Double-Chamber Rotating Bioreactor for Dynamic Perfusion Cell Seeding of Large-Segment Tracheal Allografts: Comparison to Conventional Static Methods

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Tracheal transplantation with a long-segment recellularized tracheal allograft has previously been performed without the need for immunosuppressive therapy. Recipients' mesenchymal stromal cells (MSC) and tracheal epithelial cells (TEC) were harvested, cultured, expanded, and seeded on a donor trachea within a bioreactor. Prior techniques used for cellular seeding have involved only static-seeding methods. Here, we describe a novel bioreactor for recellularization of long-segment tracheae. Tracheae were recellularized with epithelial cells on the luminal surface and bone marrow-derived MSC on the external surface. We used dynamic perfusion seeding for both cell types and demonstrate an increase in both cellular counts and homogeneity scores compared with traditional methods. Despite these improvements, orthotopic transplantation of these scaffolds revealed no labeled cells at postoperative day 3 and lack of re-epithelialization within the first 2 weeks. The animals in this study had postoperative respiratory distress and tracheal collapse that was incompatible with life.

Introduction

TRACHEAL PATHOLOGIES requiring surgical resection include stenosis, malignancy, and traumatic injury. Lesions involving less than half of the tracheal length in adults and one third in children can be resected and reanastomosis can be achieved.¹ Longer lesions require novel strategies preventing patients from long-term dependence on tracheostomies, which are complicated with frequent infections, hospital admissions, and complicated by death due to plugging from secretions and accidental decannulation in children.

All attempts at developing tracheal replacements with synthetic prosthesis or scaffolds have led to inflammation, mucous build up, granulation tissue, and further stenosis.¹ Biological scaffolds, composed of decellularized material, have shown some promise for clinical transplantation of long-segment tracheal allografts. One of the major limitations, however, continues to involve the integrity of the epithelium that is required for normal mucociliary function of the trachea. Although the epithelium can grow from the edges of the wound, migration is limited, abrogated, or delayed over long distances^{2,3} rendering dependence on airway stents. Recellularization techniques are therefore imperative for preventing airway collapse.

Lessons learned from tracheal epithelial biology of airway regeneration suggest that repopulation of donor allografts with recipient epithelial cells can lead to a normal epithelial lining, abrogating much of the damage mediated by immune rejection.⁴ Recellularization of decellularized biologic scaffolds with recipients' own cells is preferable and may potentially allow for transplantation without the need for immunosuppression.⁵⁻⁷ Techniques utilized for recellularization of tracheal allografts include "*in vivo*" (or "*in situ*") recellularization, which involve harvesting total mononuclear cell populations from the bone marrow and islands of epithelial cells and seeding them on the tracheal allograft simultaneously the day of the transplantation.⁸ This is not ideal as stem cell populations are rare in both the bone marrow and the epithelium and therefore specific isolation procedures would offer better enrichment of these cells.⁹⁻¹³

The second method used for recellularization involved a double-chamber rotating bioreactor.^{5,7,14} Bioreactor recellularization may be a better alternative to "*in vivo*" or "*in situ*" recellularization as it allows isolation and selection of specific subpopulations of cells with known properties. A bioreactor system also enables the expansion of these cells to yield the large numbers required for recellularization of large organs and permits evaluation of the scaffold before transplantation.

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Bioreactors require *ex vivo* manipulation, the appropriate design and parameters are specific to the tissue and organ and are frequently determined through trial and error. In addition, the best results have been obtained for small scaffolds only with very few advances for larger scaffolds. Certain parameters such as cell-seeding techniques, however, can be manipulated. These include manual pipetting techniques that rely on gravity for cell settlement and adhesion to pores, termed “static seeding”. This technique was utilized in the double-chamber bioreactor described for recellularization of a long-segment tracheal scaffold^{5,7,14} and often yields heterogeneous results.^{15–19}

We evaluated a cell-seeding technique, which relies on dynamic perfusion for generation of homogenous long-segment tubular structures and more specifically decellularized tracheal scaffolds. “Perfusion seeding” has been a reliable approach for seeding small but thick scaffolds of low porosity and has been used for various tissue-engineering applications enabling decellularization and subsequent recellularization of heart valves,²⁰ vein grafts,²¹ and hearts and lungs.^{22,23} We investigated perfusion cell-seeding for recellularization of long-segment tracheal scaffolds and compared our novel bioreactor techniques to traditional static-seeding methods.

Materials and Methods

Experimental design

Outbred male Yorkshire pigs (40–50 kg) were utilized. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care of Laboratory Animals” published by the National Institutes of Health. The Animal Care Committee of the Toronto General Research Institute approved all studies. For the *ex vivo* studies (nontransplanted animals), tracheae were harvested from 20 animals and allocated into five equally sized experimental groups (to be explained below): native trachea $n=4$; Protocol I static ($n=4$); Protocol II static ($n=4$); Protocol I perfusion ($n=4$); and Protocol II perfusion ($n=4$). All but native tracheae were decellularized using Protocols I and II. Mesenchymal stromal cells (MSC) and tracheal epithelial cells (TEC) were obtained for cell seeding by bone marrow aspiration and bronchoscopy, respectively. For the *in vivo* study ($n=12$), trachea were harvested from donor animals ($n=6$) and decellularized using protocol II. Recipients animals ($n=6$) had bone marrow aspiration and bronchoscopy for isolation of MSC and TEC respectively. Recellularization of donor trachea with recipient cells was performed using a static-seeding method ($n=3$) or a perfusion-seeding method ($n=3$).

Decellularization protocols

Decellularization protocols have been previously reported by our group.^{24,25} Briefly, Protocol I had been used for xenogeneic patch tracheoplasty.²⁶ It was adapted from the original protocol to a hydrated form.²⁷ Each circumferential 6–9 cm trachea was subjected to 48 h immersion in 3% Triton X-100 solution at 4°C on a mechanical shaker, changing solution after 24 h. After 48 h, a 0.1% peracetic acid, 4% ethanol, and 96% deionized water (diH₂O) wash

was administered for 2 h at 300 rpm on a mechanical shaker to decellularize and disinfect the tissue. The tracheae were then rinsed for 15 min in 0.9% NaCl and diH₂O on a mechanical shaker to remove any residual solution.

Protocol II had been used for acellular bladder matrix²⁸ and was later adapted by Brown *et al.*²⁹ Circumferential segments of tracheae measuring 6–9 cm were separately washed in sterile phosphate-buffer saline (PBS) and then stirred on a mechanical shaker in a hypotonic solution of 10 mM Tris HCl, pH 8.0, 5 mM EDTA (Sigma), 1% Triton X-100 (Sigma), PefablocPlus™ (protease inhibitor) (Sigma) 0.1 mg/mL, and antibiotics/antimycotics at 4°C for 24–48 h to lyse all cellular components. Tissue was placed in a hypertonic solution containing 10 mM Tris HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, and 1.5 M KCl and stirred for 24 h at 4°C to denature residual proteins. Tissue was then washed in Hanks’ Balanced Salt solution for 30 min on a shaker at room temperature four times prior to an overnight enzymatic digestion with 2 U/mL of benzonase (DNase/RNase; Sigma) at room temperature. A final 48 h extraction was performed at 4°C in 50 mM Tris HCl, pH 8.0, 0.25% CHAPS, 1% Triton X-100, and penicillin/streptomycin/amphotericin B with shaking. The tracheae were finally washed four times in sterile diH₂O at 4°C.

Cell culture

As previously described by our group,²⁵ MSC were isolated from bone marrow aspirates by mononuclear cell isolation following density gradient centrifugation. Total mononuclear cells were plated in 75 cm² plastic dishes (T75) and media was changed the next day. Adherent cells were expanded in 175 cm² dishes (T175) up to a cell density of $\sim 6 \times 10^7$ – 1×10^8 cells and selected for a CD45- (CD45-PE AbD Serotec) CD90+ (CD90-APC BD Pharmingen) population using magnet-assisted cell sorting bead selection. The cells conformed to the International Society for Cell Therapy criteria, including the ability to differentiate into osteoblasts, chondrocytes, and adipocytes.²⁵ MSC were cultured in high glucose DMEM (Gibco), supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (P/S). For chondrogenic differentiation, the media was changed to MEM alpha (Gibco) supplemented with porcine TGF- β 1 (10 ng/mL; Sigma), dexamethasone (100 nM; Sigma), ascorbic acid 2-phosphate (50 μ g/mL; Sigma), thyroxine (50 ng/mL; Sigma), and ITS+1 (Sigma). MSC were exposed to chondrogenic conditions for at least 14–20 days after CD90+ selection and were used between passages 4–6.

TEC were isolated from long segments of tracheae. Tracheae were collected in MEM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 50 μ g/mL gentamycin, and 2.5 μ g/mL amphotericin B. Tracheal tissue was cut into segments to expose the inner lumen containing epithelial cells and incubated for 48 h on a shaker at 4°C in MEM supplemented with 5 mg of DNase I (Sigma) and 56 mg of Pronase (Sigma). Following digestion, the segments were rinsed with PBS and added to MEM solution containing the cells and centrifuged for 10 min at 1000 rpm. The pellet was collected and rinsed in PBS. The cells were counted and plated. All TEC were plated in 175 cm² dishes (T175) at a density of 1500 cells/cm² to allow for expansion to a cell

density $\sim 6 \times 10^7$ cells. The plastic dishes were initially coated with 48 $\mu\text{g/mL}$ in 0.01 N HCl of Purcol (Advanced Biomatrix) for 2 h at 37°C before cell seeding and cultured in BEGM (Lonza) supplemented with bullet kit (Lonza). All cells used were between passage 1 and passage 2. Cell surface markers including CD44 were verified on TEC by flow cytometry.

Cell labeling

All cells were labeled with CellTracker™ probes for long-term tracing of living cells as per the manufacturer's instructions (Invitrogen). Briefly, once cells have reached confluence, the media was removed from plastic dishes, washed with PBS, and cells trypsinized and collected in serum-free media. Cell pellets were obtained following centrifugation and resuspended in a dye working solution at a concentration of 25 μM per 1×10^6 cells in PBS. Tubes were covered with foil and placed in an incubator at 37°C for 30 min and subsequently resuspended with an equal volume of prewarmed serum-containing media and incubated for another 30 min at 37°C. Tubes were centrifuged, cell pellets resuspended in full-media twice, counted, and seeded in the bioreactor. The cell trackers used for TEC and MSC were CellTracker OrangeCMTMR (Invitrogen) and CellTracker GreenCMFDA (Invitrogen) respectively.

Bioreactor design and components

The bioreactor was made of a translucent, autoclavable polymer, polyphenylsulfone (commercial name Radel®, Ensinger Hyde, Montreal) (Fig. 1). It includes a cylindrical culture chamber that holds the tracheal scaffold (Fig. 1D, E). Once the scaffold was in place, the graft wall was configured to isolate the inner chamber from the outer culture chamber permitting seeding and culturing of different cell types on the luminal and exterior surfaces of the scaffold. The confined inner chamber allowed select media to be used on either side of the scaffold to optimize proliferation of the different cell types. Tapered grooved anchors at each end, preventing slippage, secured the scaffold. The inlet rotated with the scaffold. The long end of the inner chamber was inserted into the motor casing with a sealed interior end. Silicone tubing was connected to a swivel joint, which, along with an anchor attaching to a metal bar, prevented twisting of the tubing during rotation. The scaffold rotated around two conduits: one for initial perfusion of a cell suspension and subsequent media flow delivery and another that can be used in the future for circulating air in and out of the chamber. A large pulley fitted onto this cylinder, linked to a smaller pulley via a belt and attached to a programmed stepper motor with speed control permitted adjustable rotation of the scaffold around its longitudinal axis. Two ports on top of the outer chamber permitted the inflow and outflow of cell suspensions and culture media. With the exception of the motor, pulleys, and belt, all components could be autoclaved separately before assembly. These features allowed for continual replenishment of fresh culture media in both the inner and outer chambers. Further, the media collected from both output ports may, for example, be re-used and/or analyzed for changes in metabolites, pH, nutrients, and dead cells.

Bioreactor set-up

The bioreactor components were assembled using sterile conditions under a tissue-culture safety cabinet.

Static seeding. Methods for static cell seeding were similar to those described by Asnaghi *et al.*¹⁴ The trachea was inserted in the inner chamber, which was filled with media. TEC were injected into the inner chamber with a syringe. Next, $\sim 6 \times 10^7$ MSC were seeded on the external surface of the trachea after placement into the outer chamber. The bioreactor components were assembled and silicone tubing was connected between the swivel joint and the inlet conduit and another connected between the two outer chamber ports to allow for media exchange. The bioreactor was then placed in the incubator. The cells were allowed to adhere for the first 24 h after which the chambers were half-filled with media and motors were started to allow rotation of the scaffold. The internal media was changed every 24 h and the external media every 48 h. The bioreactor was left in the incubator (37°C and 5% CO_2) for 72 h. Cell concentration in seeding suspension (1×10^6 cells/ cm^2) and rotation of the inner chamber (five revolutions per minute) were the parameters selected.

Perfusion seeding. Perfusion seeding involved two phases: a cell-seeding phase utilizing bidirectional alternating flow and a subsequent culture phase with unidirectional flow (Fig. 2). The bioreactor components were initially assembled using sterile techniques in a tissue-culture safety cabinet. Silicone tubing was connected between the swivel joint and the inlet via connection to a peristaltic flow pump and TEC media reservoir (Erlenmeyer) (Fig. 2A–D). The outer chamber ports were also connected to a peristaltic flow pump, causing media to bidirectionally flow through the tubing while changing direction every 2 min, and MSC media reservoir (Erlenmeyer) (Fig. 2A–D). The peristaltic flow pumps were started and the TEC were injected into the inner chamber and $\sim 6 \times 10^7$ MSC were injected into the outer chamber through the stopcocks connected to silicone tubing for each respective chamber. Peristaltic flow (Fig. 2B) and dynamic rotation of the scaffold was utilized for the first 2 h allowing for cell adherence following which unidirectional flow (Fig. 2D) was utilized and the stopcocks (Fig. 2C*) were opened toward the Erlenmeyer flasks containing media. The media within the TEC reservoir was changed every 24 h and the MSC reservoir every 48 h. The bioreactor was left in the incubator for 72 h. The parameters selected were cell concentration in seeding suspension of 1×10^6 cells/ cm^2 , medium flow rate of 1.5 mL/min, and rotation of inner chamber of five revolutions per minute.

Toxicity and performance assays

CD90+MSC were plated in multiple T75 dishes at a density of 5×10^5 with media change every 48 h. Media used included control media (fresh DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin), heated media (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin heated in incubator at 37°C for at least 2 days), or heated media with polyphenylsulfone (DMEM supplemented with 10% FBS,

FIG. 1. Bioreactor design. (A) Computer Aided Design (CAD) drawings of bioreactor (B) Exterior view of bioreactor showing outlet (C) Exterior view of bioreactor showing inlet (D) View of the inner chamber (E) Trachea post 72 h in bioreactor.

FIG. 2. Bioreactor setup for perfusion flow. (A) Bioreactor setup in incubator (B) Peristaltic bidirectional flow (black and blue arrows) allows for cell seeding within the inner chamber (1) and the outer chamber (2) (C) Setup for media reservoirs: seeding phase bypasses the Erlenmeyer flasks while culture phase allows recirculation of new media. This is made possible by the use of stopcocks (*) at different locations (D) Unidirectional flow allows the culture phase and exchange of media within the reservoirs.

100 U/mL penicillin, and 100 mg/mL streptomycin containing a $2 \times 2 \times 0.02$ cm piece of polyphenylsulfone (bioreactor polymer) heated in an incubator at 37°C for at least 2 days). Cells from different dishes were trypsinized and counted at 24, 48, 96, or 144 h following cell seeding. Media flowing through the bioreactor without a trachea in place (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) was also used for CD90+MSC seeding and media change in T75 dishes. Cells were plated at an initial density of 5×10^5 cells, trypsinized, and counted at 24, 48, or 96 h and compared to control media (fresh DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin).

Fresh, nondecellularized 9 cm native tracheae were placed in a media-filled tube or in a bioreactor supplemented with media by perfusion. Histology and staining with hematoxylin and eosin (H&E) were performed on sections from tracheae following a 72 h incubation at 37°C .

Cell counts and homogeneity scores

Following 72 h of recellularization within a bioreactor, tracheae ($n=4$ for each protocol and each group (static and perfusion)) were divided into three different blocks representing the middle, inlet, and outlet side as shown in Figure 3A. Each block was fixed in 10% neutral buffered formalin at room temperature and transferred to 70% ethanol the next day. All blocks were paraffin-embedded and sections of $5 \mu\text{m}$ were stained with hematoxylin and eosin (H&E). Histology pictures were taken of both the luminal and external surfaces of three different tracheal rings representing the middle of the trachea and inlet and outlet ends. Images of three different areas per section (Fig. 3B orange squares) were taken for homogeneity scores (Fig. 3C) and cell counts (Fig. 3D). Images obtained for homogeneity were $1100 \times 800 \mu\text{m}$ in length by height. Images for cells counts were of higher magnification and measured $500 \times 400 \mu\text{m}$. The mean of all three areas (orange squares) was used to represent the section as a whole. All homogeneity scores and cell counts were obtained from three independent blinded observers. The mean $\pm$ SEM was obtained for cell counts. For homogeneity scores, the coverage represented the total percentage (%) of cells that were present in images. Scores were rounded to the nearest above percentage except for scores of 0 that equal 0% and scores of 4 that equal 100% coverage (0=0%; 1=25%; 2=50%; 3=75%; and 4=100%).

Immunofluorescence

Following deparaffinization, sections were stained with DAPI at a dilution of 1:300 in H_2O , incubated for 30 min at room temperature, mounted with fluorescent mounting media (DAKO), and coverslipped. An Olympus FV1000 Laser Scanning Microscope and FV1-ASW1.6 software were used for confocal images capture.

Orthotopic transplantation of recellularized tracheae

Tracheae were harvested from donor pigs and decellularized using Protocol II. Autologous TEC and MSC were harvested from recipients ($n=3$ for each group; static and perfusion). The timeline until orthotopic transplantation is described in Figure

4. Cells were expanded for about 6 weeks before recellularization. Bronchoscopy was performed on multiple occasions to obtain adequate cell densities for recellularization. Recellularization was performed for 72 h within a bioreactor through a static or perfusion-seeding process.

Bronchoscopy. Intubation was performed using an 8Fr endotracheal tube and a flexible endoscope and 3.0 brush were inserted. The brush was passed along the walls of the large airway. Following a few passages, the brush was capped and removed from the bronchoscope and rinsed in sterile PBS to collect the adherent cells. The brush was then placed again in the bronchoscope and the process repeated 10 times. Tracheal brushings were collected in sterile saline. Brushings and brush tip were vortexed to free the cells for 1 min on medium speed. Sterile forceps were used to remove the brush. Media was used to wash the brush and the tube was then rinsed with fresh media and centrifuged at 600 g for 6 min. The rest of the cell isolation and culture steps were as described above.

Bone marrow aspiration. Recipient pigs were anesthetized and complete aseptic technique, including shaving, surgical prepping, and sterile gloves was used. With the pig placed in a supine position, a standard bone marrow collection needle (11 gauge Jamshidi needle) was inserted in the medial aspect of the proximal tibia at the level of the distal portion of the tibial tuberosity in the middle of the bone. The stylet was passed through the skin and rotated to penetrate the cortex of the bone. The bone marrow was withdrawn following removal of the stylet. Once the sample was removed, the needle was rotated out of the bone and skin. Digital pressure with a sterile gauze allowed for closure of the wound.

Orthotopic transplantation, resection, and reanastomosis. The recellularized trachea was removed from the bioreactor a few minutes before transplantation using aseptic technique and carried to the operating room in a media-filled sterile tube. A vertical incision was performed and extended to expose the cervical trachea. The strap muscles were laterally reflected and held by a self-retaining retractor. The thyroid isthmus or its remnants were divided, suture-ligated, and retracted. The anterior surface of the trachea was dissected at starting at two rings below the cricoid and above the sternal notch. The dissection was carried out circumferentially close to the tracheal wall, the cuff of the endotracheal tube was deflated and two sutures of 2-0 Vicryl were placed vertically in the midlateral line of the distal trachea on either side, through the full thickness of the tracheal wall encircling one ring. A transverse incision was next made in the trachea, at the distal end of the segment to be removed. The distal trachea was intubated with another tube and the cuff inflated. The trachea was elevated and the esophagus dissected away from the trachea. A 6 cm tracheal segment was then circumferentially resected. The donor trachea was removed from the media-filled tube and the proximal and distal anastomosis were performed with a 4-0 Biosyn (Covidien) running suture for the posterior membranous segment and interrupted sutures for the anterior segment. Animals were extubated in the operating room at the end of the procedure.

At postoperative day 3 (POD3), the wound was reopened and the donor allograft exposed. A biopsy was obtained by resecting a 1 cm segment (two rings) from the middle of the

FIG. 3. Representation of sections obtained for analysis. (A) Tracheal biopsies were obtained from different locations (Middle, Inlet, and Outlet). Each block was paraffin embedded and stained with H&E. (B) A section from each block was divided into three (orange squares). (C) Images of each square were taken over the luminal and external surface for homogeneity scores and (D) for cell counts. Images were scored by three independent blinded individuals. The mean of all three squares for each surface was obtained as a representation of each section.

donor trachea and subsequent reanastomosis were performed as described above. The wound was also closed in a similar fashion. Biopsies thus obtained were fixed and sectioned for analysis by histology and immunofluorescence.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 statistical software. Static-seeding groups were compared to perfusion-seeding groups using Tukey's Multiple

Comparison Test ANOVA. A p -value of <0.05 was considered significant.

Results

Bioreactor assembly

The rotating double-chamber bioreactor was easy to assemble, fits in an incubator and allowed seeding of decellularized tracheae and culturing without any leaks or

FIG. 4. Isolation of tracheal epithelial cells (TEC) and mesenchymal stromal cells (MSC), cell seeding, and orthotopic transplantation. TEC and MSC were isolated by bronchoscopy and bone marrow biopsy respectively and expanded in culture for 6 weeks up to a cell density of 6×10^7 . Cells were labeled with CMTMR (TEC) and CMFDA (MSC) and seeded (by static or perfusion) on donor long-segment circumferential tracheae for 72 h before orthotopic transplantation. Recipient pigs had a resection and re-anastomosis of this donor trachea at post-operative day 3 (POD3) to examine presence of cells.

evidence of gross contamination such as media color change. The final design is shown in Figure 1.

Toxicity and performance assays of perfusion bioreactor

Proliferation curves of CD90+MSC in T75 plastic dishes were similar for cells cultured in control media, heated media, and heated media containing a piece of polyphenylsulfone with no statistical difference in cell density at 24, 48, 96, and 144 h (Fig. 5A). Similarly, the proliferation curves of cells cultured in media incubated in the bioreactor also showed no significant differences in cell density at 24, 48, and 96 h (Fig. 5B). Interestingly, the pseudostratified epithelium was lost when a native trachea was placed in a media-filled tube for 72 h (Fig. 5C). The pseudostratified epithelium of the native trachea was maintained when placed in our bioreactor and perfused with media for 72 h (Fig. 5D).

Cell counts and homogeneity scores

Prior to recellularization, histology was performed on decellularized scaffolds, which appeared to be as previously described in our article²⁴ showing loss of cells within the epithelium and submucosa and presence of cells within the cartilage.²⁴ Following recellularization, histology (H&E staining) of luminal (Fig. 6A, B) and external (Fig. 6E, F) surfaces showed presence of nuclei (blue arrows) following perfusion seeding (Fig. 6Bi, Fii), which

were not observed following static seeding (Fig. 6A, E). Quantification of cell counts per field of view were evaluated by three independent blinded observers and showed higher cell counts over the luminal surface (Fig. 6C) and the external surface (Fig. 6G) following perfusion seeding compared to static seeding. This increase in cell counts over the internal surface (Fig. 6C) was significant for perfusion-seeded Protocol II decellularized grafts compared with static-seeded Protocols I and II recellularized grafts ($p < 0.05$). There was also a significant increase in cell counts over the external surface (Fig. 6G) when perfusion-seeded Protocol I recellularized tracheae were compared with static-seeded grafts ($p < 0.01$) and perfusion-seeded Protocol II recellularized grafts compared with static-seeded grafts ($p < 0.001$). This significant increase in cell counts was observed for all three sections (inlet, middle and outlet) (Fig. 6C, G). Homogeneity scores for the luminal surface were also significantly higher when perfusion seeding of Protocol I ($p < 0.01$) and Protocol II ($p < 0.01$) allografts was compared with static seeding (Fig. 6D). For the external surface (Fig. 6H), perfusion-seeded Protocol I recellularized grafts resulted in higher homogeneity scores compared with static-seeded Protocol I recellularized grafts ($p < 0.05$) and static-seeded Protocol II recellularized grafts ($p < 0.01$). Perfusion-seeded Protocol II recellularized grafts resulted in higher homogeneity scores compared with static-seeded Protocol I recellularized ($p < 0.01$) and static-seeded Protocol II recellularized

FIG. 5. Toxicity and performance assays. (A) Proliferation curve of CD90+ bone marrow-derived mesenchymal stromal cells in T75 plastic dishes with heated media containing a piece of polyphenylsulfone and (B) in media from the bioreactor. (C) Hematoxylin & Eosin (H&E) of native trachea after 72 h in media-filled tube and (D) after 72 h in perfusion bioreactor filled with media. Scale bar = 150 μ m. Data are representative of three independent experiments.

FIG. 6. Histology of luminal and external surfaces, cell counts, and homogeneity scores. H&E of luminal surface (A, B) and external surface (E, F) showed no cells following static seeding (A, E), however, nuclei were present following perfusion seeding (B, F) (arrows) as shown at higher magnification (i, ii). The graphs showed higher cell counts (C, G) and homogeneity scores (D, H) following perfusion seeding. Counts were represented as mean $\pm$ SEM. Scale bar on A, B, E, F = 150 μ m; Scale bar on higher magnification (i, ii) = 50 μ m. Data are representative of a minimum of four independent experiments; * p < 0.05; ** p < 0.01; *** p < 0.001.

(p < 0.001) grafts (Fig. 6H). This was again observed for all three sections (inlet, middle, and outlet).

Cell labeling

Fluorescent images of both the luminal surface (Fig. 7A, B) and external surface (Fig. 7C, D) of perfusion-seeded tracheae were obtained. CMTMR-labeled TEC were present on the luminal surface only (Fig. 7A) and CMFDA-labeled MSC were present on the external surface only (Fig. 7D). No CMTMR-labeled cells were found over the external surface and no CMFDA-labeled cells were found over luminal surface (Fig. 7B, C).

Orthotopic transplantation

Biopsies of the middle of the allografts were obtained at POD3 from perfusion-seeded animals and showed very few

cells within the epithelium (Fig. 8A) and cartilage (Fig. 8B). None of the cells were found to be labeled with CMTMR or CMFDA (Fig. 8i, ii). Static-seeded animals displayed symptoms of respiratory distress at POD 4 ± 1 day and were sacrificed. Perfusion-seeded animals showed similar symptoms at POD 8 ± 2 days and were also sacrificed. On intraoperative examination, tracheae either looked infected with presence of collections over the donor allografts or appeared to have some degree of collapse. Tracheal stenosis was estimated at 30% at proximal and distal anastomosis and 0% at the middle anastomosis. Histology of allografts at sacrifice revealed no cells within the epithelium (Fig. 8C) or surrounding the cartilage (Fig. 8D) in either the section closest to the middle anastomosis or the epithelium closest to the proximal anastomosis (Fig. 8E). Some infiltrative cells were found to be present near the cartilage at the proximal end (Fig. 8F). The epithelium (Fig. 8G) and

FIG. 7. Immunofluorescence postrecellularization following perfusion seeding. CMTMR labels TEC, which were present on the luminal surface only (A) and CMFDA labels MSC present on the external surface only (D). No CMFDA labeling (MSC) were found on the luminal surface (B) and no CMTMR labeling (TEC) were found on the external surface (C). Scale bar = 100 μ m. Data are representative of a minimum of three independent experiments.

FIG. 8. H&E of tracheal allografts following orthotopic transplantation of a perfusion-seeded allograft. At POD3, very few cells were found within the epithelium (A) and cartilage (B) in a biopsy obtained from the middle of the allograft. None of the cells found were labeled with CMTMR or CMFDA (i, ii). At POD10, no cells were present within the epithelium (C) or surrounding the cartilage (D) in the middle of the graft or the epithelium closest to the proximal anastomosis (E). Some infiltrative cells were present near the cartilage at the proximal end (F). The epithelium (G) and cartilage (H) closest to the distal anastomosis appeared to have some cells that might resemble epithelial cells (iii). Scale bar on (A–H) images = 150 μ m. Scale bar on immunofluorescence (i, ii) = 100 μ m. Scale bar on higher magnification (iii, iv) = 50 μ m. Data are representative of a minimum of three independent experiments.

cartilage (Fig. 8H) closest to the distal anastomosis appeared to have some cells that might resemble epithelial cells (Fig. 8iii), however, the phenotype of these cells remains unknown.

Discussion

Recellularization of large tubular scaffolds presents with many difficulties due to requirements for very high cell densities and a robust nutrient supply. Previous methods for recellularization of a long-segment circumferential decellularized tracheal scaffold have relied on static cell seeding, which was thought to promote cellular adhesion.^{5,7,14} Pre-clinical and clinical models using these techniques have reported that culture of tracheal epithelial on the luminal surface and MSCs on the external surface was required for long-term successful results.³⁰ However, very few qualitative and no quantitative measurements of cell adhesion or fate following recellularization and before transplantation have been reported. Moreover, these techniques that rely on diffusion for nutrient and cell delivery tend to yield non-homogenous results.^{31,32} The objective of this study was to investigate recellularization of long-segment decellularized tracheal scaffolds using a perfusion-seeding method with our newly designed bioreactor in comparison to the described static-seeding method. We seeded TEC on the luminal surface to promote re-epithelialization and MSC on the external surface. We demonstrated that our bioreactor was biologically safe and maintained the pseudostratified tracheal epithelium in native tracheal segments that had not been subjected to decellularization. Perfusion-seeding methods resulted in higher cell counts and homogeneity scores. Evaluation of allografts following recellularization and before transplantation showed no migration of TEC and MSC between the two seeded compartments. Despite these encouraging improvements, no labeled cells were found *in vivo* as early as postoperative day three despite animals surviving longer than with static cell-seeding methods.

First, we assessed biological safety and effectiveness of our perfusion bioreactor. The polymer used for bioreactor manufacturing, polyphenylsulfone, was shown to be biocompatible and nontoxic, and allowed proliferation of bone marrow stromal cells similar to fresh media. Biological safety results are important and required clinical application of our device. Interestingly, the perfusion bioreactor maintained the pseudostratified columnar epithelium of the native trachea, which was lost in the absence of perfusion, thereby strengthening our hypothesis that perfusion allows effective oxygen and nutrient delivery by convective flow.

We then assessed cellular homogeneity and quantified the cellular content on tracheal scaffolds before transplantation. Our perfusion bioreactor yielded long-segment tracheal scaffolds with higher cell counts and homogeneity scores in comparison with static methods. The presence of adherent cells on the luminal and external surface of perfusion-seeded tracheal scaffolds was observed even after a short incubation period of 72 h on allografts decellularized using two different protocols. These results were observed over the whole length of the trachea suggesting that our perfusion bioreactor allowed for uniformity in cellular adhesion over a 6 cm scaffold. Similar results have been obtained with dynamic perfusion of smaller scaffolds, which aimed to increase the

reproducibility of the seeding process.^{31,33,34} This method has been used to expand articular chondrocytes, differentiate bone marrow stromal cells in a 3D environment,^{35,36} allow spatial organization of many different cell types,³⁷ allow exposure to different environmental stimuli,³⁸ and for production of clinically useful volumes of engineered tissue.³⁹ Thus, significantly higher cell counts and homogeneity scores obtained from perfusion-seeded scaffolds compared with static-seeding scaffolds are consistent with work in other organs.

In addition, we tracked the fate of adherent cells following recellularization and orthotopic transplantation. Perfusion-seeded tracheal allografts were repopulated with TEC (over the luminal surface) and MSC (over the external surface) with each cell type remaining within its respective compartment. There was no migration of cells between the luminal and external surface suggesting that decellularized scaffolds were either nonporous or that the driving forces (mechanical or biological) within this bioreactor failed to stimulate penetration of these cells. The recellularized scaffolds displayed individual cells, which appeared squamous in morphology with no presence of ciliated cells over the luminal surface; a finding that would not be expected following 72 h incubation. Although a few cells were observed within the allograft *in vivo* at POD3, none of these were labeled with CMTMR (TEC) or CMFDA (MSC) suggesting that they were recipient infiltrating cells. Obviously, our perfusion-recellularization or transplantation techniques require further improvements. Longer incubation times may be beneficial for generation of an epithelium resembling that of native trachea favoring eventual re-epithelialization from the host. Moreover, phenotype proliferation and differentiation capacity of the adherent cells following recellularization remain to be evaluated.

Further, we expected that *in vivo* re-epithelialization would be more efficient on perfusion-seeding grafts due to an increase in homogeneity and cells counts. Despite presentation of respiratory symptoms in both groups, animals receiving perfusion-seeded tracheal allografts survived longer than animals receiving static-seeded allografts. Histological assessment of tracheal allografts showed signs of revascularization and re-epithelialization over the distal segment of the graft. Revascularization was not observed over the middle portion. Upon intraoperative examination, the allografts appeared to be infected or had some degree of collapse. This could be due to contamination at the time of surgery *per se* or caused by contamination during recellularization (within bioreactor) or for technical reasons (anastomotic leak); although the latter is unlikely given all three anastomoses appeared intact. Further, acute rejection could also be a cause of their symptoms although histology showed no mononuclear cell infiltrate except possibly around the external surface surrounding the cartilage. We can speculate that the partial improvement in recellularization in perfusion-seeded grafts caused an improvement in the immunosuppressive effects of recellularization we have recently noted.²⁵ Despite the improvements that we have made *ex vivo* to the recellularization process using perfusion seeding, further progress in bioreactor recellularization techniques are required to advance these results *in vivo*. These include identifying the ideal cell types, using co-culture techniques to allow synergy, thus, improving cellular

adhesion, proliferation, and differentiation and using our bioreactor to enhance the revascularization process to maintain epithelial integrity and prevent restenosis of decellularized tracheal scaffolds.

Conclusion

In conclusion, this study provides a detailed assessment of tracheal scaffolds following recellularization prior to transplantation using histological and cell-tracking techniques. It describes a method allowing dynamic perfusion seeding within a bioreactor. This bioreactor design can be used for long-segment tracheal scaffolds and potentially other tubular structures. Generally, any tube-like scaffold, whether biological or synthetic, ranging in various lengths (e.g., from 6 to 12 cm or more) can be placed in this bioreactor for recellularization. This may translate into applications for various tissues and organs including esophageal, bronchial, intestinal, and vascular grafts. The perfusion-seeding method used within our bioreactor confirmed adherence of two different cell types and achieved higher cell numbers and homogeneous structures compared with traditional static-seeding methods. Despite such results, the cell adherence achieved was insufficient in accelerating *in vivo* re-epithelialization of long-segment tracheal allografts, which remains a significant challenge in this field.

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Disclosure Statement

No competing financial interests exist.

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