Decellularized Tracheal Extracellular Matrix Supports Epithelial Migration, Differentiation, and Function

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Tracheal loss is a source of significant morbidity for affected patients with no acceptable solution. Interest in engineering tracheal transplants has created a demand for small animal models of orthotopic tracheal transplantation. Here, we examine the use of a decellularized graft in a murine model of tracheal replacement. Fresh or decellularized tracheas harvested from age-matched female donor C57BL/6 mice were transplanted into syngeneic recipients. Tracheas were decellularized using repeated washes of water, 3% Triton X-100, and 3 M NaCl under cyclic pressure changes, followed by disinfection with 0.1% peracetic acid/4% ethanol, and terminal sterilization by gamma irradiation. Tracheas were explanted for immunolabeling at 1, 4, and 8 weeks following surgery. Video microscopy and computed tomography were performed to assess function and structure. Decellularized grafts supported complete reepithelialization by 8 weeks and motile cilia were observed. Cartilaginous portions of the trachea were maintained in mice receiving fresh transplants, but repopulation of the cartilage was not seen in mice receiving decellularized transplants. We observed superior postsurgical survival, weight gain, and ciliary function in mice receiving fresh transplants compared with those receiving decellularized transplants. The murine orthotopic tracheal transplant provides an appropriate model to assess the repopulation and functional regeneration of decellularized tracheal grafts.

Introduction

TRACHEAL DEFECTS or stenosis can result from congenital abnormalities, trauma, or pathologies such as cancer or infection. Partial tracheal loss in patients is debilitating and life-threatening.¹ In pediatric patients, surgical approaches, including slide tracheoplasty, have been employed with some success.² In adults, trachea mobilization has enabled post-resection repair in select cases. Long-term stenting, dilation, and tracheostomy have also been employed as palliative care. However, regardless of the approach, complication rates remain very high and long-term morbidity is common.^{3,4} In addition, there remains a cohort of patients for which none of the standard approaches can be employed. Therefore, a functional tracheal replacement graft is still desirable.

Initially, engineered tracheal grafts consisted of purified collagen sponges around a stent or synthetic scaffold.^{5,6} Although widely studied in preclinical models, these have had multiple

deficiencies. Failure of the first engineered tracheas resulted from several causes, including infection, stenosis, and complete tissue disintegration.³ Current engineered tracheal grafts and patches are considerably more complex and employ both multiple graft modifications and recipient treatments. Common to many is a foundation built upon a decellularized tracheal allograft^{7,8} or a synthetic polymer/nanofiber scaffold.⁹ In at least one case, an aortic allograft was employed as the tubular scaffold¹⁰ to provide similar structural support. These scaffolds are then seeded with various cell types to provide functionality, as the size of these grafts limits the degree to which cellular invasion can occur. A number of cell populations have been suggested for reseeded, including basal cells of the lower trachea and induced pluripotent stem cells.¹¹ Scaffolds used in clinical case reports have typically been reseeded with autologous airway cells isolated through bronchoscopic biopsy and/or chondrocytes differentiated from bone marrow mesenchymal stem cells.^{7,8,12–14} Finally, pedicle soft tissue flaps from the abdomen or chest are

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then wrapped around the engineered tracheal transplant to promote angiogenesis and to sequester the transplant from the mediastinum.⁶ Recipients may in some cases be treated pre- and posttransplant with growth factors to maintain the cell population. Current strategies have shown modest success based upon mortality rates, arguably more so with the decellularized allografts than with the synthetic scaffolds.

Despite clinical reports of transplantation of bioengineered tracheas,^{7,15–20} the molecular and cellular processes controlling the survival of the tracheal grafts remain incompletely defined. We tested the hypothesis that decellularized tracheal scaffolds allow cellular invasion/repopulation and functional epithelialization following orthotopic transplantation. Employing a murine tracheal transplant model, we report the first evidence that decellularized tracheal scaffolds allow rapid functional cellular restoration and provide a rationale for continued development of this technology.

Materials and Methods

Animals

All animal experiments were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee and were performed in compliance with the Guide for the Care and Use of Laboratory Animals, as published by the NIH.²¹ Age-matched female C57BL/6 mice (~12 weeks old and 20 g in weight) were used in the study, half as donors and half as recipients. Donor tracheas were either immediately transplanted into a recipient or were decellularized (with and without vacuum assistance) and sterilized for subsequent implantation as a tracheal graft. In the first cohort, mice receiving fresh ($n=7$) or nonvacuum decellularized ($n=17$) tracheal grafts were weighed at regular intervals over 4 weeks to assess failure to thrive in animals during the postoperative period. Surgeries were performed in groups of two or three animals over several sessions. At the end of the study period, specimens were harvested for histological analysis, and high-quality slides were selected for quantification. Tracheas in the second cohort were decellularized with vacuum assistance before transplantation, and surviving mice were sacrificed after 1 or 8 weeks. Histologic examination was performed on at least four tracheas in each treatment group at both the 1- and 8-week time points. At 8 weeks, three additional specimens underwent fresh video microscopy to examine the ciliary beat frequency (CBF). In three other tracheas, microcomputed tomography (micro-CT) analysis was performed.

Tracheal harvest

Donor animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg) and euthanized by exsanguination. The trachea was exposed through a midline neck incision with extension through the proximal sternum. The trachea was harvested from the carina to proximal to the larynx. The larynx was included to facilitate determination of proximal to distal orientation of the tracheal graft, which was particularly important for the fresh transplants. All tracheas were immediately placed in chilled saline and stored on ice. The fresh transplants were prepared for implantation within 15 min of harvest.

Decellularization

Tracheas were trimmed of excess tissue under a dissecting microscope (Zeiss StemiDV4) and then frozen at -80°C until time for further processing in preparation for surgery. The tracheas were thawed in deionized water at room temperature. Tracheas were then decellularized with fourteen 90-min cycles each consisting of deionized water, 3% Triton X-100, and 3 M NaCl treatments, leaving a decellularized tracheal scaffold. Tracheas in late cohorts were subjected to cyclical pressure changes between room atmosphere and 94% vacuum (6.325 kPa absolute pressure) during these washes in a custom apparatus. Vacuum initially degasses the scaffold, removing any microbubbles that might serve as a barrier to interactions between cells and the decellularization reagents. The cyclical pressure changes are hypothesized to provide superior infiltration of detergents and removal of cellular debris. Following decellularization, scaffolds were disinfected through agitation on a shaker in a 0.1% peracetic acid (PAA)/4% ethanol solution for 90 min at room temperature followed by three 30-min rinses in phosphate-buffered saline (PBS). Scaffolds were then individually packaged in physiologic saline and terminally sterilized by exposure to 20 kGy gamma irradiation.

Orthotopic tracheal transplantation

Recipient animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Animals were placed in a supine position and maintained on a heating pad throughout the surgery. The tracheal reconstruction was performed with microscopic assistance, as described previously.^{22–25} The ventral cervical trachea was exposed through a midline incision. The graft was then prepared by removing any loose connective tissue from the surface and liquid from the lumen and cut to a length of five cartilaginous rings from the proximal end of the graft. Care was taken to maintain the proximal–distal orientation of the grafts, particularly for the fresh transplants. A segment of three recipient tracheal rings was dissected from the surrounding connective tissue circumferentially starting approximately four rings below the larynx, with care not to damage the recurrent laryngeal nerves. Once the tracheal segment was freed, a transverse cut was made in the intracartilaginous tissue until a complete transection was performed. A second transection was performed to remove two complete rings of the trachea. Meticulous hemostasis was performed through the process. The distal anastomosis was performed first followed by the proximal anastomosis. In both cases, the anastomosis was performed with two interrupted 10-0 prolene sutures placed near the dorsal ends of the cartilage rings and one or two sutures placed on the ventral aspect of the tracheal repair. The strap muscles were approximated, and the skin incision was then closed with interrupted 7-0 PDS sutures. Animals were monitored until fully recovered from anesthesia. The operative time averaged 20 min.

Postoperative care

After surgery, mice were housed in groups of four to five in standard cages, and food and water were supplied *ad libitum*. The following medications were administered as

subcutaneous injections for 5 days following surgery: buprenorphine (0.1 mg/kg) twice daily for pain relief and gentamicin (8 mg/kg) once daily for infection prophylaxis. At 1, 4, and 8 weeks following surgery, animals were humanely euthanized with intraperitoneal injections of ketamine/xylazine followed by immediate exsanguination, and the tracheas were harvested for analysis. For CBF evaluation, animals were euthanized by exposure to carbon dioxide and cervical dislocation.

Histology

Hematoxylin and eosin staining was performed on decellularized grafts before implantation using standard techniques. Briefly, deparaffinization of tissue sections was performed with two changes of xylene for 3 min each followed by rehydration in an ethanol series. Sections were exposed to hematoxylin for 2 min, rinsed with water, exposed to eosin, and rinsed with water a second time. Finally, slides were dehydrated with an ethanol series, exposed to xylene, and sealed with a coverslip. Explants selected for immunofluorescence were embedded in wax and likewise deparaffinized in two xylene washes, followed by rehydration in an ethanol series. Antigen retrieval was performed using a 10 mM citrate buffer in double distilled water. Five percent bovine serum albumin in PBS was used as a blocking reagent. For the keratin-5 (K5)/keratin-14 (K14) dual stains, the following primary antibodies were applied: mouse anti-K14 (1:500 in blocking reagent) (Thermo/Neomarkers MS-115-P0) and rabbit anti-K5 (1:1000) (Covance PRB-160P). The following secondary antibodies were employed: Alexa-Fluor 488-conjugated goat anti-mouse IgG₃ (1:500) (Invitrogen A21151) and AlexaFluor 594-conjugated donkey anti-rabbit (1:500) (Invitrogen A21207). Likewise, for acetylated tubulin (ACT)/club cell secretory protein (CCSP) dual stains, the following antibodies were applied: mouse anti-ACT IgG_{2b} (diluted 1:20,000) (Sigma T6793) and goat anti-CCSP (1:1000) (kindly provided by Dr. Peter Di, University of Pittsburgh). These were detected with donkey anti-mouse IgG (H+L) 594 (1:500) (Jackson Immuno 715-485-150) and donkey anti-goat IgG (H+L) 488 (1:500) (Jackson Immuno 715-515-150). All slides were counterstained with the VectaShield Mounting Medium with DAPI (Vector Laboratories H-1200). Completed slides were examined with an Olympus IX71 fluorescence microscope (Nikon), and the images were captured with Nikon cellSens Dimension (version 1.5). A single section from each explant was selected for imaging and quantification. Sections were selected for quality based on the orientation of the cutting plane and the integrity of the epithelium. Multiple overlapping images were taken of each explant and the Adobe Photoshop CS5 was used to prepare a photomosaic of all the images from a given slide, which allowed visualization of the entire trachea. Multiple measurements were taken from each photomosaic for quantification.

Quantification

ImageJ (NIH, Bethesda, MD) was used to measure the length of several basement membrane segments along each explanted tracheal lumen. For each measured segment, DAPI-stained and antibody-immunolabeled cells along the segment were hand counted to determine cell densities

(cells/ μ m). Mean cell densities were calculated for each explanted trachea at each time point.

CBF evaluation

Three tracheas from each treatment group were harvested 8 weeks after surgery. Strips of tracheal tissue were secured luminal side down on a 35-mm glass-bottomed culture dish (Willco Wells) using a glass coverslip covered with a silicone sheet (0.5 mm thick; AAA Acme Rubber Co.), from which a small window had been cut to form a shallow chamber. Cilia dynamics were captured at room temperature with a $\times 100$ differential interference contrast oil objective and a Leica inverted microscope (Leica DMIRE2). Movies were captured at 200 frames/s (fps) with a Phantom v4.2 camera (Vision Research). To quantify CBF, ImageJ was used to examine cyclic variations in pixel intensities corresponding to the ciliary stroke. More than three randomly selected areas were imaged from each trachea to calculate the mean native and graft CBF for each treatment group.

Ciliary beat axis evaluation

To quantify variance in ciliary beat axis (CBA), an average of 35 cells were selected in each available *en face* video. ImageJ was used to determine a beat axis for each selected cell, with zero degrees representing an axis parallel to the left-right axis of the frame and 90 degrees representing the perpendicular axis. The mean beat axis (between zero and 90 degrees) was determined for each video. The degree to which each cell beat axis deviated from the video mean was calculated. These deviations were pooled for each animal and plotted as a histogram.

Microcomputed tomography

Three-dimensional image acquisition of explanted tracheas was carried out using a high-resolution micro-CT (Siemens; Inveon Multimodality) at 12 μ m image resolution at 80 kV and 500 μ m X-ray. Three-dimensional surface volume renderings were reconstructed using OsiriX software.

Statistics

Statistical analysis was performed using GraphPad Prism 6 (GraphPad). Data are presented as mean $\pm$ one standard deviation for each group. For weight change and survival analysis, Student's *t*-tests and log-rank (Mantel-Cox) tests were performed, respectively. Differences in cell counts and CBFs between untreated controls, fresh transplants, and decellularized transplants were assessed with two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. For CBA, differences in the variance of each distribution were quantified by the F-test. Statistical significance was defined as $p < 0.05$.

Results

Orthotopic tracheal transplantation and decellularized tracheal reconstruction rescue mice following tracheal loss

Tracheal loss is often fatal, whereas lack of robustly phenotyped preclinical models has hindered tracheal replacement development.²⁶ We tested the hypothesis that

FIG. 1. Orthotopic transplantation of fresh tracheal grafts is associated with superior recovery postoperatively. Orthotopic transplants of fresh and decellularized tracheal grafts were performed on wild-type female C57BL/6 mice. **(a)** Still frames from video recording of tracheal transplant performed in a wild-type mouse. Arrows denote the site of the tracheal defect (in the first panel) and the transplanted graft (in the second). **(b)** Survival (fresh: $n=7$, decellularized: $n=17$, $p=0.165$) and **(c)** weight gain ($n>3$ at each time point, $p<0.001$ at time points denoted with asterisks) over 4 weeks following surgery shown, for early model development studies.

orthotopic decellularized tracheal transplant alone would rescue mice following full thickness tracheal loss. We performed orthotopic transplantation using fresh or decellularized grafts in age-matched female mice, as described (Fig. 1a). A summary of the harvested tracheas and analyses performed on them is provided in Table 1. Excellent healing of the fresh tracheal transplants was observed, rescuing mice from full thickness tracheal loss (Fig. 1b, c). Although mortality rates were initially higher for animals receiving the decellularized grafts, particularly in the model development phase, they approached those for fresh transplants as experience with the model increased. Mortality typically occurred within the first week posttransplant and was generally associated with obstruction secondary to damage to the recurrent laryngeal nerves, stenosis, or mucous build-up in the tracheal lumen. Mean weight gain was determined as a physiologic relevant marker of overall health. Importantly, surviving animals receiving fresh transplants were found to have a $15.1\% \pm 3.4\%$ increase in weight over the 28-day

period following surgery (Fig. 1c). In contrast to animals receiving fresh transplants, surviving animals receiving decellularized grafts experienced an approximate 11% decrease in weight ($89.2\% \pm 16.0\%$ of preoperative weight) during the postoperative interval.

Vacuum-assisted tracheal decellularization effectively eliminates cells

We subjected fresh murine tracheas to a range of decellularization cycles (as detailed in the Materials and Methods section) during the model development phase. Interestingly, tracheas treated with fewer cycles displayed the same degree of decellularization as tracheas treated with higher number of cycles. Early hematoxylin and eosin stains demonstrated total removal of the epithelial layer and no nuclei in the intercartilaginous segments of the tracheal wall, although some nuclear material was maintained within the chondrocyte lacunae of the cartilaginous rings themselves (Fig. 2).

TABLE 1. DESCRIPTION OF ANIMAL TREATMENT GROUPS

	Fresh ("intact")	Decell
1 week (cohort 2)	$n=4$ tracheas collected for histology	Vacuum decell. $n=5$ tracheas collected for histology
4 weeks (cohort 1)	$n=7$ surgeries; weight and survival tracked for 4 weeks $n=2$ (of 7) died $n=5$ (of 7) survived 4 weeks $n=4$ (of 5 survivors) selected for histology. Slides selected based on quality.	Nonvacuum decell. $n=17$ surgeries, weight and survival tracked for 4 weeks $n=11$ (of 17) died $n=6$ (of 17) survived 4 weeks $n=4$ (of 6 survivors) tracheas selected for histology. Slides selected based on quality.
The survival data from this cohort are used in Figure 1.		
8 weeks (cohort 2)	$n=14$ tracheas $n=8 \rightarrow$ histology $n=3 \rightarrow$ CBF $n=3 \rightarrow$ microCT	Vacuum decell. $n=10$ tracheas $n=4 \rightarrow$ histology $n=3 \rightarrow$ CBF $n=3 \rightarrow$ microCT

CBF, ciliary beat frequency; micro-CT, microcomputed tomography.

FIG. 2. Decellularization effectively removes cells from explanted tracheal tissue. Representative hematoxylin and eosin stains from native (**a**) and decellularized (**b**) tracheas following 14 cycles of detergent osmotic shock treatment with cyclic pressure changes, as described in this study. Magnification = 20 $\times$. Scale bar = 50 μ m.

Decellularized tracheal scaffolds display epithelial restoration following orthotopic transplantation

Our finding that decellularized tracheal scaffolds rescued mice from full-thickness tracheal defects suggested that the decellularized scaffolds facilitated reconstitution of the cellular population. Immunofluorescent labeling of fresh and decellularized grafts posttransplantation showed complete resurfacing of the internal surface of decellularized grafts by 1 week postoperatively (Figs. 3 and 4 and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Time course quantification of cell-specific repopulation of the tracheal scaffold was performed. Within the first week following surgery, we observed rapid repopulation of the luminal surface with a large number of dual-expressing keratin-5/keratin-14 (K5+/K14+) cells (Fig. 3d, h, i and Supplementary Fig. S1). There was no evidence of secretory cells or ciliated cells by 1 week (Fig. 4c, d). Over successive time points, the population of K5+/K14+ cells declined, whereas the proportion of K5+/K14- and ACT+ cells increased (Figs. 3n, 3o, and 4n).

This same pattern was observed at the anastomotic site in animals receiving the fresh tracheal transplant (Fig. 3a, e, i). The middle of the fresh transplants showed increased numbers of K5+/K14- and ACT+ cells compared with the repopulated decellularized graft. At 8 weeks posttransplant, the repopulated decellularized grafts contained equivalent numbers of K5+/K14+ and K5+/K14- cells compared with fresh orthotopic tracheal transplants (Fig. 3n, o). Total numbers of secretory cells per micrometer, although initially not significantly different from numbers in fresh transplants, were decreased in decellularized grafts after 8 weeks (Fig. 4o). The cartilaginous rings of the decellularized tracheal grafts did not repopulate with chondrocytes over the course of the study period (Figs. 3 and 4, rightmost columns).

Cilia function is diminished in decellularized tracheal transplants

An important feature of the healthy trachea is the ability to handle secretory load, and a robust coordinate ciliated cell response is a requirement for engineered tracheal transplants. We used state-of-the-art real-time microscopic imaging to assess cilia function in our decellularized and fresh tracheal transplants (Supplementary Movie S1) and calculate the CBF. We observed the presence of functional cilia in both decellularized and fresh orthotopic tracheal grafts. In both fresh and decellularized transplant groups, the CBF for cells along the graft was not significantly different from adjacent native tissue. However, the CBF in both fresh and

decellularized grafts was significantly lower compared with the native untreated trachea (Fig. 5b and Supplementary Movie S1).

We observed that the ciliary movement in repopulated decellularized transplants appeared less uniformly oriented than in the native tissue (Supplementary Movie S1). A mean beat axis was calculated for each *en face* video, and an analysis of the deviations from the means was performed for each of the three animals. We observed a significantly greater variability in the decellularized graft's repopulated epithelium compared to the adjacent native tissue (Fig. 5c).

Tracheal patency is maintained in orthotopic tracheal transplants

The current generation of engineered tracheal transplants has been complicated by the loss of structural integrity and an inability to maintain the tracheal diameter.² We assessed tracheal morphology using micro-CT (Fig. 6 and Supplementary Movie S2). Cartilaginous rings were visible as radioopaque structures in fresh transplants (Fig. 6b) as they were in wild-type untreated controls (Fig. 6a), but could not be visualized in decellularized transplants (Fig. 6c). Both the fresh and decellularized transplants experienced a moderate degree of concentric narrowing at 8 weeks postsurgery (Supplementary Fig. S2). The time course of this narrowing was not immediately clear.

Discussion

The present study showed quantification and functional assessment of the first ever murine model of orthotopic decellularized tracheal transplant. Although more technically challenging than established heterotopic transplantation models, the orthotopic model carries a reduced risk of luminal obliteration.²² Furthermore, orthotopic transplantation provides the opportunity to observe physiologic healing through epithelial migration and proliferation from native tissue adjacent to the anastomosis site. Most important, it mimics the clinical situation and demands functional responses from the transplant if the animal is to both survive and thrive.

A wide variety of techniques have been proposed to decellularize tissues and organs, including chemical, enzymatic, and mechanical.^{14,27} In this study, the decellularized trachea processed with Triton X-100, 3 M NaCl, deionized water, and 0.1% PAA supported rapid cellularization with epithelial cells on the lumen of the graft and mesenchymal cells within the parenchyma of the scaffold. By 8 weeks, the lumen was covered with a site-appropriate pseudostratified

FIG. 3. Histologic quantification of basal cell protein expression in mouse trachea transplants. Mice receiving fresh (**a, b, e, f, i, j**) or decellularized (**c, d, g, h, k, l**) transplants were sacrificed 1 (**a–d**), 4 (**e–h**), or 8 (**i–l**) weeks following surgery. Immunofluorescent labeling for basal cell markers keratin-5 (K5, red) and keratin-14 (K14, green) was performed, followed by DAPI staining (blue). Dual-expressing (K5+/K14+) cells are indicated in yellow. Images are shown at a site of anastomosis (**a, e, i, c, g, k**) or at a midgraft region (**b, f, j, d, h, l**). Arrows denote interface between graft and native tissue; arrowheads denote decellularized graft rings (where visible). Scale bar in panel (**a**) = 100 μm. (**m**) Higher resolution image demonstrates prototypical K5+/K14- (arrowhead) and K5+/K14+ dual-expressing (arrow) cells. Scale bar = 50 μm. Single-positive K5+/K14- (**n**) and dual-expressing K5+/K14+ (**o**) cells along the basement membrane were counted to determine cell densities (cells/μm). Columns labeled “Ctrl” denote cell densities for untreated native trachea from C57BL/6 mice. Columns with error bars represent mean ± one standard deviation with $n \geq 3$ mice in each group. Pairs of columns denoted with an asterisk (*) indicate statistically significant differences ($p < 0.05$). Columns denoted with a dagger (†) are statistically different relative to the untreated control. Multiple photomicrographs were captured, merged, and adjusted for ease of counting. Magnification = 10×. Orientation mark given in (**d**) applies to all images.

epithelium with ciliated and secretory cells present. By 8 weeks, the epithelium showed motile cilia, although the CBF was less than a native mouse trachea, and showed variations in the orientation of ciliary movement as compared to the normal synchronized beating found in the native trachea. An analysis of longer time points will determine if ciliary function normalizes in transplanted grafts.

The trachea is potentially an ideal candidate for repair using a decellularized graft. A mature epithelium, in particular, is a necessary component of any tracheal graft, to¹ act as a barrier defense and² to provide mucociliary clearance. It has been reported that a confluent epithelial layer can reduce or even prevent fibrosis and subsequent stenosis of a tracheal graft.^{5,6} To promote the development of an

FIG. 4. Histologic quantification of ciliated and secretory cell maturation in mouse trachea transplants. Mice receiving fresh (a, b, e, f, i, j) or decellularized (c, d, g, h, k, l) transplants were sacrificed 1 (a–d), 4 (e–h), or 8 (i–l) weeks following surgery, and immunofluorescent labeling for mature epithelial cell markers acetylated tubulin (ACT, red) and club cell secretory protein (CCSP, green) was performed, followed by DAPI staining (blue). Images are shown at a site of anastomosis (a, e, i, c, g, k) or at a midgraft location (b, f, j, d, h, l). Arrows denote interface between graft and native tissue; arrows denote decellularized graft rings (where visible). Scale bar in panel (a) = 100 μm. Orientation mark given in panel (d) applies to all images. (m) Higher resolution image demonstrates prototypical ACT+ (arrowhead) and CCSP+ (arrow) cells. Scale bar = 50 μm. Cells expressing ACT (n) and CCSP (o) along the basement membrane were counted to determine cell densities (cells/μm). Columns labeled “Ctrl” denote cell densities for untreated native trachea from C57BL/6 mice. Columns with error bars represent mean ± one standard deviation with $n \geq 3$ mice in each group. Pairs of columns denoted with an asterisk (*) indicate statistically significant differences ($p < 0.05$). Columns denoted with a dagger (†) are statistically different relative to the untreated control. Multiple photomicrographs were captured, merged, and adjusted for ease of counting. Magnification = 10×.

epithelial layer, decellularized tracheas are frequently seeded with epithelial cells, and cartilaginous rings are repopulated with chondrocytes derived from bone marrow stem cells. Such grafts have been shown to maintain this cell population during the recovery period.^{8,12,28} Furthermore, the decellularized matrix derived from porcine tracheal tissue promotes reepithelialization in a canine model of patch

tracheoplasty.^{29,30} Porcine decellularized tracheal tissue grafted into pigs and mice in heterotopic models showed no immune rejection.³¹ These findings suggest that the decellularized matrix provides an appropriate substrate for epithelial migration, differentiation, and function.

The native tracheal epithelium is complex and composed of heterogeneous cell populations, some of which act

FIG. 5. Ciliated cell function of orthotopic fresh and decellularized tracheal transplants. Real-time video microscopy was employed to quantify ciliary activity 8 weeks after transplantation. **(a)** A contrast-adjusted still frame depicting beating cilia within a decellularized graft is shown; arrows denote ciliated cells. **(b)** Calculated ciliary beat frequencies (CBFs) are shown, with $n=3$ mice in each group. Column labeled “Ctrl” denotes CBF for untreated native trachea from C57BL/6 mice, based on historical data ($n=41$). Columns denoted with a typographical dagger ($\dagger$) indicate statistically significant differences ($p<0.05$) relative to the control column. **(c, d)** ImageJ was used to examine variability in ciliary beat axis (CBA) within the epithelium of repopulated decellularized grafts. Shown are contrast-adjusted still frames of decellularized **(d)** and adjacent native **(c)** tissue. See also Supplementary Movie S1. **(e)** Distributions of CBA (relative to mean beat axis) in three mice receiving decellularized grafts. (*) Indicates a statistically significant difference between the native and graft variances ($p<0.0001$).

FIG. 6. Microcomputed tomography (CT) analysis of fresh or orthotopic tracheal transplants. Representative images shown from CT performed on explanted trachea from wild-type untreated C57BL/6 mice **(a)**, mice receiving fresh transplants **(b)**, and mice receiving decellularized transplant **(c)**. Orientation in **(a)** applies to all images. Arrows in **(c)** denote the boundaries of the decellularized graft.

as progenitor cells.^{32–34} Although multiple studies have included histologic evaluation of tracheal allografts following transplantation, none has examined invasion, proliferation, and regression of various populations of basal cells over time. Herein, we examined the kinetics of epithelial healing and quantified the spatiotemporal rate of epithelial repopulation through immunofluorescent labeling. We observed complete resurfacing of the decellularized tracheal lumen by the end of the first week posttransplant, following early proliferation of K5+/K14+ basal cells. Previous studies have demonstrated that K5+/K14+ basal cells represent a precursor cell population with the capacity to develop into ciliated (ACT+) and secretory (CCSP+) cells.^{33,35} This finding is supported by our histological data, which shows a correlation between the depletion of the K5+/K14+ cell population and the generation of a mature differentiated epithelium (Figs. 3 and 4). Similarly, in animals receiving fresh transplants, the presence of K5+/K14+ cells near sites of anastomosis suggests that this cell population plays a role in epithelial healing (Fig. 3a).

Histologically, we observed that cartilaginous portions of the decellularized trachea remain acellular throughout the course of the 8-week healing period, while chondrocytes within the fresh tracheal transplants are maintained. CT (Fig. 5) did not demonstrate radioopaque cartilaginous rings within decellularized grafts after 8 weeks, in contrast to fresh transplants. Scans performed on decellularized grafts before transplantation similarly demonstrated a lack of visible radioopaque cartilage (data not shown). These findings suggest that cartilaginous rings lose their molecular structure during the process of decellularization and are not repopulated after transplantation. Despite the presumptive loss of the mechanical structure associated with degradation of the cartilaginous rings, decellularized tracheal grafts maintained their patency over 8 weeks (Supplementary Fig. S2). Additional studies will assess chondrocyte viability and repopulation in orthotopic transplants.

The present study had several limitations. First, the murine orthotopic tracheal transplant is technically challenging, and these challenges contributed to the decreased postoperative survival observed in the early model development studies. The technical challenges involved in the surgery were mitigated with experience, as demonstrated by the improved survival seen in later cohorts. Second, histologic analysis was limited by the selection of time points. Rapid repopulation of the luminal surface of each tracheal graft prevented observation of basal cell behavior within the first week, during the period of initial proliferation. This will be addressed by the addition of early time points in future studies. Third, the diameter and cross-sectional area of the decellularized tracheal grafts could not be accessed by means of CT, as originally predicted. We hypothesize that exposure to PAA and/or gamma irradiation during the decellularization process disrupts the molecular structure of the cartilaginous rings, making it difficult to identify the lumen of each graft through micro-CT. Given that the cartilaginous rings maintain the structural integrity of the trachea and patency is an absolute requirement for a tracheal graft, the functional consequences of this loss of cartilage must be further investigated. Furthermore, we observed moderate narrowing of the decellularized tracheal explants, which could be secondary to this loss of cartilage.

Conclusions

These data demonstrate the potential for an orthotopic murine tracheal transplant model to be performed for the evaluation of decellularized grafts. The data presented suggest that this model is a reliable preclinical platform for research. The use of mice will allow for the procedure to be economic and efficient. This model will also allow for harnessing the power of mutant murine models to test the role of specific genes (in knockout or overexpressing strains) in the process of matrix restoration and tracheal transplant healing. The role of innate and acquired immunity in the setting of tracheal transplantation may also be investigated by applying this technique to several well-characterized murine systems of immunologic activation.

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Disclosure Statement

J.S.I. is the Chairman of the Scientific Advisory Boards of Vasculox, Inc. (St. Louis, MO) and Radiation Control Technologies, Inc. (Rockville, MD) and holds equity interests in the same. T.W.G. is the Vice President of Research and Development at ACell, Inc. (Columbia, MD). No other competing financial interests exist.

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