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Epidermal growth factor mediated healing in stem cell-derived vocal fold mucosa

Liliana Palencia, BS,[a] *Amritava Das, MEng,*[b] *Sean P. Palecek, PhD,*[b] *Susan L. Thibeault, PhD,*[c,*] *and Ciara Leydon, PhD*[c,1]

[a] *School of Medicine and Public Health, University of Wisconsin–Madison, Madison, Wisconsin*
[b] *Department of Chemical and Biological Engineering, University of Wisconsin–Madison, Madison, Wisconsin*
[c] *Division of Otolaryngology-Head and Neck Surgery, Department of Surgery, University of Wisconsin–Madison, Madison, Wisconsin*

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ABSTRACT

Background: The goal of vocal fold wound healing is the reconstitution of functional tissue, including a structurally and functionally intact epithelium. Mechanisms underlying reepithelialization in vocal folds are not known, although it is suspected that healing involves the interplay between several growth factors. We used a three-dimensional human embryonic stem cell-derived model of vocal fold mucosa to examine the effects of one growth factor, exogenous epidermal growth factor (EGF), on wound healing.

Materials and methods: A scratch wound was created in the *in vitro* model. Rate of wound healing, epidermal growth factor receptor (EGFR) activation, and cell proliferation after injury were analyzed with and without application of both exogenous EGF and an EGFR inhibitor, gefitinib.

Results: Wound repair after injury was significantly hastened by application of exogenous EGF (13.3 μm/h, ±2.63) compared with absence of exogenous EGF (7.1 μm/h ±2.84), but inhibited with concurrent addition of Gefitinib (5.2 μm/h, ±2.23), indicating that EGF mediates wound healing in an EGFR-dependent manner. Immunohistochemistry revealed that EGFR activation occurred only in the presence of exogenous EGF. Although not statistically significant, increased density of Ki67 staining in the epithelium adjacent to the scratch wound was observed after treatment with EGF, suggesting a tendency for exogenous EGF to increase epithelial cell proliferation.

Conclusions: Exogenous EGF increases the rate of wound healing in an EGFR-dependent manner in a three-dimensional stem cell-derived model of vocal fold mucosa. This model of wound healing can be used to gain insight into the mechanisms that regulate vocal fold epithelial repair after injury.

* *Corresponding author*. Division of Otolaryngology-Head and Neck Surgery, Department of Surgery, University of Wisconsin–Madison, 5107 WIMR, 1111 Highland Avenue, Madison, WI 53705. Tel.: +1 608 263 6751; fax: +1 608 252 0939.
E-mail address: thibeault@surgery.wisc.edu (S.L. Thibeault).
[1] Department of Speech-Language Pathology, Sacred Heart University, 5151 Park Avenue, Fairfield, CT
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1. Introduction

Vocal folds are vulnerable to injury from trauma, penetrating neck wounds, surgical intubation, laryngeal infections, and inflammation and other mechanical, biological, and chemical challenges. Aberrant healing from injury, or scarring, can result in the loss of the vibratory function of the vocal folds, negatively affecting quality and efficiency of voice production. The personal and public health impact of voice disorders is significant; voice disorders, in general, are associated with psychosocial costs for the individual [1], as well as short-term disability costs and work productivity losses comparable with asthma, heart disease, and depression [2]. Meanwhile, effective techniques to prevent and remediate voice disorders, including those resulting from injury, are limited. Importantly, our ability to develop treatments to maximize healthy wound healing in human vocal folds *in vivo* is significantly compromised by the physical inaccessibility and ethical constraints associated with studying human vocal folds. Furthermore, the lack of human vocal fold epithelial cells from primary sources, their reduced proliferative capability, and the absence of vocal fold epithelial cell lines have created few opportunities to study the pathophysiology of vocal fold wound healing *in vitro*. In the present study, for the first time, we used a novel three-dimensional (3D) model of vocal fold mucosa to study wound healing in cells of human origin. Here, we examined the role for a potential treatment, exogenous epidermal growth factor (EGF), in hastening vocal fold epithelial wound healing. Furthermore, we explored the role for the epidermal growth factor receptor (EGFR), in mediating EGF-dependent effects on wound healing.

After injury to the vocal fold epithelium, a continuous, functionally intact epithelium must be restored. Regeneration of a complete epithelium occurs rapidly after resection of the vocal fold mucosa in animal models; within 3 d, a multilayered epithelium is restored in a rat model [3]. However, it takes up to 5 wk for restoration of epithelial barrier function [4]. The cellular mechanisms underlying epithelial structural and functional regeneration are largely unknown. Likely, epithelial proliferation and migration after injury is guided by autocrine and paracrine signaling through growth factors, chemokines, and cytokines by epithelial cells and fibroblasts, the most abundant cell type in vocal fold lamina propria. Recent work suggests that epithelial cells secrete growth factors that are important for wound healing. For example, epithelial cells secrete transforming growth factor beta 1 (TGF-β1) and TGF beta 3 after injury in an animal model (TGF-β3) [5]. We have reported previously that EGF is secreted by epithelial cells *in vitro* in the absence of injury, and *in vivo* after injury [6]. EGF has been shown in a variety of tissues to promote epithelial proliferation and migration, and research has indicated that EGF increases epithelial wound closure and shortens healing time [7,8]. Furthermore, the EGFR, a receptor for ligands including EGF, is activated after vocal fold injury [6]. It has been proposed that EGFR, a member of the family of tyrosine kinase receptors, increases wound healing via second-messenger signaling [9]. Specifically, EGF-EGFR interactions promote receptor dimerization, activation of the receptor kinase domain, and downstream phosphorylation of signaling molecules that promote cell proliferation and migration [10,11].

An *in vitro* model of vocal fold mucosa offers the opportunity to explore epithelial cell signaling during wound healing in a controlled, simplified environment. We previously created and characterized a human embryonic stem cell model of vocal fold mucosa [17]. The model mimics key morphologic and phenotypic features of an *in vivo* mucosa; it contains a multilayered epithelium and a basement membrane overlying a collagen gel containing fibroblasts. Epithelial cells showed presence of stratified, squamous cell markers (keratin 13 and keratin 14), as well as intercellular junctions (tight junctions, adherens junctions, gap junctions and desmosomes). In the present study, we exploited the model to examine epithelial regeneration after a scratch injury. Our aim was to explore how EGF and its receptor, the EGFR, mediate reepithelialization by cell proliferation in an *in vitro* model. We sought to determine if application of exogenous EGF after scrape injury increased wound healing in stem cell-derived epithelial cells of our 3D model of vocal fold mucosa. Moreover, we sought to establish if reepithelialization after a scrape injury depended on EGFR activation in stem cell-derived epithelial cells. We hypothesized that exogenous EGF would increase EGFR activation and cell proliferation resulting in more rapid wound closure. In addition, we hypothesized that wound healing would be slowed or incomplete in the absence of EGFR activation.

2. Methods

2.1. Derivation of simple epithelial cells and creation of 3D tissue construct

Nine 3D stem cell-derived constructs of vocal fold mucosa were created as described previously [17]. Briefly, simple epithelial cells were differentiated from a human embryonic stem cell line (WA09) via retinoic acid treatment [18,19]. The resulting keratin 18 (K18) and p63-expressing cells were placed on a collagen I gel seeded with vocal fold fibroblasts that were characterized elsewhere (T21 cell line) [20]. The gels were submerged with flavonoid adenine dinucleotide medium and placed in a 37°C incubator. The medium contained Ham F-12/Dulbecco's Modified Eagle's Medium (3:1 ratio), fetal bovine serum (2.5%), hydrocortisone (0.4 μg/mL), cholera toxin (8.4 ng/mL), insulin (5 μg/mL), adenine (24 μg/mL), EGF (10 ng/mL), penicillin (100 U/mL), and streptomycin (0.01 mg/mL). After 2 d, medium was removed from the gel surface to create an air liquid interface. Medium was refreshed every 2 d.

2.2. Scratch wound assay

After 19–21 d at the air liquid interface, three 3D constructs were submerged overnight in a low-serum medium (Dulbecco's Modified Eagle's Medium with 0.5% fetal bovine serum). A scratch wound of approximately 0.5 mm in thickness was created along the engineered epithelium to the depth of the collagen substrate using a 100-μL sterile pipette. The constructs were gently rinsed with low-serum medium to remove