Epidermal growth factor receptor activity is necessary for mouse basal cell proliferation

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Brechbuhl HM, Li B, Smith RW, Reynolds SD. Epidermal growth factor receptor activity is necessary for mouse basal cell proliferation. Am J Physiol Lung Cell Mol Physiol 307: L800–L810, 2014. First published September 12, 2014; doi:10.1152/ajplung.00201.2014.—ERB family receptors (EGFR, ERB-B2, ERB-B3, and ERB-B4) regulate epithelial cell function in many tissue types. In the human airway epithelium, changes in ERB receptor expression are associated with epithelial repair defects. However, the specific role(s) played by ERB receptors in repair have not been determined. We aimed to determine whether ERB receptors regulate proliferation of the tracheobronchial progenitor, the basal cell. Receptor tyrosine kinase arrays were used to evaluate ERB activity in normal and naphthalene (NA)-injured mouse trachea and in air-liquid interface cultures. Roles for epidermal growth factor (EGF), EGFR, and ERB-B2 in basal cell proliferation were evaluated in vitro. NA injury and transgenic expression of an EGFR–dominant negative (DN) receptor were used to evaluate roles for EGFR signaling in vivo. EGFR and ERB-B2 were active in normal and NA-injured trachea and were the only active ERB receptors detected in proliferating basal cells in vitro. EGF was necessary for basal cell proliferation in vitro. The EGFR inhibitor, AG1478, decreased proliferation by 99%, and the Erb-B2 inhibitor, AG825, decreased proliferation by ~66%. In vivo, EGFR-DN expression in basal cells significantly decreased basal cell proliferation after NA injury. EGFR and EGFR are necessary for basal cell proliferation. The EGFR/EGFR homo- and the EGFR/ERB-B2 heterodimer account for ~34 and 66%, respectively, of basal cell proliferation in vitro. Active EGFR is necessary for basal cell proliferation after NA injury. We conclude that EGFR activation is necessary for mouse basal cell proliferation and normal epithelial repair.

epidermal growth factor; EGFR; ERB receptors; basal cells; proliferation

CHEMICAL EXPOSURES [chlorine gas (30), hyperoxia (35), sulfur dioxide (5), fire smoke (52)], biological agents [Pseudomonas aeruginosa (1) and respiratory syncytial virus (18)], and lung injury is a logical target for interventions focused on healing the asthmatic epithelium. Ultrastructural analysis of the asthmatic epithelium identified residual basal cells in regions of epithelial sloughing (29). The concept that these basal cells could repair the epithelium is supported by reports that human basal cells proliferate in response to acute injury (reviewed in Ref. 37) and serve as the progenitor for nascent secretory and ciliated cells in vitro (8, 14). Collectively, these data suggested that basal cells are the cellular mediator of repair following acute epithelial injury.

We reported that naphthalene (NA) injury of the mouse tracheal epithelium results in club cell (Clara cells) necrosis and sloughing of ciliated cells (6). As demonstrated for other chemical injuries in mice, the basal cell mitotic index (MI) increased 10-fold after NA injury and lineage tracing demonstrated that basal cells were the progenitor for nascent club cells and ciliated cells (11, 19, 20). Thus NA-induced tracheal injury modeled the histological features of acute injury in humans and the post-NA regenerative process utilized the progenitor cell type that is likely to repair the human airway epithelium.

The molecular signaling pathway that regulates basal cell proliferation has not been defined. Immunohistochemical evidence suggests that epidermal growth factor receptor (EGFR) signaling plays a role in airway diseases including asthma (3, 15), chronic obstructive pulmonary disease (53), chronic bronchitis (28, 44), and cystic fibrosis (51). Furthermore, increased EGFR expression was detected in basal cells located in regions of repairing epithelium in asthmatic patients (33, 45) and in basal cells of chronic smokers (25, 40). A specific role for EGFR in basal cell proliferation is supported by the finding that successful culture of basal cells from normal adult human bronchial and nasal tissue (26, 55) and from hamster (54), rat (46), and mouse trachea require epidermal growth factor (EGF). In this study, we tested the hypothesis that EGFR signaling is necessary for basal cell proliferation following acute injury.

MATERIALS AND METHODS

Animal strains. Mice were cared for according to procedures approved by the National Jewish Health Institutional Animal Care and Use Committee. All experiments used 6- to 8-wk-old mice. C57Bl/6 (B6) mice were used for receptor tyrosine kinase (RTK) profiling and in vitro experiments. Transgenic mice harbored the keratin (K) 14 promoter regulated-reverse tetracycline transactivator (K14-rTA) (38) and/or the tetracycline responsive element-regulated EGFR–dominant negative (TRE-EGFR-DN, The Jackson Laboratory no. 010575) transgenes. Monotransgenic mice harbored only the K14-rTA transgene (TA−/DN+). Bitransgenic mice harbored both transgenes (TA+/DN+).

NA exposure. NA-induced injury exhibits a sexual dimorphism with female mice being more sensitive than male mice (48). Consequently, the NA-injury studies were limited to female mice. B6 mice were treated with 275 mg/kg ip NA and recovered 3, 6, or 8 days. NA-injured mice decrease their intake of food and water for ~3 days (6). Consequently, activation of the EGFR-DN transgenic system requires pretreatment with doxycycline (dox), TA−/DN+ and TA+/DN+ mice were fed dox chow (625 mg/kg Harlan) for 6 days prior to...
NA exposure and throughout the recovery period. These mice were treated with 300 mg/kg ip NA on day 0 and with 30 mg/kg 5-bromo-2’-deoxyuridine (BrdU) on recovery days 3, 4, and 5. Tissue recovery time points are indicated in RESULTS. Experiments used six mice per genotype per group for mRNA analysis and three mice per genotype per group for histological analysis.

**ALI cultures.** Air-liquid interface (ALI) cultures used previously published methods (4, 57). These cultures have distinct proliferation periods (4) and permit independent analysis of the signals that regulate these processes. The proliferation period extends from day 0 (plating) through days 5–6. During this period, the transepithelial resistance (TER) is ~66 Ω-cm² on day 1, ~400 Ω-cm² on days 3–4, and ~1,500 Ω-cm² on day 5 or 6 (42). On days 5–6 growth factors are removed and the cultures are air lifted. These processes initiate basal cell differentiation to club-like and ciliated cells.

Base medium contained DMEM/F12 1:1 with 15 mM HEPES and 2.5 mM l-glutamine (GIBCO 1130); l-glutamine (Cellgro 25-005-C1), 2 mM final concentration; amphoterin B (Cellgro 30-003-CF), 0.25 μg/ml final concentration; 7.5% (wt/vol) sodium bicarbonate (Sigma S5761), 0.03% (vol/vol) final concentration; penicillin/streptomycin (Cellgro 30-002-CI), 1 × 1,000 IU penicillin and 1,000 μg/ml streptomycin final concentration; insulin/transferrin/selenium (ITS, GIBCO 41400-45), 10 μg/ml insulin, 5.5 μg/ml transferrin, 6.7 × 10⁻³ μg/ml selenium final concentration; cholera toxin (CTx, Sigma S8052), 0.1 μg/ml final concentration; hydrocortisone (HC, MP Biomedicals 194569), 0.1 μg/ml final concentration; and retinoic acid (RA, Sigma R2625), 0.05 μM final concentration.

Culture media varied with experimental design (Table 1). Additives included bovine pituitary extract (BPE, GIBCO 13028014), 0.03 mg/ml final concentration; fetal bovine serum (FBS, HyClone SV30014.03), 0.5–5.0% final concentration; EGF (BD 354001), 0.5–5.0% final concentration; and retinoic acid (RA, Sigma R2625), 0.05 μM final concentration.

**Gene expression analysis.** Standard reagents and methods were used to purify RNA and for gene expression (6). Briefly, RNA was isolated by using Qiagen RNeasy Mini Kits (Qiagen 74104) in combination with Qiashredders (Qiagen 79654). Tracheal RNA was isolated by ground-glass homogenization in RNA lysis buffer. RNA from ALI cultures was isolated by removing the Transwell membranes, placing them into RNA lysis buffer, and vigorous vortexing. Pools of RNA from normal trachea or proliferating NIH-3T3 cells were used as a calibrator for tracheal and ALI gene expression analysis. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of gene expression was calculated as previously reported (6). Assays on demand were purchased from Life Technologies (Carlsbad, CA) and included Ccnd1 (alias cyclin D1) (Mm00432359_m1), Ccnd3 (alias cyclin D3) (Mm01612362_m1), Myc (alias c-myec) (Mm00487803_m1), and FoxJ1 (Mm00807215_m1). Standard reagents and protocols from Life Technologies were used. Relative gene expression was calculated by the delta-delta cycle threshold method (17).

**Immunofluorescence staining.** Tissue and cell culture immunostaining used previously reported methods and validated antibodies (6). Images were captured by using a Zeiss AxioVision microscope (Carl Zeiss MicroImaging, Jena, Germany).

**Morphometry.** For cell culture experiments, regions of interest were identified from the DAPI channel followed by imaging in the red or green channels. At least three images were acquired for each membrane at ×200 magnification. MI represents the number of BrdU+ nuclei divided by the number of DAPI+ nuclei and cell density the number of DAPI+ nuclei per unit area.

For in vivo experiments, the ventral trachea was imaged from cartilaginous ring 4 through ring 12 at ×200 magnification. Epithelial subregions were defined as those overlying the cartilage rings (mid-cartilaginous) and regions between the cartilage rings (intercartilaginous) (6). Epithelial cells were classified by K5 and/or BrdU expression and data were normalized to the length of basement membrane (12). MI was calculated as indicated above. Basal cell MI is the number of K5+/BrdU+ cells divided by the number of K5+ cells. Basal cell contribution to the mitotic pool is the number of K5+/BrdU+ + cells divided by the number of BrdU+ cells. The nonbasal cell specific MI is the number BrdU+/K5− divided by the number of BrdU+ cells.

**Statistical analysis.** Data are presented as means ± SE. All statistical analysis used GraphPad Prism version 5 (GraphPad Software, San Diego, CA). The F-test was used to determine whether data sets were normally distributed. Unpaired two-tailed t-tests and one-way ANOVA with Tukey’s posttest were used to evaluate normally distributed data sets. The Mann-Whitney test was used to evaluate skewed data sets.

**RESULTS**

**ERB Receptors Are Active in the Steady-State and Repairing Trachea**

The ERB/HER RTK family includes four receptors: EGFR, ERB-B2, ERB-B3, and ERB-B4 (2). EGFR, ERB-B3, and ErbB4 bind ligand whereas an ERB-B2 ligand has not been identified. EGFR, ERB-B2, and ERB-B4 are RTKs and ERB-B3 is transactivated by the other ERB receptors (41). Phosphorylated (p) ERB receptors are the active form of the proteins.

ERB activation has not been previously reported for the mouse trachea. Thus we used RTK arrays to determine whether pERB were present in the steady-state and/or NA-injured trachea. Whole tracheal homogenates, which included protein from all tracheal cell types, were utilized.

pEGFR, pERB-B2, pERB-B3, and pERB-B4 were detected in steady-state trachea (Fig. 1, A and B, “Control”). In contrast, ERB activation varied as a function of time after NA injury.

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Table 1. Test media

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Base medium contained insulin, transferrine, cholera toxin, hydrocortisone, and retinoic acid. Additives were fetal bovine serum, bovine serum albumin, bovine pituitary extract, epidermal growth factor, and neuregulin-1.
pEGFR and pERB-B2 were detected on NA-recovery days 3–8 (Fig. 1B). pERB-B3 was present on recovery days 6–8. pERB-B4 was only present on day 6. This study demonstrated that 1) all four ERB family members were active in steady-state trachea; 2) EGF and ERB-B2 were active throughout the repair process; and 3) ERB-B3 and ERB-B4 were activated only at later recovery time points.

**EGFR and ERB-B2 Are Activated During Basal Cell Proliferation In Vitro**

Although the ERB activity study (Fig. 1B) used total tracheal proteins, it was notable that pEGFR and pERB-B2 were detected when epithelial repair is characterized by rapid basal cell proliferation (12). In contrast, ERB-B3 and ERB-B4 were activated later when basal cells began to differentiate. These data suggested a specific role for EGFR and ERB-B2 during the proliferation phase of repair.

To focus on basal cell proliferation, we evaluated ERB activity in ALI cultures. On culture day 3, basal cells proliferate rapidly (4). EGFR and ERB-B2 were the only active ERB receptors detected at this time point (Fig. 1C). In contrast, pEGFR, pERB-B2, and pERB-B3 were detected throughout the differentiation period (differentiation days 4–8). ERB-B4 activation was limited to differentiation day 8. These data also supported the concept that EGFR and ERB-B2 played a role in basal cell proliferation.

**EGF Promotes Basal Cell Proliferation**

The standard ALI proliferation medium (Medium-1, Table 1) contains multiple potential basal cell growth factors. Thus our first goal was to determine which additives were necessary for basal cell proliferation. Basal cell MI was used to evaluate basal cell proliferation and basal cell density was used to detect changes in cell survival.

We reasoned that BPE, EGF, and FBS were the most likely basal cell growth factors. Thus medium containing ITS, CTx, HC, and RA was defined as base medium. We then compared basal cell MI and density in Medium-1 and in base medium supplemented with FBS and EGF (Medium-2), or FBS (Medium-3) (Fig. 2A). On day 3, the MI for basal cells cultured in Medium-1 was 37 ± 7% (Fig. 2, B and G) and cell density was 1.075 × 10^6 ± 1.67 × 10^5 cells/cm² (Fig. 2, B and H). The MI and cell density for cells cultured in Medium-2 (Fig. 2, C, G, and H) was similar to Medium-1. However, the MI and cell density decreased by approximately half when cells were cultured in Medium-3 (Fig. 2, D, G, and H). These data indicated that BPE was not necessary for basal cell proliferation and suggested that EGF promoted proliferation.

We next evaluated FBS as a source of growth- and/or survival-factors. Initial studies used base medium in which 2% bovine serum albumin (BSA) served as a protein source. However, cells plated in this medium did not survive to day 3 (data not shown). In contrast, cells cultured in base medium supplemented with 0.5% FBS and 2% BSA (Medium-4) were viable on day 3 (Fig. 2E). MI and cell density in Medium-4 were significantly less than that observed for Medium-3 (Fig. 2, G and H). Addition of EGF (Medium-5) restored the MI and cell density to Medium-3 levels (Fig. 2, F–H). We conclude that 1) FBS contains factor(s) that are necessary for basal cell survival, 2) growth factor restriction in the presence of 0.5% FBS attenuates basal cell proliferation but does not alter survival, and 3) optimal basal cell proliferation requires EGF.

**EGF Promotes Cell Cycle Reentry**

We next used a growth factor starvation approach to determine whether EGF induces basal cell proliferation. Basal cells were plated in Medium-3 and incubated for 3 days (Fig. 3A). The cultures were then changed to Medium-4 for 24 h. This serum and growth factor starvation resulted in a cell cycle distribution in which ~40% of cells were in G1-phase, 10% were in S-phase, and 50% were in G2/M-phase (Fig. 3B). No apoptotic cells were detected.

A second set of cultures were fed Medium-4 or changed to Medium-5 on day 4. Cell cycle analysis was performed 48 h later (day 6). The cell cycle profile for cells cultured in Medium-4 shifted significantly toward G1-phase between days 4 and 6. In contrast, cells that were switched to Medium-5 exhibited increased mitotic activity as indicated by a 2.5-fold increase in the frequency of S-phase cells. Apoptotic cells were not detected. We conclude that 1) EGF stimulated basal cell cell-cycle progression and 2) EGF restriction did not cause cell death.

**EGF but not NRG-1 Promotes Basal Cell Proliferation**

The RTK array demonstrated that EGFR and ERB-B2 were active during the proliferation phase. EGF is activated by category 1 ligands including EGF, transforming growth factor-α, and amphiregulin (39). ERB-B2 does not bind ligand but is transactivated by ERB-B3 (13), its preferred heterodimerization partner. ERB-B3 is activated by neuregulins (NRG)-1 and NRG-2 (39). Thus the previous studies implicated EGFR as a regulator of basal cell proliferation but raised the possibility that ERB-B2 and -B3 were also involved. To evaluate roles for ERB-B2 and -B3 in basal cell proliferation we compared the promitotic activity of EGF and NRG1.
To establish a standard for basal cell proliferation, we first determined whether EGF caused a dose-dependent proliferative response. Basal cells were cultured in Medium-3 for 3 days followed by serum and growth factor starvation for 1 day (Medium-4) (Fig. 4A). Sets of cultures were then fed Medium-4 or switched to media containing 1/1000 EGF (Medium-5), 0.5/1000 EGF (Medium-6). Cell proliferation was assayed on day 6. EGF caused a dose-dependent increase in the basal cell MI (Fig. 4B).

To determine whether NRG-1 could replace EGF as a growth factor, cells were cultured in Medium-3 for 3 days followed by serum and growth factor starvation for 1 day (Medium-4) (Fig. 4A). Sets of cultures were then fed Medium-4 or changed to medium containing 1× NRG-1 (Medium-7), 2× NRG-1 (Medium-8), or 3× NRG-1 (Medium-9) (Fig. 4A). In contrast with EGF, NRG-1 did not support basal cell proliferation even at concentrations that were six times the standard (1×) EGF dose (Fig. 4B). These
data indicated that ERB-B3 did not play a role in basal cell proliferation.

Cell density varied slightly but significantly between the minus and plus EGF groups and between the 1× EGF and NRG-1 treatment groups (Fig. 4C). To determine whether these differences were biologically relevant, we used TER to compare cell polarization on days 5 and 6. Resistance varied between cells cultured in Medium-7 and Medium-4 or -5 on day 5. On day 6, TER was significantly increased in cultures containing Medium-7 relative to Medium-4 or -5 (Fig. 4D). Taken together these data suggested that EGF specifically induced basal cell proliferation and that this proliferation was necessary for polarization.

**EGFR Activity Is Necessary for Basal Cell Proliferation**

We used the EGFR-specific inhibitor AG1478 (27) and the ERB-B2 specific inhibitor AG825 (32) to determine whether EGFR and/or ERB-B2 kinase activity was necessary for basal cell proliferation. Cells were plated in Medium-3. On day 3, cultures were changed to Medium-3 containing vehicle (2% DMSO), 10 μM AG1478, 50 μM AG825, or both inhibitors. MI and cell density were assayed 24 or 48 h after treatment (Fig. 5A).

AG1478 treatment decreased the basal cell MI by 96% (1.0 ± 0.85%) at 24 h and 99% (0.36 ± 0.51%) at 48 h (Fig. 5, B and C). AG825 treatment decreased the basal cell MI by 69% (5.2 ± 2.4%) at 24 h and 56% (5.6 ± 3.9%) at 48 h. When the cells were treated with both inhibitors, the MI decreased by 94% (1.1 ± 1.0%) and >99% (0.12 ± 0.29%) relative to vehicle at the 24- and 48-h time points (Fig. 5, B and C).

Cell density in cultures treated with AG1478 was not significantly different relative to vehicle control at either time point (Fig. 5, D and E). However, cell density in cultures treated with AG835 or both inhibitors was significantly decreased relative to control at 48-h time point.

Taken together, these data indicated that EGFR kinase activity was a major determinate of basal cell proliferation and that ERB-B2 kinase activity played a lesser role in this process. Since these studies were done in the absence of added EGF, they also indicated that an EGF-class ligand was the critical growth factor supplied by FBS. Ligand-depletion studies will be needed to identify the specific ligand(s) found in FBS. Finally, the significant effect of AG825 on cell density sug-
gested that ERB-B2 might also play a role in basal cell survival.

Validation of the EGFR-DN Transgenic System

Autophosphorylation of the EGFR cytoplasmic domain generates docking sites for second messenger proteins that initiate a subsequent downstream signaling cascade (39). Xie et al. (56) generated mice harboring a COOH-terminal truncated EGFR (EGFR-TD, aa 1–690) that lacked most of the cytoplasmic domain including the entire tyrosine kinase region. They demonstrated that EGFR-TD functioned as a dominant-negative inhibitor of EGF-initiated EGFR autophosphorylation in vitro. Subsequently, Roh and colleagues (38) generated transgenic mice in which EGFR-TD was expressed under regulation of the tetracycline responsive element (TRE). Generation of bitransgenic mice harboring the TRE-EGFR-TD (renamed EGFR-dominant negative, EGFR-DN) and a second transgene that is composed of a cell-type selective promoter and the tetracycline transactivator or reverse-transactivator (rtTA) allows analysis of EGFR function using the dox off or on strategies (34).

To determine whether basal cell proliferation requires EGFR signaling in vivo, we used transgenic mice that harbored the K14-rtTA (TA) transgene (23) and/or the TRE-EGFR-DN (DN) transgene (38). Preliminary studies evaluated dox-dependent changes in epithelial cell type frequency, genotype-dependent variation in NA-mediated injury, and specificity of the K14-promoter-regulated transgenic system.

For the first study, TA−/DN− and TA+/DN+ mice were treated with dox for 6 days and epithelial cell type frequency was evaluated. Dox exposure did not alter epithelial cell frequency in TA−/DN− and TA+/DN+ mice (not shown). These data are in contrast with our previous study that reported dox-dependent changes in K14-rtTA/TRE-cre/β-cateninexon3 mice (43).

To determine whether NA injury exhibited genotype-dependent differences, TA−/DN− and TA+/DN+ mice were pretreated with dox for 6 days and then treated with 300 mg/kg NA on day 0. Trachea and lung tissues were harvested on day 4 and club-like and club cell depletion was evaluated by measuring club cell secretory protein (CCSP) gene expression. In both the trachea and lung, CCSP mRNA levels were significantly decreased relative to untreated control (Fig. 6, A and B) but did not exhibit genotype-dependent differences. These data indicated extensive epithelial injury at the 300 mg/kg dose (6) and that injury was similar in TA−/DN+ or TA+/DN+ mice.

Since previous studies indicated that basal cells are extremely rare in the intrapulmonary airways of mice (19), the
K14-regulated transgenic system should not alter EGFR activity in the lung. To test this supposition, we determined whether expression of EGFR target genes [cMyc and cyclin D1 (16, 24)] and a nontarget gene [cyclin D3 (24)] varied according to genotype in the lung. No differences in gene expression were detected (Fig. 6, D, F, and H). These data indicated that the transgenic system did not alter EGFR function in the lung.

In contrast with the lung, cMyc and cyclin D1 mRNAs were significantly decreased in TA/DN+ tracheal relative to TA+/DN+ controls (Fig. 6, C and E). However, tracheal cyclin D3 mRNA abundance did not vary significantly between TA−/DN+ and TA+/DN+ mice (Fig. 6G). These data indicated that the transgenic system was activated specifically in tracheal basal cells.

**EGFR Inhibition Decreases Basal Cell Proliferation After NA Injury**

To evaluate roles for EGFR in proliferation, TA−/DN+ and TA+/DN+ mice were treated with dox and NA as indicated in Fig. 7. EGFR is necessary for basal cell proliferation in vivo. EGFR-DN monotransgenic mice (TA−/DN+) and bitransgenic mice that coharbored the K14-rTA driver (TA+/DN+) were fed doxycycline (dox) chow for 6 days prior to NA exposure (300 mg/kg). The animals were maintained on dox and recovered for 5 days. Tracheal (A, C, E, G) and lung (B, D, F, H) homogenates were assayed by qRT-PCR for club cell secretory protein (CCSP; A and B); cMyc (C and D); cyclin D1 (E and F); and cyclin D3 (G and H). Data are presented as means ± SE. N = 6 mice per time point. ***P < 0.001 relative to untreated control. Other P values are indicated in each panel.
above and with BrdU on recovery days 3, 4, and 5. Tissue was recovered on day 5 and stained for the pan-basal cell marker Keratin (K) 5, BrdU, and DAPI (Fig. 7, A–D). The ventral trachea from cartilaginous rings 4 through 12 was subdivided into the midcartilaginous and intercartilaginous regions as previously reported (6).

MI in the midcartilaginous region of TA+/DN+ mice was significantly decreased compared with TA−/DN+ con-
trols (Fig. 7E). This parameter did not vary according to genotype in the intercartilaginous region. The basal cell MI in the midcartilaginous region decreased (2.3-fold) in TA+/DN+ mice compared with TA−/DN+ controls (Fig. 7F) but did not differ in the intercartilaginous region. The basal cell contribution to the mitotic pool was significantly decreased in the midcartilaginous region (2.1-fold) and intercartilaginous region (2.3-fold) of TA+/DN+ mice relative to TA−/DN+ controls (Fig. 7G). The nonbasal cell specific MI was increased in the midcartilaginous region (1.5-fold) of TA+/DN+ mice compared with TA−/DN+ mice (Fig. 7H). However, this parameter did not vary by genotype in the intercartilaginous region.

Cell density in the midcartilaginous region was significantly decreased in TA+/DN+ mice relative to TA−/DN+ mice (Fig. 7I). These data demonstrated that EGFR activity was necessary for a basal cell proliferation during repair of the NA-injured epithelium and that decreased basal cell proliferation resulted in epithelial hypoplasia.

**DISCUSSION**

**EGFR Promotes Basal Cell Proliferation**

This study used NA injury to model columnar epithelial sloughing. We show that EGFR inhibition decreases overall epithelial proliferation, basal cell proliferation, and basal cell contribution to the mitotic pool by ~50%. These data demonstrate that EGFR activity is necessary for basal cell proliferation and implicate EGFR as a promitotic stimulus for epithelial repair following injuries and diseases characterized by epithelial sloughing.

**Complementation of the EGFR Proliferation Signal**

Several lines of evidence suggest that ERB-B2 contributes to the EGFR-dependent mitotic signal in basal cells. We show that both EGFR and ERB-B2 are active on post-NA recovery day 6 (Fig. 1) and that ERB-B2 kinase activity is a minor component of the basal cell mitotic stimulus in vitro (Fig. 5). Similarly, Fischer and colleagues (10) demonstrated that ERB-B2 was necessary for human basal cell proliferation following neutrophil elastase-mediated damage in vitro. Finally, a recent report by Vermeer et al. (50) indicated that ERB-B2 inhibitor treatment of a breast cancer patient led to a lung epithelial repair defect. Collectively, these data suggest that ERB-B2 activation via EGFR contributes to the EGFR-dependent proliferation signal in basal cells.

**Additional Roles for EGFR in Basal Cell-Mediated Epithelial Repair**

EGFR inhibition exhibited a more striking effect on proliferation in the midcartilaginous region than in the intercartilaginous region. These data lead to the suggestion that EGFR contributes to other aspects of the reparative process.

**Migration.** Kim and colleagues (22) showed that EGFR signaling was necessary for cell migration during wound healing in guinea pig tracheal epithelial cells in vitro. Furthermore, Shaykhiev and colleagues (40) reported that EGF induced a basal cell phenotype consistent with induction of a migratory phenotype (e.g., epithelial-mesenchymal transition). Our published work demonstrated that the basal cell MI is similar in the inter- and midcartilaginous regions following NA injury (6). In contrast, EGFR inhibition resulted in a significant decrease in overall and basal cell specific proliferation that was limited to the midcartilaginous region. The finding that EGFR inhibition decreased cell density in the midcartilaginous region, but did not significantly alter cell density in the intercartilaginous region (Fig. 7), suggests that EGFR may regulate basal cell migration after NA injury.

**Survival.** Tyner and colleagues (47) reported that EGFR activity was necessary for ciliated cell survival in vitro. The present study demonstrates that EGFR inhibition does not alter basal cell survival in vitro (Fig. 5). In contrast, ERB-B2 inhibition in vitro decreased cell density at the 48-h time point. These data suggest that ERB-B2 rather than EGFR may be a basal cell survival factor.

**Polarization.** We report that EGFR is necessary for increased TER (Fig. 4D). These data, in combination with our previous demonstration of a postconfluence mitosis in mouse ALI (42), suggest that the EGFR-regulated increase in TER may be due to an EGFR/EGFR-dependent increase in basal cell number. However, we cannot exclude the possibility that increased TER is due to EGFR-dependent changes in claudin gene expression (reviewed in Ref. 58).

In contrast with our results, Shaykhiev and colleagues (40) reported that EGF exposure of differentiating human ALI cultures decreased the TER approximately threefold. Extrapolation of our mouse studies suggests that the decreased TER was due to an increase in basal cell proliferation. However, our demonstration of pERB-B3 and pERB-B4 in differentiating mouse ALI (Fig. 1C) and the knowledge that EGFR transactivates ERB-B3 and that EGFR can heterodimerize with ERB-B4 suggest a more complex scenario that should be tested experimentally.

**Differentiation.** Studies in nonrespiratory epithelia suggest that EGFR activity is necessary for differentiation of basal cell progenitors to luminal cell types (9). Our in vivo studies demonstrate that nonbasal cell proliferation was increased in the midcartilaginous region of TA+/DN+ mice (Fig. 7D). Since these mitotic cells do not express the club-like cell marker CCSP, it is possible that they are immature club-like cells that have yet to accumulate detectable levels of CCSP (36). Alternatively, these cells may be the “intermediate” cell type that has been described in some tracheal injury/repair studies (31). Regardless of this cell’s identity, such cells are not normally identified in the NA-injured tracheal epithelium (6). These data suggest a potential role for EGFR in basal cell differentiation to club-like and/or ciliated cells.

**Modeling Repair in Diseases Characterized by Epithelial Sloughing**

The present study demonstrates that EGFR promotes mouse basal cell proliferation in response to loss of columnar epithelial cells following NA injury. By extension, identification of pEGFR positive basal cells in diseased airways may indicate a promitotic signal. However, interpretation of such single-time point studies is complicated by potential roles for EGFR in basal cell migration, polarization, and differentiation. This study demonstrates that NA injury can be used in combination with genetic manipulation to identify specific roles for ERB family members in healing the sloughed epithelium in vivo.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

H.M.B. and S.D.R. conception and design of research; H.M.B., B.L., R.M.S., and S.D.R. performed experiments; H.M.B. and S.D.R. analyzed data; H.M.B. and S.D.R. interpreted results of experiments; H.M.B. and S.D.R. prepared figures; H.M.B. and S.D.R. drafted manuscript; H.M.B. and S.D.R. edited and revised manuscript; H.M.B., B.L., R.M.S., and S.D.R. approved final version of manuscript.

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