The Laryngoscope

Epithelial Differentiation of Adipose-Derived Stem Cells for Laryngeal Tissue Engineering

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Objectives/Hypothesis: One potential treatment option for severe vocal fold scarring is to replace the vocal fold cover layer with a tissue-engineered structure containing autologous cells. As a first step toward that goal, we sought to develop a three-dimensional cell-populated matrix resembling the vocal fold layers of lamina propria and epithelium.

Study Design: Basic science investigation.

Methods: Adipose-derived stem cells were cultured in fibrin hydrogels with various growth factors. At the end of the culture period, matrices were sectioned and labeled with immunomarkers to identify cell phenotype.

Results: Adipose-derived stem cells survived, attached, and populated three-dimensional fibrin matrices. Under select conditions, a superficial layer of cells expressing epithelial marker proteins overlay a deeper mesenchymal cell layer.

Conclusions: A three-dimensional structure of fibrin and adipose-derived stem cells was created as a prototype vocal fold replacement. Two segregated cell phenotypes occurred, producing a bilayered structure resembling epithelium over lamina propria. This preliminary work demonstrates the feasibility of tissue engineering to produce structures for vocal fold replacement.

Key Words: Adipose-derived stem cells, vocal folds, epithelium, lamina propria, fibrin, bilayer, epidermal growth factor.

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INTRODUCTION

Vocal fold scarring remains a clinical challenge, with few reliable treatment options available. Approaches have focused on injecting fillers or cells with the hope of restoring normal vibratory properties to the superficial lamina propria layer. Tissue engineering efforts also center on the lamina propria, by encapsulating vocal fold fibroblasts within a variety of scaffolds.[1–3] The impaired vibration does stem from disrupted lamina propria extracellular matrix, with increased collagen deposition and loss of elastic fibers.[4] However, the end result is tethering of mucosal epithelium to the vocal ligament, so that the entire cover layer is involved by the scar. Evidence that cell or matrix injection can modify mature scar matrix and restore vocal fold mucosal wave has been limited.[5,6] Also, the feasibility of harvesting fibroblasts from the human vocal fold remains uncertain.

This report introduces a new type of tissue-engineered vocal fold cover replacement that restores the lamina propria and vibratory epithelium en bloc. Excision and replacement of the scarred, adynamic vocal fold cover segment would address the fundamental derangement by introducing new, organized extracellular matrix and epithelium. Transplanted cells could prevent rescarring and maintain the structure in vivo. We embedded human adipose-derived stem cells (ASC) in a fibrin scaffold derived from cryoprecipitate for a completely autologous implant, and investigated ASC differentiation in that prototype vocal fold cover replacement.

Adipose-derived stem cells are multipotent cells isolated from adult lipoaspirate. They have been widely studied as an autologous mesenchymal cell source to produce chondrocytes, osteoblasts, and fibroblasts.[7] ASCs are genetically similar to bone-marrow–derived mesenchymal stem cells (BM-MSC), which are known to transdifferentiate across lineages to ectodermal and endodermal cell types. Cross-lineage pluripotentiality has been less extensively defined for ASC, although epithelial and neural progenitor cells have been reported.[8,9] Unlike embryonic stem cells that can produce complex geometric structures of dual lineages,[10] adult-derived stem cells, such as ASC or BM-MSC, have not been reported to exhibit organized dual lineage differentiation. We hypothesized that ASCs require an air interface and specific growth factor signals for epithelial differentiation, and that cells away from the air interface default to a fibroblastic phenotype. A bilayered structure of epithelial cells and fibroblasts, such as those found in the vocal fold cover, would thus be achieved.

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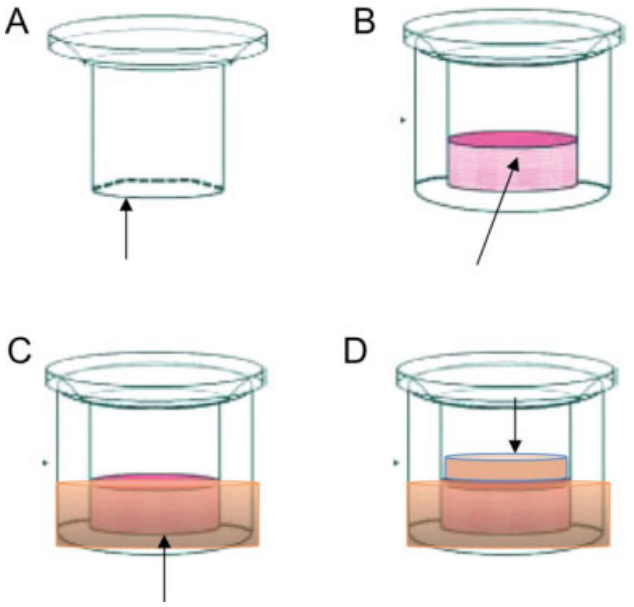

Fig. 1. Fibrin–adipose-derived stem cell (ASC) culture in Transwell inserts. (A) Empty insert; arrow indicates porous membrane base. (B) Insert suspended in culture well; arrow indicates fibrin-ASC gel within insert. (C) Culture medium in well and gel with air interface; arrow indicates pathway for liquid diffusion to gel within insert. (D) Submerged gel condition; arrow indicates additional culture medium on top of gel creating a gel-liquid interface. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MATERIALS AND METHODS

Adipose-Derived Stem Cell Harvest and Culture

The University of California–Los Angeles (UCLA) Institutional Review Board approved the collection and use of donated human lipoaspirate. Adipose-derived stem cells were isolated from human lipoaspirate as described previously.[7] Briefly, lipoaspirate was washed with sterile phosphate buffered saline (PBS), digested in 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C, and combined with Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) to halt digestion. The infranatant was collected and centrifuged, and the pellet was resuspended and filtered. The resulting cells were cultured on standard tissue culture plates in control medium (DMEM with 10% FBS, 1% amphotericin B, and 1% penicillin/streptomycin) at 37°C and 5% CO_2. Cells were passaged when confluent, and harvested for use on the third passage. Each experiment was repeated with ASC from three different donors.

Fibrin-ASC Construct Formation

Human cryoprecipitate, pooled from 10 donors, was obtained from the UCLA blood bank and kept frozen at −70°C until used as a fibrinogen source. Bovine thrombin (Sigma-Aldrich) was prepared at 2 U/mL in 20 mM Hepes buffered saline with 15 mN $CaCl_2$. Thawed cryoprecipitate was mixed in a 4:1:1 ratio with thrombin solution and cell suspension to form fibrin-ASC gels. Initial cell density was 2×10^6 cells/mL, chosen to approximate the cell density in human vocal fold lamina propria. The liquid mixtures were pipetted into Transwell (Cole-Parmer, Vernon Hills, IL) inserts for 24-well culture plates, with a porous polyester membrane (pore size 0.4 μm) at the base (Fig. 1A and 1B). Gelation was complete within 30 minutes at 37°C. An additional 50,000 ASCs were pipetted directly onto the gel surface to replicate an epithelial layer. Resultant neotissue constructs were cylindrical, measuring approximately 6 mm in diameter and 2 mm thick. After gelation, 1 mL of culture medium was added to the wells, and reached the tissue constructs only through the porous insert base (Fig. 1C). For experiments of the submerged gel condition, an additional 100 μL of culture medium was added within the insert, on top of the gel (Fig. 1D). Samples were incubated at 37°C and 5% CO_2.

Growth Factor Supplementation

Control medium was as described above for ASC harvest. Individual growth factors added to control medium were: epidermal growth factor (EGF) at 10 ng/mL, fibroblast growth factor (FGF) at 10 ng/mL, or retinoic acid at 5 μM. Retinoic acid was initially suspended in dimethyl sulfoxide at 10 mM before diluting in control medium. Medium was changed every 2 or 3 days until harvest on day 8 or 14. Initially, duplicate samples of each growth factor treatment were cultured for 8 days in two separate experiments. After refining the growth factor conditions, samples with EGF or control medium were cultured for 8 and 14 days in three separate experiments with different lipoaspirate donors.

Human Vocal Fold Controls

The UCLA Institutional Review Board approved the collection and use of cadaveric human laryngeal specimens. Larynges were obtained from autopsy performed within 48 hours of death, and were frozen at −80°C until use. After thawing, true vocal folds were dissected free from the surrounding cartilage. They were embedded, sectioned, and stained as described below.

Immunohistochemistry

Tissue constructs were harvested with care to maintain their orientation. Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA) was used to embed the samples, and the blocks were flash-frozen in liquid nitrogen vapor. Sections were cut at 4 μm on a microtome, and stored at −20°C until use. Hematoxylin and eosin staining was performed on one slide from each construct by the pathology department using standard clinical staining techniques. Masson trichrome staining was also performed by the pathology department on selected sections.

For fluorescent immunohistochemistry, sections were thawed at room temperature and fixed in 4% paraformaldehyde. Triton X-100 at 0.5% in PBS was applied for 5 minutes to sections labeled for intracellular antigens; no detergent was used for membrane-bound antigens. Nonspecific binding was blocked with 2% goat serum for 30 minutes. Mouse monoclonal primary antibodies were applied at a dilution of 1:50 overnight at 4°C in a humidified chamber. After washing with PBS, secondary antibodies were applied for 1 hour at room temperature. Antibodies included E-cadherin (Abcam 1416, Cambridge, MA), cytokeratin 8 (Abcam 28050), vimentin (Sigma-Aldrich V5255), and goat anti-mouse IgG conjugated to fluoroisothiocyanate (FITC) fluorophore (Invitrogen M30101, Carlsbad, CA). Sections were washed again and coverslipped with VectaShield mounting agent with diamidino-2-phenylindole (DAPI) nuclear stain (Vector Labs, Burlingame, CA).

Fluorescence was viewed the same day as staining, with a Zeiss Axiovert (Carl Zeiss MicroImaging, Inc., Thornwood, NY) inverted fluorescent microscope. FITC and DAPI images were collected separately with a computer-controlled charge-coupled device camera at either 20× or 40× magnification and merged with SPOT image processing software (Diagnostic Instruments, Sterling Heights, MI). All experiments included a slide with no

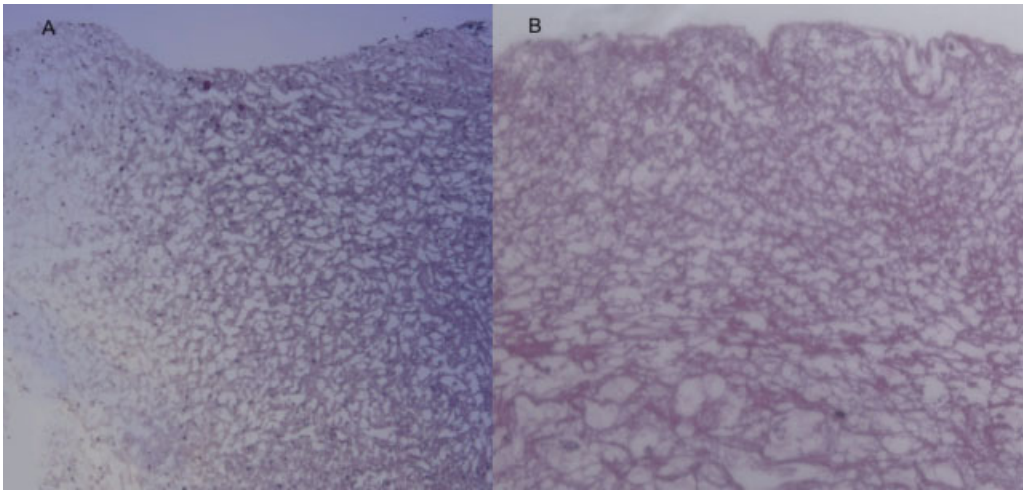

Fig. 2. Hematoxylin and eosin stains. (A) Human vocal fold, coronal section. (B) Fibrin–adipose-derived stem cell construct after 10 days of culture with epidermal growth factor and an air interface.

primary antibody as a negative control. Human vocal fold sections were used as positive controls to confirm expected antibody reactivity.

RESULTS

Fibrin-ASC Construct Fabrication and Microstructure

ASC embedded within fibrin attached and survived over the entire time course investigated, up to 2 weeks in culture. Light microscopy demonstrated cells spread within the gels treated with EGF, FGF, or no growth factors. Retinoic acid treatment reduced cell viability and attachment, so was not pursued further. Resultant constructs could withstand handling, manipulation, and placement of 4-0 chromic sutures, irrespective of treatment group or culture period. Hematoxylin and eosin microscopy showed a fibrin lattice with similar gross morphology to the vocal fold lamina propria. Cell nuclei were identified throughout the construct (Fig. 2). Trichrome staining showed no collagen deposition after 2 weeks of submerged liquid culture without growth factors (Fig. 3A). Culture with EGF and an air interface produced faint collagen staining throughout the gel and stronger fibrillar collagen staining beneath the surface (Fig. 3B).

Growth Factor Induction of Epithelial Marker Proteins in Fibrin-ASC Constructs

Immunohistochemistry demonstrated expression of the epithelial marker protein E-cadherin under certain culture conditions. Constructs with an air-gel interface and EGF treatment showed positive labeling for E-cadherin (Fig. 4A). E-cadherin labeling was diminished in constructs treated with FGF (Fig. 4B). Control constructs without growth factors did not express E-cadherin (Fig. 4C). Figure 4 shows results at 8 days of culture; similar results occurred at 14 days. Results were reproduced with cells from three different lipoaspirate donors.

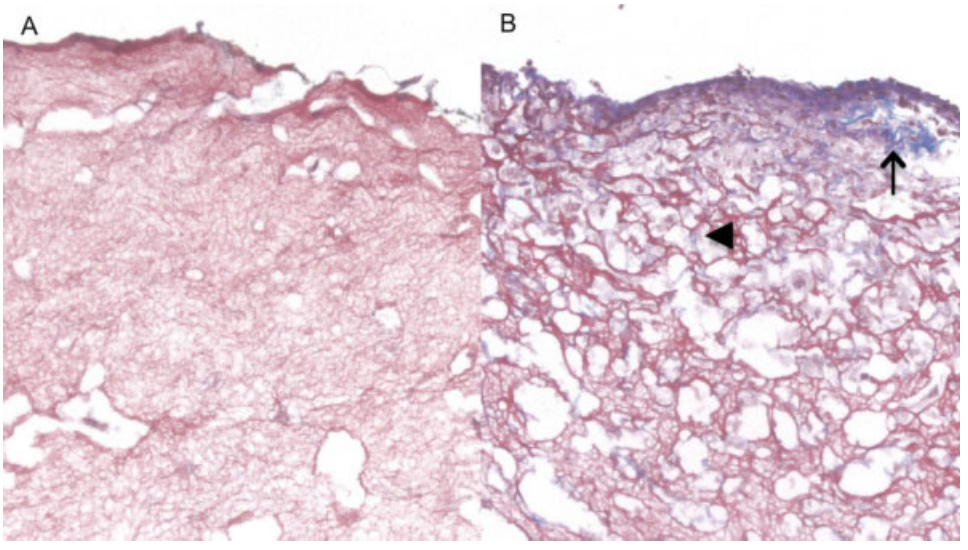

Fig. 3. Trichrome stains of fibrin–adipose-derived stem cell constructs after 14 days of culture. Collagen appears blue. (A) Control case without growth factors and submerged under liquid shows no collagen staining. (B) Construct treated with epidermal growth factor and cultured with an air interface shows collagen fibers beneath the surface (arrow) and light staining throughout the gel (arrowhead).

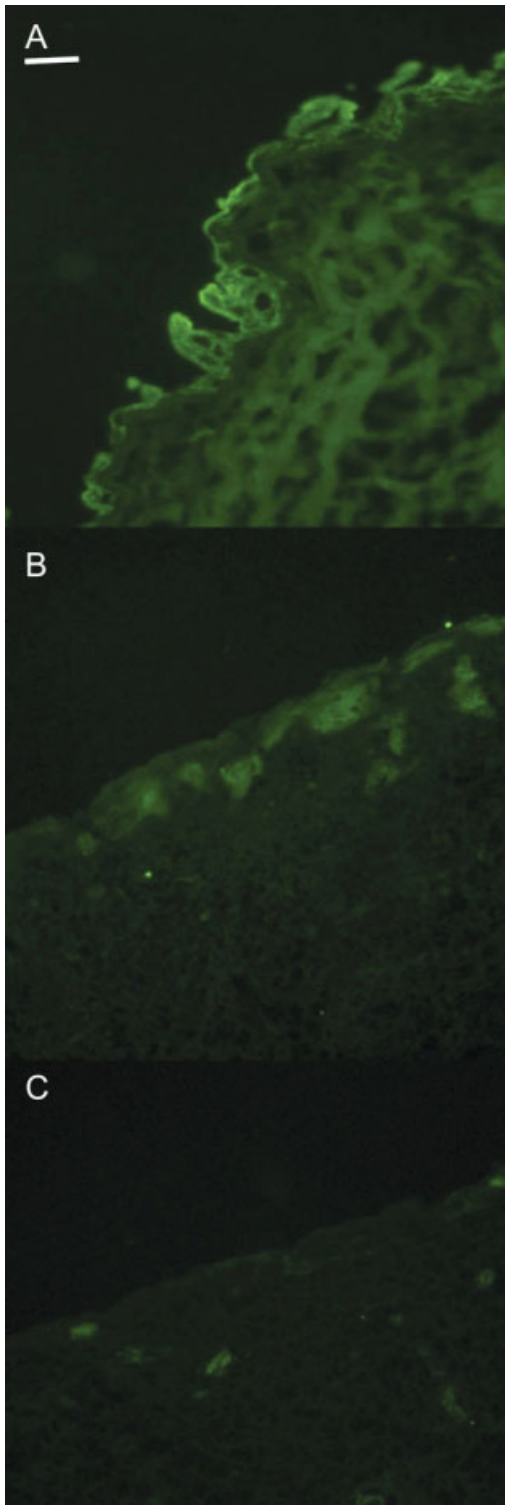

Fig. 4. Immunohistochemistry for E-cadherin in fibrin–adipose-derived stem cell constructs, cultured for 8 days with an air-liquid interface. E-cadherin is labeled with fluoroisothiocyanate fluorophore appearing green. Cell nuclei appear blue with diamidino-2-phenylindole labeling. Scale bars 100 μm. (A) Epidermal growth factor-treated construct shows cellular E-cadherin labeling near surface. (B) Fibroblast growth factor-treated construct shows weak E-cadherin labeling at surface. (C) Control construct without growth factors shows no E-cadherin labeling.

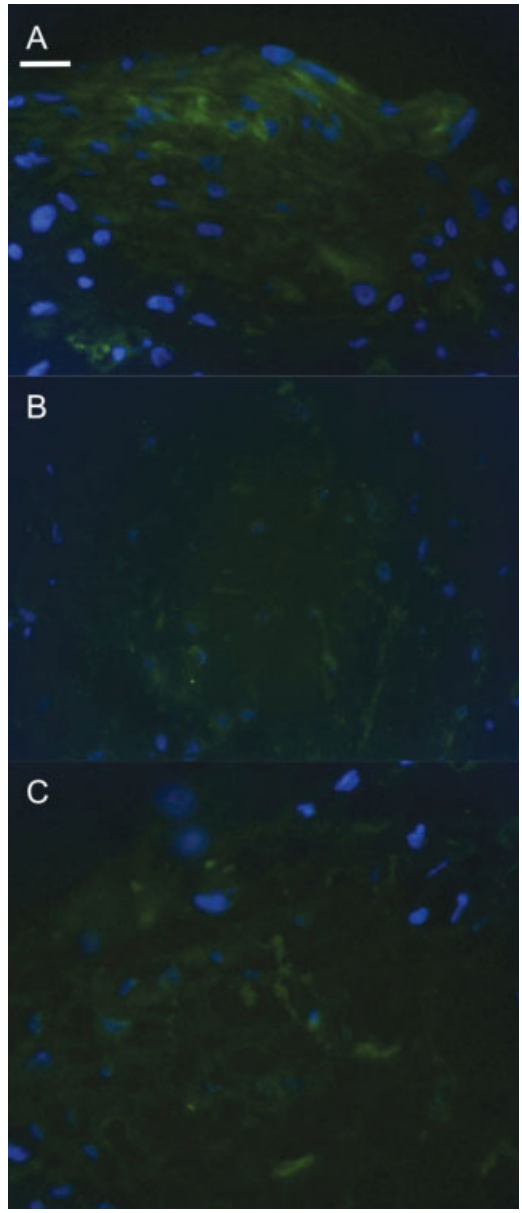

Fig. 5. Immunohistochemistry for cytokeratin 8, appearing green. Scale bar 100 μm. (A) Human vocal fold epithelium shows cytokeratin 8 labeling along the entire surface. (B) Adipose-derived stem cells (ASC) in fibrin, cultured for 8 days with an air-gel interface and with epidermal growth factor, express cytokeratin 8 near the surface. (C) ASC in fibrin, cultured for 8 days with an air-gel interface but without growth factors, shows rare cytokeratin 8 labeling throughout the gel.

To further outline the degree and subtype of epithelial differentiation, immunohistochemistry for cytokeratin 8 was performed. Labeling was less extensive than in human vocal fold epithelium (Fig. 5A). Constructs cultured with an air-gel interface and EGF showed positive labeling for cytokeratin 8 at the surface after 8 days (Fig. 5B). Labeling was diminished in control constructs without growth factors but with an air-gel interface, and cytokeratin 8-positive cells did not cluster at the surface (Fig. 5C). Similar results occurred at 6 and 14 days.

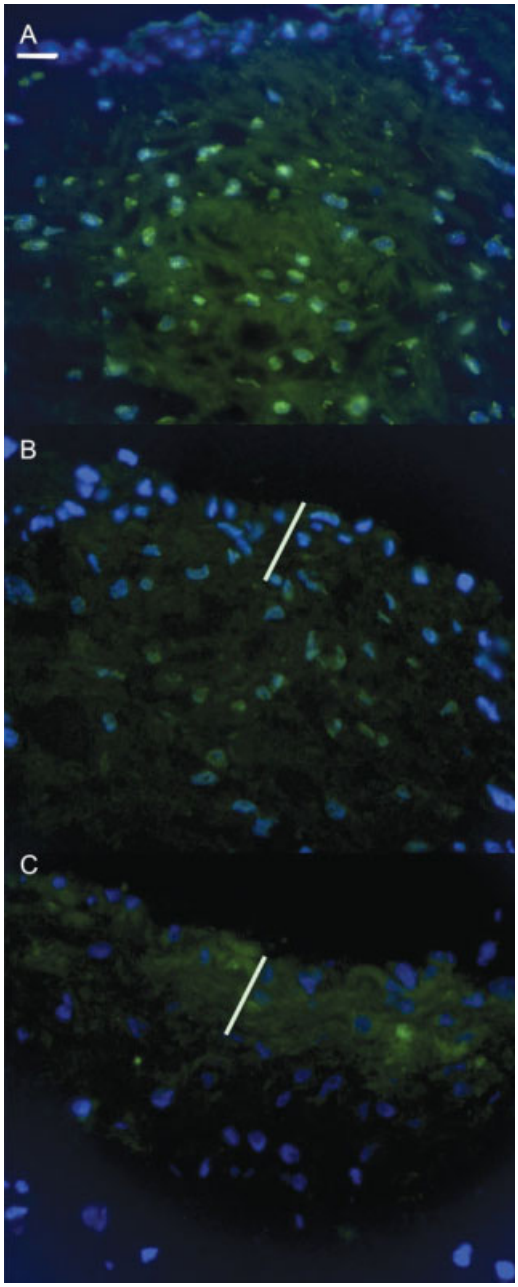

Fig. 6. Immunohistochemistry for E-cadherin in fibrin–adipose-derived stem cell constructs, cultured for 14 days with epidermal growth factor. E-cadherin appears green; cell nuclei appear blue. Scale bar 100 μm. (A) Construct cultured with an air-gel interface shows positive E-cadherin labeling at the surface. (B) The same construct as in A shows diminished E-cadherin labeling within the bulk of the construct, away from the surface. (C) Construct submerged under liquid during culture shows diminished E-cadherin labeling at the surface.

Geometric Dependence of ASC Phenotype in Fibrin Culture

Immunohistochemistry after 14 days of culture with EGF demonstrated that marker protein expression varied with the interface condition and with cell position within the gel. Cells located near an air-gel interface showed more labeling for E-cadherin than cells embedded within the bulk of the same constructs (Fig. 6A and 6B). Submerged constructs with a liquid interface showed diminished E-cadherin expression (Fig. 6C).

ASC embedded within the bulk of the gel, not expressing E-cadherin, did express vimentin. Vimentin is a cytoskeletal protein found in the mesenchymal lineage; it is expressed by lamina propria fibroblasts

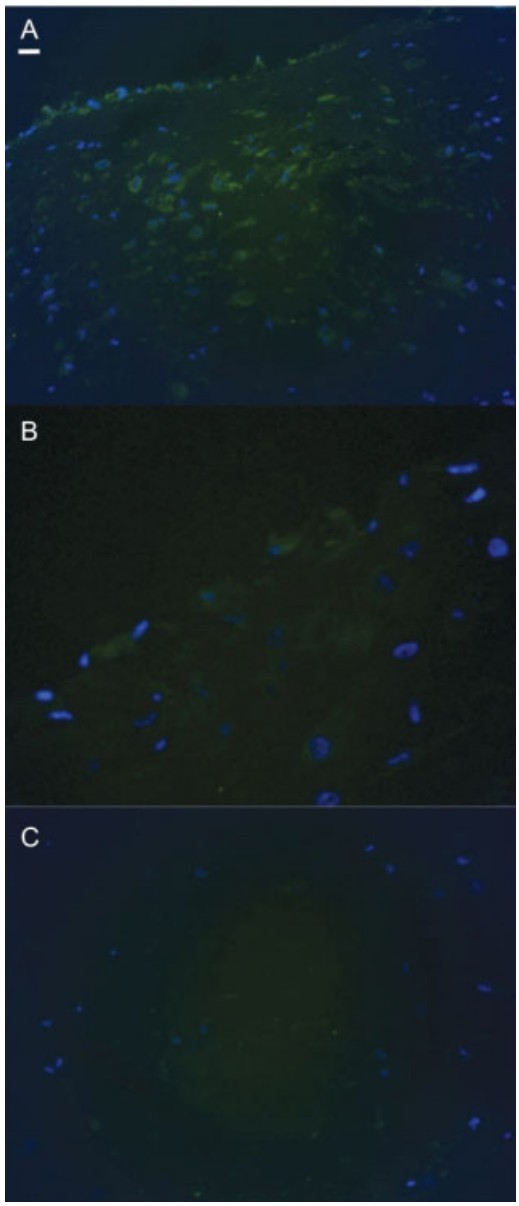

Fig. 7. Bilayered cell phenotypes in fibrin–adipose-derived stem cell (ASC) constructs. Labeling appears green; cell nuclei appear blue. Scale bar 100 μm. (A) Human vocal fold labeled for vimentin shows perinuclear expression in the lamina propria. (B) ASC in fibrin, cultured for 14 days with an air interface and epidermal growth factor, show perinuclear labeling for vimentin within the bulk of the construct. The greatest labeling occurs away from the surface. (C) An adjacent section of the gel in Fig. 6B, labeled for E-cadherin, shows expression at the surface. White bars mark the same level in Fig. 6B and 6C.The area of greatest vimentin labeling in Fig. 6B is underneath the region of E-cadherin expression in Fig. 6C.

(Fig. 7). Vimentin-expressing ASC in fibrin gels were located deep to E-cadherin–expressing ASC.

DISCUSSION

Tissue engineering of any organ is a multifaceted process combining cells, extracellular environment, and chemical signals to produce a structure with similar function as the target tissue. The key vocal fold property to replicate is the pliable connection between the epithelial layer and the deeper muscle, because it is that lamina propria layer that permits mucosal vibration and phonation. This study introduces an implantable construct that morphologically and phenotypically resembles the vocal fold cover with its layered structure of lamina propria and epithelium. We propose that this bilayered construct may effectively treat severe vocal fold scarring by replacing the entire vibratory unit.

In this tissue-engineered vocal fold cover, adult adipose-derived stem cells differentiated concomitantly to two cell lineages with a stratified geometric organization. ASC treated with EGF near the surface of a three-dimensional fibrin gel differentiated to an epithelial phenotype expressing E-cadherin and cytokeratin 8. Deeper cells within the gel expressed vimentin, signifying a mesenchymal phenotype. Producing both epithelial and mesenchymal lineages from ASC reduces the donor morbidity to a single lipoaspiration procedure, rather than two separate biopsies as needed to harvest mature differentiated cells. The single cell source also simplifies manufacturing, because all cells can be cultured together during cell expansion and construct maturation.

One prior study reported epithelial differentiation from ASC. In that work, ASC in monolayer culture with retinoic acid expressed cytokeratin 18 but not other cytokeratins.[9] Cytokeratin 18 is inconsistently expressed in the larynx, whereas cytokeratin 8 as investigated here is expressed in most normal larynges.[11] A follow-up study by the same group treated ASC in monolayer culture with EGF, finding increased cell proliferation and chemotaxis but no differentiation.[12] In our three-dimensional system, retinoic acid impaired cell viability, and therefore was not pursued. Also, in the fibrin culture system, EGF alone was inadequate to produce organized epithelial differentiation; an air interface was a requisite additional signal to optimize the epithelial phenotype and surface localization.

Under optimized conditions for 2 weeks, the newly differentiated epithelial cells congregated at the surface of the three-dimensional construct, but did not form a confluent layer. Collagen fibers were localized beneath the upper cell layer in some segments, but again did not extend along the entire gel surface. A longer culture period or higher seeding cell density could improve the surface cell coverage. Further microscopic examination is required to determine if the collagen fibers seen on trichrome staining represent early basement membrane formation. However, complete epithelialization and a basement membrane are not essential in vitro. The cells may continue to divide and reach confluence after implantation, and epithelial cells from surrounding normal tissue are expected to migrate onto the graft. It is our hope that providing some cells as seed material will hasten re-epithelialization, which has been a limiting step in vivo for tissue-engineered airway constructs. A prior report of undifferentiated ASC implanted into rat tracheal defects did show better early epithelialization than acellular implants.[13] The mild improvement seemed to be due to host-cell recruitment rather than ASC differentiation; both factors would be expected to contribute to epithelialization in our construct.

Fibrin, although not present in the normal vocal fold, offers promise as a scaffold because of its favorable mechanics. The fibrin-based constructs developed here were soft and pliable, but maintained good physical integrity, suggesting suitable handling and performance properties for ultimate vocal fold implantation. During slow degradation by proteolytic enzymes, the fibrin serves as a template for deposition of new extracellular matrix by embedded cells or host tissue. Here, modest collagen deposition occurred after 2 weeks in the optimized culture case, with substantial amounts of fibrin still present. Quantitative mechanical testing is required to assess the collagen's impact and to compare with native vocal folds. Further matrix remodeling is expected to occur in vivo.

A model mechanism for ASC differentiation in this system would include both chemical and physical signals. For example, a synergistic differentiation model has been shown to control mesenchymal stem cell myogenic differentiation on matrices of variable stiffness.[14] In that work, inductive media produced a baseline differentiated marker protein expression, whereas addition of a matrix with physiologic stiffness upregulated myogenic nuclear transcription factors and increased differentiated protein expression. Extrapolating to our system, EGF signaling may act in conjunction with mechanotransduction to influence gene expression and produce the observed epithelial marker proteins. EGF receptors on all cells in the construct are stimulated, with subsequent intracellular signaling. Cells at the surface also sense the air interface, as evidenced by microarray profiling of bronchial epithelial cells. Expression of numerous genes was altered in interfacial culture versus submerged culture, with cytoskeletal elements among the most significant.[15] If ASCs behave similarly, the combination of EGF receptor signaling with cytoskeletal rearrangement may trigger epithelial differentiation in those cells near the air interface. Specific matrix inputs from the fibrin may also contribute. Further molecular experiments are required to test this multisignal hypothetical model of ASC differentiation.

CONCLUSION

A tissue-engineered replacement for the vocal fold cover was fabricated. The three-dimensional hydrogel of fibrin with adipose-derived stem cells resembled the vocal fold lamina propria and mucosa layers in microstructure and handling properties. In this report of stratified dual differentiation, adult stem cells differentiated to epithelial and mesenchymal lineages organized

by position. Epidermal growth factor and an air interface were required to produce epithelial differentiation.

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