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Review

Repairing the vibratory vocal fold

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CONFLICT OF INTEREST: none

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Abstract**OBJECTIVES**

A vibratory vocal fold replacement would introduce a new treatment paradigm for structural vocal fold diseases such as scarring and lamina propria loss. This work implants a tissue-engineered replacement for vocal fold lamina propria and epithelium in rabbits, and compares histology and function to injured controls and orthotopic transplants. Hypotheses were that the cell-based implant would engraft and control the wound response, reducing fibrosis and restoring vibration.

STUDY DESIGN

Translational research

METHODS

Rabbit adipose-derived mesenchymal stem cells (ASC) were embedded within a three-dimensional fibrin gel, forming the Cell-based Outer Vocal fold Replacement (COVR). 16 rabbits underwent unilateral resection of vocal fold epithelium and lamina propria, and reconstruction with one of three treatments: fibrin glue alone with healing by secondary intention, replantation of autologous resected vocal fold cover, or COVR implantation. After four weeks, larynges were examined histologically and with phonation.

RESULTS

15 rabbits survived. All tissues incorporated well after implantation. After one month, both graft types improved histology and vibration relative to injured controls. Extracellular matrix (ECM) of the replanted mucosa was disrupted, and ECM of the COVR implants remained immature. Immune reaction was evident when male cells were implanted into female rabbits. Best histologic and short-term vibratory outcomes were achieved with COVR implants containing male cells implanted into male rabbits.

CONCLUSIONS

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Vocal fold cover replacement with a stem cell-based tissue-engineered construct is feasible and beneficial in acute rabbit implantation. Wound modifying behavior of the COVR implant is judged to be an important factor in preventing fibrosis.

KEY WORDS: adipose-derived stem cell, tissue engineering, vocal fold scarring, mucosa, phonation

LEVEL OF EVIDENCE: NA

For Peer Review

INTRODUCTION

Regenerative medicine is being considered for difficult clinical problems throughout the body. The irreplaceable nature of the vibratory vocal fold (VF) is one of those challenges. Normal voicing requires a mobile epithelium overlying a specialized lamina propria. That unique microstructure is limited to a very small geography, and does not match any other tissue. Injury or resection alters the extracellular matrix (ECM) making it stiff and inelastic, and requiring greater effort to phonate. Implanted tissue replacements are subject to the same scar formation. Thus, a functioning VF replacement that heals without scarring would open new avenues of treatment for structural VF disorders.

Repairing injured VF lamina propria has also proven difficult. Injected steroids may improve function in mild-moderate scars, but the impact if any on underlying ECM has not been studied¹. Synthetic implants and autologous tissue grafts improve voice only modestly, and do not reduce the aerodynamic effort for phonation². Basic fibroblast growth factor has shown promise, improving voice in small uncontrolled human reports of presbylaryngeous and scarring^{3,4}. Suggested mechanisms include increased hyaluronic acid synthesis, and other ECM effects possibly mediated via hepatocyte growth factor upregulation⁵. The improvements with growth factors, which act upon multiple cellular pathways, hint at the utility of broad therapeutic targeting for VF scarring. Implanting cells can further extend the reach of therapy, by targeting multiple pathways both simultaneously and longitudinally through time, and by responding to host signals. This concept that cellular activity can best lead the repair work forms the basis for considering regenerative medicine over other, simpler, therapies.

Broadly speaking, regenerative medicine "replaces or regenerates human cells, tissue, or organs, to restore or establish normal function"⁶. Newly introduced cells control the repair process, via an assemblage of actions involving multiple pathways. It thus differs fundamentally from traditional pharmaceuticals that have narrower mechanisms of action and are unresponsive to host factors⁷. At least three manifestations of autologous cell injections for VF repair have been tested in human clinical trials: bone marrow-derived mesenchymal stem cells⁸, adipose stromal cells⁹, and fibroblasts¹⁰. A phase 1 trial

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3 of autologous fibroblasts demonstrated voice improvement in 4 of the 5 patients with scarring¹¹.

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5 However, the authors reported that two patients with full-thickness scars involving epithelium and lamina
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7 propria did not recover mucosal mobility. Such patients, for example those with no normal lamina
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9 propria remaining or with inadequate response to injectable treatments, may benefit from more extensive
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11 therapy.

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14 A more comprehensive therapy, for the most severe scars or tissue loss, could involve complete
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16 removal of the dysfunctional tissue and replacement with a new structure. Tissue engineering, or creation
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18 of new tissue from cells *in vitro*, may offer a means to reproduce the otherwise unmatched vibratory VF.
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20 Success would require that the tissue replacement incorporate well in the larynx, restore vibratory
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22 function, and not suffer from scar formation during wound healing. Prior work developed an adipose-
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24 derived multipotent stem cell-based structure that resembled VF epithelium and lamina propria in cell
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26 phenotype, cell density, ECM fiber density, and mechanical stiffness¹²⁻¹³. This structure is termed the
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28 Cell-based Outer Vocal fold Replacement (COVR), in distinction from a complete VF replacement which
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30 would include thyroarytenoid muscle. The purpose of this study was to implant the COVR in a rabbit VF
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32 resection model, assessing its function relative to injured controls and to autologous VF mucosa
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34 replantation. The hypothesis was that the cell-based implant would temper the initial wound healing
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36 response, ultimately leading to reduced fibrosis.
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41 **METHODS**

42 *COVR development in vitro*

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45 Inguinal fat from male rabbits was digested in 0.1% type I collagenase, as previously described¹⁴,
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47 ¹⁵. After centrifugation, infranatant was washed free of lipid and red blood cells. The resulting
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49 stromovascular fraction was plated in culture. Adherent adipose-derived stem cells (ASC) were harvested
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51 at third passage. Cell multipotency was confirmed by differentiation to osteogenic and adipogenic
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53 phenotypes^{14,15}.
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3 Cell-based constructs were formed within 12 mm Corning Transwell culture inserts¹². Rabbit
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5 fibrinogen at 5 mg/ml was mixed in a 4:1:1 ratio with bovine thrombin (2 units/mL in HBS with CaCl₂)
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7 and cell suspension (6x10⁶ cells/ml) to form fibrin gels with embedded ASC. After gelation, additional
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9 ASC were pipetted onto the surface to replicate an epithelial layer. Culture medium within the annulus
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11 supplied the tissue constructs only through the insert base. Medium contained 10% fetal bovine serum
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13 and 10ng/ml epidermal growth factor, and was changed every 2-3 days for 2 weeks.
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18 *Rabbit laryngeal surgeries*

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20 Sixteen New Zealand white rabbits, weighing 2.8-3.5 kg, underwent survival surgeries as
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22 summarized in Table I and as detailed previously^{16,17}. After laryngofissure, the membranous cover layer
23
24 was resected from the left true vocal fold (known in rabbits as the inferior division of the thyroarytenoid
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26 fold) by sharp dissection. Cordectomy extended from the anterior commissure to the vocal process of the
27
28 arytenoid cartilage, at the interface between lamina propria and thyroarytenoid muscle, equivalent to
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30 European Laryngological Society (ELS) type 2 cordectomy. All right vocal folds remained as uninjured
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32 controls. Immediately following resection, one of three reconstructions was performed. Six rabbits had
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34 immediate replacement of the resected cover¹⁶. Four sutures of 6-0 plain gut at the corners of the
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36 replanted tissue secured it in position, and fibrin glue prepared from rabbit fibrinogen was applied at the
37
38 interface to ensure adhesion. Eight rabbits had COVR implants with male ASC, similarly secured. Two
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40 rabbits served as injured controls and were treated only with fibrin glue over the defect. Five rabbits (one
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42 autologous replant, two COVRs, and two controls) underwent endoscopy at two weeks post-op. All were
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44 euthanized after four weeks and larynges harvested.
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50 *Phonation*

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52 Larynges were phonated at 4 weeks post-surgery, either *in vivo* or after euthanasia and larynx
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54 harvest, as indicated in Table I^{16,17}. Two animals (#7 and 8) had both methods for direct comparison. *In*
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56 *vivo* phonation was performed under anesthesia with VF exposed via thyrohyoid pharyngotomy. An
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3 endotracheal tube supplied airflow through the glottis, and manual thyroid cartilage pressure adducted the
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5 vocal folds. Later implants underwent fresh excised larynx phonation immediately after harvest for
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7 experimental simplicity. An adduction suture was placed through both vocal processes, and larynges were
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9 mounted on a PVC pipe with regulated and humidified airflow. In both settings, a high-speed digital
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11 videocamera recorded vibration at 8,000 frames per second, and acoustic output was recorded. A Matlab
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13 algorithm converted two-second vibration segments to a kymogram, performed at the midpoint of the
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15 membranous vocal folds. Qualitative assessment was made of vocal fold appearance, glottic closure, and
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17 mucosal wave symmetry.
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20 21 22 23 *Microscopy*

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25 Larynges were harvested maintaining their orientation, formalin-fixed, paraffin-embedded, and
26
27 sectioned. Histologic stains included H&E, Masson's trichrome, elastin von Gieson, Alcian blue for
28
29 mucopolysaccharides, Von Kossa for mineralization, oil red O for lipids, and PTAH for fibrin.
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31 Immunohistochemistry assessed vimentin (AMF17B, DSHB, University of Iowa) and cytokeratin
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33 (Abcam ab961) expression, using normal rabbit larynx for comparison.
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38 **RESULTS**

39 40 *In vitro phenotypes*

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42 Rabbit ASC deposited lipid and mineral under adipogenic and osteogenic culture conditions,
43
44 confirming their multi-potency¹⁵. No deposits were detected in three-dimensional COVR culture,
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46 confirming that unwanted spontaneous differentiation did not occur. All rabbit ASC throughout the
47
48 COVR maintained vimentin expression without significant cytokeratin expression, indicating
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50 mesenchymal phenotype.
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55 56 *Rabbit survival surgeries*

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Treatments and assessments of 16 rabbits are summarized in chronological order in Table 1. Operations performed were autologous mucosa replantation (n=6), COVR implant (n=8), and unilateral cordectomy only (n=2). One animal died from inadequate hemostasis causing a tracheal hematoma. Remaining animals survived without complication, maintained oral intake, and gained weight. Five had endoscopy after two weeks (Figure 1).

Phonatory vibration

One month after surgery, phonatory vibration was recorded with high-speed video either *in vivo* or after larynx excision. Two pilot animals undergoing both methods confirmed that results were similar. All larynges produced sound in response to transglottic airflow. Autologous replant and COVR animals consistently achieved closure of the membranous glottis, whereas injured controls only closed the superior vocal folds (Figure 2). Injured controls demonstrated significant phase asymmetry. Contralateral vocal fold vibration was also severely disrupted. This is attributed to poor entrainment of the normal vocal fold with the injured side, and has also been observed in an excised larynx injury model¹⁸. In contrast, both vocal folds participated in phonation after autologous replants or COVR implants; COVR symmetry appeared improved.

Histology

By 4 weeks, all vocal folds had re-epithelialized, and cells populated the entire tissue (Figure 3). Unreconstructed cordectomy showed superficial edema overlying dense collagenous fibrosis, seen as blue fibers on Masson's trichrome staining. Thickened elastic fibers remained at the base of the resection near the thyroarytenoid muscle.

After autologous mucosa replant, two of the five animals had increased collagen throughout the lamina propria relative to contralateral uninjured controls. Elastic fibers were present in all replanted

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3 mucosae, although some had a disorganized appearance with disruption of the normal fine transverse
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5 elastic fibers. Mucopolysaccharides were preserved.
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8 Four weeks after COVR, the implant was difficult to distinguish from surrounding vocal fold
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10 tissue. Residual suture securing the implant corners positively identified the implant site in some
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12 sections. In all sex-matched animals (male ASC implanted into male rabbits), collagen content appeared
13
14 equivalent to contralateral controls and inflammatory cells were not appreciated. All sex-mismatched
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16 implants (male ASC in female rabbits) demonstrated increased collagen and notable inflammatory
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18 infiltrate in both the operated and control sides. In both genders, elastic fibers within adjacent native vocal
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20 fold were discontinued at the injury edge. Mature elastic fibers were not identified with elastin von
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22 Gieson staining within the definitive implant sites. Elastic fibers occasionally detected in deep tissue near
23
24 the thyroarytenoid muscle may have been spared from resection. Those fibers appeared similar to
25
26 unoperated vocal folds and were not thickened. Mucopolysaccharide staining was present within COVR
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28 implants to a lesser degree than in autologous mucosa replants (Figure 4).
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32 The fibrin-ASC COVR prior to implant showed a bland, uniform structure without detectable
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34 collagen or elastic fibers. That provisional fibrin matrix was partially detected four weeks after implant in
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36 two of eight animals. PTAH staining shows fibrin as fine bluish fibers within the granulation tissue of the
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38 implant site (Figure 5).
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43 **DISCUSSION**

44 *Study summary*

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47 This work furthers the development of a cell-based replacement for the vocal fold mucosa. A
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49 tissue-engineered structure containing adipose-derived mesenchymal stem cells was successfully
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51 engrafted onto rabbit vocal folds wounded by complete epithelium and lamina propria resection. The
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53 COVR implant (Cell-based Outer Vocal fold Replacement) produced excellent short-term function after
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3 four weeks of integration. Results were superior to autologous replantation of structurally intact vocal
4 fold mucosa, and greatly improved over healing by secondary intention.
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8 This work hypothesized that implanted cells and scaffold would influence host wound healing,
9 manipulating the cellular response and ECM remodeling to result in a functional vibratory mucosa
10 without fibrosis. Although the four-week timepoint studied here only represents acute results, it is
11 apparent that the early wound healing process is tempered. Cordectomy injury produced edema, collagen
12 deposition, and distortion of residual deep lamina propria ECM. Those processes did not occur in gender-
13 matched COVR implants. Reconstructing with autologous mucosa replants did abrogate scar formation,
14 but to a lesser degree than with the tissue-engineered COVR. Comparing the COVR with complete
15 mucosal replant suggests that undifferentiated ASC in fibrin may guide wound healing with less fibrosis
16 than the differentiated vocal fold cells can. Also, the freshly resected mucosal transplant contained a
17 mature and ideal ECM structure. That ECM was disrupted during the first month after surgery, and its
18 vibration impaired relative to normal. Thus, implanting a complete ECM is only beneficial if wound
19 healing can be tailored to prevent damage.
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34 An unanticipated sex difference was identified in the COVR implant animals. Female rabbits
35 receiving male cells showed clearly more inflammation and fibrosis than male rabbits receiving
36 allogeneic male cells. It is unclear from this early finding whether the greater inflammatory activity in
37 females was due to the antigenicity of male cells, or to an inherently more inflammatory phenotype in the
38 female rabbits.
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47 *Tissue-engineering the vocal folds*

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49 The concept of a cell-populated, three-dimensional, tissue-engineered structure for vocal fold
50 reconstruction was first described in 2009¹². It was proposed as an alternative to injectable therapies of
51 biomaterials and/or cells for lamina propria regeneration. Motivation for this more radical approach came
52 from the observation that remodeling existing scar was likely to fail in a subset of severely affected
53 patients¹⁹. Also, injectables alone are not feasible for acute reconstruction after vocal fold cordectomy.
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3 Other variations of three-dimensional tissue-engineered vocal fold replacement have since been proposed,
4 using alternate cell sources and materials²⁰⁻²². The encouraging findings from multiple investigators
5 support the feasibility of this general approach. The optimal composition can now be fine-tuned, and may
6 depend on clinical practicality as much as results from animal models. For example, cell availability,
7 simple processing, and minimal risk of serious adverse events are all practical requirements for this
8 elective therapy.
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11 The COVR proposed in this work aims to regenerate vibratory vocal fold mucosa with an
12 autologous-derived implant. It would be applied after complete scar resection, with repair controlled by
13 both the fibrin scaffold and the embedded cells. Fibrin, the acellular component of blood clots, is a
14 bioactive protein supporting numerous actions including angiogenesis, elastogenesis, and cell migration²³⁻
15²⁵. The provisional fibrous structure guides deposition of new matrix in an orderly manner²⁶. As it
16 degrades by enzymatic fibrinolysis, the released peptide fragments act upon surrounding cells in a
17 feedback loop to control the remodeling rate²⁷. The adipose-derived stem cells are essential regulators of
18 matrix remodeling in this system. ASC downregulate mRNA for collagens I and III and α -smooth muscle
19 actin in vocal fold fibroblasts²⁸. The native fibroblasts therefore becomes less fibrotic and less contractile,
20 both protecting the airy lamina propria matrix. These ASC effects translated to reduced collagen
21 deposition when implanted within canine lamina propria²⁹.
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41 ASC also have broad and strong immunosuppressive actions that influence, but do not eliminate,
42 the local host response. Through a number of cytokine effects and direct cell-cell contact, they reduce
43 cytotoxic T-cell proliferation, prevent dendritic cell differentiation, inhibit natural killer cell function, and
44 induce regulatory T cell and B cell pathways which in turn promote immune tolerance³⁰⁻³². In this study,
45 male ASC implants in male rabbits had very little inflammatory infiltrate at 4 weeks. In contrast, male
46 ASC implants in female rabbits had significant inflammatory cells and greater collagen deposition,
47 consistent with the widespread association between inflammation and fibrosis³³. The findings do confirm
48 that cell- or antibody-mediated immune reactivity can target ASC in the vocal fold implants (as elsewhere
49 in the body), and adversely effects graft outcomes. All study animals received allogeneic transplants (cells
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3 from non-host animals), but perhaps the narrow genetic profile of New Zealand white rabbits shielded the
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5 same-sex implants from immune recognition. Alternatively, female rabbits could be predisposed to
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7 greater immune reactivity and fibrosis. The difference is relevant to the therapeutic success in females,
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9 and warrants further study. Sex is only beginning to be appreciated as a biological variable, and the
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11 literature at this point is too sparse to extrapolate to the VF.
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14 15 16 *Future directions*

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18 These studies demonstrate promising wound healing with a tissue-engineered vocal fold mucosa
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20 implanted in rabbits at the time of acute injury. This scenario most resembles a cordectomy resection of
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22 extensive scar or of epithelial carcinoma. Further investigation is required to better model both of those
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24 human conditions. First, this study is not immediately generalizable to the clinical condition of severe
25
26 established vocal fold scar. Altered phenotype of scar fibroblasts, or vascular insufficiency in the wound
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28 bed, may induce re-fibrosis or impede graft integration. An animal model with injury, scar maturation,
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30 then delayed COVR implantation would be relevant. Also, longer monitoring is required to assess the
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32 ultimate ECM structure, which is still evolving at four weeks post-implant.
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36 Additional concerns face this approach for vocal fold reconstruction in the wake of cancer
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38 resection. Stem cell reconstruction has been most extensively studied in breast cancer, and remains
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40 intensely controversial³⁴. An influential mouse study with ductal breast carcinoma cells found increased
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42 metastases when MSC were administered³⁵; similar results occurred with head and neck cancer cell
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44 lines³⁶. Nonetheless numerous conflicting animal and human studies have led to uncertainty about long-
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46 term outcomes³⁷. The first likely cancer patients will be those seeking delayed scar treatment years after
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48 therapy. Additional careful animal research is required before considering stem cell therapy for immediate
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50 laryngeal cancer reconstruction.
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53 54 **CONCLUSIONS**

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Regenerative medicine for the vocal fold is progressing. This work describes a cell-based outer vocal fold replacement (COVR) produced from adipose-derived multi-potent stromal cells and fibrin. Its implantation after type 2 cordectomy in rabbits reduced scarring and enabled symmetric mucosal waves during short-term phonation. Function was better than replanted autologous vocal fold mucosa after one month. Long-term evaluation is needed to fully assess functional outcomes.

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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 local Institutional Animal Care and Use Committee.

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FIGURE LEGENDS

Figure 1. Endoscopy two weeks after vocal fold cordectomy surgery in rabbits. All images obtained from a 0-degree rigid endoscope with CCD camera. A) Normal unoperated cadaveric rabbit; black arrows indicate the ventricle dividing the inferior and superior divisions of the thyroarytenoid folds. B) Left-sided type 2 cordectomy control; early formation of an anterior glottic web demonstrated. C) Autologous mucosa replant; white asterisk indicates the surgical site. D) COVR implant; white arrow marks the superior edge of the implant site.

Figure 2. Kymograms extracted from high-speed videolaryngoscopy segments. Kymograms were performed at the mid-portion of the membranous vocal fold, within the injury region. Operated left vocal fold appears as the bottom tracing in each segment. A) Cordectomy control showing markedly irregular vibration. B) Autologous mucosa replantation showing regular but asymmetric vibration. C) COVR implant with regular and symmetric vibration.

Figure 3. Rabbit vocal fold histology, all at 20X. Scale bars are 200 microns. Left column is standard H&E stain, middle column is Masson's trichrome showing collagen in blue, and right column is elastin von Gieson stain with elastic fibers appearing black. Row A, normal unoperated rabbit vocal fold with epithelium in the left of each panel and thyroarytenoid muscle in the top right corner. The lamina propria network of fine collagen and elastin fibers is demonstrated. Row B, four weeks after ELS type 2 cordectomy, showing superficial edema and deep fibrosis. Row C, four weeks after cordectomy with autologous mucosa replant. Elastic fibers appear thickened relative to unoperated control side, and lamina propria is compressed. Row D, four weeks after cordectomy with COVR implantation. Collagen appears similar to unoperated control side; mature elastic fibers are not detected.

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3 Figure 4. Alcian blue stain for mucopolysaccharides in rabbit vocal folds, four weeks after surgery. A)
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5 COVR implant with sparse staining, most prevalent in the superficial region. B) Autologous mucosa
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7 replant, with preserved staining throughout the tissue.
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11 Figure 5. Fibrin gel histology. A) H&E stain of COVR *in vitro* at time of implant, 20X. B) PTAH stain
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13 showing fine bluish fibers suggestive of fibrin, within the COVR implant site after four weeks. Cell
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15 nuclei also appear blue. 40x. C) Corresponding H&E stain of rabbit COVR implant shown in B, 40X.
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17 Fibrin is indistinguishable from other matrix on standard stain.
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Table 1. Operative treatments of 16 rabbits.

<u>Animal</u>	<u>Sex</u>	<u>Reconstruction</u>	<u>Phonation</u>
1	M	transplant	in vivo
2	M	transplant	died
3	M	transplant	in vivo
4	M	transplant	in vivo
5	M	transplant	in vivo
6	M	transplant	in vivo
7	M	COVR	in vivo, excised
8	M	COVR	in vivo, excised
9	M	COVR	excised
10	M	COVR	excised
11	F	COVR	excised
12	F	COVR	excised
13	F	COVR	excised
14	F	COVR	excised
15	M	fibrin glue	excised
16	M	fibrin glue	excised

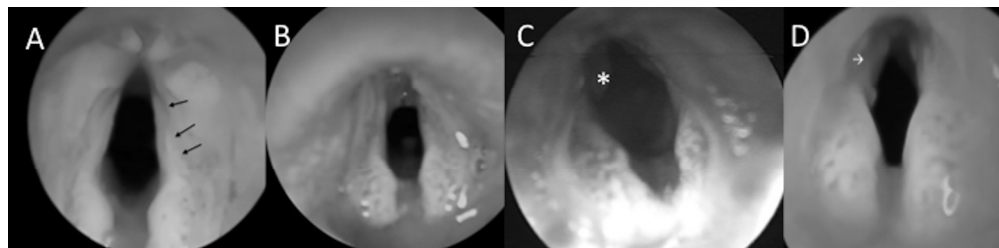


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152x37mm (300 x 300 DPI)

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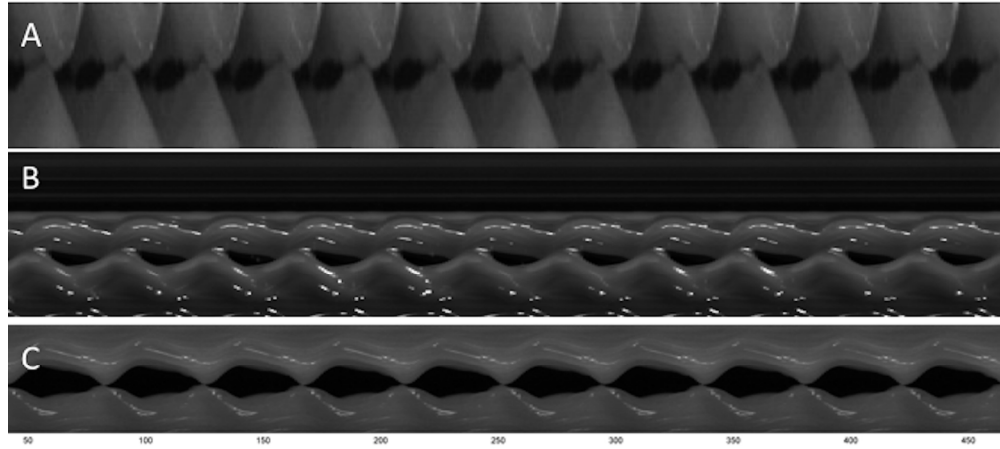
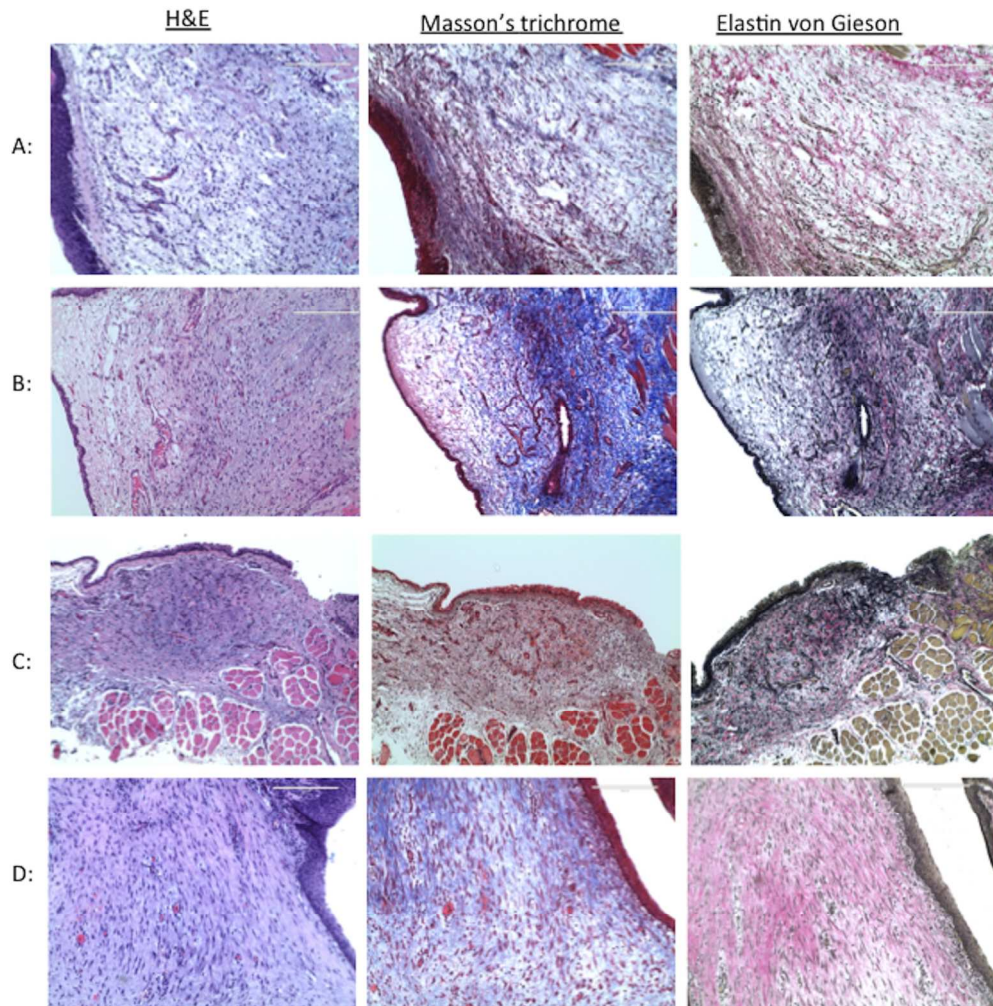


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152x69mm (300 x 300 DPI)



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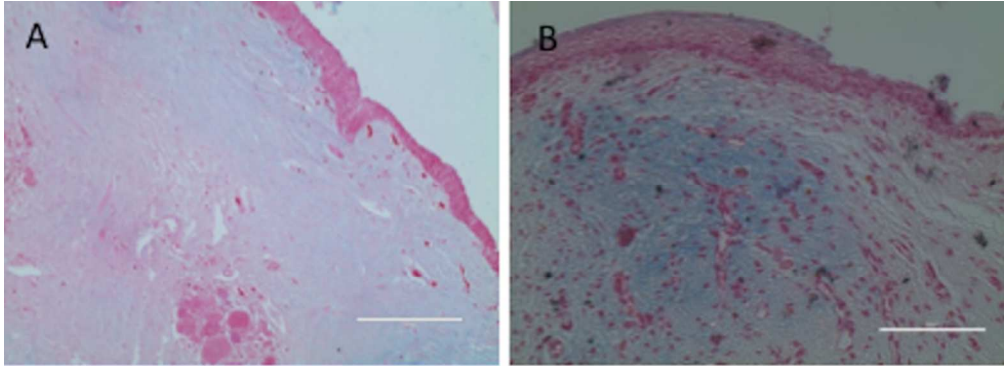


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76x28mm (300 x 300 DPI)

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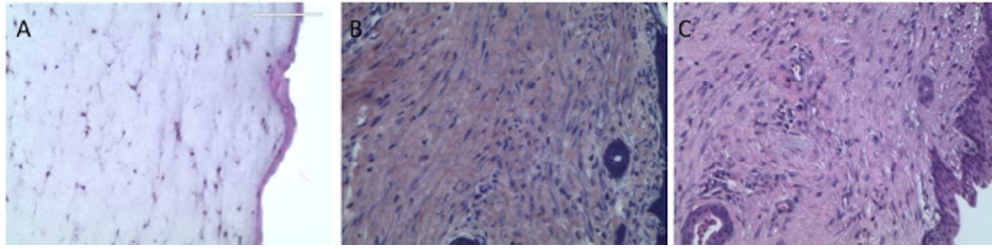


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114x28mm (300 x 300 DPI)

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