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USE OF AUTOLOGOUS ADIPOSE-DERIVED MESENCHYMAL STEM CELLS FOR CREATION OF LARYNGEAL CARTILAGE

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Abstract

Objective—Adipose derived mesenchymal stem cells (ASCs) are an exciting potential cell source for tissue engineering because cells can be derived from the simple excision of autologous fat. This study introduces a novel approach for tissue engineering cartilage from ASCs and a customized collagen oligomer solution, and demonstrates that the resultant cartilage can be used for laryngeal cartilage reconstruction in an animal model.

Methods—ASCs were isolated from F344 rats, seeded in a customized collagen matrix, and cultured in chondrogenic differentiation medium for 1, 2 and 4 weeks until demonstrating cartilage-like characteristics *in vitro*. Large laryngeal cartilage defects were created in the F344 rat model, with the engineered cartilage used to replace the cartilage defects, and the rats followed for 1–3 months. Staining examined cellular morphology and cartilage-specific features.

Results—*In vitro* histological staining revealed rounded chondrocyte-appearing cells evenly residing throughout the customized collagen scaffold, with positive staining for cartilage-specific markers. The cartilage was used to successfully repair large cartilaginous defects in the rat model, with excellent functional results.

Conclusion—This study is the first study to demonstrate, in an animal model, that ASCs cultured in a unique form of collagen oligomer can create functional cartilage-like grafts which can be successfully used for partial laryngeal cartilage replacement.

Level of Evidence—N/A

Keywords

Larynx; cartilage; tissue engineering; collagen; oligomer; chondrocytes; adipose stem cell

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Introduction

With recent advances in tissue engineering, a variety of cartilage engineering approaches have been described in the literature.[1–7] Perhaps the most renowned cartilage engineering in the otolaryngology literature include the reports by Macchiarini et al using bone marrow-derived mesenchymal stem cells (MSCs) on either decellularized cartilage or polyurethane scaffold for tracheal replacements in patients.[8] The major limitation with decellularized cartilage and polyurethane scaffold for laryngeal cartilage engineering is that the cells coat the surface of the scaffold but fail to fully integrate. Since polyurethane is not a natural biomaterial, such as collagen, there is also increased risk of an inflammatory or foreign body reaction.

Ideally, engineered laryngeal cartilage would have cells evenly distributed throughout the scaffold, the ability to fuse with adjacent tissue (laryngeal muscle) without inducing an inflammatory response, and the ability to mature and stabilize *in vivo*. In the current study, we explore the use of a patented oligomeric collagen, which uniquely retains natural collagen intermolecular cross-links. This model supports suprafibrillar assembly to yield a highly interconnected D-banded collagen-fibril network that is more mechanically stable and resistant to proteolytic degradation compared to those formed by conventional monomeric collagens.[9–12] The oligomer collagen scaffold is in the form of a solution that rapidly self-assembles into a solid contiguous regeneration matrix when activated. The methodology allows stem cells and collagen oligomers to be suspended in solution prior to polymerization into a solid state, and has been shown to promote rapid vascularization *in vivo*.[10,12] The physical properties can be modulated to provide the proper cell-matrix mechanotransduction cues for guiding ongoing cartilage development and regeneration. This approach also fosters in-vivo tissue integration by creating a cellular microenvironment that captures the cellular processes and cell-matrix signaling of embryonic cartilage development rather than mature cartilage. In short, this process involves the formation of a pre-cartilagenous state in which mesenchymal cells, interfacing with type I collagen, differentiate into chondrocytes that produce cartilage-specific ECM components including type II collagen and glycosaminoglycans.[12] Since this approach mimics native cartilage development, one would anticipate implanted cartilage constructs to become increasingly stable *in vivo*, rather than weakening or degrading over time.

The aim of this study was to determine if a unique oligomeric collagen[9–12] could be combined with adipose derived stem cells (ASCs) to create 3-dimensional laryngeal cartilage. We hypothesized that the cartilage grafts created from ASCs in collagen could be used to consistently repair partial laryngeal defects in a rat model. If successful, this approach could be readily translatable, as cartilage constructs could be derived from a simple fat biopsy under local anesthesia, with autologous tissue engineered cartilage derived within one month in culture.

Materials and Methods

ASC Isolation and Culture

ASCs were obtained from 12-week-old male Fischer 344 rats (Envigo, Indianapolis, IN). Adipose tissue was collected from surrounding epididymis and cut into less than 1 mm^3 pieces. Tissue then was digested in 0.2% Collagenase IV (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°C. After centrifugation at 1000 rpm for 5 minutes, the pellet was resuspended in ASC culture medium (Dulbecco's Modified Eagle's Medium, DMEM supplemented with 10% FBS, 1% penicillin/streptomycin/amphotericin,) and filtered through a 300 μm and 70 μm filter sequentially. The final cell pellet was resuspended in ASC culture medium and cultured at 37°C and 5% CO_2. Medium was freshened twice a week and cells were passaged when confluent.

Chondrocyte Differentiation

4×10^4 ASC cells were seeded in 24 well tissue culture plates in culture medium overnight. Then medium was replaced with either ASC culture medium or chondrogenic medium (Hyclone Advance stem Chondrogenic Differentiation Medium, SH30889.02, Thermo Scientific). Medium was changed twice a week. After 2 weeks, morphological change was evaluated with microscopy. To detect type II collagen deposition, cells were fixed in 3% PFA for 10 minutes. After blocking with 1% BSA, cells were incubated with rabbit polyclonal to collagen II antibody (ab34712, Abcam Inc. Cambridge, MA) for 15 hours. The cells were then incubated with a secondary goat anti-rabbit antibody Alexa Fluor 488 conjugate (A-11034, Thermo Fisher Scientific, Carlsbad, CA) and counterstained with DAPI nuclear marker (4', 6-diamidino-2-phenylindole, dihydrochloride, 62248, Thermo Fisher Scientific, Rockford, IL). For control chondrocyte cultures and constructs, human chondrocytes were attained (NHAC-kn) (Lonza Walkersville, MD,CC-250) and cultured in the supplied culture medium (Lonza, CC-3217) at 37°C and 5% CO2. Medium was refreshed twice a week and cells were passaged when confluent. For control cell differentiation, 2×104 NHAC-kn cells were seeded in 24 well plates in culture medium overnight, then medium was replaced with either supplied medium or chondrogenic medium (Hyclone Advance Stem Chondrogenic Differentiation Medium, SH30889.02, Thermo Scientific).

Preparation of ASC Seeded Constructs

F344 rat ASCs were grown in DMEM complete medium until they were ready to use. 5×10^5 cells were mixed with 320 μl of neutralized collagen and added to a well of 96 well plate (Figure 1). After polymerizing, they were compressed to 0.5mm thickness scaffolds. Chondrogenic Differentiation Medium was added to scaffolds. Scaffolds were maintained in 37°C, 5% CO_2 incubator for up to 4 weeks. Medium was freshened twice a week.

Histological Analysis in vitro

ASCs/collagen constructs were harvested at 1, 2 and 4 weeks. Scaffolds were rinsed twice with PBS and fixed in 3% PFA for 1 hour, and then frozen in O.C.T Compound (4585, Fisher HealthCare, Houston, TX) for cryostat sectioning. Sections were cut into thickness of 20 μm by cryotome. Standard H&E staining was used to visualize cell distribution (and

morphology). Safranin-o (S8884, Sigma-Aldrich, St. Louis, MO) staining was used to observe accumulation of sulfated proteoglycans.

Mechanical Test of Engineered Constructs

Five acellular collagen scaffolds and 5 ASC seeded collagen scaffolds were cultured in chondrogenic differentiation medium for 3 weeks and then submitted for compression testing. Compression testing was performed with samples fully hydrated, with an unconfined compression device set at 5.6 lb/24.9 N load cell.

In vivo study

Partial Laryngectomy Procedures—The animal study protocol was approved by Purdue University Animal Care and Use Committee, and institutional guidelines, in accordance with the National Institutes of Health guidelines, were followed for the handling and care of the animals. Twelve Fischer 344 male rats (Envigo, Indianapolis IN) were anesthetized with isoflurane via face mask (1–5%). A midline vertical skin incision measuring 1.0 cm was made over the larynx, with the incision carried through the skin, bluntly dividing the submandibular glands, and then incising and retracting the strap muscles inferolaterally to expose the underlying larynx. The beveled tip of an 18 gauge needle was used to create a window in the left thyroid cartilage, and then continued dissection used to resect the left lateral thyroid cartilage anteromedially (a portion of cartilage was preserved posteriorly over the pyriform sinus to avoid mucosal injury). The defect size was consistent with our prior published model,[4] and the defect was large enough that repair was necessary, as control animals (leaving defect unrepaired) did not survive the immediate postoperative period. A piece of ASCs/collagen scaffold (pre-cultured in chondrogenic differentiation medium for 1 week) of appropriate size was implanted into the defect, the strap muscles were re-approximated and the skin was closed with 5–0 Vicryl suture.

Larynges were fixed with 4% paraformaldehyde in PBS for 24 hours, then changed to 30% sucrose in PBS solution until tissues sunk to the bottom. Tissues were frozen by cryo-embedding media (OCT). The larynges were cut at the axial position to sections with thickness of 12μm. Hematoxylin and eosin (H& E) staining was performed to show the structure of the scaffolds relative to the native laryngeal tissue as well as inflammation. To detect negatively charged sulfated proteoglycans in the implant (characteristic of cartilage), sections were stained with Alcian Blue (KTABP2.5, American Mastertech Scientific, Lodi, CA).

Results

Chondrocyte Differentiation

Within two weeks, the ASCs in ASC medium demonstrated characteristic ASC morphology (elongated/spindle shaped cells throughout), while the ASCs in chondrocyte differentiation medium (ASC-CH) became rounded and pyramidal shaped suggesting an overall change in outer cellular morphology (Figure 2). By three weeks, the cells were depositing collagen II (suggesting chondrocyte transformation) while the ASCs were not (Figure 3). These findings

suggested that the ASC phenotype had differentiated into that of a chondrocyte *in vitro* within a timeline of three weeks.

Histological Analysis in vitro

The ASC seeded scaffolds in ASC medium rapidly decreased in size over time, while the acellular scaffolds remained stable in size. However, the acellular scaffold progressively lost rigidity over time, becoming increasing soft and pliable to touch.. In contrast, ASC-CH scaffolds stayed consistent in size over time and became increasingly rigid (Figure 4, *left*). Mechanical testing supported the relative difference in stiffness between the acellular construct and the ASC-CH constructs (Figure 4, *right*). H&E staining demonstrated that, as the 3-dimensional ASC-CH constructs mature, the cells become increasingly round and the matrix demonstrates more notable lacunae-like changes consistent with maturing cartilage (Figure 5). Safranin-o stain, a marker for accumulated sulfated proteoglycans (characteristic of cartilage), became more intense over time, with dense Safranin-o positive areas noted throughout the ASC-CH constructs by week four (Figure 6).

In Vivo Response to ASC-CH for Cartilage Replacement

In the postsurgical period, animals steadily gained weight, and there were no problems with airway compromise, difficulty handling secretions, or other signs of laryngeal dysfunction. On gross post-mortem laryngeal examination at 1 month, the engineered graft and native laryngeal cartilage were in close approximation, and by 3 months the engineered graft had fused and healed into the native cartilage, such that the laryngeal shape was well preserved and it was difficult to identify the original site of the hemilaryngeal defect (Figure 7A). Alcian blue staining of postmortem laryngeal sections demonstrated the engineered graft to be weakly positive at 1 month, while at 3 months the engineered graft demonstrated blue stained negatively-charged sulfated proteoglycans consistent with cartilage formation (Figure 7B). On H&E staining, the graft maintained adequate thickness, and there was no evidence of any inflammatory or foreign body reaction, with adjacent myofibers healing and fusing within the collagen matrix of the graft (Figure 8).

Discussion

Numerous previous studies have investigated the use of chondrocyte seeded scaffolds for laryngotracheal airway reconstruction. For example, Kamil et al demonstrated that chondrocytes (derived from auricular cartilage) in polyethylene oxide/polypropylene oxide copolymer could be matured in vivo (in pig dorsum) and then used for laryngotracheal reconstruction.[13] Goldstein et al used 3-dimensional printing to create a chondrocyte-coated poly-lactic acid (PLA) scaffold, and used it for laryngotracheal reconstruction in an animal (rabbit) model.[14] While both these approaches are novel, if clinically translated, a generous cartilage biopsy (for example, a piece of septal cartilage or auricular cartilage) would be needed to attain chondrocytes, so it is not necessarily an advantage over the traditional surgical approach to harvest cartilage grafts from nasal or auricular donor sites for laryngotracheal reconstruction. Other approaches to engineering tracheal cartilage have focused on use of bone marrow derived mesenchymal stem cells for tracheal cartilage

reconstruction, based on either decellularized cartilage or a polymer based scaffold such as polyurethane.[3,15]

A less invasive approach would entail taking a small biopsy of adipose tissue under local anesthesia alone, and using these cells to create cartilage grafts for laryngotracheal reconstruction and/or laryngeal tissue engineering. Hashemibeni and colleagues found that they could repair small defects of the anterior tracheal wall with grafts which had been engineered from differentiated adipose-derived stem cells in an alginate-bead based biodegradable scaffold.[16] While the grafts did develop into cartilage *in vivo*, the grafts were not well differentiated into cartilage implants *in vitro*, and defects were too small to determine if the engineered cartilage was providing adequate physiologic support. This is the first study to demonstrate that ASCs in a unique-developed collagen matrix can be used to create functional cartilage-like grafts for partial laryngeal cartilage replacement. Our laboratory has previously investigated other scaffold materials such as polycaprolactone (PCL), PLA and polyglycolic acid (PGA) with a variety of cell types, with contraction of the implants being a consistent problem (*data unpublished*). When standard collagen polymer is used as scaffold, cells tend to rest on the surface of the scaffold rather than being evenly distributed throughout the construct. The current study incorporated a unique oligomer-based collagen as scaffold material, thereby allowing cells to evenly distribute throughout the scaffold, and obviating the implant contraction problems that we encountered in prior models. Furthermore, because the collagen is still recognized as native, there were no issues with inflammatory or foreign body response. Since viable cells are distributed evenly throughout the collagen scaffold and do not require a prefabricated mold, the implants can be readily customized in size and shape. Future studies will involve using the grafts in larger defects and larger animal models for airway laryngotracheal reconstruction, and combining the implants with engineered muscle and vibratory surface for hemilaryngeal replacement.

Conclusion

We demonstrate a unique, yet simple approach using a novel collagen scaffold and chondrocyte differentiation medium to differentiate ASCs into chondrocytes, creating cartilage grafts *in vivo* that mimic native cartilage. Future studies will be necessary to better define potential clinical applications of this approach to engineering laryngeal cartilage.

Acknowledgements

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This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Purdue University.

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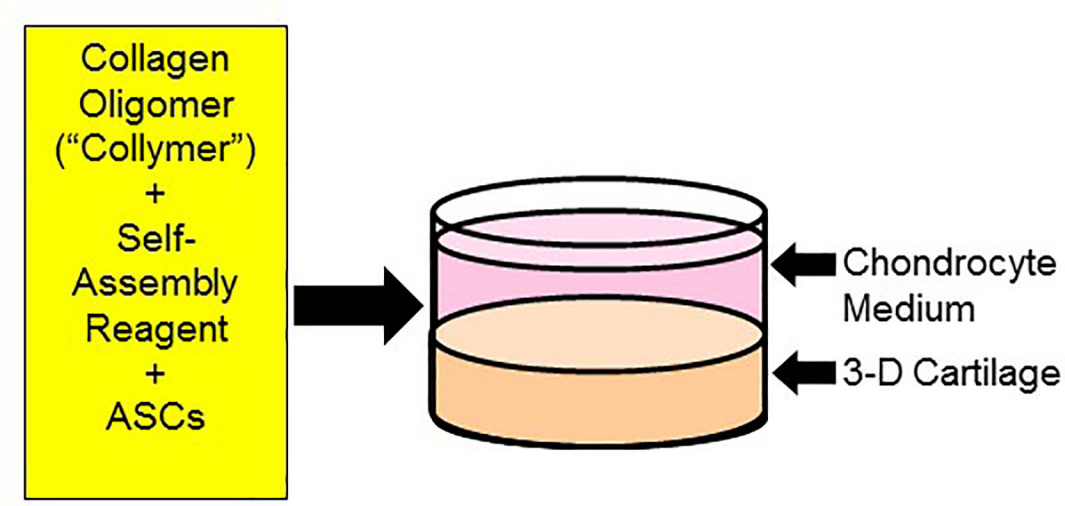

Figure 1.

Basic overview of steps to create 3-dimensional tissue engineered cartilage from adipose-derived mesenchymal stem cells (ASCs). This novel model of using collagen oligomer ("Collymer") and self-assembly reagent allows ASCs to be evenly distributed throughout the tissue engineered cartilage, unlike traditional models of engineering in which begins with cells cultured onto a scaffold in the polymerized form, thereby limiting penetration/ distribution of cells.

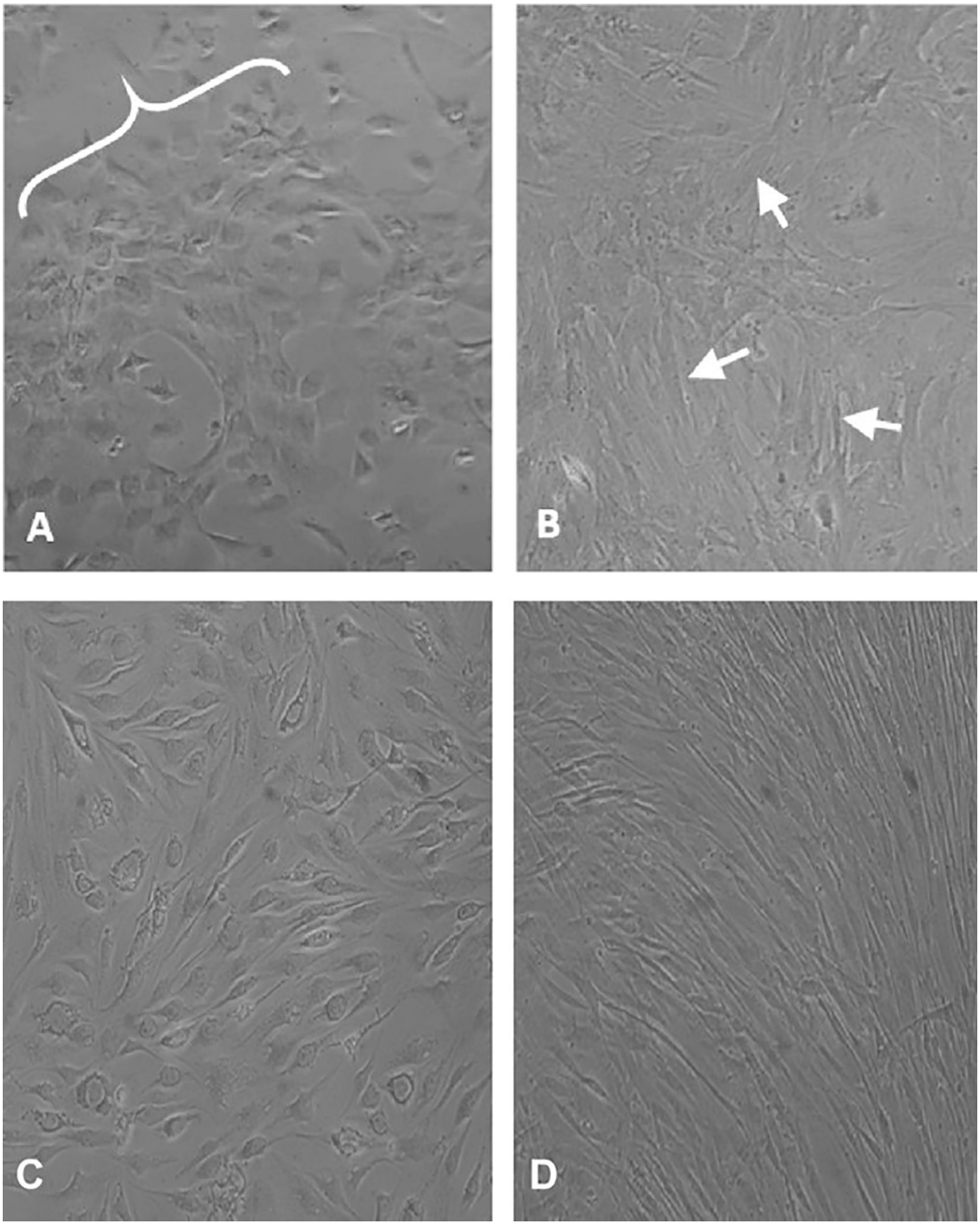

Figure 2.

(A) Phase contrast image taken at 10X magnification at 2 weeks demonstrates rounded cell morphology (bracket) in the adipose stem cells (ASCs) incubated in chondrocyte medium suggesting transformation into a chondrocyte phenotype. (B) In contrast, in the control medium the ASCs demonstrate more characteristic spindle, elongated morphology. (C) In the differentiation medium, chondrocyte change to round morphology. (D) In the control medium the chondrocytes demonstrate spindle and elongated morphology.

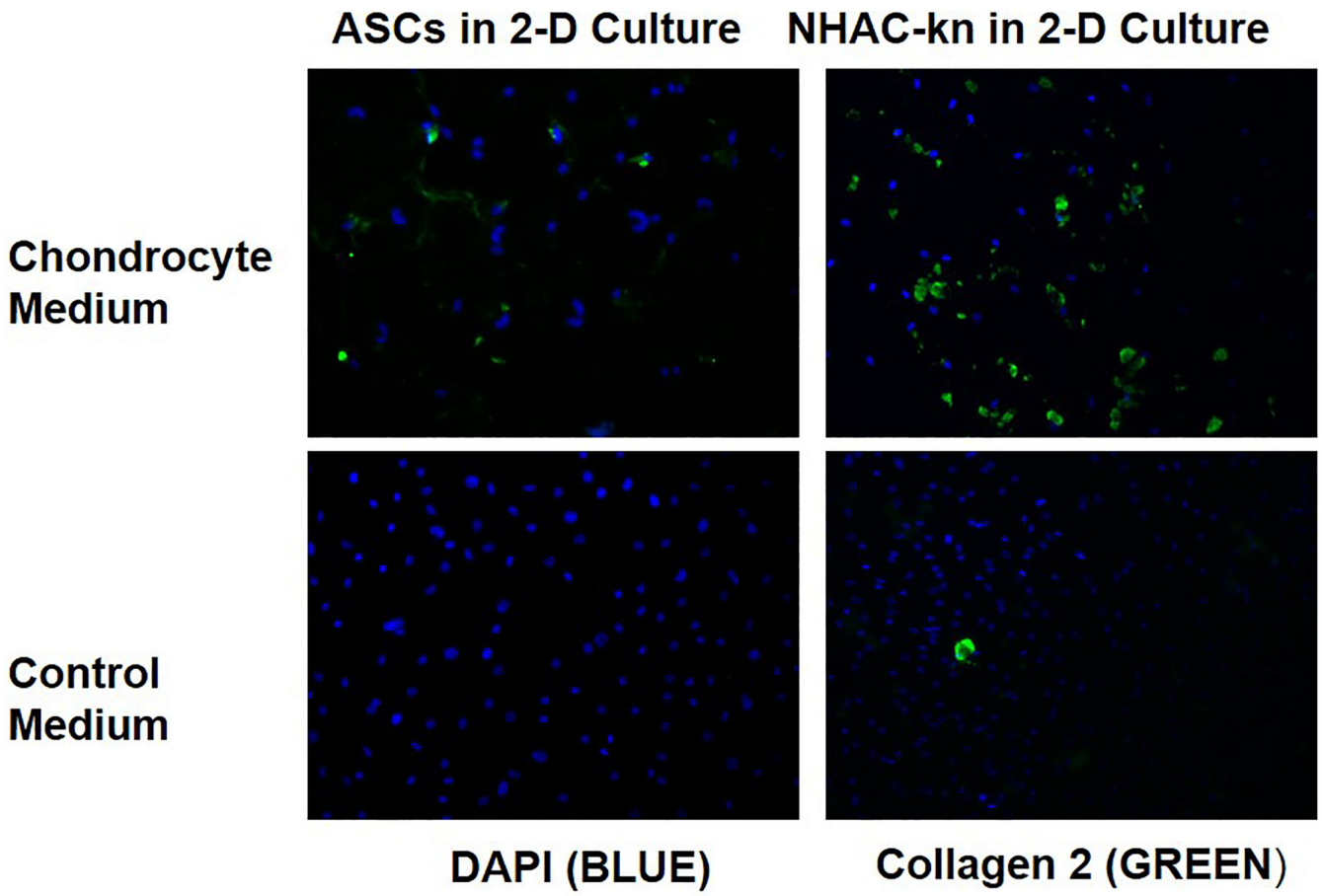

Figure 3.

Adipose-derived mesenchymal stem cells (ASCs) and chondrocyte in culture demonstrate abundant cells in both the chondrocyte medium (above) or control medium (below). DAPI stain demonstrates cell nuclei in blue (fluorescent microscopy, 10x). Corresponding image revealing collagen 2 (green) demonstrates abundant collagen 2 in the ASCs and chondrocyte within the chondrocyte medium, while no collagen 2 is detected from the ASCs, and less collagen 2 is detected from chondrocyte in the control medium.

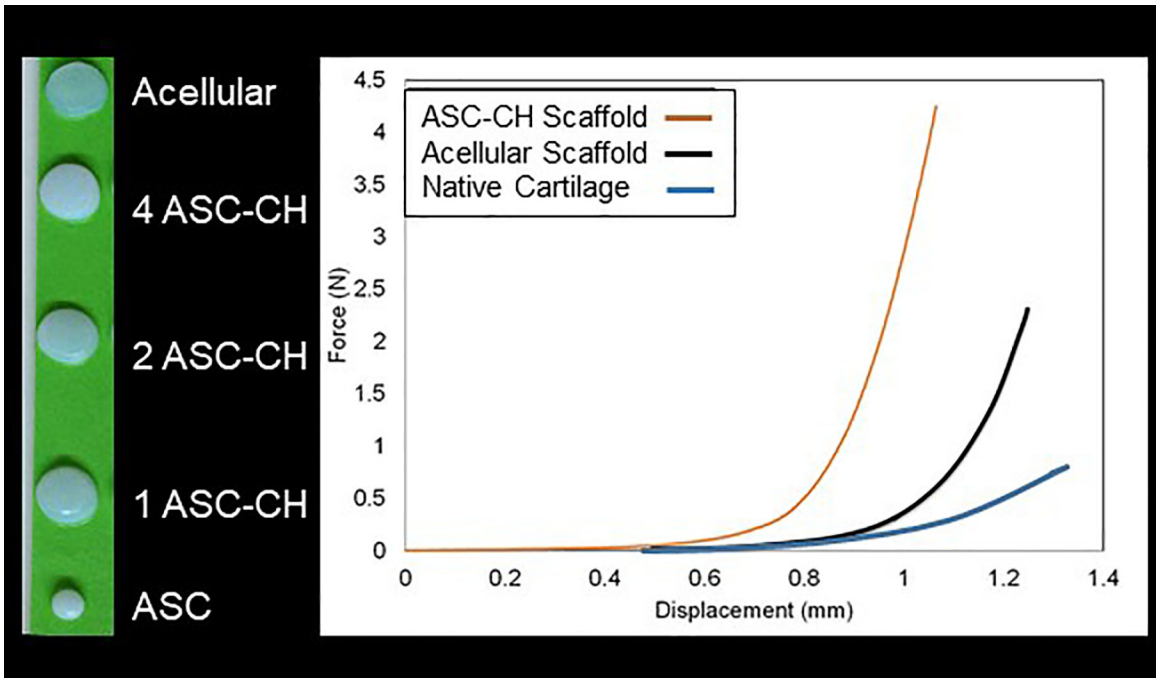

Figure 4.

Three-dimensional constructs (left) with the ASC (bottom) construct representing the ASCs in collagen scaffold grown in ASC medium at 4 weeks; the ASC construct was initially the same size as the other constructs, but decreased in size over time. The Acellular (top) construct represents the collagen scaffold cultured without cells. The middle ASC-CH constructs are demonstrated at 1 week (1 ASC-CH), 2 weeks (2 ASC-CH), or 4 weeks (4 ASC-CH) in vitro; the constructs did not change in size over time, and become subjectively more rigid. Compression testing (right) suggests a relative difference in stiffness of the constructs at 4 weeks, with the acellular construct (black line) and samples of native rat laryngotracheal cartilage (blue line) being displaced with compression force more than the ASC-CH construct (red line) when the same force is applied. Graph represents mean results from at least 2 samples per condition, with differences not reaching statistical significance.

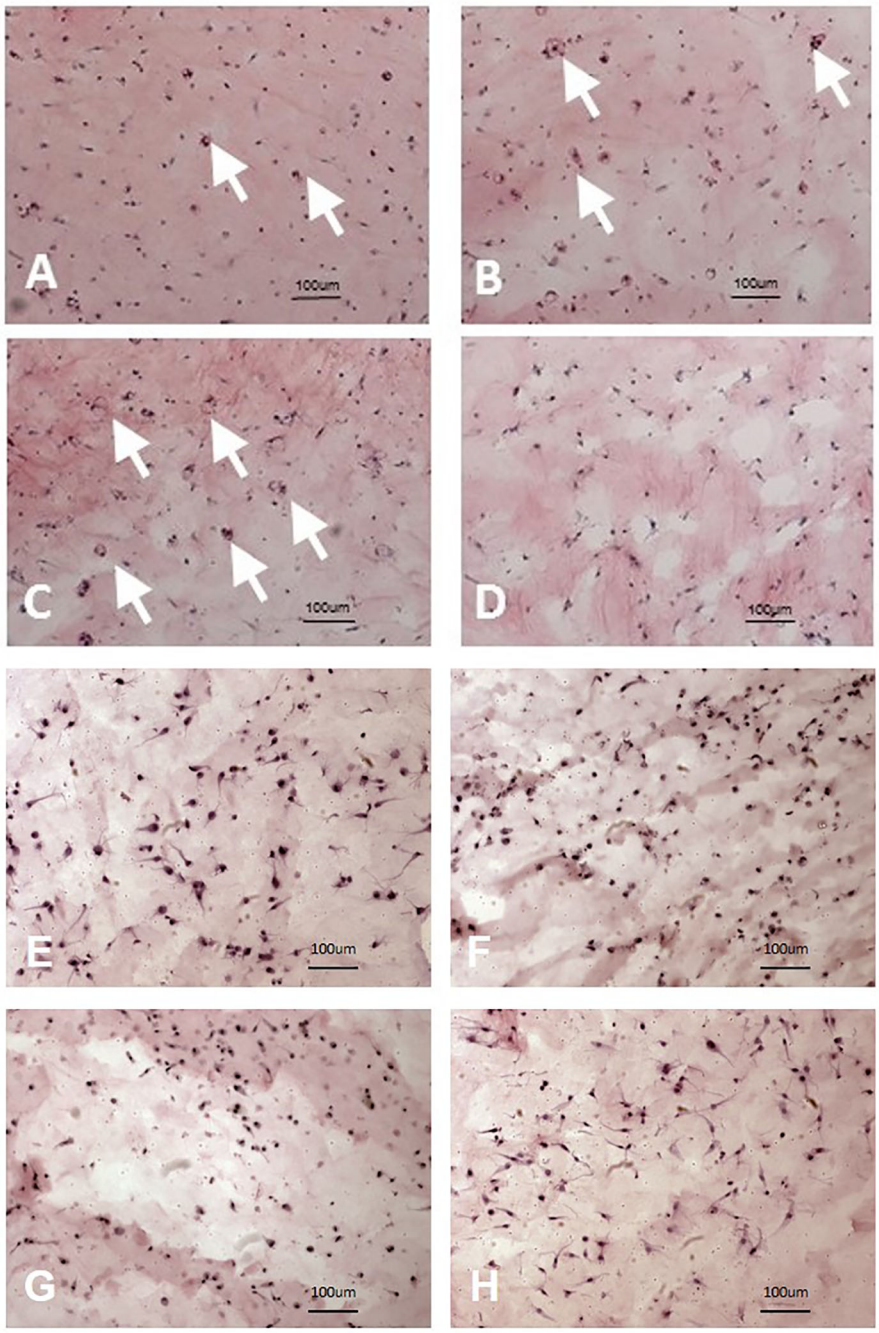

Figure 5.

H & E stained 20 um sections of three-dimensional construct [consisting of ASCs (A-C) and NHAC-kn (E-G) within collagen within chondrocyte differentiation medium] at (A&E) 1 week, (B&F) 2 weeks, and (C&G) 4 weeks in vitro [brightfield imaging at 10x]. Note that as the ASCs differentiate into chondrocytes, many cells become associated with lacunae-like changes (arrows) within the matrix; this process begins at 1 week (A) and becomes increasingly more defined by 4 weeks (C). Control ASCs (D) and control chondrocyte (H) in the same collagen matrix which have been cultured in control medium (ASC medium), with

cells remaining spindle-like and demonstrating no comparable cartilage-like changes in the matrix.

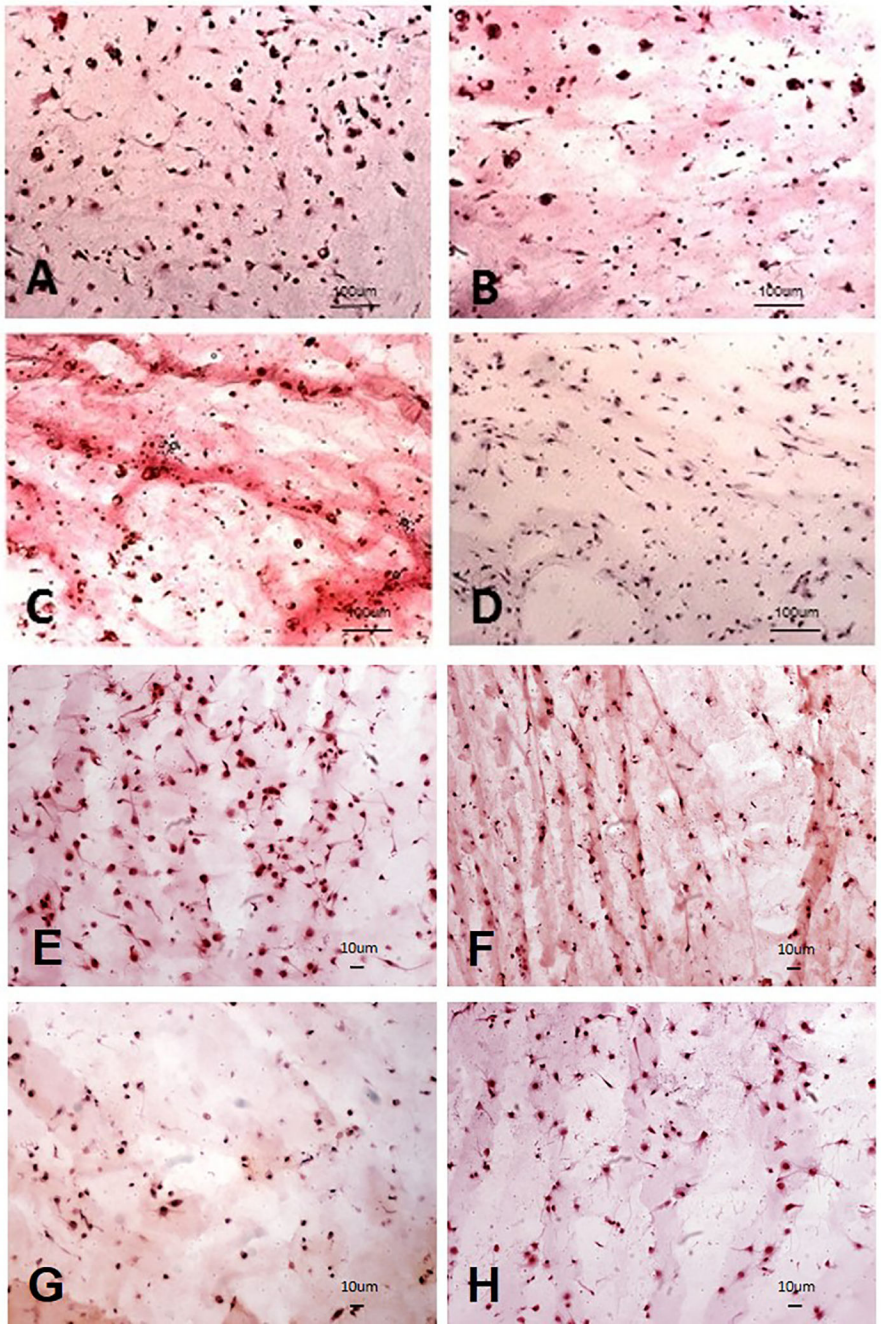

Figure 6.

Cartilage marker, safranin O, has been used to stain these 20 um sections of three-dimensional construct [consisting of ASCs (A-C) and chondrocyte (E-G) within collagen within chondrocyte differentiation medium] at (A&E) 1 week, (B&F) 2 weeks, and (C&G) 4 weeks in vitro [brightfield imaging at 4x for ASCs and 10x for chondrocyte]. By 4 weeks the implant demonstrates many areas of bright orange safranin O staining consistent with maturing cartilage. Control ASCs (D) and control chondrocyte (H) in the same collagen

matrix which have been cultured in control medium (ASC medium) and do not stain orange with safranin O.

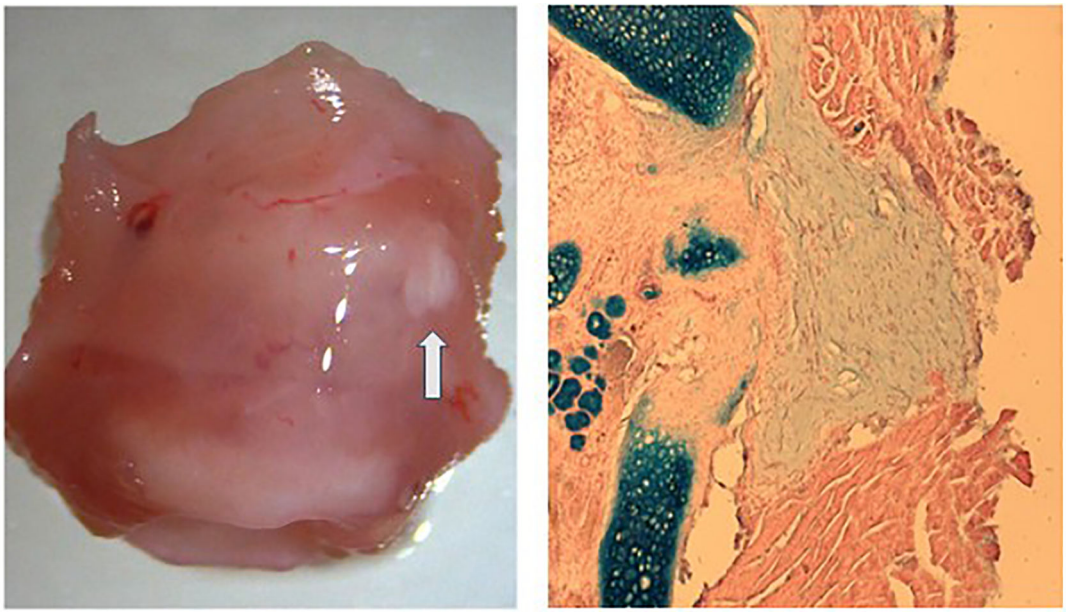

Figure 7.

Rat larynx after explantation. Anterior view of laryngeal cartilage demonstrate normal overall anatomy, with just a small area of remaining white fullness externally where the left hemilaryngeal cartilage defect was replaced with the tissue engineered cartilage, and no overall distortion of the gross laryngeal anatomy.

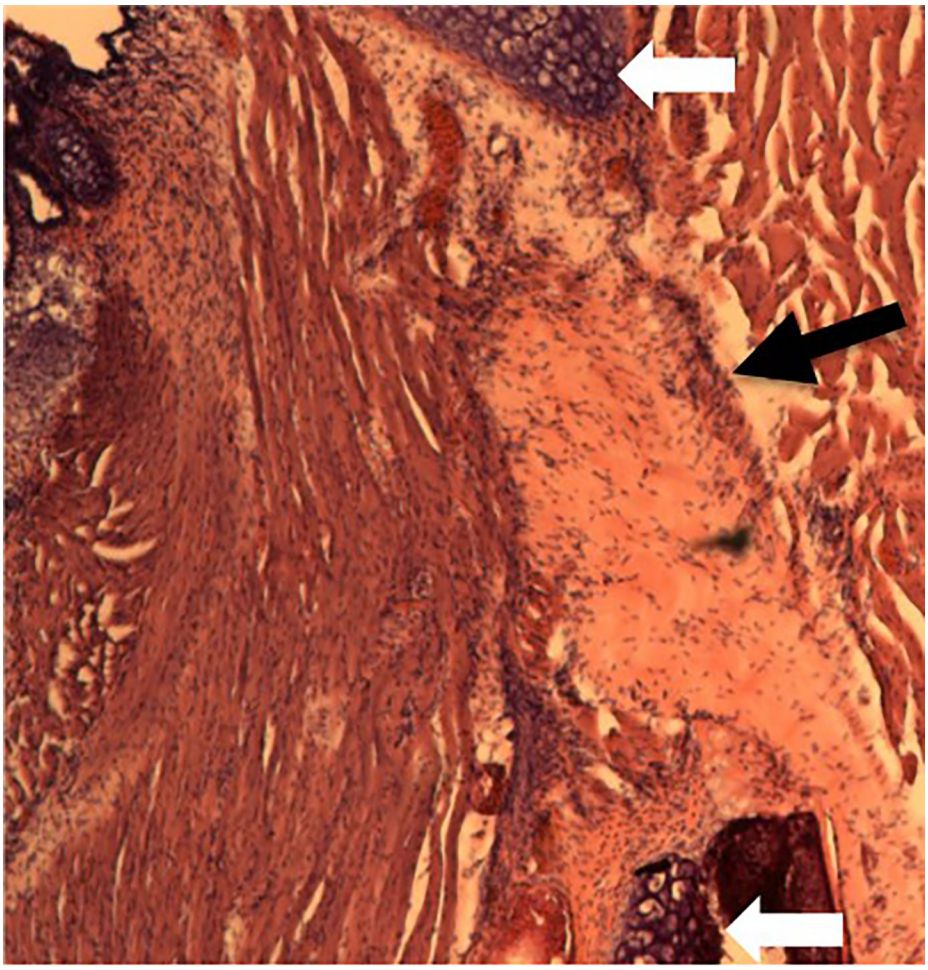

Figure 8.
H & E stain of implant spanning across laryngeal cartilage defect (black arrow). Edges of the remaining native cartilage are demarcated with white arrows.