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Motor Endplate-Expressing Cartilage-Muscle Implants for Reconstruction of a Denervated Hemilarynx

Sarah Brookes, DVM; Sherry Voytik-Harbin, PhD; Hongji Zhang, MD; Lujuan Zhang, MD; Stacey Halum, MD, FACS

Objective: Tissue engineering of the larynx requires a complex, multiple tissue layer design. Additionally, spontaneous reinnervation of the larynx after recurrent laryngeal nerve (RLN) injury is often disorganized, resulting in subpar function. This study investigates use of tissue-engineered cartilage and motor endplate-expressing (MEE) tissue-engineered skeletal muscle implants for laryngeal reconstruction and the promotion of organized reinnervation after RLN injury.

Methods: F344 rat primary muscle progenitor cells (MPCs) were isolated. Three-dimensional muscle constructs were created by encapsulating MPCs in type I oligomeric collagen under passive tension. Constructs were then cultured in differentiation medium (MPC control constructs) or induced to form motor endplates (MEE constructs) with neurotrophic agents. Three-dimensional cartilage constructs were created with adipose stem cells differentiated in chondrocyte medium. The muscle and cartilage constructs were implanted into surgically created myochondral defects in the F344 rat larynx with injured or intact (control) RLN. At 1-, 3-, and 6-month timepoints, videolaryngoscopy, electromyography (EMG), histology, and immunohistochemistry were used to assess outcomes.

Results: At all timepoints, cartilage-muscle implants were well integrated into host tissue. Functionally, there was increased vocal fold adduction and EMG activity in nerve-injured endplate constructs compared to those treated with the MPC control constructs. Motor endplate-expressing constructs had increased myofiber cross-sectional area compared to MPC control constructs.

Conclusion: Although our laboratory previously demonstrated that muscle and cartilage constructs could be used separately for hemilaryngeal reconstruction, this study suggests combining them with the modification of MEEs rather than MPCs, resulting in improved muscle recovery after recurrent laryngeal nerve injury.

Key Words: Muscle progenitor cells, type I oligomeric collagen, tissue-engineered skeletal muscle, laryngeal reconstruction, tissue-engineered cartilage, recurrent laryngeal nerve injury.

Level of Evidence: NA

INTRODUCTION

Laryngeal cancer is one of the most commonly diagnosed airway cancers, with over 13 thousand new cases diagnosed annually in the United States alone. Standard treatments for advanced cancer, which involve surgical resection and/or chemoradiation, are life-saving but may render patients dependent on a tracheostomy and/or feeding tube with poor quality of life. A recent review by Hamilton and Birchall indicated that one treatment strategy for advanced laryngeal cancer is to tissue engineer a functional laryngeal replacement. This, they postulate, can be broken down into a number of design considerations, including restoration of 1) structure through scaffold design and 2) function by promoting skeletal muscle regeneration with associated vascularization and innervation. Autologous grafts, such as myocutaneous flaps and buccal mucosa, can be too bulky and come with donor site availability and morbidity problems. Approaches aimed at recapitulating laryngeal structure have included using decellularized larynx, decellularized cartilage, or various natural and synthetic biomaterials seeded with cells. Major limitations of these approaches have been inadequate integration with the surrounding host tissue, significant inflammatory response, and poor vascularization/tissue survival. Another vital component to engineering a functional larynx is to restore nerve and muscle function. Because laryngectomy surgery is often associated with injury of the recurrent laryngeal nerve (RLN), it is important to include denervation in the preclinical animal model. Brookes et al. and Paniello et al. have shown that use of muscle progenitor cells (MPCs) induced to express motor endplates improves reinnervation and function post-RLN injury.

To begin to address these challenges, our group has previously shown that we can replace thyroid cartilage with a novel oligomeric collagen-adipose stem cell (ASC)
implant\textsuperscript{13} that enhances cartilage healing with no inflammatory foreign body reaction. We have also shown that replacement of resected adductor muscle complex with a tissue-engineered (TE) skeletal muscle implant not only is well tolerated by the animal, similar to the cartilage implant, but that it also improves function (on electromyogram [EMG]) when compared to controls.\textsuperscript{11} We now extend this work by investigating the integrated use of TE muscle-cartilage implants for reconstruction of partial laryngectomy defects in the presence and absence of RLN injury in a rodent model. This preclinical model was designed to recreate the clinically relevant surgical reconstruction scenario where neoplastic resection is performed with RLN injury. The purpose of this study was to examine the microscopic, structural, and functional outcomes of TE muscle-cartilage implants for repair of a denervated hemilarynx after partial laryngectomy (resection of outer cartilage/adductor muscle).

**MATERIALS AND METHODS**

**Primary Muscle Progenitor Cell Isolation and Culture**

Primary MPCs were generated from skeletal muscle biopsies obtained from 12-week-old male Fischer 344 rats (Envigo, Indianapolis, IN), as previously described.\textsuperscript{11} Briefly, fresh muscle tissue was minced in myogenic growth medium (MGM); 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 0.1% chick embryo extract (Accurate Chemicals, Westbury, NY) and digested in 0.2% collagenase type I (EMD Millipore, Temecula, CA) at 37 °C for 2 hours. Digested tissue was filtered through a 100-μm cell strainer, plated onto untreated 100-mm petri dishes (Fisher Scientific, Chicago, IL), and cultured overnight at 37 °C with 5% CO\textsubscript{2}. The supernatant was removed the next morning and transferred to culture flasks (Corning Life Sciences, Corning, NY). Cells were cultured to 70% confluence and used in experiments at passages 3 to 5.

**Fabrication of Engineered Skeletal Muscle Constructs**

Muscle implants were fabricated as previously described.\textsuperscript{11} Briefly, MPCs from F344 rats were suspended in type I oligomeric collagen (4.0 mg/mL) and polymerized. They were then compressed to 0.5-mm-thick constructs (75 mg/mL) 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\textsubscript{2} incubator for up to 4 weeks. The medium was changed twice per week.

**Fabrication of Engineered Cartilage Constructs**

Cartilage constructs were fabricated as previously described.\textsuperscript{13} Briefly, F344 rat ASCs were suspended in type I oligomeric collagen (4.0 mg/mL) and polymerized. They were then compressed to 0.5-mm-thick constructs (75 mg/mL) 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\textsubscript{2} incubator for up to 4 weeks. The medium was changed twice per week.

**Laryngectomy, Recurrent Laryngeal Nerve Injury, and Implantation of Engineered Muscle**

Engineered muscle constructs were implanted in our established rat partial laryngectomy model.\textsuperscript{14} 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. In brief, 12 Fischer 344 rats were anesthetized with intraperitoneal injection of xylazine and ketamine and then maintained on 1% to 4% isoflurane. The ventral larynx was exposed via a midline incision. The sternohyoid muscle was incised and reflected to expose the thyroid cartilage. A section (approximately 2 mm × 2 mm) of thyroid cartilage and associated adductor muscle was removed from the left side. Animals were randomized into groups receiving implants made with

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**Fig. 1.** (A) Schematic diagram of surgical procedure. Alcian blue staining shows progressive healing and differentiation of the cartilage construct over the 1-month (B), 3-month (C), and 6-month (D) timepoints. Increase in blue staining indicates progressive cartilage regeneration, proteoglycan deposition, and maturation. *Edges of surgically created defect. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]**

Brookes et al.: MEE Cartilage-Muscle Implants
MPC or MEE cells with and without RLN injury. All groups received identical cartilage implants with endpoints at 1, 3, and 6 months. The muscle construct was placed into the defect (similar to a medialization laryngoplasty implant), followed by the cartilage construct over the top (Fig. 1), with extrusion prevented by suturing overlying sternohyoid muscles over the cartilaginous defect. For groups with RLN injury, the left recurrent laryngeal nerve was cauterized as it entered the larynx. The subcutaneous tissue and skin were then closed with 5-0 Vicryl suture.

**Video Laryngoscopy and Laryngeal Electromyography**

At 1-, 3-, and 6-month endpoints, video laryngoscopy and laryngeal electromyography were performed under isoflurane anesthesia as previously described. Video laryngoscopy was performed using a rigid endoscope with attached camera. EMG recordings (Niking Viking Quest electromyography machine, Madison, Wisconsin) were collected with a 25-gauge bipolar concentric needle, 50- to 100-μV amplitude, 10- to 100-ms sweep speeds, and a grounding clamp at the exposed lateral sternocleidomastoid muscle. The EMG recording needle was inserted directly into the center of the defect/implant site, adductor muscle complex, and posterior cricoarytenoid muscle during laryngospasm (produced via lightening the isoflurane anesthesia and stimulating with needle) and at rest. Immediately following EMG, rats were humanely euthanized and tissue was collected.

**Histopathological and Histochemistry Assessment**

After euthanasia, rat larynges and associated implants were harvested en bloc, fixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose at 4 °C for an additional 24 hours. Cryosections (25-μm thickness) were prepared on a Thermo Cryotome FE (Fisher Scientific, Kalamazoo, MI). Sections were stained with hematoxylin & eosin (H&E) and Alcian blue for histopathological analysis. Slides were viewed on a Nikon microscope (Eclipse E200, Nikon, Melville, NY) and images captured with a Leica camera (DFC480, Leica, Buffalo Grove, IL). As previously described, myofiber diameter was evaluated by measuring the lesser fiber diameter using Image J software (NIH, Bethesda, MD).

For histochemistry analysis, all specimens 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. For staining tissue explant cryosections, beta III tubulin conjugated primary antibody (1:10 NL555 Molecular Probes) was applied and incubated overnight at 4 °C. After rinsing extensively, slides were incubated with Alexa Fluor 488 (Molecular Probes) conjugated α-bungarotoxin (1:100) 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). The number of motor endplates with nerve contact were counted and divided by the total number motor endplates to give percent innervation, as previously described.

**RNA isolation and Reverse Transcription and Quantitative Polymerase Chain Reaction**

Total RNA was extracted using the TRIzol method. The cDNA was synthesized using 100 μg of RNA and random hexamers in SuperScript-III kit (Invitrogen, Carlsbad, CA) according to manufacturer recommendations. The resulting cDNA was then amplified by polymerase chain reaction (PCR) using Go Taq DNA Polymerase system (Promega, Madison, WI). Primers used are listed in Table I. A single cycle consisted of 15-second denaturation at 94 °C, 30-second annealing at 55 °C, and 60-second extension at 72 °C. 38 cycles were used for GAPDH, NTN-1, and NT-4. 35 cycles were used for NT-3 and GDNF. A 10-μL sample of reaction mixture was electrophoresed in a 2% agarose gel containing ethidium bromide to evaluate the amplification and determine the size of the generated fragments. A 100 base pair DNA ladder was used as a standard size marker. Densitometry was performed for relative band quantification. Intensity was measured using Image J software (NIH) and results normalized to GAPDH.

**Statistical Analysis**

Student t test was used for all analysis, and P < 0.05 was considered significant.

**RESULTS**

**Muscle and Cartilage Implant Integration**

All animals survived the postsurgical period with no life-threatening complications. Some RLN-injured animals showed mild stridor, but this resolved with time. All animals steadily gained weight over the study period. Postmortem gross pathological exam showed integration of the cartilage and muscle implants into host tissue with no macroscopic signs of inflammation. Alcian blue staining of cryosectioned specimens was weakly positive at 1 month (Fig. 1B). At 3- and 6-month timepoints, sections demonstrated darker blue staining consistent with glycosaminoglycan deposition and cartilage formation (Fig. 1C–D). H&E staining at 1 month displayed immature muscle neighboring native adductor muscle with no foreign body inflammatory response (Fig. 2A). At 3 months, maturation of the muscle within the implant was demonstrated by development of cross-striations and myofiber alignment.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
</tr>
</thead>
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<tr>
<td>NTN-1</td>
<td>CTTCATGTTTCATCCTCAGCTTTCCTT</td>
<td>CTTGATTTAAGATCTCTGTAGCG</td>
</tr>
<tr>
<td>NT-3</td>
<td>ACATACCTGTGTCTACCTGTA</td>
<td>GTCCACCTTTCCTCTGCATGTG</td>
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<td>NT-4</td>
<td>CCCCTGCTGACATCTTCTCCAGAC</td>
<td>CTGGACGTACGCAAAGCGCCGCA</td>
</tr>
<tr>
<td>GDNF</td>
<td>GGTCTACGGGAGACCGGATCCGAGGTGC</td>
<td>AGATAAACAGCCGCGGCGCAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGTCTACGGGAGACCGGATCCGAGGTGC</td>
<td>AACCTCCCTAGATTGTGAGCA</td>
</tr>
</tbody>
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PCR = polymerase chain reaction.
(Fig. 2B). By 6 months, little to no immature muscle was visible within the defect area, suggesting complete integration with native tissue (Fig. 2C).

**Muscle Implant Functional Results After RLN Injury**

α-bungarotoxin and beta III tubulin staining demonstrated that MEE implants showed a greater number of motor endplates and robust innervation (Fig. 3). In addition, both the thyroarytnoid and posterior cricoarytnoid muscles showed significantly increased motor endplate-to-nerve contact in the MEE-treated group ($P < 0.01$) (Fig. 4). Qualitative EMG measurements of the adductor muscle complex of the MPC-treated animals showed low-level activity (Fig. 5A), whereas the MEE-treated animals showed near normal muscle activity, with interference and recruitment patterns (during laryngospasm) that mimicked normal adductor muscle (Fig. 5C). This finding was further corroborated by adductor muscle complex myofiber diameter measurements, with values for MEE-treated animals significantly greater than those for MPC-treated animals ($P < 0.001$) (Fig. 5E).

In nerve-injured animals, video laryngoscopy showed function recovery in all animals receiving the MEE implant with definitive although slightly asymmetric adduction/abduction relative to the normal side. In contrast, none of the animals receiving the MPC implant showed definitive movement at any timepoint (Fig. 6; Supporting Video S1).

**Polymerase Chain Reaction**

Quantification of PCR expression based on band intensity revealed statistical differences between differentiated MPCs and MEEs (Fig. 7). Specifically, significant
Fig. 4. MEE implants induce an increased percentage of motor endplate with nerve contact compared to MPC implants, in abductor and adductor muscles. Motor endplate (α-bungarotoxin, pink) and nerve (beta III tubulin, green) staining of PCA and TA muscles in the MPC-treated (A) and MEE-treated (B) groups. The number of motor endplates with nerve contact were counted and divided by the total number of motor endplates to give percent innervation of each muscle compared to an uninjured control (C). *P < 0.05. **P < 0.01. Scale bar: 20 μm. MEE = motor endplate-expressing; MPC = muscle progenitor cells; TA = thyroarytenoid; PCA = posterior cricoarytenoid. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

Fig. 5. RLN-injured animals treated with MEE implants showed increased EMG activity and myofiber diameter compared to MPC implants. EMG of the adductor muscle complex showed increased activity in MEE-treated (C) animals compared to MPC-treated (A) animals. Histology showed myofiber atrophy and inflammatory cell infiltrate in MPC (B) animals and normal architecture in MEE (D) animals. (E) Myofiber diameter of adductor muscle showed significant increase in MEE-treated animals (Mean ± standard deviation, P < 0.001, 2 animals per group, minimum 36 measurements per animal). Scale bar: 100 μm. EMG = electromyography; MEE = motor endplate-expressing; MPC = muscle progenitor cell; RLN = recurrent laryngeal nerve. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]
differences ($P < 0.05$) were detected between the MPC and MEE expression of NT-3, NTN-1, NT-4, and GDNF (2 vs. 3, respectively). Thus, the MEE induction was associated with increased neurotrophic factor expression in all the factors tested.

DISCUSSION

We have previously demonstrated that aligned muscle implants$^{11}$ and cartilage implants$^{13}$ individually integrate with host tissue and differentiate, with no foreign body reaction in a rat model of partial hemilaryngectomy. With the long-term goal of developing new therapeutic options for restoring or replacing damaged or diseased airway (larynx), we extended those studies with the first application of combined tissue-engineered cartilage-muscle implants following partial hemilaryngectomy with and without RLN injury. Study results showed that the two-layer implant demonstrated viability and structural integrity comparable to the respective single layers. Such findings are important because this brings us closer to the ideal multi-layer functional tissue-engineered larynx for clinical applications. Of course, this study is limited in that it represents an early stage of investigation using a small animal model; potential clinical applicability cannot be determined until functional survival is demonstrated in large animal models, followed by clinical trials.

Differentiating features inherent to our TE larynx design include the use of a 1) self-assembling type I oligomeric collagen for scaffold customization and cell encapsulation, and 2) specialized muscle progenitor cells chemically induced to express motor end plates. Scaffolds and tissue constructs fabricated with oligomeric collagen take advantage of collagen’s inherent capacity for molecular self-assembly and natural intermolecular crosslinks, which serve to physically stabilize resulting fibril network and slow collagen turnover (resist proteolytic degradation)$^{16,17}$. As a result, the collagen-fibril scaffold, together with its resident cells, rapidly integrate with host tissue such that both physical and biochemical cues (e.g., mechanical forces) inherent to the local tissue micro-environment can be effectively transmitted to guide cell phenotype and function. The significance of this mechano-chemical signalling was evidenced by the progressive maturation of the muscle and cartilage implants in the absence of a significant inflammatory reaction. By contrast, conventional biodegradable biomaterials and decellularized tissues are often not amenable to cell encapsulation and yield tissue responses characterized by inflammatory-mediated degradation and/or fibrotic capsule formation, which are known to delay or prevent tissue vascularization and regeneration.$^{18,19}$ Additionally, the no treatment controls, reported previously, showed scarring of the defect and a marked inflammatory response.$^{11}$

Our TE construct incorporates MEE muscle cells with the goal of accelerating and guiding more organized neuromuscular junction formation.$^{20}$ Our previous study$^{11}$ with intact RLN showed enhanced innervation, based on motor endplate-to-nerve contact and EMG activity, of engineered muscle constructs when MEE muscle cells were used to create the implants. Similarly, Paniello

![Fig. 6. Still images from video laryngoscopy show recovery of left vocal fold movement in animals treated with MEE implants (A). Animals treated with the MPC implant (B) showed little to no movement in the left vocal fold at any timepoint. MEE = motor endplate expressing, MPC = muscle progenitor cell. See online Supporting Video S1. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]](image-url)
et al. showed that injection of MEE muscle cells in dogs with RLN injury resulted in increased innervation based on motor endplate-to-nerve contact, which in turn contributed to increased muscle mass and measured laryngeal adductor pressure. Our current results suggest that animals receiving MEE constructs with complete RLN transection injury show increased laryngeal re-innervation (based on motor endplate-to-nerve contact and EMG) and qualitatively functional (based on video laryngoscopy) gains of both adductor and abductor muscle complexes when compared to animals treated with MPC implants. Notably, the presence of MEE implants attenuated atrophy of muscle, allowing for improved function postinjury. Our hypothesis is that the chemical signaling by the MEE cells improves innervation and thus function post injury. Our RT-PCR data further supports this hypothesis, showing that MEE cells have increased expression of multiple neurotropic factors (Fig. 7).

Whereas tissue-engineered implants are still clinically in their infancy, one advantage of an autologous cell-based implant is that no immunosuppression is required—potentially a major safety advantage because the first laryngeal transplant conducted for laryngeal adductor pressure. Our current results suggest that animals receiving MEE constructs with complete RLN transection injury show increased laryngeal re-innervation (based on motor endplate-to-nerve contact and EMG) and qualitatively functional (based on video laryngoscopy) gains of both adductor and abductor muscle complexes when compared to animals treated with MPC implants. Notably, the presence of MEE implants attenuated atrophy of muscle, allowing for improved function postinjury. Our hypothesis is that the chemical signaling by the MEE cells improves innervation and thus function post injury. Our RT-PCR data further supports this hypothesis, showing that MEE cells have increased expression of multiple neurotropic factors (Fig. 7).

Whereas tissue-engineered implants are still clinically in their infancy, one advantage of an autologous cell-based implant is that no immunosuppression is required—potentially a major safety advantage because the first laryngeal transplant conducted for laryngeal cancer resulted in rapid tumor recurrence and patient death due to the required immunosuppression. Other advantages to the use of autologous stem cells include the promotion of tissue regeneration, promotion of angiogenesis, and inhibition of inflammation. Many of these effects are mediated via the release of a wide array of cytokines from the cells, leading to an autocrine-mediated acceptance/integration/revascularization of the implanted tissue. These same effects raise safety concerns about using the implants in an oncologic setting. However, investigations suggest cells such as ASCs have antineoplastic effects with malignancies such as pancreatic adenocarcinoma, hepatocarcinoma, colon, and prostate cancers—and there are no reports of tumorigenesis in comparable tissue-engineered tissue—so we anticipate that oncologic safety will not be a major hurdle if appropriate surgical margins are attained at the time of initial resection.

Whereas we anticipated possible restoration of adductor function after reconstruction with the tissue-engineered implants, the finding of restored abduction and adduction in the MEE implants was an unexpected and exciting finding. Histology (percentage of motor endplates with nerve contact) supported strong reinnervation of both the PCA and adductor complex in the MEE-implanted animals after RLN injury, although the PCA consistently had a greater percentage of motor endplates with nerve contact, likely suggesting earlier reinnervation due to the closer anatomic proximity of the PCA to regenerating RLN. This concept of obviating synkinesis by enhancing the speed of axonal regeneration was originally described with nimodipine therapy by Mattsson et al. and has been further supported with clinical investigations by Rosen et al. Thus, we hypothesize that the cytokine effects (expression of trophic factors from MEEs as detected with PCR) may be
improving the efficiency of axonal regeneration, thereby obviating synkinesis. Future studies in large animal models will be needed to better understand the reproducibility and physiology involved.

CONCLUSION

Overall, we were able to show that a two-layer, muscle-cartilage implant integrates into host tissue with no foreign body inflammatory reaction. Additionally, we were able to improve function in a RLN-injured larynx with a muscle implant induced to express motor endplates. Future studies in a large animal model will be needed to better assess the clinical applicability of this model.

ACKNOWLEDGMENT

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This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the 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.

BIBLIOGRAPHY