



# Laryngeal Reconstruction Using Tissue-Engineered Implants in Pigs: A Pilot Study

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**Objective/Hypothesis:** There are currently no treatments available that restore dynamic laryngeal function after hemilaryngectomy. We have shown that dynamic function can be restored post hemilaryngectomy in a rat model. Here, we report in a first of its kind, proof of concept study that this previously published technique is scalable to a porcine model.

**Study Design:** Animal study.

**Methods:** Muscle and fat biopsies were taken from three Yucatan minipigs. Muscle progenitor cells (MPCs) and adipose stem cells (ASCs) were isolated and cultured for 3 weeks. The minipigs underwent a left laterovertical partial laryngectomy sparing the left arytenoid cartilage and transecting the recurrent laryngeal nerve. Each layer was replaced with a tissue-engineered implant: 1) an acellular mucosal layer composed of densified Type I oligomeric collagen, 2) a skeletal muscle layer composed of autologous MPCs and aligned oligomeric collagen differentiated and induced to express motor endplates (MEE), and 3) a cartilage layer composed of autologous ASCs and densified oligomeric collagen differentiated to cartilage. Healing was monitored at 2 and 4 weeks post-op, and at the 8 week study endpoint.

**Results:** Animals demonstrated appropriate weight gain, no aspiration events, and audible phonation. Video laryngoscopy showed progressive healing with vascularization and re-epithelialization present at 4 weeks. On histology, there was no immune reaction to the implants and there was complete integration into host tissue with nerve and vascular ingrowth.

**Conclusions:** This pilot study represents a first in which a transmural vertical partial laryngectomy was performed and successfully repaired with a customized, autologous stem cell-derived multi-layered tissue-engineered implant.

**Key Words:** Larynx, cartilage, tissue engineering, collagen, oligomer, chondrocytes, adipose stem cell.

**Level of Evidence:** N/A

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## INTRODUCTION

Devastating dysphonia or aphonia impacts thousands of individuals in the United States each year undergoing traumatic or oncologic partial laryngectomies.<sup>1,2</sup> Currently, there are no options for laryngeal reconstruction, which add muscle volume and restore dynamic motion. Tissue-engineered myochondral

implants (MIs) that are derived from the patient's own adult stem cells (via a biopsy under local anesthesia) and customized to address patient deficits would assist in addressing these unmet needs in laryngeal reconstruction. In addition, since the recurrent laryngeal nerve (RLN) may or may not be intact in these reconstructive scenarios, the ideal MIs would provide viable and functional reconstruction in environments with or without RLN integrity.

Over the past decade our laboratory has developed a novel method of constructing MIs with motor endplate-expressing muscle cells (MEEs) to promote innervation of MIs in vivo.<sup>3–11</sup> When the muscle cells are induced to express motor endplates, the cells release an array of neurotrophic factors (NFs); in vivo, these NFs promote axonal regeneration and innervation of the motor endplates, and associated muscle constructs.<sup>10</sup> Our group has also developed a patented unique self-assembling liquid collagen that, together with scalable biomanufacturing methods (e.g., extrusion, compression molding), is ideal for fabrication and customization of autologous MIs.<sup>12–17</sup> Our previous investigations using these MIs have been successful in a rodent model of partial laryngectomy. While "scaling up" has always been a tremendous hurdle in the field of laryngologic tissue engineering, we herein report approaches to overcome this hurdle with resultant creation of large-volume MIs, adequate for reconstruction in a large animal model. Thus,

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

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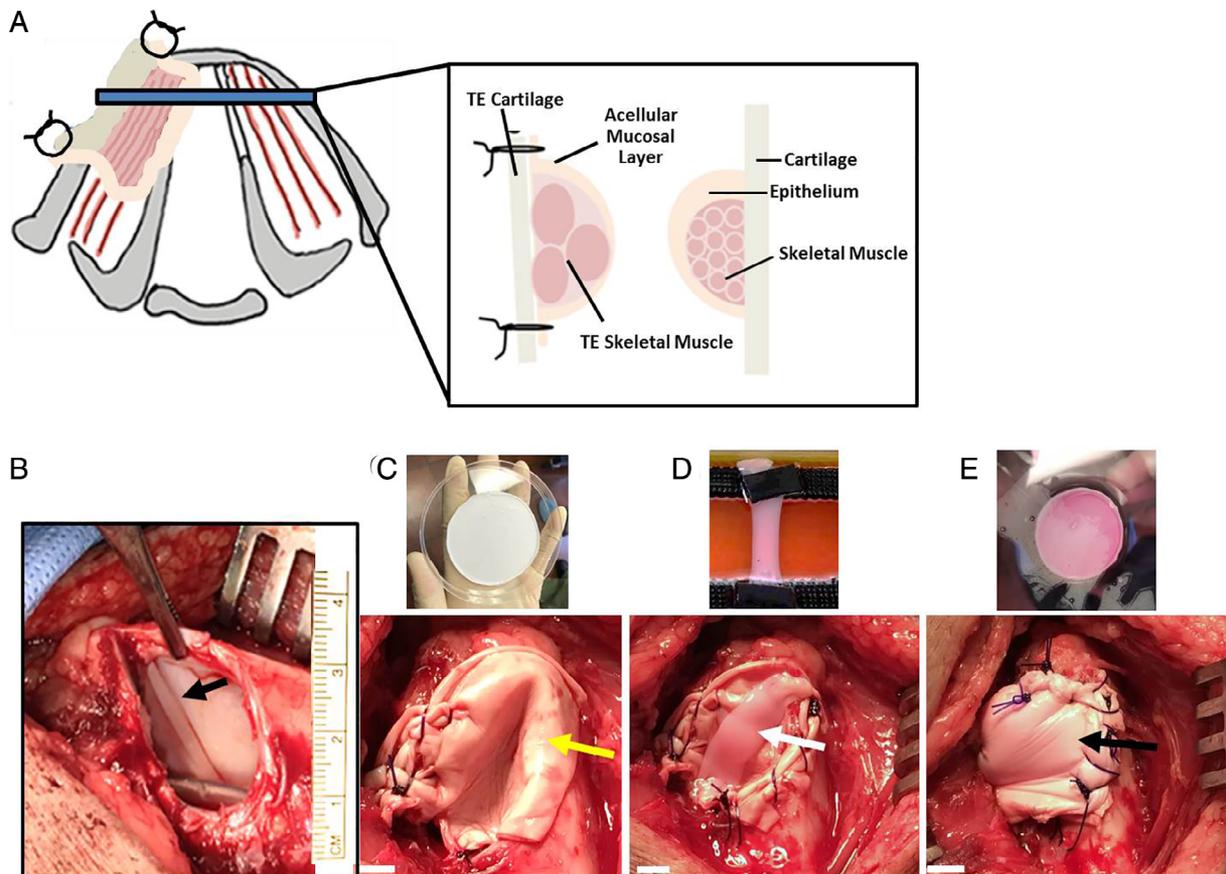


Fig. 1. (A) Schematic drawing of left transmurular laterovertical partial laryngectomy with tri-layer tissue-engineered implants. The entire true vocal fold and false vocal fold were excised from the vocal process of the arytenoid to the anterior commissure, leaving only the arytenoid and small posterior thyroid cartilage strut as anchor points for the implant. Surgical Procedure: (B) Transmurular surgical defect was created in left hemilarynx showing contralateral (right) vocal fold. (C) Mucosal sheet (acellular), (D) cellularized muscle, and (E) cellularized cartilage constructs before (top) and during (bottom) surgical implantation. Scale bars: 500  $\mu$ m

the goal of the following investigations is to determine if autologous MEEs and ASCs can be used along with self-assembling collagen to create MIs that provide functional replacement for partial laryngectomy defects *in vivo* in a porcine model. Specifically, we will investigate if the MIs: 1) demonstrate strong innervation, adequate muscle volume and fiber alignment/organization, and visible adductor activity; and 2) provide restoration of basic laryngeal protective reflexes for cough, swallowing, and airway maintenance over a follow-up period of up to 2 months.

## MATERIALS AND METHODS

### Primary Muscle Progenitor Cell and Adipose Stem Cell Isolation and Culture

Yucatan minipigs (S&S Farms, Malta, IL) were anesthetized under isoflurane, and skeletal muscle and fat biopsies were obtained from the dorsal neck area. Fresh muscle and adipose tissue were minced in growth medium (GM); Dulbecco's Modified Eagle Medium (DMEM), 1% penicillin, streptomycin, amphotericin B (PSF-1, Hyclone, Logan, Utah), 20% fetal bovine serum (FBS, Hyclone, Logan, Utah), and digested in 0.2% collagenase type I (EMD Millipore, Temecula, CA) at 37°C for

2 hours. Digested muscle tissue was filtered through a 100- $\mu$ m cell strainer, plated onto untreated 100-mm petri dishes (Fisher Scientific, Chicago, IL), and cultured overnight at 37°C with 5% CO<sub>2</sub>. The supernatant was removed the next morning and transferred to culture flasks (Corning Life Sciences, Corning, NY). Digested adipose tissue was filtered through a 70- $\mu$ m cell strainer and plated in a T-75 cell culture flask. Cells were cultured to 70% confluency and used in experiments at passages 1 to 2.

### Fabrication of Engineered Skeletal Muscle Constructs

Muscle implants were fabricated as previously described.<sup>3-10</sup> Briefly, MPCs from biopsies were suspended in type I oligomeric collagen (GeniPhys, Zionsville, IN) (2.0 mg/mL) and flowed through a 10-mm cylindrical mold. Once polymerized, implants were cultured under passive tension in DMEM supplemented with 1% PSF-1, and 10% FBS at 37 C and 5% CO<sub>2</sub> for 2 days, with medium changes every 2 days. On day 3, medium was changed to differentiation medium, representing DMEM supplemented with 2% horse serum (HyClone) and 1% PSF-1, and constructs were cultured for an additional 5 days to induce myotube formation, at which point acetylcholine chloride (40 nM;

TABLE I.  
Basic Acoustic Analysis of Porcine Phonation.

Acoustic Properties	Immediately Preoperatively	Intermediate Postoperative Period (2 weeks)	Late Postoperative Period (8 weeks)
Relative Amplitude	1.00 ± 0.15	0.54 ± 0.03	1.06 ± 0.11

To selectively analyze glottic phonation (rather than supraglottic noise), maximum amplitude for glottic phonation was analyzed at 2000 Hz, with 60 second audio recordings attained for each animal. Specifically, the amplitude of each glottic phonation was visualized and measured by Audacity software under waveform view (<https://www.audacityteam.org/>). The amplitude of each group was normalized by the normal glottic phonation obtained preoperatively.

Tocris Bioscience, Bristol, England), agrin (10 nM; R&D Systems, Minneapolis, MN), and neuregulin (2 nM; R&D Systems) were added to the medium to induce motor endplate formation. Constructs were cultured an additional 5 days with medium changes every 3 days. Motor endplate expression was confirmed by immunostaining with Alexa Fluor 594 conjugated  $\alpha$ -bungarotoxin (Molecular Probes, Eugene, Oregon).

### **Fabrication of Engineered Cartilage Constructs**

Cartilage constructs were custom fabricated based on previously described methods.<sup>10,12,17</sup> Briefly, porcine ASCs were suspended in type I oligomeric collagen and polymerized. They were then compressed to constructs (412 mg total collagen, 3.4 cm in diameter, 5 mm in thickness) and cultured in chondrogenic differentiation medium (Hyclone Advance Stem Chondrogenic Differentiation Medium, SH30889.02; Thermo Scientific, Waltham, MA) and were maintained in a 37°C, 5% CO<sub>2</sub> incubator for 2 weeks. The medium was changed twice per week.

### **Fabrication of Engineered Vocal Fold Mucosa**

Densified collagen scaffolds, custom fabricated using an adapted confined compression technique,<sup>12, 14–16</sup> were obtained from GeniPhys. Briefly, acid-soluble type I oligomeric collagen was neutralized and added to sterile cylindrical compression chambers. After collagen self-assembly, the resulting collagen scaffold was compressed to achieve a construct that was 315 mg in total collagen, 6.3 cm in diameter, and 0.55 mm in thickness. The construct was aseptically removed from the chamber and stored in sterile, sealed airtight containers prior to use in testing or surgery.

### **Partial laryngectomy, RLN Injury, and Reconstruction with the MI**

The animal study protocol was approved by Purdue Animal Care and Use Committee; institutional guidelines, in accordance with the National Institutes of Health (NIH), were followed for the handling and care of the animals. Three Yucatan minipigs were used for the experiments, all receiving cell based implants. After 3 weeks of stem cell expansion and differentiation, the pigs underwent a partial laryngectomy surgery in which over a 3 cm<sup>2</sup> trans-mural defect was created, resecting the entire inner true vocal fold and false vocal fold with associated adductor muscle and outer supporting cartilage (Fig. 1) with the defect extending from the anterior commissure to the unilateral arytenoid. Only the arytenoid and overlying posterior laryngeal cartilage strut were spared on the reconstructed side to provide anchoring sites for the MI. Each layer was replaced with a tissue engineered implant: an acellular mucosa layer composed of densified collagen, a skeletal muscle layer composed of aligned oligomeric collagen and autologous MPCs differentiated and induced to express motor endplates (MEE), and a cartilage layer composed of densified oligomeric collagen and autologous ASCs differentiated to cartilage (Fig. 1). Each layer was sutured to the thyroid cartilage using running 2–0 Vicryl. The left RLN was ligated and a 1 cm section removed. The subcutaneous tissue and skin were then closed with 2–0 Vicryl suture.

### **Videolaryngoscopy and Laryngeal Electromyography**

At 2, 4, and 8 weeks after hemilaryngeal reconstruction, animals were returned to the operating room to be examined with videolaryngoscopy under anesthesia with inhalational isoflurane. Videolaryngoscopy was performed while titrating the anesthetic to permit stimulation-induced laryngospasm. The endotracheal tube (ETT) was briefly withdrawn and the videolaryngoscope was introduced orally. As needed, additional

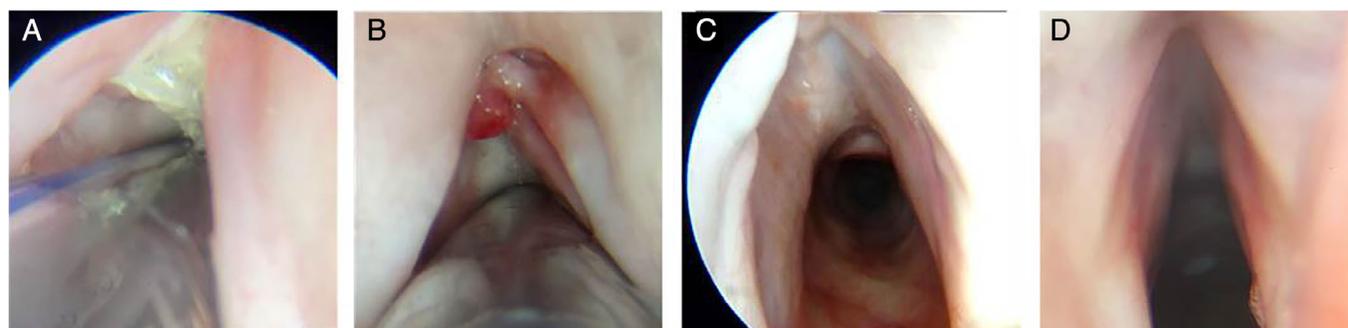


Fig. 2. Representative endoscopic images of progressive healing of vocal fold mucosal surface on left side at (A) 2 weeks, (B) 4 weeks, and (C) 8 weeks following surgery compared to (D) normal. Vascularization and epithelialization is noted at 4 weeks. At 8 weeks, the mucosal surface appears completely healed and the implant is not identifiable within the airway lumen. Asterix indicates location of repair.

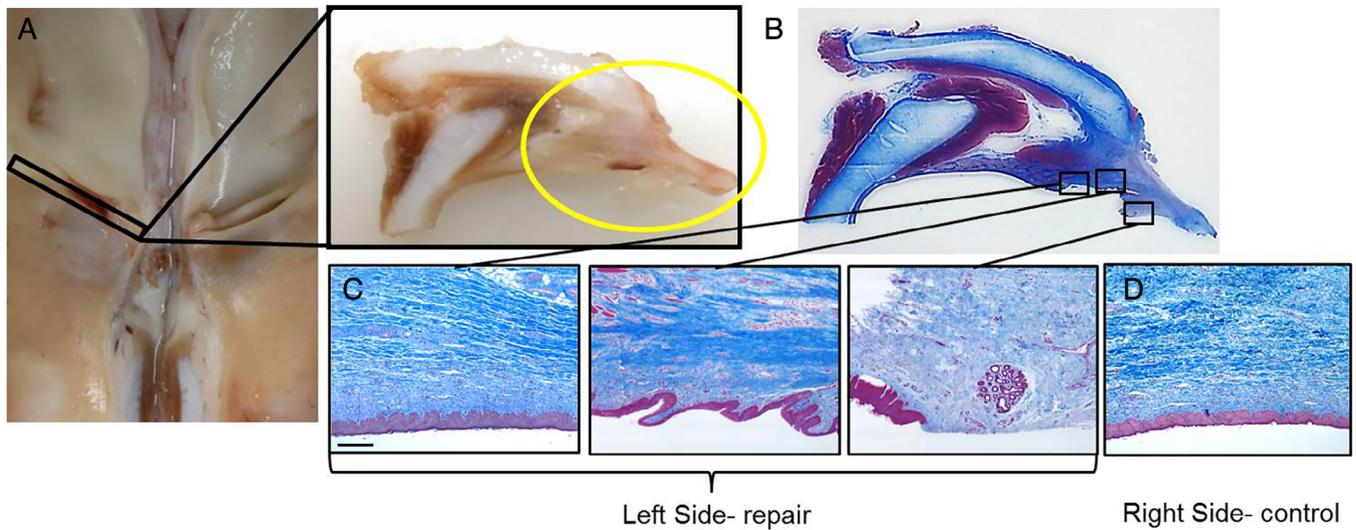


Fig. 3. Regenerated mucosal epithelium as seen at 8 weeks in the post mortem larynx that is (A) unstained and (B and C) stained with Mas-son's Trichrome compared to normal (D). Yellow circle outlines location of original defect, much of which is grossly indistinguishable from the native hemilarynx now. Scale bar: 200  $\mu$ m

isoflurane anesthesia was provided via nosecone. The animals were assessed for stimulation induced laryngospasm (adduction) as well as spontaneous respiration related abduction. At the 8-week assessment, evoked laryngeal electromyography (LEMG) of the adductor complex was performed in addition to performing videolaryngoscopy during RLN stimulation. LEMG was performed with hooked wire electrodes placed in the right sided thyroarytenoid muscle or left sided engineered muscle while stimulating the ipsilateral RLN at the entrance into the larynx to determine firing patterns and recruitment of the MI muscle relative to that of the native laryngeal muscles. Despite the prior

transection injury, the RLN fibers could be identified at the entrance in the larynx (near the cricothyroid joint) in all animals bilaterally. After videolaryngoscopy and LEMG were completed, the animals were euthanized while still under anesthesia according to NIH and ACUC-approved methods.

**Voice Data Collection and Analysis**

Voice collected by 96 k/24-bit Portable Stereo Recorder DR-05 (Tascam Linear). The recorder was placed approximately

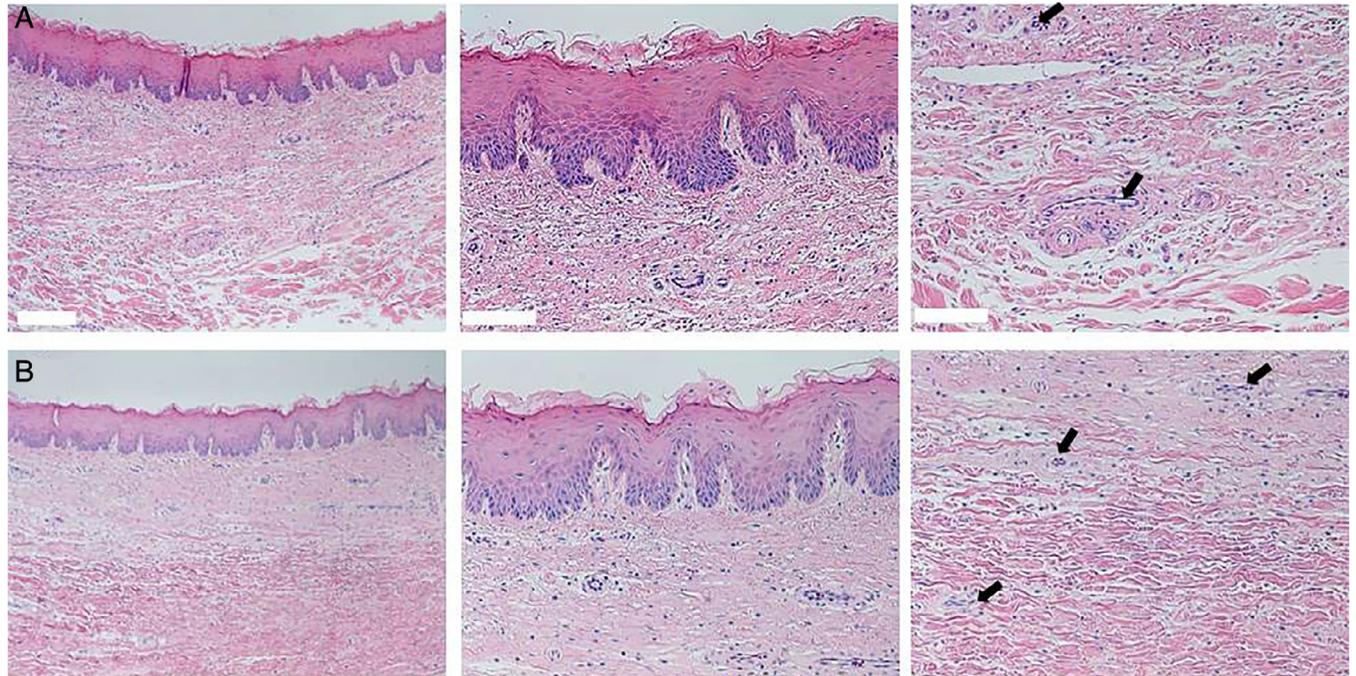


Fig. 4. H&E staining of (A-C) mucosal layer showing regrowth of epithelium at 8 weeks following surgery compared to (D-F) untreated control. Arrows indicate vasculature. Scale bars: (A and D) 200  $\mu$ m; (B-C and E-F) 100  $\mu$ m

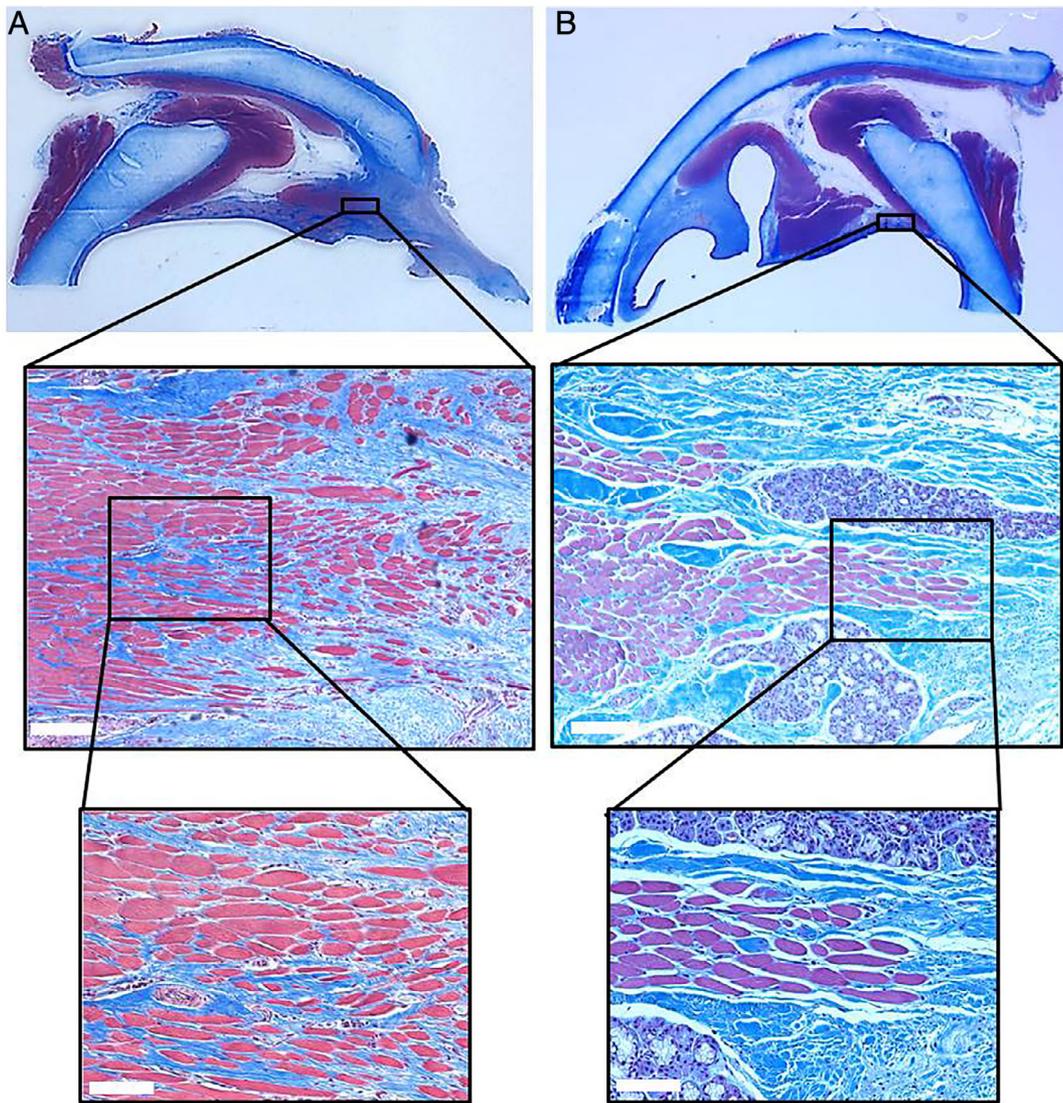


Fig. 5. Masson's Trichrome stain of skeletal muscle interface in (A) reconstructed hemilarynx and (B) native control hemilarynx. Early stages of muscle regeneration are noted in the reconstructed larynx, with more mature skeletal muscle fibers noted in the untreated control.

50 cm directly in front of the pig's mouth and 60 second audio recordings were attained for each animal. The squeal is a long, high-pitched cry, or noise that is the true sound from the vocal fold. The squeals of both pigs were recorded before the nerves were injured, and it was seen that the squeals occurred at a frequency of 2.0 kHz. "Audacity" was used to analyze the maximum amplitudes. Once the right RLN was severed, audio samples were taken every 2 weeks for a total of 8 weeks.

### **Histological Assessment**

After euthanasia, pig larynges and associated implants were harvested *en bloc* and fixed in 10% neutral buffered formalin and paraffin embedded. Sections were stained with hematoxylin and eosin (H&E) and Mason's trichrome by the Purdue Histology Lab. Slides were viewed on a Nikon microscope (Eclipse E200, Nikon, Melville, NY) and images captured with a Leica camera (DFC480, Leica, Buffalo Grove, IL).

Muscle and cartilage constructs cultured *in vitro* were fixed in 4% paraformaldehyde overnight, and then transferred to 30%

sucrose at 4°C for an additional 24 hours. Cryosections (25- $\mu$ m thickness) were prepared on a Thermo Cryotome FE (Fisher Scientific, Kalamazoo, MI). For histochemistry analysis, cryosections were washed with phosphate buffered saline three times, permeabilized with 0.1% Triton X-100 for 20 minutes, and then blocked with 1% bovine serum albumin for 2 hours. To stain for motor endplates and cytoarchitecture, slides were incubated with Alexa Fluor 594 (Molecular Probes) conjugated  $\alpha$ -bungarotoxin (1:100) and Alexa Fluor 488 phalloidin (F-actin), respectively, for 2 hours at room temperature. Slides were rinsed and mounted with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA) for imaging on a Zeiss LSM 880 confocal microscope (Oberkochen, Germany).

## **RESULTS**

### **Videolaryngoscopy**

Animals demonstrated appropriate weight gain, no aspiration events, and audible phonation, initially

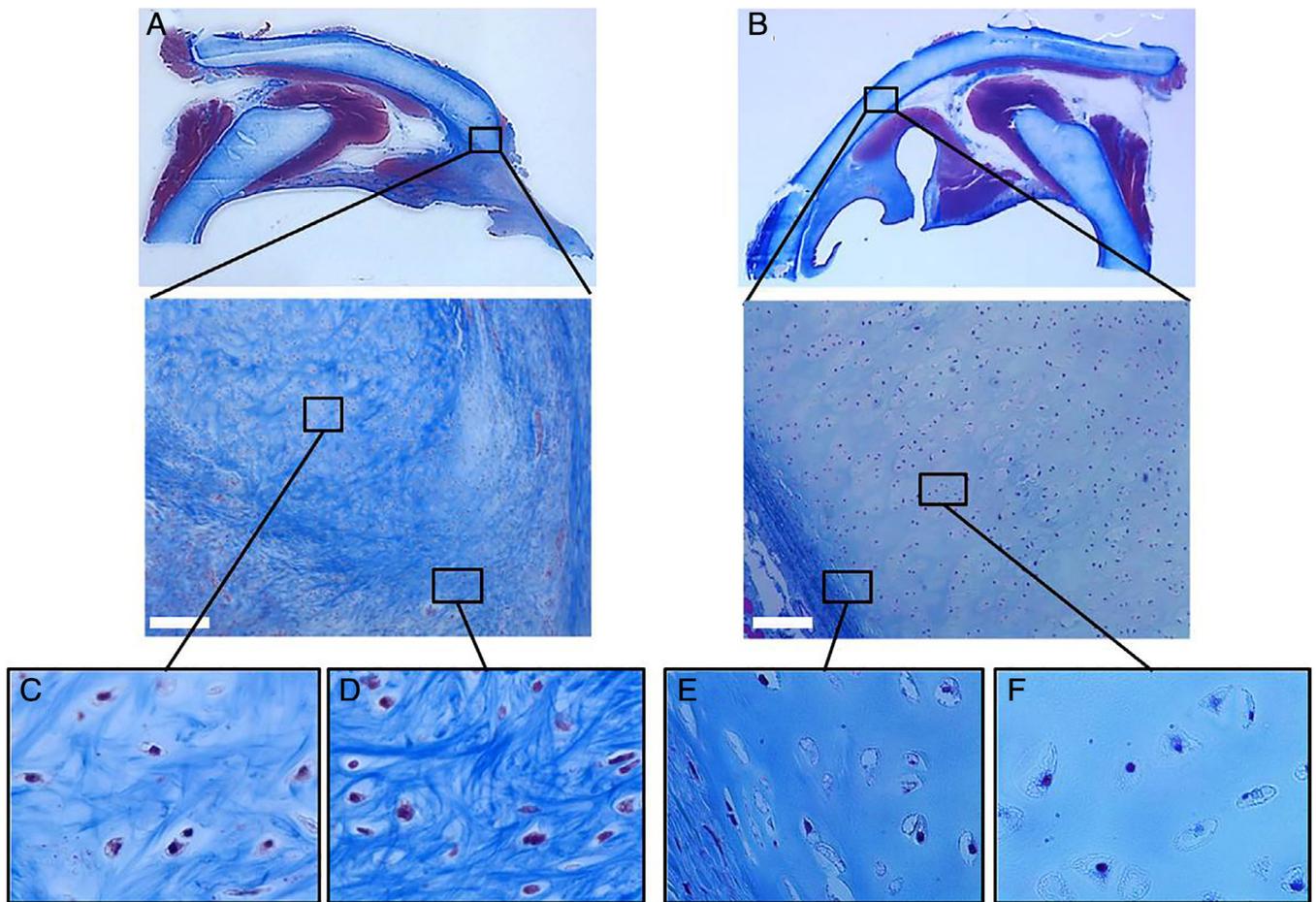


Fig. 6. Masson's trichrome stain of cartilage interface in (A) reconstructed larynx and (B) untreated control. Neocartilage with different levels of maturation are evident within the reconstructed larynx, with (C) less dense collagen fibrils and cells within larger lacunae toward the center and (D) denser collagen fibrils with more cells within small lacunae found near the periphery. (E and F) Normal cartilage features chondrocytes within small lacunae surrounded by a highly uniform collagen matrix.

extremely soft and then gradually increasing in amplitude over time (see Table I). Video laryngoscopy at 2, 4, and 8 weeks showed healthy, viable MI tissue, initially with exudate (week 2) at the anterior anastomosis, which was then gradually replaced with healthy mucosa and small granulomas at the anastomotic site (week 4), and by 8 weeks there was complete re-epithelialization with no induration or inflammation (Fig. 2).

### ***Histological Assessment***

Histology confirmed re-vascularization of the implants supporting the observations of the viable tissue appreciated on videolaryngoscopy (Fig. 3). The mucosal layer showed complete coverage with stratified squamous epithelium and vascularization along with glandular growth (Fig. 4). The skeletal muscle layer showed complete integration, with good alignment and no inflammatory response (Fig. 5). The cartilage layer showed significant neocartilage formation, with the implant portions adjacent to the native cartilage most closely replicating normal cartilage (Fig. 6). Of particular interest

was the finding that all three layers demonstrated complete integration of the engineered tissues not only with the native tissue but also with between MI layers, with no inflammatory foreign body reaction or rejection. Furthermore, the defect dramatically decreased in size over time, such that it became difficult to identify the original defect by 8 weeks because much of the MI became morphologically indistinguishable from native tissue. Finally, as part of the histological analysis, the tissue-engineered MIs were assessed for neuromuscular junctions via neuron (beta-3 tubulin) and motor endplate ( $\alpha$ -bungarotoxin) immunohistochemistry, which confirmed the presence of innervated neuromuscular junctions throughout the engineered muscle (Fig. 7).

### ***LEMG***

Evoked LEMG waveforms could be obtained bilaterally in all of the animals. At 8 weeks, the MEE implant animals also demonstrated visible adduction on direct RLN stimulation, while the acellular control animal

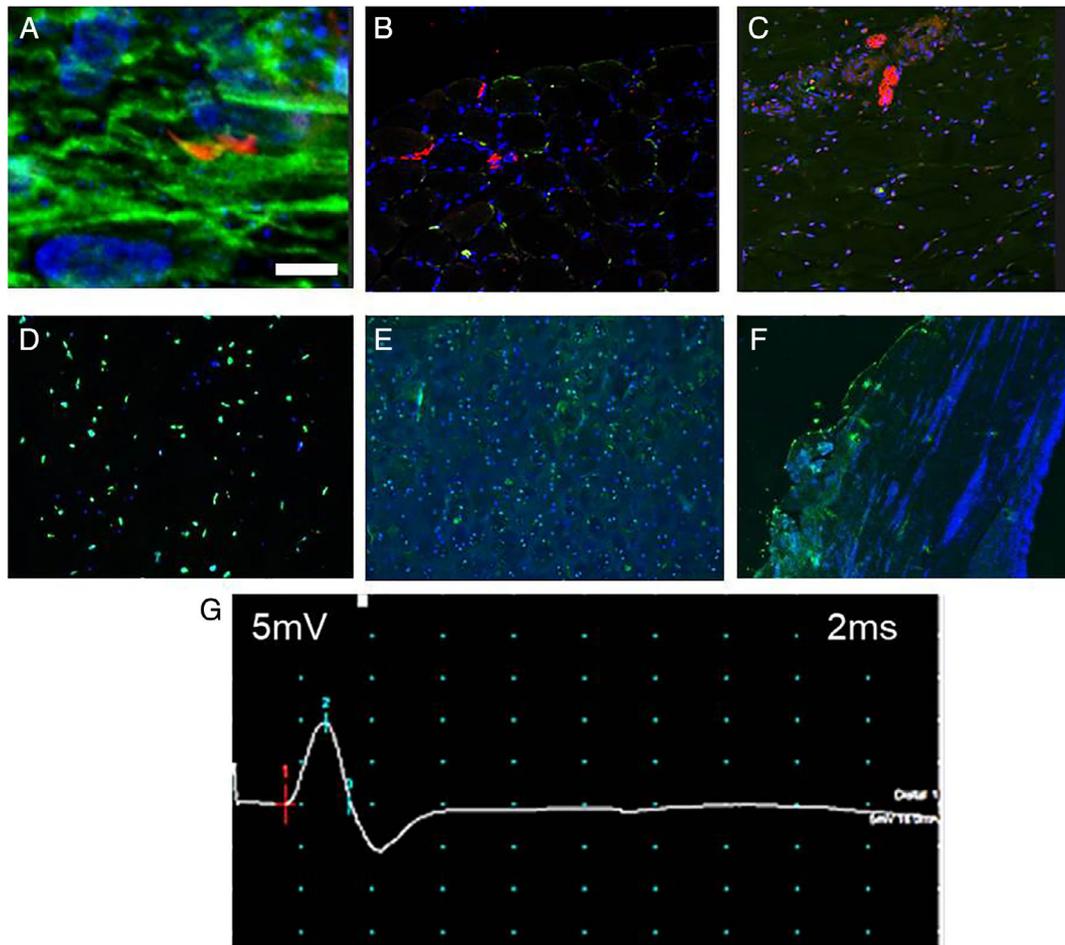


Fig. 7. (A) in vitro MEE induction (Red- motor endplates, Green- F-Actin, Blue- nuclei) (B and C) in vivo innervation (B: Normal muscle, C: Innervated implant; red: Nerve fiber, green: Motor endplate, blue: Nuclei). (D) in vitro cartilage differentiation. (E and F) in vivo cartilage formation (E: Normal cartilage, F: Implant; green: Collagen II, blue: Nuclei). (G) Evoked LEMG with representative maximal stimulated motor unit action potential (MUAP) when transected RLN was stimulated distal to the point of injury shows early reinnervation in the MEE implanted animal at 8 weeks post surgery. Scale bar: 100  $\mu$ m.

actually demonstrated abduction but no clear adductor activity(Fig. 7).

## DISCUSSION

Our previous investigations have used MIs to reconstruct the larynx in a rodent model of partial laryngectomy.<sup>7,8,10,17</sup> However, because the rodent larynx has a very small glottic airway and lacks protective reflexes such as cough, it is not a good model for transmural defects (only myochondral defects could be created). Considering that a transmural defect needs to be created to better simulate a neoplastic resection, these experiments focused on a large-animal translational model involving the porcine larynx. Not only did this permit reconstruction of a transmural defect, but the porcine laryngeal anatomy and physiology also closely mimic that of the human larynx.<sup>18,19</sup> This initial porcine study demonstrates that autologous MEEs and ASCs can be used within unique self-assembling collagen to create MIs that provide restoration of basic laryngeal protective reflexes for cough, swallowing, and airway maintenance

over a follow-up period of up to 2 months. Furthermore, we found that the MIs developed strong innervation, adequate muscle volume, and visible adductor activity on laryngoscopy, likely explaining the favorable post-surgical physiologic responses that the animals displayed.

While the ideal laryngeal replacement strategy has not been clearly established to date, there have been a wide variety of approaches, including the concept of an artificial larynx<sup>20</sup> as well as the mesenchymal stem cell-coated decellularized larynx for laryngeal replacement.<sup>21</sup> Unfortunately, none of these investigational options add muscle volume and restore dynamic motion. As viable, aligned muscle is critical for restoration of laryngeal function, our laboratory has extensively studied approaches for engineering autologous laryngeal muscle,<sup>7,8,10</sup> and used the engineered muscle as the foundation for the MIs described in this study. Because of the complexity of the larynx and anatomic variability between animals, a multilayered construct was created so that each layer could be individually manicured/trimmed at the time of surgery, and then secured to the surrounding structures and

other implant layers. The three layers included: 1) an acellular collagen vocal fold mucosa replacement (inner layer), 2) tubular MEE muscle long enough to layer upon itself (middle layer), and 3) an ASC-populated cartilage (outer layer). We removed the entire unilateral true and false vocal fold, including the inner mucosa, adductor muscle, and outer laryngeal cartilage-sparing only one arytenoid and a thin posterior laryngeal cartilage strut to facilitate anchoring the MIs. The engineered muscle was also secured in a manner such that the vector of muscle contraction would mirror that of the native adductors (running from the anterior commissure to vocal process of the arytenoid). Indeed, using this approach we have attained innervated engineered muscle with functional adduction based on videolaryngoscopy. Most importantly, the pigs have had no loss of function, maintaining airway, phonation, and swallowing (normal weight gain/no aspiration events). In fact, while phonatory quality was initially quite soft/breathy and weak in amplitude, the amplitude was actually restored to near-normal by 8 weeks.

While findings from the current investigation are encouraging, they are still very preliminary in nature. Future studies will be needed to adjust the MI structural integrity and function based on the size of the defect, and to create larger, possibly bilateral partial laryngectomy defects. Furthermore, other investigators such as Long et al<sup>22</sup> have been highly successful in tissue engineering vibratory mucosa to optimize voice quality, while our model used a nonvibratory collagen sheet for structural support. Thus, additional structural optimization will be important before clinical translation will become feasible.

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