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

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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 post-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/hour, ±2.63) compared to absence of exogenous EGF (7.1 μm/hour ±2.84), but inhibited with concurrent addition of Gefitinib (5.2 μm/hour, ±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. While not statistically significant, increased density of Ki67 staining in epithelium adjacent to the scratch wound was observed following

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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 following injury.

Keywords

vocal fold; epithelium; wound healing; scratch assay; epidermal growth factor; epidermal growth factor receptor

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 to 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 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. Further, we explored the role for the epidermal growth factor receptor (EGFR), in mediating EGF-dependent effects on wound healing.

Following injury to the vocal fold epithelium, a continuous, functionally intact epithelium must be restored. Regeneration of a complete epithelium occurs rapidly following resection of the vocal fold mucosa in animal models; within 3 days, a multilayered epithelium is restored in a rat model [3]. However, it takes up to five weeks for restoration of epithelial barrier function [4]. The cellular mechanisms underlying epithelial structural and functional regeneration are largely unknown. Likely, epithelial proliferation and migration following 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 following 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]. Further, the epidermal growth factor receptor (EGFR), a receptor for ligands including EGF, is activated following 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 following a scratch injury. Our aim was to explore how epidermal growth factor (EGF) and its receptor, the epidermal growth factor receptor (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 three-dimensional model of vocal fold mucosa. Moreover, we sought to establish if reepithelialization following 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.

Methods

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

Nine three-dimensional 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 flavinoid adenine dinucleotide (FAD) medium and placed in a 37°C incubator. The medium contained Ham's F-12/DMEM (3:1 ratio), FBS (2.5%), hydrocortisone (0.4μg/ml), cholera toxin (8.4 ng/ml), insulin (5 μg/ml), adenine (24 μg/ml), epidermal growth factor (10 ng/ml), penicillin (100 U/ml) and streptomycin (0.01 mg/ml). After two days, medium was removed from the gel surface to create an air liquid interface (ALI). Medium was refreshed every two days.

Scratch Wound Assay

Following 19–21 days at the ALI, three 3D constructs were submerged overnight in a low-serum medium (DMEM with 0.5% FBS). 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 debris and assigned to one of three experimental conditions. Under the control condition the scratched gels were immersed in low-serum medium only. Under the EGF condition, scratched gels were immersed in medium supplemented with exogenous epidermal growth factor (100 ng). Under the EGF, Gefitinib condition, gels were pretreated for one hour before injury with Gefitinib (1 μM) [21], an epidermal growth factor (EGFR) inhibitor. Subsequently, these gels were submerged in medium containing EGF and Gefitinib (1 μM) after injury. Experiments were completed in triplicate.

Wound Closure

Gels were imaged immediately and 24 hours after injury using an Olympus CKX41 microscope and Spot software (Version 4.6; Diagnostic Instruments). Wound width (microns) and rate of healing (microns per hour) were measured by three raters using ImageJ [22]. At 24 hours, gels were fixed in formalin and processed for routine immunohistochemistry.

Immunohistochemistry

Cultures were fixed in 4% paraformaldehyde, processed, and embedded in paraffin. Standard hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) analyses were performed. For IHC, antigen retrieval was achieved using heat-activated 10 mM sodium citrate buffer (pH 6). Sections were incubated overnight at 4°C using antibodies directed against the proliferation marker, Ki67 (1:100; ab16667, Abcam, Cambridge, MA) and a marker of phosphorylated epidermal growth factor receptor, an anti-tyrosine 1068 antibody (Tyr1068, 1:100; #3777, Cell Signaling Technology, Danvers, MA). A Ki67 labeling index was used to measure rate of proliferation of epithelial cells. The index was calculated by dividing the number of Ki67-positive epithelial cells by the total number of epithelial cells within a ten - cell width of either side of the wound.

Statistical analyses

Three raters measured wound width, Ki67 labeling, and identified presence or absence of EGFR staining. Statistical significance between treatment means for rate of healing (μm/hr) and Ki67 labeling was determined through use of a one- way analysis of variance (ANOVA). In addition, intraclass and interclass coefficients were included in the data analysis of rate of healing and Ki67 labeling to assess intra and inter rater reliability. Percent agreement in terms of identification of staining was used to determine intra- and inter-rater reliability for EGFR data. Inter-rater reliability for 100% of images was obtained for rate of judgments of wound healing and Ki67 labeling. Intra-rater reliability was completed on 20% of the sections selected at random. Data for each treatment variable were obtained as follows:

Wound width—Change in wound width over time, rate of healing, for each treatment was determined by visually identifying the area of most closure in each wound at 24 hours post-injury. The original wound width of that area was measured, as well as the wound width at 24 hours post-injury. Rate of healing in μm/hr was then determined.

Proliferation rate—Ki67 labeling index was determined by finding the wound edge and counting 10 cells along the basal layer in the direction away from the injury. This was repeated for both sides of the wound. The cell area contained within that 10-cell width was assessed for ki67 labeling. The percentage of cells labeled for Ki67 was determined for each side of the wound and an average of the two wound edges was calculated.

EGFR staining—Determination of the presence of staining for activated EGFR was conducted in a binary manner. Raters answered either “yes” or “no” to the presence of staining around the wound edge.

Results

We examined the roles of exogenous EGF and its receptor, EGFR, in a stem cell-derived model of vocal fold epithelium following injury. Scratch wounds were created in mucosa (Figure 1). Epithelial regeneration was observed following scratch wound injury in the presence and absence of exogenous EGF (Table 1). However, the rate of healing was significantly faster following addition of EGF compared to absence of EGF or concurrent treatment with EGF and an EGFR inhibitor, Gefitinib (ANOVA, $p=0.019$). Intra-rater and inter-rater correlation coefficients were 0.92 and 0.88, respectively.

Cell proliferation was observed in all epithelium following scratch injury (Figure 2). At 24 hours post injury, an increased density and heterogeneous distribution of Ki67, or number (%) of positive cells across all epithelial layers, was observed in the epithelium surrounding the scratch wound area under all conditions (Figure 3; Table 2). However, Ki67 labeling index was greatest in the EGF-treated samples (ANOVA, $p=0.16$). Intra-rater and inter-rater reliability for measurement of the Ki67 index were high, at 0.96 and 0.95, respectively.

Activated EGFR was observed only following EGF treatment at 24 hours post-injury (Figure 3). EGFR activation was found in all layers of the epithelium under that condition. EGFR activation was not observed when EGF was omitted from the medium or when both EGF and the EGFR inhibitor, Gefitinib, were present. Inter-rater and intra-rater reliability were 0.89 and 0.89, respectively.

Discussion

Vocal fold mucosa is composed of a stratified, squamous epithelium and a lamina propria containing multiple cell types, including fibroblasts. Vocal fold wound healing likely involves complex interactions between the various cell types, as well as between cells and the extracellular matrix. As a step towards elucidating the cellular mechanisms underlying epithelial repair after vocal fold injury, we examined the role of a growth factor, EGF, and its receptor, EGFR, in reepithelialization in a human embryonic stem cell-derived three-dimensional *in vitro* model of vocal fold injury. We observed that exogenous EGF hastened

the rate of wound healing at 24 hours post-scratch injury. This effect was not observed following inhibition of the EGFR, indicating that EGF mediated its effects in an EGFR-dependent manner.

Elucidating mechanisms underlying early epithelial regeneration is an important prerequisite for understanding the role of epithelial cells in normal and aberrant healing, as well as identifying treatment to induce rapid and complete healing. Following vocal fold injury, the fundamental goal of wound healing is the reconstitution of functional mucosa, including the epithelium. Restoration of a continuous, multilayered epithelium occurs within days following wound healing, while return of a functional barrier takes up to five weeks post-injury [4]. The lag in restoration of barrier function theoretically renders the epithelium vulnerable to damage from chemical, biological and mechanical insults during this time. Consequently, efforts towards elucidating and manipulating healing at a cellular level have high therapeutic relevance.

Exogenous growth factors have been shown to reduce scarring in lamina propria in animal models following injury [13–16]. Exogenous application of TGF-β3 suppresses vocal fold scar formation in dog [2] and rat [5] models. Similarly, exogenous hepatocyte growth factor (HGF) [13], 5-amino acid deleted type HGF (dHGF) [14], and basic fibroblast growth factor (bFGF) [15,16] improve outcomes post-injury in animal models. Consequently, application of growth factors offers a promising approach to enhancing the rate and completeness of wound healing. To understand the mechanisms by which growth factors suppress scarring, several studies examined the effects of exogenous growth factors at the cellular level, specifically using cultured vocal fold fibroblasts. Exogenous HGF, EGF, bFGF and TGF-β1 increased hyaluronan synthesis by fibroblasts [23]. Elsewhere, exogenous HGF upregulated production of endogenous HGF, TGF- β1, as well as other growth factors in rat fibroblasts [24]. In the single paper that explored the effects of EGF on vocal fold fibroblast behavior in a scratch assay, no appreciable increase in fibroblast migration was reported [25]. Likely, the effects of EGF on vocal fold fibroblast proliferation and migration are cell, dose and substrate specific [26]. To the best of our knowledge, there are no reports on the effects of growth factors on vocal fold epithelial cells. This work suggests that epithelial cells provide a responsive target for treatment with growth factors.

We acknowledge several limitations to this study. First, EGFR serves as a receptor for various growth factors, only one of which, EGF, was studied in the present work. Consequently, the present study does not attempt to capture the complexity of inhibitory and excitatory effects induced by competing ligand growth factors such as HGF which is expressed in vocal folds following injury [27]. Second, vocal fold response to injury likely involves complex, undefined epithelial-mesenchymal interactions. By focusing on epithelium, we gleaned important, yet incomplete insight into the effects of exogenous EGF on cell proliferation and rate of wound healing. EGF may alter behavior of other cells, including fibroblasts [23,25], which play a critical role in determining the biomechanical properties of lamina propria post-injury through their impact on the composition of the extracellular matrix. Future study of the effects of exogenous EGF on fibroblasts and epithelial-fibroblast interactions using our model could provide important insights into lamina propria remodeling, a common and undesirable effect of injury. Third, epithelial

healing begins immediately after wound healing and continues until a complete epithelium has been restored. Here we examined the early phase of wound healing. We selected 24 hours as an end point for this study; while additional time points may have provided insight into the speed of EGFR-activation on wound healing, epithelial proliferation has been shown to peak in an animal model at one day post injury {28]. Finally, scratch injury to our in vitro model has not been characterized previously, consequently, the ability of the model to mimic the *in vivo* response to injury is unknown. However, our observations of EGFR activation and cell proliferation in response to injury in the present study are consistent with those reported elsewhere in an in vivo model of injury [6]. These findings lend support the use of the *in vitro* model to explore vocal fold wound healing in a controlled environment.

Notwithstanding the above limitations, this study has provided us with important and clinically relevant insights into EGF-mediated vocal fold healing. Specifically, our study shows that EGF hastens wound healing in an EGFR-dependent manner. Further, we validated that the stem cell-derived 3D model of vocal fold mucsoa can be used to explore mechanisms that enable and regulate epithelial repair following mechanical injury. This work provides necessary groundwork for developing treatments that hasten reepithelialization and, consequently, improve host tissue protection following mechanical injury.

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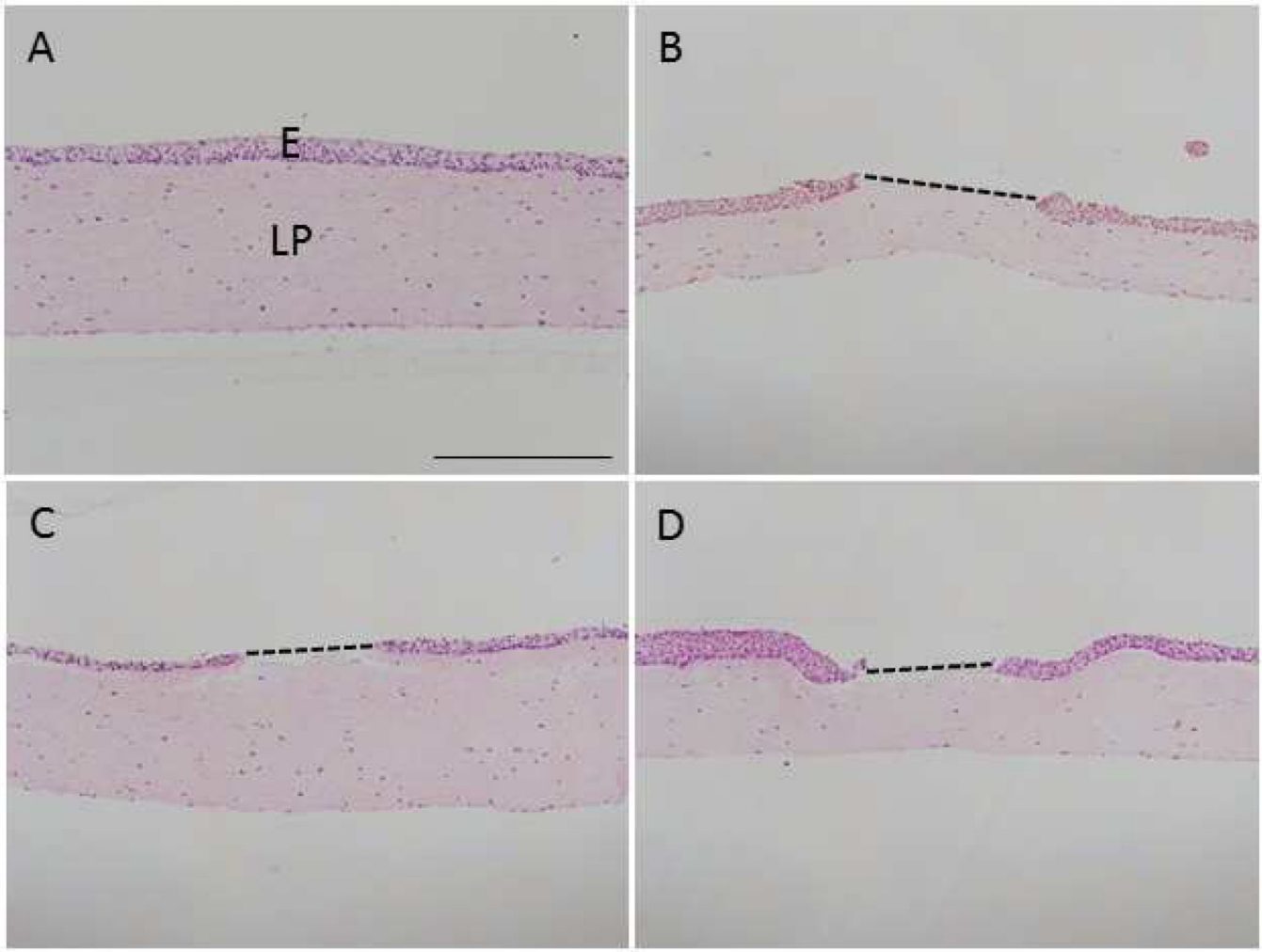

Figure 1. Epithelial wound 24 hours after scratch injury

Hematoxylin and eosin (H&E) staining showing epithelium (E), collagen gel seeded with fibroblast (blue) to mimic lamina propria (LP) in an uninjured model of vocal fold mucosa (A). The mucosa is shown 24 hours after scratch injury under in the presence of the growth factor, EGF, only (B), absence of EGF (C), and EGF with the EGFR inhibitor Gefitinib (D). A dashed line indicates scratch injury. Experiments were completed in triplicate. Images of representative scratch wounds are shown here. Scale bar: 500 μm.

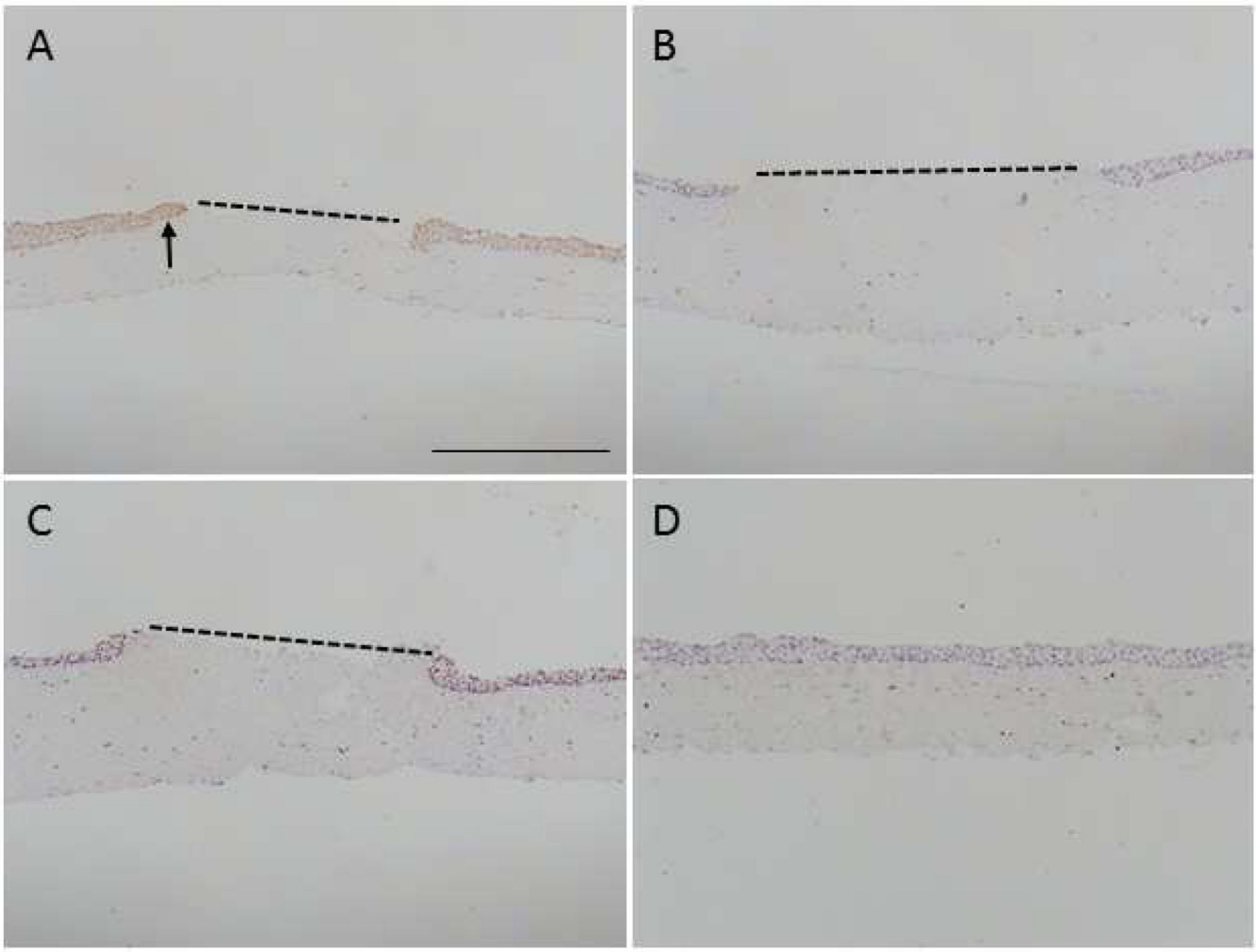

Figure 2. EGFR activation and epithelial cell proliferation 24 hours after scratch injury

Presence of brown staining (arrow) indicates EGFR activation in epithelial cells adjacent to the scratch in a multilayered stem cell derived epithelium following treatment with EGF (A). No EGFR activation was observed following treatment with EGF and the EGFR inhibitor, Gefitinib (B), in the absence of exogenous EGF treatment (C), and in uninjured mucosa (D). A dashed line indicates scratch injury. Experiments were completed in triplicate. Images of representative scratch wounds are shown here. Scale bar: 500 μm.

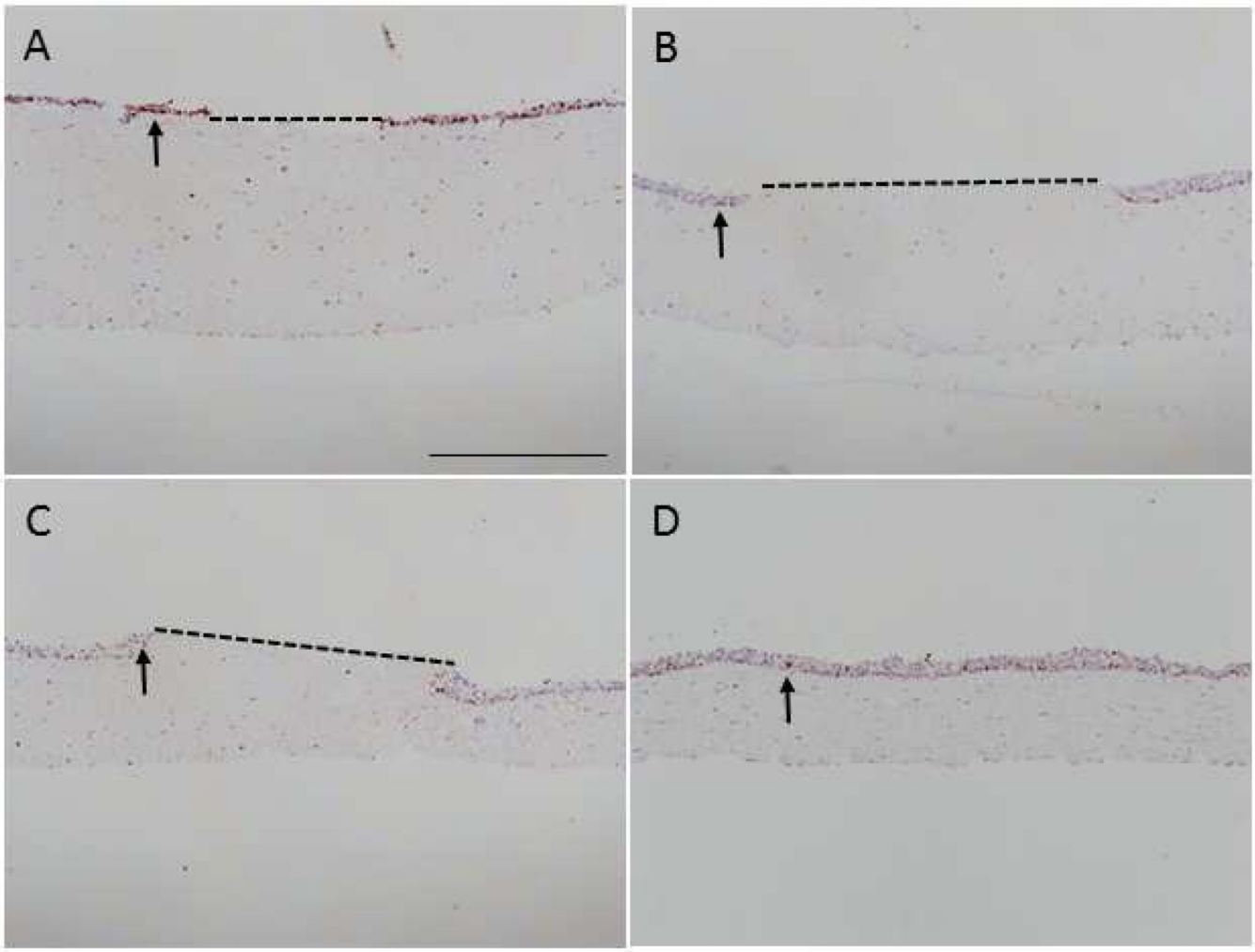

Figure 3. Epithelial cell proliferation 24 hours after scratch injury

Immunohistochemistry for the cell proliferation marker Ki67 shows prolific brown staining (arrow) in epithelial cells under the EGF only condition. Staining was observed, albeit at lower levels, in the absence of EGF (B) and following simultaneous treatment of EGF and Gefitinib (C). Staining was absent in uninjured mucosa (D). A dashed line indicates scratch injury. Experiments were completed in triplicate. Images of representative scratch wounds are shown here. Scale bar: 500 μm.

Table 1

Healing rate under three experimental conditions

Treatment condition	Mean rate of healing in microns/hr (± 1 SD)
EGF, No Gefitinib	13.3 (±2.63)
EGF, Gefitinib	5.2 (±2.23)
No EGF, No Gefitinib	7.1 (±2.84)

The mean rate of healing was significantly higher under the EGF condition at 24 hours post-scratch assay (p=0.019) compared to absence of EGF or presence of both EGF and the EGFR inhibitor, Gefitinib. Standard deviations are shown in parentheses. Experiments were conducted in triplicate.

Table 2

Ki67 labeling index under three experimental conditions

Treatment condition	Mean Ki67 labeling index (± 1 SD)
EGF, No Gefitinib	0.363 (±.05)
EGF, Gefitinib	0.125 (±.20)
No EGF, No Gefitinib	0.210 (±.10)

The mean rate of healing showed a higher, while not significantly significant (p=0.16), Ki67 labeling index in the presence of EGF at 24 hours post-injury compared to absence of EGF or presence of both EGF and the EGFR inhibitor, Gefitinib. Standard deviations are shown in parentheses. Experiments were conducted in triplicate.