

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