Tissue engineering for treatment of vocal fold scar

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Purpose of review

Creating a neovocal fold or lamina propria by tissue engineering is a potential scheme for treating severe vocal fold scar. Although still investigational, multiple approaches have recently been described in tissue culture or animal models.

Recent findings

Proposed cell types for vocal fold application have been native vocal fold fibroblasts, autologous fibroblasts from nonlaryngeal tissues, and adult-derived stem cells. Scaffolds of interest include decellularized matrix, biological polymers, and synthetic or chemically modified biopolymers. Chemical, mechanical, and spatial signals have been applied, such as hepatocyte growth factor, cyclic stretch, and air interface. Cells, matrix, and signals are combined in an effort to replicate normal vocal fold tissue as closely as possible. Each of these components of vocal fold tissue engineering is discussed here.

Summary

Multiple tissue engineering approaches hold promise for reproducing functional vocal fold tissue. Scar prevention techniques have been the most successful. Modifying existing scar is more difficult and may necessitate complete scar excision and replacement with a three-dimensional neotissue. Functional assessment *in vivo* is essential to the ongoing evaluation of techniques.

Keywords

dysphonia, growth factor, matrix, mechanics, stem cell, sulcus vocalis, tissue engineering, vocal fold scar

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Introduction

'Tissue engineering' generally refers to the manipulation of viable cells to heal or replace damaged tissues. A spectrum of therapeutics can be envisioned within this paradigm. At its most complex, a functional living tissue indistinguishable from its target is created *in vitro*. As a less controlled example, injecting cells into damaged tissue triggers healing *in situ*. Injecting matrix or growth factors without cells does not meet this definition and instead may be considered under the broader title 'regenerative medicine.' Many such therapies are discussed elsewhere in this volume.

Implanting a three-dimensional tissue-engineered construct may be a new treatment for challenging vocal fold scars. Several models have been reported recently and are reviewed here by considering their key features. Variables typically controlled during tissue engineering include cell type, extracellular matrix (ECM) or scaffold, and physical or chemical maturation signals. Each of these variables is considered separately in this review. In reality, there is considerable interdependence among them. For example, ECM influences cell behavior, cells remodel their surrounding matrix, and maturation factors can alter cell behavior and matrix structure.

Cells

In the traditional paradigm of tissue engineering, cells assist with initial construct development and maintain function after implantation. For the vocal fold, that may involve resorption of the original scaffold, deposition of proteoglycans and elastic fibers, and long-term 'housekeeping' of the structure in response to normal function and aging processes. Ideally, autologous cells phenotypically identical to lamina propria fibroblasts would populate an implanted lamina propria replacement. Epithelial cells, also critical for phonation via their mucosal wave, must also eventually resurface the construct in a watertight barrier.

Actual vocal fold fibroblasts have been studied in animal models where cells are harvested on initial injury, cultured, and reimplanted. In a controlled comparison on rabbit scars, Thibeault *et al.* [1••] injected either physiologic saline, autologous vocal fold fibroblasts, hyaluronic acid-based matrix, or fibroblasts with matrix. After 2 months,

only the autologous vocal fold fibroblasts produced statistically significant rheological improvement over controls. This process may not be feasible in humans, where lamina propria biopsy is morbid and may supply phenotypically altered fibroblasts from a scar environment. After harvest, human vocal fold fibroblasts can be immortalized by transducing a telomerase reverse transcriptase gene to produce a potentially implantable cell line [2]. They also share markers and multipotent behavior with mesenchymal stem cells (MSC) [3••], which may allow their expansion *in vitro*. Still, autologous vocal fold fibroblasts may be difficult to obtain and the immune rejection risk of implanting allogeneic donor vocal fold fibroblasts is not yet well defined.

Because of the limited availability of human vocal fold fibroblasts, similar cells from more accessible tissues are of interest. If using another fibroblast source, it is hoped that the vocal fold environment induces lamina propria-specific behavior and appropriate ECM remodeling. Autologous buccal fibroblast injection did improve both histology and functional mucosal waves in scarred canine vocal folds [4]. Dermal fibroblasts are another accessible cell type, but they have less inherent ability to produce hyaluronic acid than vocal fold fibroblasts and did not improve gross histology in a small short-term rabbit study [5].

Stem cells have received intense attention for vocal fold application. Embryonic stem cells demonstrated some protective ability when injected into rabbits at the time of scarring [6], but technical and regulatory hurdles currently preclude widespread investigation of these allogeneic cells. Autologous adult-derived cells are more practical. In-vivo trials have been reported with bone marrow-derived MSC, whereas adipose-derived stem cells (ASC) from liposuction or fat biopsy have been investigated *in vitro*. Bone marrow MSC injected before or during injury prevented the gross appearance of scar in dogs [7] and improved rheology in rabbits [8]. Improvement of existing scar is more elusive than scar prevention. Bone marrow MSC injected with a synthetic hyaluronic acid-based matrix into scarred rat vocal folds modestly increased expression of fibronectin and collagen III mRNA relative to saline-treated controls, but differentiated to a myofibroblast phenotype [9•]. Scar histology and rheology were not reported. Fibronectin and collagen III were previously associated with scar prevention [10], but the myofibroblast phenotype is considered to be profibrotic [11]. Regarding ASC, in-vitro studies support their ability to prevent scars. ASC secreted hepatocyte growth factor (HGF) that attenuated collagen production and fibroblast proliferation in coculture [12•]. In three-dimensional ASC culture, disorganized type I collagen deposition was ameliorated by conditions promoting epithelial differentiation [13••]. ASC can also produce elastic fibers, which typically does not occur in a scar environment [14••]. Whether this constellation of in-vitro findings translates to reduced scar formation *in vivo* has yet to be determined.

To summarize, only fibroblasts have demonstrated improvement in existing scars after cell injection therapy [1••,4]. Complete excision and replacement of severely scarred vocal folds may be more successful. Building a complete vocal fold cover for implantation requires both fibroblastic and epithelial cells. Both ASC and bone marrow MSC were traditionally thought to produce mesenchymal lineages only, but recent evidence of epithelial differentiation supports their pluripotentiality. Green fluorescent protein-labeled bone marrow MSC injected into scarred rat vocal folds differentiated into both epithelial and muscle cells *in vivo* [15]. ASC in fibrin culture with an air interface and epidermal growth factor produced a bilayered construct of epithelial cells at the surface with mesenchymal cells found deeper, resembling the vocal fold cover's structure of mucosa overlying lamina propria [16•]. Using either of these cell types to create an epithelialized vocal fold replacement would simplify manufacturing and healing. The single pluripotent cell eliminates the need to harvest and supply mature epithelial cells or a mucosal flap, or to await reepithelization *in vivo*. Those latter methods risk rescarring at the lamina propria–mucosal junction, a theoretical concern that has not been addressed with homogeneous lamina propria replacements.

Scaffolds

The structure–function relationship is a key tenet of tissue engineering; investigators aim to replicate normal tissue structure as closely as possible with the expectation that proper function will follow. However, precisely recreating the complex lamina propria ECM of collagen, elastin, hyaluronic acid, and numerous supporting molecules would be impractical *in vitro*. Instead, most approaches aim to capture selected key features in a provisional matrix. The temporary scaffold is then modified and degraded by embedded cells *in vitro* or by host cells *in vivo*. Decellularized organ matrix, biologic polymers, synthetic biomimetic hydrogels, and synthetic polymers have all been described as scaffolds for three-dimensional lamina propria replacement and are reviewed here.

Decellularized human or animal tissue scaffolds have been used in airway tissue engineering because of their ease of manufacturing and good replication of native architecture. Processing steps remove all viable cells and soluble molecules, leaving a largely collagenous scaffold that is theoretically free of antigenic material and, therefore, suitable for implantation between individuals and even across species.

After rehydration, the fibrous ECM supplies some molecular information as well as appropriate pore dimensions to facilitate cell invasion and attachment. For a review of the production, characterization, and application of this technology, see Badylak *et al.* [17]. In notable examples, decellularized scaffolds have been implanted to replace rat lungs [18] and a human trachea [19]. Each of those trials used cadaveric ECM derived from the organ of interest to recreate complex airway geometry and microstructure. Autologous or allogeneic cells were then seeded and cultured in a bioreactor for several days prior to implantation. In contrast, early attempts at lamina propria replacement with acellular scaffolds have not demonstrated clear improvement in scarring. Acellular liver ECM containing endogenous HGF was sutured to deeply injured canine vocal folds, but only modestly improved the scar histology after 3 months [20•]. In a rat scar model, acellular bovine lamina propria ECM was indistinguishable from injured controls on histology and image analysis after 3 months [21]. Note that these two scaffolds for lamina propria replacement were implanted without cells, a significant difference from the lung and trachea engineering methods. As a next iteration *in vitro*, Chan *et al.* [22] seeded vocal fold fibroblasts onto decellularized umbilical vein and found mechanical properties similar to those of lamina propria.

Fibrous biopolymers such as collagen can also be easily produced in hydrogel form, resulting in a gel of randomly oriented fibers with a high water content. The degradable gel serves as a template for new matrix deposition, then releases harmless degradation products during resorption *in vivo*. Collagen has been frequently studied for other tissue engineering applications due to its physiologic relevance, widespread availability, and good biocompatibility. For three-dimensional vocal fold replacement, mixed hydrogels of collagen with other substances have been more common [14••,23]. One reason may be that collagen hydrogels are aggressively contracted and degraded by fibroblasts, rapidly causing near-disappearance of the gel. Also, embedded cells deposit more new ECM in other materials such as fibrin [14••,24]. Finally, collagen's stiff mechanical properties do not match the very soft behavior of the vocal fold cover [25].

Hyaluronic acid has been extensively studied due to its prevalence in lamina propria; other articles in this volume describe injectable hyaluronic acid preparations in more detail. Hyaluronic acid alone cannot form a three-dimensional structure, so for tissue engineering it has been chemically modified to form copolymers or cross-links [1••,9•,14••,26–28,29•]. Farran *et al.* [29•] encapsulated fibroblasts in two different hydrogel mixes of collagen and hyaluronic acid, and then measured mechanical properties. When hyaluronic acid was chemically modified to cross-link with collagen fibers, the elastic modulus was similar to that of native vocal folds. Hyaluronic acid modified to cross-link to itself, however, interfered with collagen fiber formation and produced markedly weaker hydrogels. This experiment demonstrates the influence of microstructure on function, underscoring that matching the molecular composition alone inadequately replicates native tissues.

Fibrin, polymerized from the circulating blood protein fibrinogen, naturally occurs as a fibrous hydrogel in the initial stages of blood coagulation. A key advantage is that it can be produced from autologous cryoprecipitate by adding the enzyme thrombin. When employed as a vocal fold scaffold *in vitro*, fibrin with embedded adipose-derived stem cells exhibited appropriate elastic modulus on indentation and vibrated under physiologic conditions [13••]. Furthermore, it is known to promote elastin mRNA transcription and mature elastic fiber deposition by entrapped cells [14••,24].

Scaffolds discussed thus far have all been biologically based. Synthetic scaffolds are a reasonable alternative offering more reproducibility and control. For example, poly-(ethylene glycol)-diacrylate hydrogels allow some structural control by varying the composition. This translates to control over mechanical properties as well, and, in turn, cell behavior and matrix remodeling [30,31]. Synthetic hydrogels do lack the inherent complexity of a biologic matrix; embedded cells could therefore require additional signaling molecules to guide their behavior.

Synthetic elastomeric polymers can be conceived to replace the elastic recoil function of the vocal folds. True elastic fibers cannot be extracted from animal or human tissue for processing into new structures, so incorporating them into a tissue-engineered construct requires their production by embedded cells, a process that can be challenging and slow. Traditional stretchy materials such as natural and synthetic rubbers are not candidates for implantation due to relatively short life span, inadequate extensibility, and potential cellular toxicity. Biodegradable block copolymers that mimic the secondary structure of elastic fibers have recently been examined with cell adhesion tests, short-term mechanical characterization, and limited animal implantation [32–34]. As these materials degrade, new elastic matrix must be synthesized in order to maintain function; this has not been demonstrated. Also, long-term vibratory cycling at vocal frequencies has not yet been replicated in mechanical tests of these innovative materials.

None of the scaffolds described here mimics the aligned microstructure of the vocal fold lamina propria. Advanced techniques such as electrospinning, micropatterning, and bioprinting can create biomaterials with aligned or

layered fibers [35–37]. To date these approaches have not been applied to vocal fold tissue engineering.

Maturation

A cell-populated scaffold is a dynamic neotissue that matures during culture and after implantation in response to physical and chemical influences. A dominant feature of the vocal fold cover is its high-frequency straining, which is likely to impact cells and ECM. Titze *et al.* [38] first investigated the effects of short-term tensile strain on vocal fold fibroblasts. Cells cultured on a nonimplantable polymer increased expression of elastin, procollagen, and fibronectin mRNA after 6 h of vibration. Two recent reports of long-term intermittent vibration have produced contradictory results. Gene transcription by dermal fibroblasts in a cross-linked hyaluronic acid hydrogel exhibited complex temporal effects that ultimately resulted in decreased construct viscosity [39•]. Conversely, matrix stiffening occurred when laryngeal fibroblasts were vibrated within polyurethane in a similar bioreactor [40•]. Generalizing the results of mechanical conditioning to all tissue-engineered constructs is, therefore, not yet possible.

Aspects of culture geometry can also influence constructs during maturation. Bronchial epithelial cells are known to require an air interface to maintain their differentiated function [41]; a tissue-engineered tracheal transplant, therefore, was matured in a bioreactor that periodically exposed the epithelial cells to air [19]. An air interface was also needed to produce bilayered epithelial and mesenchymal cell differentiation from ASC in a fibrin tissue construct. Traditional submerged liquid culture produced a homogeneous cell phenotype, excess surface collagen, and increased elastic modulus on indentation [16•,24].

Chemical signals add another level of complexity to vocal fold tissue engineering. Notably, HGF has been well studied in cell culture and animal models, but only rarely combined with tissue engineering techniques [5,11,12•, 42]. HGF did increase hyaluronic acid production by vocal fold fibroblasts in a synthetic peptide matrix [42]. This key molecule could be useful during the maturation and implantation of any type of vocal fold replacement to minimize repeat scar formation. Growth factors have also been described in the context of stem cell differentiation, for example epidermal growth factor to induce an epithelial phenotype [16•]. As the field progresses, targeting specific cellular functions with soluble molecules will be another way to tailor the development of tissue constructs.

Other issues remain to be addressed before bringing a tissue-engineered vocal fold replacement to clinical practice. As with any cell-based therapy, safety concerns are paramount and have not yet been assuaged experimentally. Stem cells in particular pose a theoretical risk of undergoing malignant transformation or inducing it in nearby cells, especially in cases of cancer reconstruction. Manufacturing concerns include quality control and construct preservation, if shipping or storage is required before implantation. Also, the implantation method will depend on the construct characteristics and may include large bore injection, endoscopic application, or open cervical approach with laryngofissure. Topical biologic adhesive such as fibrin glue may be useful to secure the construct to the underlying vocal ligament. Minimizing rescarring will be critical when implanting a three-dimensional tissue replacement.

Conclusion

Tissue engineering techniques to treat vocal fold scarring are being developed at numerous centers, with equally numerous variations of the 'cells, scaffold, and signals' scheme. Among cell types reported, fibroblasts have demonstrated improvement in animal models of existing scar, whereas stem cells show promise for recreating the entire vocal fold cover. Several scaffolds are being investigated for either injection or three-dimensional implantation. Because ECM structure is a key determinant of phonation, careful evaluation of the microstructure, mechanical properties, and functional outcome is warranted. Adding mechanical conditioning or growth factor signaling may coax improved function from the constructs, although this aspect of the field is still relatively unexplored.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 578).

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