

Histamine may induce airway remodeling through release of epidermal growth factor receptor ligands from bronchial epithelial cells

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ABSTRACT Asthma is a chronic inflammatory disease that is associated with airway remodeling, including hyperplasia of airway epithelial cells and airway smooth muscle cells, and goblet cell differentiation. We wished to address the potential role of histamine, a key biogenic amine involved in allergic reactions, in airway remodeling through the epidermal growth factor receptor (EGFR) pathway. Here, we demonstrate that histamine releases 2 EGFR ligands, amphiregulin and heparin-binding epidermal growth factor-like growth factor (HB-EGF), from airway epithelial cells. Amphiregulin and HB-EGF were expressed in airway epithelium of patients with asthma. Histamine up-regulated their mRNA expression (amphiregulin 3.2-fold, $P < 0.001$; HB-EGF 2.3-fold, $P < 0.05$) and triggered their release (amphiregulin EC₅₀ 0.50 μ M, 31.2 ± 2.7 pg/ml with 10 μ M histamine, $P < 0.01$; HB-EGF EC₅₀ 0.54 μ M, 78.5 ± 1.8 pg/ml with 10 μ M histamine, $P < 0.001$) compared to vehicle control (amphiregulin 19.3 ± 0.9 pg/ml; HB-EGF 60.2 ± 1.0 pg/ml), in airway epithelial cells. Histamine increased EGFR phosphorylation (2.1-fold by Western blot analysis) and induced goblet cell differentiation (CLCA1 up-regulation by real-time qPCR) in normal human bronchial epithelial (NHBE) cells. Moreover, amphiregulin and HB-EGF caused proliferation and migration of both NHBE cells and human airway smooth muscle cells. These results suggest that histamine may induce airway remodeling *via* the epithelial-derived EGFR ligands amphiregulin and HB-EGF.—Hirota, N., Risse, P.-A., Novali, M., McGovern, T., Al-Alwan, L., McCuaig, S., Proud, D., Hayden, P., Hamid, Q., Martin, J. G. Histamine may induce airway

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Key Words: amphiregulin • heparin-binding epidermal growth factor-like growth factor • HB-EGF • proliferation • migration • goblet cell differentiation

ASTHMA IS A CHRONIC INFLAMMATORY disease associated with airway remodeling, including airway smooth muscle hypertrophy and hyperplasia, airway epithelial cell hyperplasia, and goblet cell differentiation (1, 2). Current therapies aim to minimize inflammation in responsive individuals, but considerable uncertainty remains as to their capacity to decrease or reverse the ongoing remodeling process. Several types of immune cells, such as eosinophils, activated T cells, and mast cells, are involved in the pathogenesis of asthma. Mast cells are present in the airway tissues and are found among bronchial smooth muscle bundles in patients with asthma (3), where they may release bronchoactive mediators and proinflammatory cytokines and contribute to the pathogenesis of asthma (4). Histamine, which is released from mast cells by degranulation, is known to contribute to allergic reactions through increase of vascular permeability, mucus secretion, and airway smooth muscle contraction. Although the roles of histamine and mast cells in asthma have been studied for many years, their effects on human cellular components pertinent to airway remodeling have not yet been demonstrated (5).

Epidermal growth factor receptor (EGFR) signaling has been reported to be involved in tissue repair and airway remodeling in asthma (6–10). Several known ligands for EGFR include epidermal growth factor

Abbreviations: AB, Alcian blue; ADAM, a disintegrin and metalloproteinase; ALI, air-liquid interface; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; hASM cell, human airway smooth muscle cell; HBE cell, human bronchial epithelial cell; HB-EGF, heparin-binding epidermal growth factor-like growth factor; LTD₄, leukotriene D₄; MMP, matrix metalloproteinase; NHBE cell, normal human bronchial epithelial cell; PAS, periodic acid-Schiff; TGF- α , transforming growth factor- α .

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(EGF), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, and transforming growth factor- α (TGF- α). Recently, the relationship of some of the above-mentioned EGFR ligands to asthma has been studied. Amphiregulin is released at higher levels in the sputum of patients with asthma compared to nonasthmatic individuals, and expression increases after exacerbations (11, 12). HB-EGF is reported to be involved in the development of airway remodeling in an experimental asthma model, and the expression of HB-EGF in airway smooth muscle cells correlates with asthma severity and the extent of airway remodeling (13, 14). However, the airway epithelium is one of the principal sources of EGFR ligands, and an increase in their expression and/or release from human airway epithelial cells in response to rhinovirus infection, insulin, leukotriene D₄ (LTD₄), LPS, cigarette smoke, and mechanical wounding or compression has been documented (9, 15–19). Based on these published data, we focused on the potential role of the little-studied ligand amphiregulin and of HB-EGF in processes relevant to airway remodeling in asthma. In this report, we showed the expression of these EGFR ligands in human asthmatic epithelial cells and investigated the effect of histamine on their release from airway epithelial cells and their effect on primary cultured human airway epithelial and smooth muscle cells.

MATERIALS AND METHODS

Materials

Tyrphostin AG1478 was obtained from Cayman Chemical (Ann Arbor, MI, USA). Human recombinant HB-EGF and human amphiregulin were obtained from R&D Systems (Minneapolis, MN, USA). GM6001 and TAPI-1 were obtained from Calbiochem (La Jolla, CA, USA). S-(+)-chlorpheniramine maleate salt, cimetidine, thioperamide maleate salt, and periodic acid-Schiff (PAS) Kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alcian blue (AB) and Mayer's hematoxylin were purchased from Electron Microscopy Sci-

ences (Hatfield, PA, USA). Phosphorylated EGFR antibody was purchased from Abcam (Cambridge, MA, USA). EGFR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Study subjects

Subjects with asthma (American Thoracic Society criteria; ref. 20) and healthy controls ($n=12$ for subjects with asthma, $n=6$ for control) were recruited as part of a Difficult Asthma Study performed at the Montreal Chest Institute of the McGill University Health Centre, and bronchoscopic biopsies were performed for all the subjects following American Thoracic Society guidelines (20). Subjects had experienced no exacerbations during the 12 wk preceding the study (see **Table 1** for clinical characteristics of asthma and control group subjects). The study was approved by an institutional ethics committee, and written informed consent was obtained.

Immunostaining for amphiregulin

Paraffin-embedded 5- μ m-thick sections of bronchial biopsy specimens were incubated with goat anti-human amphiregulin polyclonal antibody (R&D Systems) at 4°C overnight. The same concentration of isotype IgG (R&D Systems) was used as a negative control. Sections were then incubated with a biotinylated rabbit anti-goat IgG, followed by an avidin/biotin-alkaline phosphatase complex. The signal was developed with Vector Red (Vector Laboratories, Burlingame, CA, USA) and followed by hematoxylin counterstaining. A morphometric analysis of the immunoreactivity of amphiregulin in the epithelium was performed on tissue sections from control ($n=6$) and asthma group subjects ($n=12$). The area of positive staining was normalized to the total area of epithelium. Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) was used to perform the analysis.

Cell culture

The human BEAS-2B bronchial epithelial cell line (derived from adenovirus 12-SV40-transformed normal human bronchial epithelium) was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C under 5% CO₂. Following passage, cells were dissociated using

TABLE 1. Clinical characteristics of control and asthma group subjects

Group	Age (yr)	Sex (M:F)	Atopy (%)	Smoking	Treatment	FEV _{1.0} (L)	FEV _{1.0} (%)	FEV _{1.0} /FVC
Amphiregulin								
Control, $n = 6$	28.8 $\pm$ 4.5	3:3	33.3	—	—	4.43 $\pm$ 0.47	114.8 $\pm$ 4.6	82.9 $\pm$ 1.2
Asthma, $n = 12$	44.7 $\pm$ 3.5	6:6	66.7	— Ex	β A, 12/12; ICS, 12/12; NCS, 3/12; MK, 3/12; OCS, 5/12	2.44 $\pm$ 0.24	81.0 $\pm$ 7.0	72.6 $\pm$ 2.6
HB-EGF								
Control, $n = 6$	26.7 $\pm$ 3.9	2:4	50	—	—	4.13 $\pm$ 0.44	114.7 $\pm$ 4.8	85.7 $\pm$ 2.4
Asthma, $n = 12$	46.6 $\pm$ 2.9	5:7	83.3	— Ex	β A, 12/12; ICS, 12/12; NCS, 6/12; MK, 3/12; OCS, 2/12	2.56 $\pm$ 0.33	71.5 $\pm$ 7.5	65.2 $\pm$ 3.4

Data are expressed as means $\pm$ SE. M, male; F, female; FEV, forced expiratory volume; FVC, forced vital capacity; Ex, ex-smoker; β A, β -agonists; ICS, inhaled corticosteroids; NCS, nasal corticosteroids; MK, montelukast; OCS, oral corticosteroids.

trypsin (Sigma-Aldrich) and cultured in an air-liquid interface (ALI) system. BEAS-2B cells were seeded (25,000/ cm²) onto Transwell clear culture inserts (0.4 µm pore size; Costar, Cambridge, MA, USA). Cells were cultured submerged for the first 2 d, and medium was changed every day. On d 3, an ALI was established by removing the medium from the apical surface, exposing only the basal surface of the cells to medium. Cells were serum-starved 24 h before all experiments, and inserts were transferred to 6-well plates with ultra-low attachment surface (Costar) to measure amphiregulin and HB-EGF release. Medium from the bottom chamber was collected for all experiments.

Normal human bronchial epithelial (NHBE) cells were cultured in 6-well plates in a humidified incubator at 37°C and 5% CO₂ using bronchial epithelial cell growth medium (BEGM, Lonza, Walkersville, MD, USA). Cells were used between passages 1 and 4 for NHBE cells. NHBE cells and asthmatic airway epithelial cells cultured in ALI were generously provided by MatTek (Ashland, MA, USA) and were cultured on 12-well inserts in a humidified incubator at 37°C and 5% CO₂ using commercial primary epithelial cell medium (assay medium; MatTek) that was changed daily. Trans-epithelial electrical resistance of these cells was measured by World Precision Instruments (WPI, Sarasota, FL, USA) Endohm system. NHBE cells used in this work were harvested from lung tissue that was procured from disease-free, non-smoking donors.

Primary human airway smooth muscle (hASM) cells were isolated and cultured as described previously (21). Briefly, tissue digestion was performed by gently rocking the tissues in HBSS containing collagenase type IV (0.4 mg/ml), elastase (0.38 mg/ml), and soybean trypsin inhibitor (1 mg/ml) at 37°C for 90 min. The dissociated cells were then collected. Following centrifugation, the pellet was reconstituted in growth medium consisting of DMEM-Ham's F12 (1:1) medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml), and gentamicin (50 µg/ml). Cells were used between passages 2 and 5. The cells were identified as smooth muscle cells by positive immunohistochemical staining for smooth muscle α-actin and positive identification of smooth-muscle myosin heavy chain and calponin by Western blot analysis.

Reverse transcriptase-PCR and real-time quantitative PCR

Cells were cultured until confluence and starved for 24 h in 0.1% BSA-containing medium before stimulation. In the case of NHBE cells and asthmatic epithelial cells cultured in ALI, stimulation was performed in assay medium. RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA).

Reverse transcription was performed using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). Quantitative PCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen; see Table 2 for primer sequences). The cDNA was amplified in the StepOne-Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method. Individual data were normalized against the housekeeping gene (ribosomal S9). Results were expressed as a fold change in expression over the control.

ELISA for amphiregulin and HB-EGF

Protein levels for amphiregulin and HB-EGF in culture medium of BEAS-2B cells, NHBE cells, and asthmatic epithelial cells cultured in ALI were measured by ELISA. Cells were starved in 0.1% BSA-containing DMEM for 24 h prior to stimulation. At 4 h following histamine stimulation with and without inhibitors, culture medium was collected. In some experiments, AG1478 (1.0 µM), GM6001 (25 µM), TAPI-1 (10 µM), S-(+)-chlorpheniramine maleate salt (1 µM), cimetidine (10 µM), and thioperamide maleate salt (10 µM) were added to the cell cultures 30 min before the addition of histamine. The concentrations of amphiregulin and HB-EGF in the cell culture medium were determined by Human Amphiregulin DuoSet and Human HB-EGF DuoSet (R&D Systems) following the manufacturer's protocols.

Assessment of the EGFR activation by Western blotting

The expression of phosphorylated EGFR and EGFR was measured by Western blot analysis. NHBE cells were incubated with and without histamine for 24 h, then washed with ice-cold PBS and lysed in ice-cold extraction buffer containing 62.5 mM Tris-HCl, (pH 6.8), 2% SDS, 10% glycerol, and 50 mM DTT. SDS-PAGE was carried out using 8% polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 2% BSA in Tris-HCl buffer containing Tween-20 (TBST). Membranes were then incubated with primary antibody diluted in TBST containing 2% BSA (phosphorylated-EGFR antibody, 1:40,000; EGFR antibody, 1:1000) overnight at 4°C. Fluorescent secondary antibody (goat anti-rabbit IgG dylight-800, 1:15,000, Thermo-Fisher Pierce, Rockford, IL, USA) was diluted in TBST and then incubated with the membranes for 1 h at room temperature. Western blots were quantified with the Odyssey imaging system (Li-Cor, Lincoln, NE, USA).

TABLE 2. Primers for real-time quantitative PCR (for human)

Primer	Forward	Reverse
S9	CTGCTGACGCTTGATGAGAA	CAGCTTCATCTTGCCCTCA
Amphiregulin	CTGCCCTTATGTCTGCTGTG	TTTCGTTCTCAGCTTCTCC
HB-EGF	CAAGGGGCTAGGAAGAAGA	CGATGATGACCAGCAGACAGACA
EGF	AAGAATGGGGGTCAACCACT	TGAAGTTGGTTGCATTGACC
TGF-α	ACTGCCCAGATTCCCACACTC	ACGATGGAGACCACCACCAAG
Epiregulin	TTTCCATCTTCTACAGGCAGTC	GTTATTGACACTTGAGCCACAC
Betacellulin	GCTACCACCACACAATCAAAGC	TCAACTCTCTCACACCTTGCTC
CLCA1	TTCATCAGGAAATGGAGCTGT	CTGGCTGTTCTGGAGGGTTA
EGFR (ErbB1)	GGGTGACTGTTTGGGAGTTG	GGCGACTATCTGCGTCTATC
ErbB2	GGGAAACCTGGAACCTACCT	CCCTGCACCTCTGGATA
ErbB3	CACAATGCCGACCTCTCC	CACGAGGACATAGCCTGTCA
ErbB4	TTCCACTTTACCACAACATGCTA	CAGAATGAAGAGCCACCA

Staining of NHBE cells in ALI

NHBE cells grown on culture inserts were fixed with 10% formalin for 10 min. The membranes on which the cells were grown were removed from the inserts and embedded in paraffin. Sections (5 μ m) of the membrane were cut and stained with AB/PAS to identify mucous glycoconjugates. Hematoxylin was used for counterstaining. Sections were also stained with hematoxylin and eosin for the assessment of cellular morphology.

Cell proliferation assay

hASM cells and NHBE cells were grown until 70% confluency in 6-well plates with growth medium. The proliferative response was determined by flow cytometric quantification of Ki67 (FITC mouse anti-human Ki67 set; BD Pharmingen, San Diego, CA, USA) as recommended by the manufacturer. Cells were rendered quiescent by culturing for 24 h in 0.1% BSA containing DMEM-Ham's F12 (1:1) medium (for hASM cells) or 0.1% BSA containing bronchial epithelial cell basal medium (BEBM; Lonza; for NHBE cells) and incubated for 24 h. The samples were analyzed by flow cytometry (FACS Calibur; BD, San Jose CA, USA) and commercial software (Cellquest; BD).

Migration assays

hASM cell migration assays were performed as described previously with a 48-well microchemotaxis chamber (Neuro Probe, Inc., Cabin John, MD, USA; ref. 22). Cells that had been growth arrested in medium supplemented with 0.5% FBS for 24 h were harvested with 0.05% trypsin-EDTA solution (Sigma-Aldrich) and were resuspended (1.0×10^6 cells/ml) in medium containing 0.5% FBS. An 8-mm-pore polycarbonate membrane (Neuro Probe) was treated with 0.01% collagen type I (rat tail; Nalgene Culture 3D Matrix; Nalgene, Rochester, NY, USA) solution in 0.01 N HCl and placed between 2 chambers. hASM cells were added to the upper chamber, and different concentrations (0.1, 1, 10, and 100 ng/ml) of amphiregulin or HB-EGF, or medium alone, were added to the lower chamber. After 4 h of incubation at 37°C, the membrane was removed, and its upper face was scraped clear of cells. Cells that migrated to the lower side of the membrane were fixed and stained with the Hema 3 kit (Fisher, Kalamazoo, MI, USA), according to the manufacturer's instructions. The number of migrated cells was counted in 5 random fields at $\times 3200$.

Statistical analysis

Data were analyzed for statistical significance using Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). All data are expressed as means $\pm$ SE, with ≥ 3 independent observations/experiment. Statistical differences were determined by an unpaired Student's *t* test in experiments that contained only 2 groups and by ANOVA and Tukey posttest for comparisons among several means. Values of $P < 0.05$ were considered to be significant.

RESULTS

Amphiregulin and HB-EGF are expressed in asthmatic epithelium

We performed immunohistochemical staining to assess protein expression of amphiregulin and HB-EGF in

airway tissues (Fig. 1A, B). Biopsies from 6 healthy control subjects and 12 subjects with asthma were available for the study (Table 1). Amphiregulin was expressed mainly in airway epithelium and in isolated subepithelial cells but not in airway smooth muscle (Fig. 1A). HB-EGF immunoreactivity was detected in subepithelial mucous glandular cells, inflammatory cells, epithelial cells, and smooth muscle bundles (Fig. 1B). We performed a morphometric analysis of amphiregulin immunoreactivity in control and asthma group samples (Supplemental Fig. S1). A strong trend showed increase in amphiregulin expression in the subjects with asthma ($P=0.09$). However, 7 asthma group subjects were above the upper 95% confidence interval for amphiregulin expression in the airway epithelium. Previously, HB-EGF has been shown to be overexpressed in subjects with severe asthma from the same cohort (14), so we did not repeat a morphometric analysis of this ligand. Since samples were collected from the patients when they had had no recent exacerbations, these results indicate that amphiregulin and HB-EGF are expressed chronically in asthmatic airway epithelial cells and are up-regulated in certain subjects.

Histamine induces mRNA production and release of amphiregulin and HB-EGF from airway epithelial cells

To investigate the effect of histamine on amphiregulin and HB-EGF release from airway epithelial cells, we stimulated BEAS-2B cells with histamine (0.1, 1.0, 10 μ M) for 4 h and measured the concentrations of amphiregulin and HB-EGF in the culture medium by ELISA (Fig. 2A). Levels of both amphiregulin and HB-EGF in the culture medium increased with histamine stimulation in a concentration-dependent manner (amphiregulin EC_{50} 0.50 μ M, 31.2 ± 2.7 pg/ml with 10 μ M histamine, $P < 0.01$; HB-EGF EC_{50} 0.54 μ M, 78.5 ± 1.8 pg/ml with 10 μ M histamine, $P < 0.001$) compared to vehicle control (amphiregulin 19.3 ± 0.9 pg/ml; HB-EGF 60.2 ± 1.0 pg/ml). The mRNA levels of amphiregulin and HB-EGF were measured by real-time quantitative PCR. The expression of both amphiregulin and HB-EGF was up-regulated after 4 h stimulation with histamine (Fig. 2B, amphiregulin 3.2-fold, $P < 0.001$; HB-EGF 2.3-fold, $P < 0.05$). These results demonstrate that histamine induces both release and mRNA production of amphiregulin and HB-EGF in airway epithelial cells.

Histamine induces amphiregulin and HB-EGF release from airway epithelial cells through histamine receptor H1 and metalloproteinase activation

To determine the pathway of amphiregulin and HB-EGF release induced by histamine we assessed protein levels by ELISA in the presence of histamine receptor antagonists and metalloproteinase inhibitors. When BEAS-2B cells were incubated with histamine and S-(+)-chlorpheniramine maleate salt (1.0 μ M), a histamine receptor H1 antagonist, release of amphiregulin and HB-EGF decreased. By contrast, no significant

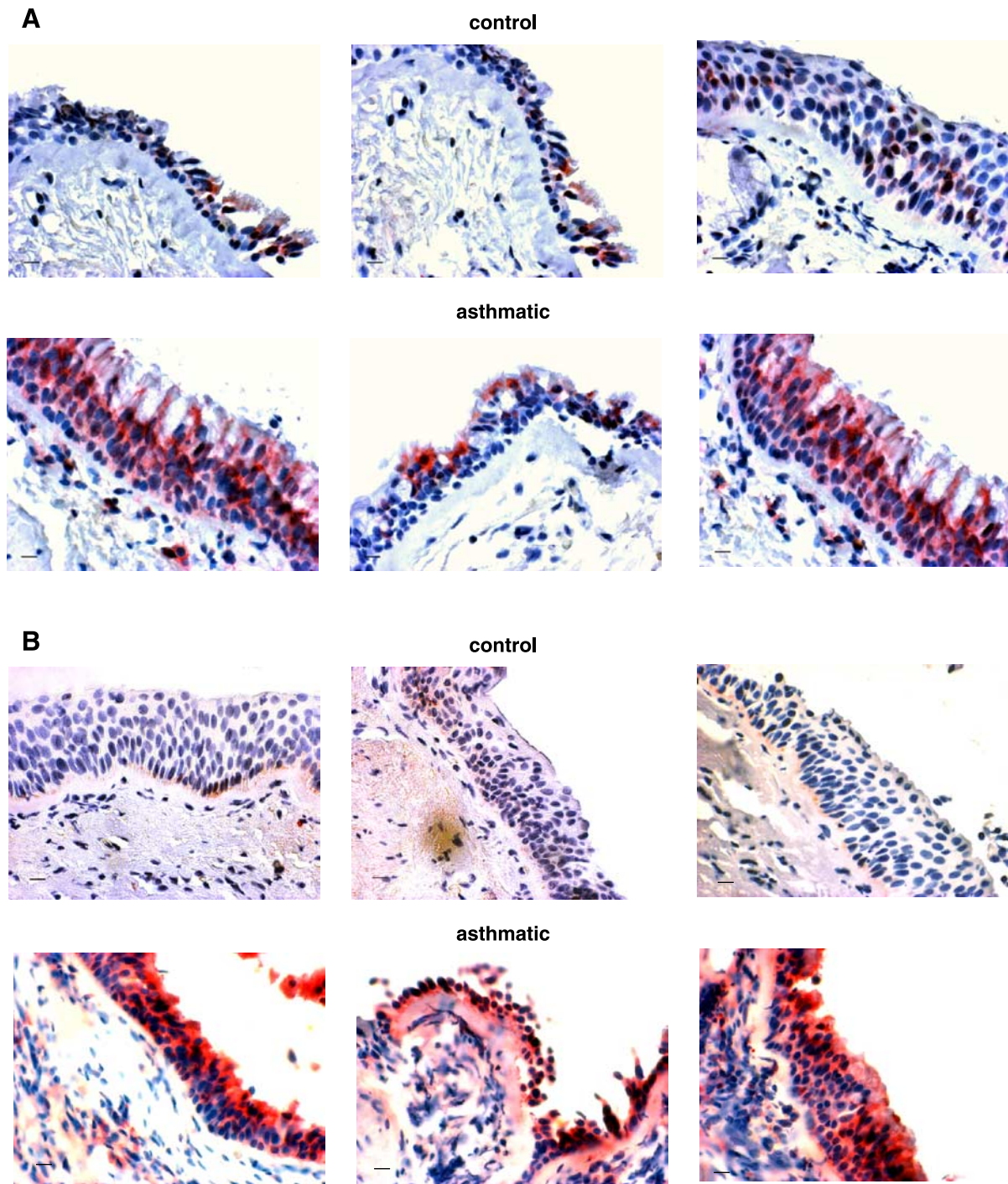


Figure 1. Amphiregulin and HB-EGF are expressed chronically in asthmatic epithelium. The expression of amphiregulin (A) and HB-EGF (B) was detected by immunohistochemical staining (red staining) in human airway biopsy samples. Amphiregulin and HB-EGF express more in some asthmatic samples (bottom panels) than control samples (top panels). Photomicrographs are 3 representatives from each group ($n=6$ for control group, $n=12$ for asthma group). Scale bar = 10 μm .

changes were observed after incubation with cimetidine (10 μM , histamine receptor H₂ antagonist) and thioperamide maleate salt (10 μM , histamine receptor H_{3/4} inhibitor), suggesting that histamine exerted its effects by stimulation of histamine H₁ receptor in airway epithelial cells (Fig. 3A). The BEAS-2B cells were then incubated with TAPI-1 (10 μM), an inhibitor of a disintegrin and metalloproteinase (ADAM) 17 and

matrix metalloproteinases (MMPs), and GM6001 (25 μM), a broad MMP and ADAM inhibitor. The release of amphiregulin and HB-EGF with histamine stimulation as well as their baseline release was blocked by TAPI-1 and GM6001 (Fig. 3B). These results indicate the involvement of metalloproteinases for both histamine-induced and constitutive amphiregulin and HB-EGF release from airway epithelial cells.

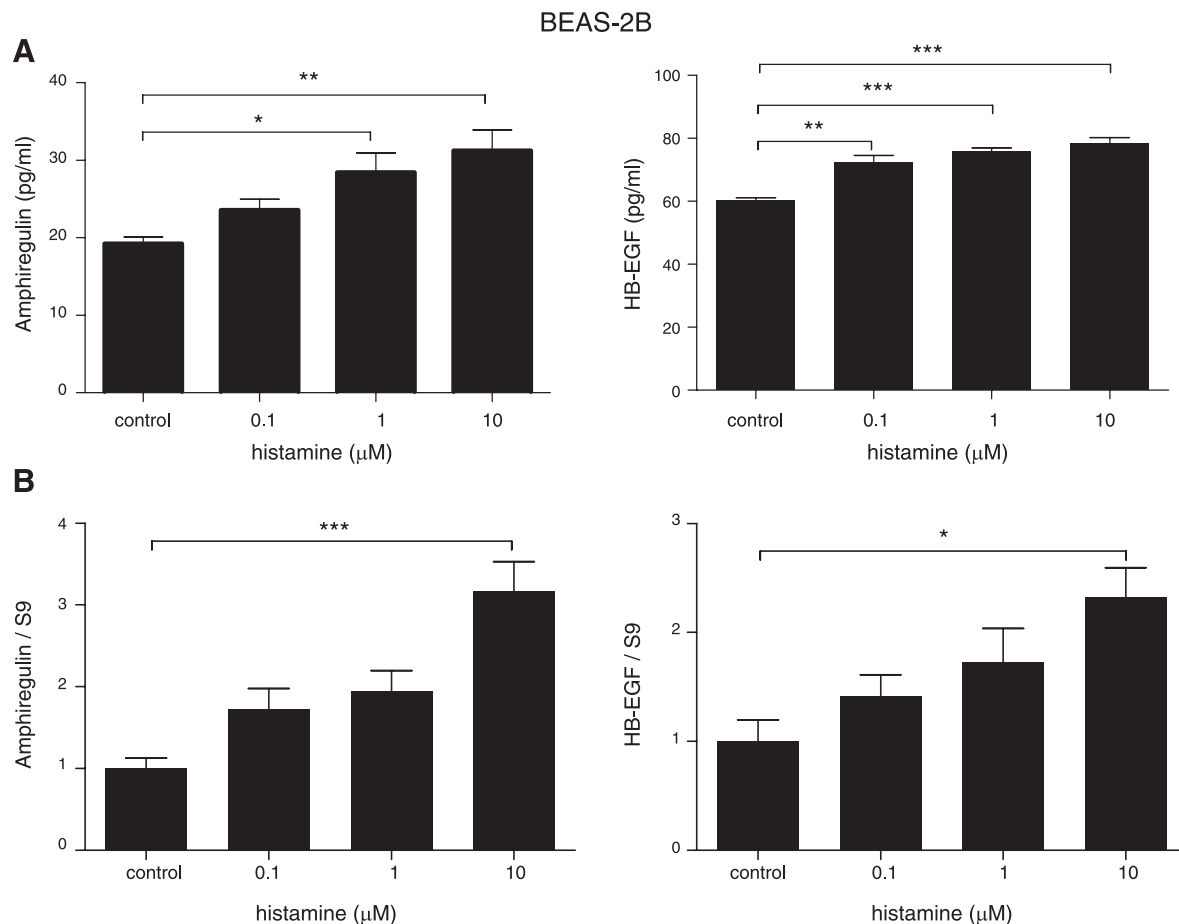


Figure 2. Histamine induces the release and mRNA expression of amphiregulin and HB-EGF in human airway epithelial cells. BEAS-2B cells were stimulated with histamine (0.1, 1.0, 10 μ M) for 4 h. **A**) Concentrations of amphiregulin (left panel) and HB-EGF (right panel) in the culture medium were measured by ELISA. **B**) mRNA of amphiregulin (left panel) and HB-EGF (right panel) were measured by quantitative PCR. Data are represented as means $\pm$ SE ($n=5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA with Tukey *post hoc* pairwise comparisons.

Histamine induces amphiregulin and HB-EGF expression in NHBE cells

To confirm the effects of histamine in primary cultured epithelial cells, we stimulated NHBE cells and hASM cells with and without histamine for 4 h. By quantitative real-time PCR analysis, mRNA of amphiregulin and HB-EGF increased with histamine in NHBE cells (Fig. 4A), while neither increased in hASM cells (Fig. 4B). We also measured mRNA of other EGFR ligands (EGF, TGF- α , epiregulin, and β -cellulin), but histamine did not induce their mRNA expression (Supplemental Fig. S2).

Histamine induces EGFR activation in NHBE cells

To detect the activation of EGFR by histamine stimulation, we performed Western blots using a specific antibody against phosphorylated EGFR in NHBE cells. NHBE cells stimulated with histamine (10 μ M) exhibited an increase of phosphorylated EGFR (Fig. 5A). The peak time point for phosphorylation was 2 h. The NHBE cells were then stimulated with histamine (10

μ M) for 2 h with and without AG1478 (1.0 μ M), a EGFR inhibitor. Phosphorylation of EGFR was up-regulated by histamine (2.1-fold) and decreased to the baseline level by AG1478 (Fig. 5B).

Histamine induces goblet cell differentiation through EGFR activation in NHBE cells

To assess the effects of histamine on epithelial remodeling, we stimulated NHBE cells cultured in ALI with and without histamine and AG1478 (1.0 μ M) for 5 d and performed quantitative analysis of mucus gene expression (CLCA1). To assess the formation of tight junctions in these cells, transepithelial electrical resistance was measured ($440 \pm 17.9 \Omega/\text{cm}^2$, $n=18$). These values were high enough to indicate the formation of tight junctions (23). mRNA of CLCA1, a marker of goblet cell differentiation, increased after 5 d of histamine stimulation, and its effect was inhibited by AG1478 (Fig. 6A). Cells were stained with AB/PAS to identify mucous glycoconjugates (Fig. 6B). More positive staining was found in NHBE cells incubated with histamine than control, and this

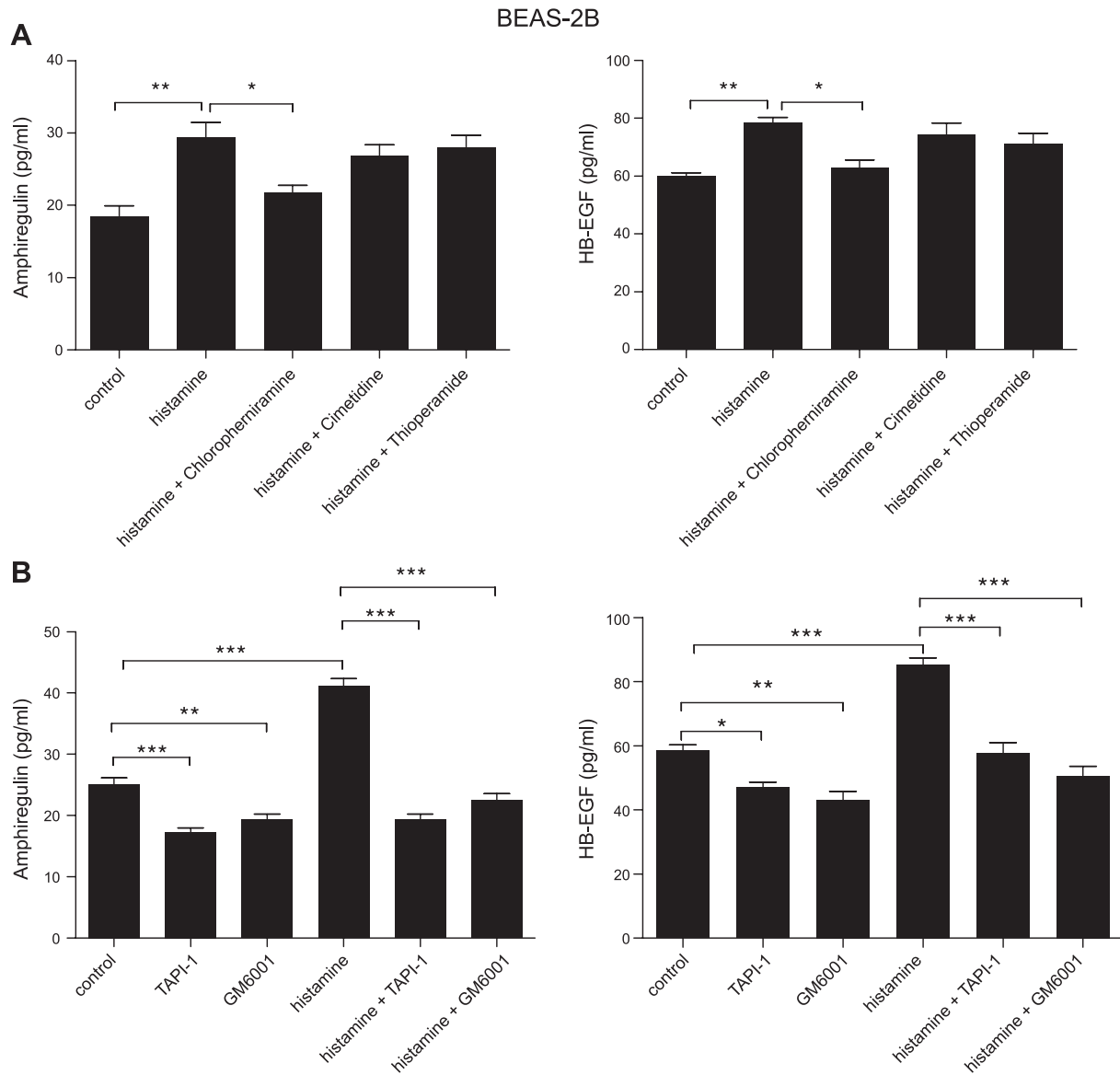


Figure 3. Release of amphiregulin and HB-EGF from airway epithelial cells by histamine was induced through the histamine receptor H1 and metalloproteinase activation. **A)** BEAS-2B cells were stimulated with and without histamine (10 μ M) and histamine receptor inhibitors for 4 h [chlorpheniramine (1.0 μ M); histamine receptor H1 inhibitor, cimetidine (10 μ M); histamine receptor H2 inhibitor, thioperamide (10 μ M); histamine receptor H3/4 inhibitor]. Concentrations of amphiregulin (left panel) and HB-EGF (right panel) were measured by ELISA ($n=5$ for amphiregulin, $n=4$ for HB-EGF). **B)** BEAS-2B cells were stimulated with and without histamine (10 μ M) and metalloproteinase inhibitors for 4 h [TAPI-1 (1.0 μ M) and GM6001 (25 μ M)]. Concentrations of amphiregulin (left panel) and HB-EGF (right panel) were measured by ELISA ($n=4$). Data are represented as means + SE. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA with Tukey *post hoc* pairwise comparisons.

effect was blocked by AG1478. These findings suggest that histamine induces goblet cell differentiation through EGFR activation.

Amphiregulin and HB-EGF induce remodeling in NHBE and hASM cells

To assess the effects of amphiregulin and HB-EGF on two of the principal tissues remodeled in asthma, we performed a cell proliferation assay using flow cytometric quantification of Ki67. When NHBE and hASM cells were stimulated with amphiregulin and HB-EGF (1, 10, 100 ng/ml), both amphiregulin and HB-EGF induced

cell proliferation (**Fig. 7A, B**). Moreover, HB-EGF and amphiregulin induced migration of hASM cells (**Fig. 7C**). This finding suggests that amphiregulin and HB-EGF, which are released from airway epithelial cells, may cause airway remodeling. In **Fig. 7A–C**, HB-EGF was more potent than amphiregulin in the induction of both proliferation and migration of hASM cells. To explore this difference, we performed real-time quantitative PCR analysis for ErbB family members, namely EGFR (ErbB1) and ErbB2–4). NHBE cells expressed EGFR (ErbB1), ErbB2, and ErbB3 (Supplemental Fig S2A), while hASM cells expressed EGFR and ErbB2 (Supplemental Fig. S3B).

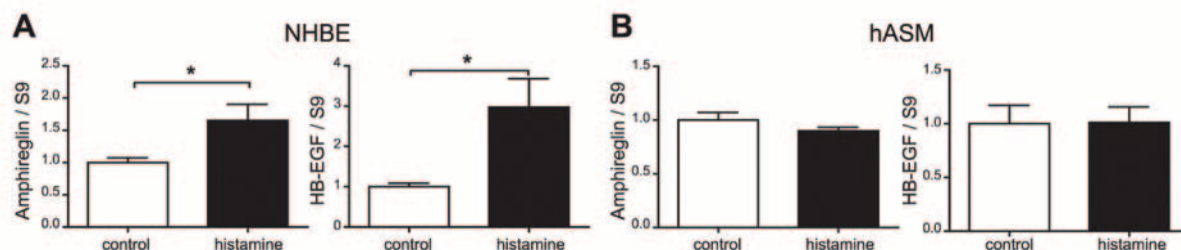


Figure 4. Histamine induces amphiregulin and HB-EGF expression in NHBE cells. NHBE cells (A) and hASM cells (B) were incubated with histamine (10 μ M) for 4 h. mRNA of amphiregulin (left panel) and HB-EGF (right panel) was measured by quantitative PCR in NHBE cells (A) and hASM cells (B). Data are represented as means + SE ($n=3$ for NHBE cells, $n=4$ for hASM cells). * $P < 0.05$; unpaired t test.

Asthmatic bronchial epithelial cells express more EGFR, amphiregulin and HB-EGF than NHBE cells

We assessed the expression of EGFR, amphiregulin, HB-EGF and the effect of histamine in asthmatic human bronchial epithelial (HBE) cells. Both NHBE cells and asthmatic HBE cells were cultured in ALI. Their

transepithelial electrical resistance was measured (NHBE cells, $440 \pm 17.9 \Omega/\text{cm}^2$ $n=18$; asthmatic HBE cells, $241 \pm 64.2 \Omega/\text{cm}^2$ $n=4$). mRNA levels of EGFR, amphiregulin and HB-EGF were measured by real time quantitative PCR. Asthmatic HBE cells exhibited more mRNA expression of EGFR, amphiregulin, and HB-EGF than NHBE cells (**Fig. 8A**, EGFR 1.7-fold; $P < 0.05$;

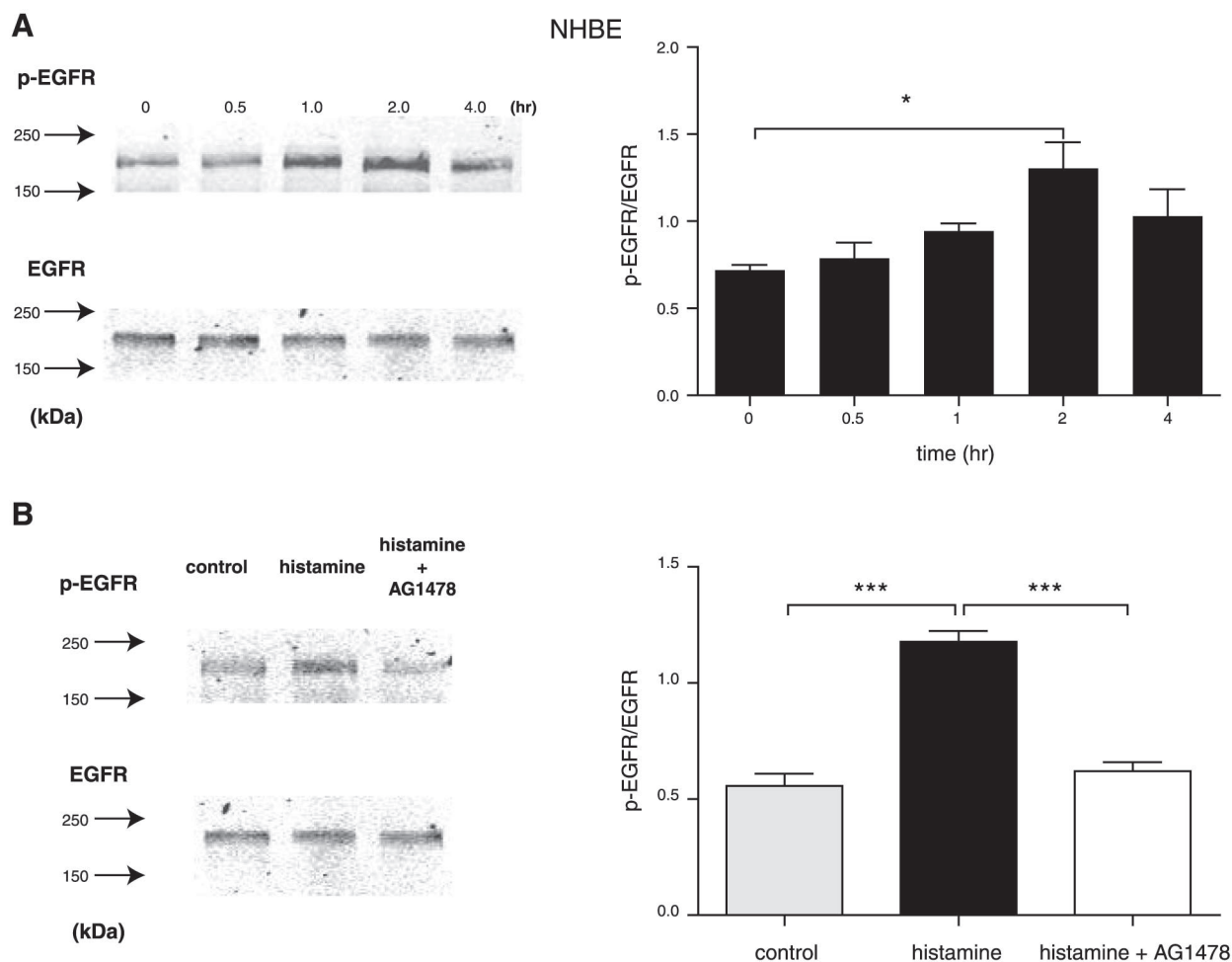


Figure 5. Histamine induces EGFR activation in NHBE cells. NHBE cells were stimulated with histamine (10 μ M), and a Western blot was performed. Data are representative of 3 independent experiments with identical results (left panels); densitometry was performed for these 3 experiments (right panels). A) NHBE cells were incubated with histamine for several different time periods (0 to 4 h). B) NHBE cells were stimulated by histamine with and without AG1478 (1.0 μ M) for 2 h. Data are represented as means + SE ($n=3$). * $P < 0.05$, *** $P < 0.001$; ANOVA with Tukey *post hoc* pairwise comparisons.

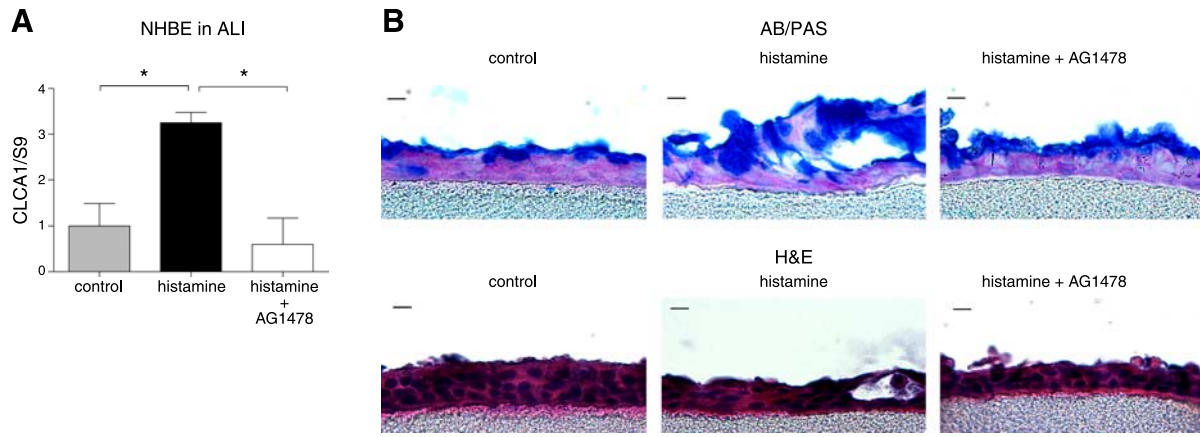


Figure 6. Histamine induces goblet cell differentiation through EGFR activation in NHBE cells. *A*) mRNA of CLCA1 was measured by quantitative PCR. NHBE cells in ALI were incubated with and without histamine (10 μ M) and AG1478 (1.0 μ M) for 5 d. Data are represented as means $\pm$ SE ($n=3$). $*P < 0.05$; ANOVA with Tukey *post hoc* pairwise comparisons. *B*) NHBE cells cultured in ALI were stained with AB/PAS to identify mucous glycoconjugates (top panels). Hematoxylin was used for counterstaining. More positive staining (blue) was found in NHBE cells incubated with histamine (middle panels) than control (left panels), and this effect was blocked by AG1478 (right panels). Cells were also stained with hematoxylin and eosin (H&E, lower bottom panels). Scale bars = 10 μ m.

amphiregulin 2.0-fold; HB-EGF 2.8-fold). Baseline concentrations of amphiregulin and HB-EGF were higher in supernatant from asthmatic HBE cells than NHBE

cells. Following 24 h of stimulation with histamine, NHBE cells and asthmatic HBE cells amphiregulin and HB-EGF were elevated in culture medium (Fig. 8B).

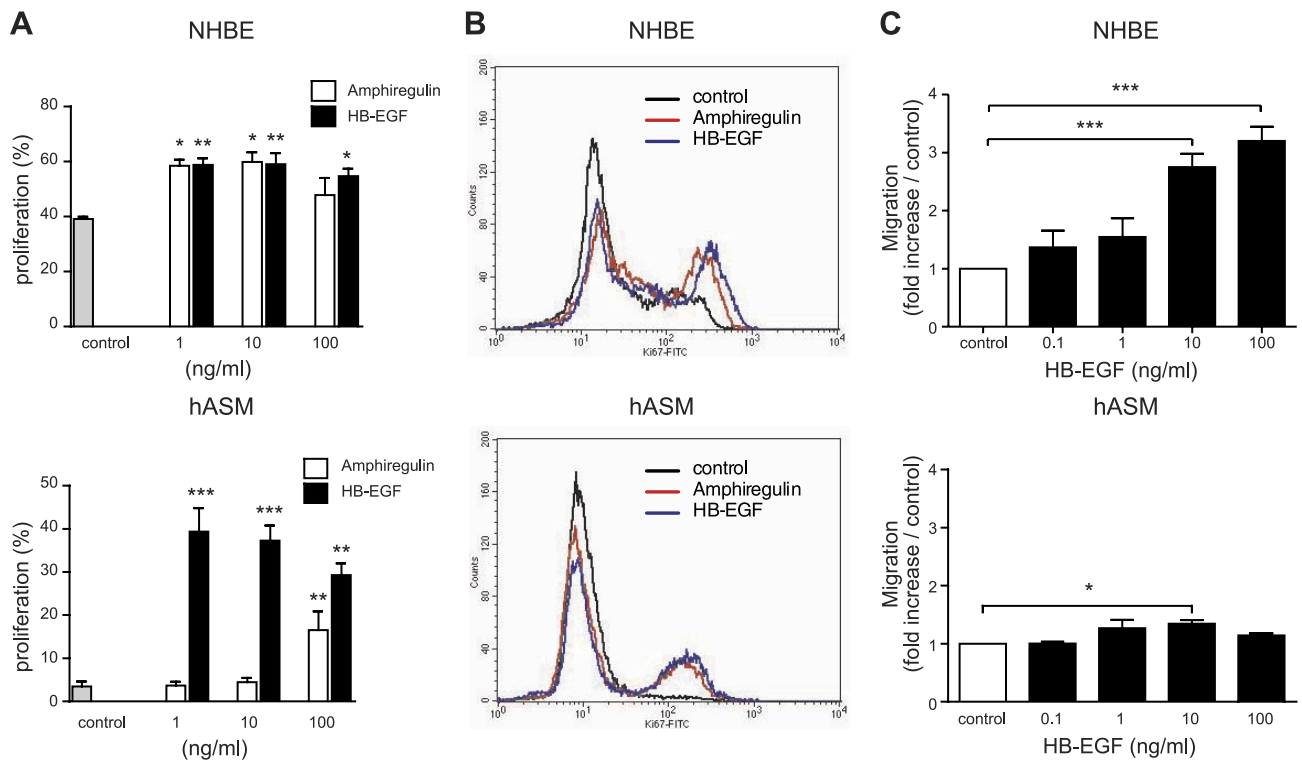


Figure 7. Amphiregulin and HB-EGF induce remodeling in NHBE and hASM cells. *A*) Cell proliferation of NHBE cells (top panel) and hASM cells (bottom panel) with and without amphiregulin and HB-EGF (1, 10, 100 ng/ml) was measured by flow cytometric quantification of Ki67. Cells were incubated with and without amphiregulin or HB-EGF for 24 h. Data are represented as means $\pm$ SE ($n=4$). *B*) Representative results from the flow cytometric analysis of Ki-67 (top panel, NHBE cells, 10 ng/ml of amphiregulin and HB-EGF; bottom panel, hASM cells, 100 ng/ml of amphiregulin and HB-EGF). Black, control; red, amphiregulin; blue, HB-EGF. *C*) Numbers of migrated hASM cells were counted after 4 h incubation with amphiregulin or HB-EGF. Fold increases of migrated cells compared to control are shown for amphiregulin (top panel) and HB-EGF (bottom panel). Data are represented as means $\pm$ SE ($n=3$ for amphiregulin, $n=4$ for HB-EGF). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs.* control; ANOVA with Tukey *post hoc* pairwise comparisons.

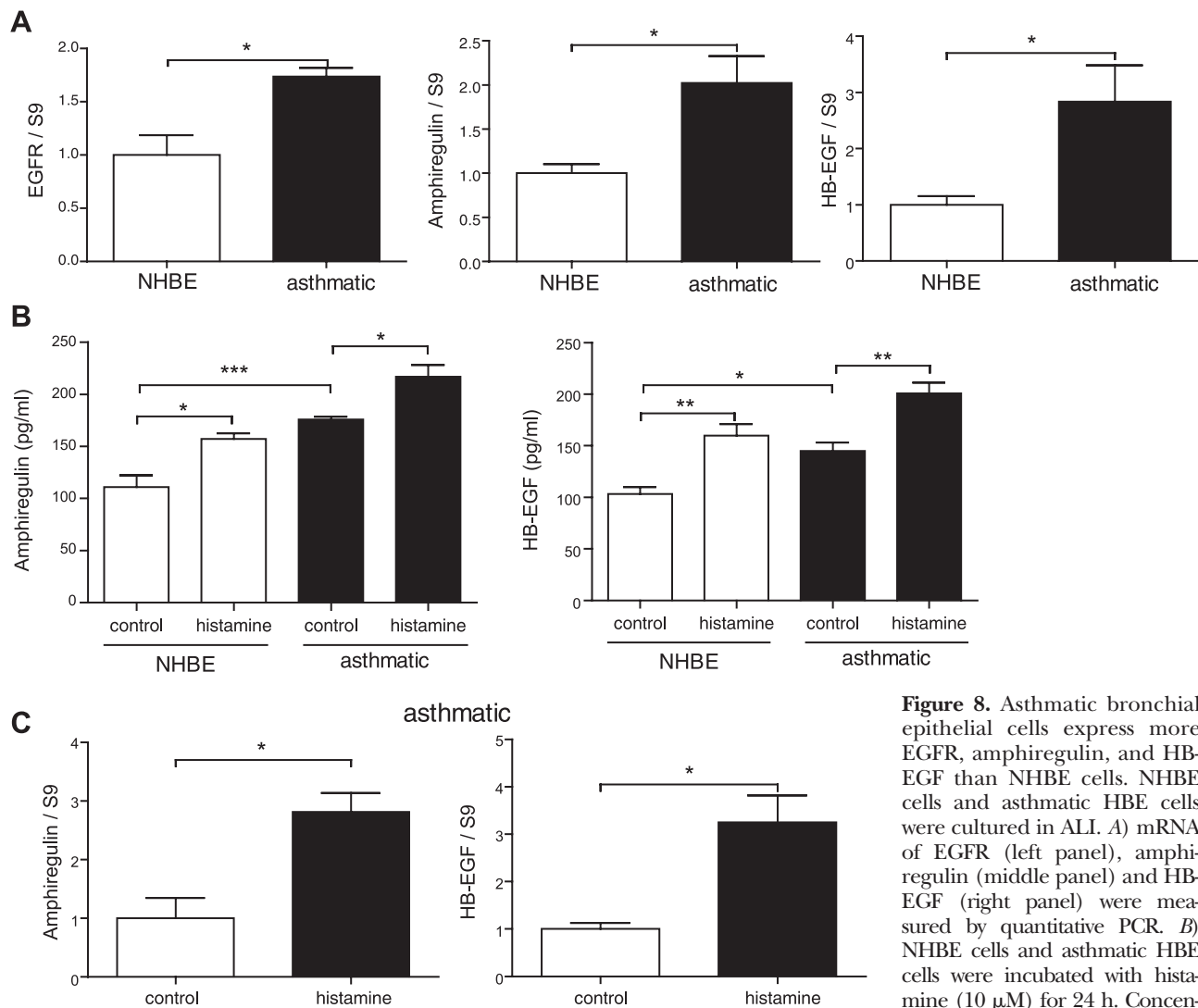


Figure 8. Asthmatic bronchial epithelial cells express more EGFR, amphiregulin, and HB-EGF than NHBE cells. NHBE cells and asthmatic HBE cells were cultured in ALI. A) mRNA of EGFR (left panel), amphiregulin (middle panel) and HB-EGF (right panel) were measured by quantitative PCR. B) NHBE cells and asthmatic HBE cells were incubated with histamine (10 μ M) for 24 h. Concentrations of amphiregulin (left

panel) and HB-EGF (right panel) in the culture medium of NHBE cells and asthmatic HBE cells were measured by ELISA. C) Asthmatic HBE cells were stimulated with histamine (10 μ M) for 4 h and mRNA of amphiregulin (left panel), and HB-EGF (right panel) were measured by quantitative PCR. Data are represented as means + SE ($n=4$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ANOVA with Tukey *post hoc* pairwise comparisons.

Four hours of stimulation of asthmatic HBE cells with histamine increased the mRNA of amphiregulin and HB-EGF (Fig. 8C). These results suggest enhancement of the EGFR axis in asthmatic epithelial cells.

DISCUSSION

It is well established that EGFR ligand precursors are present on the cell surface and they are cleaved and released by metalloproteinases, such as MMPs and ADAMs (8, 24–27). In the current study, we have shown that histamine stimulation increased mRNA levels of amphiregulin and HB-EGF in airway epithelial cells as well as their release. Histamine induces the synthesis of amphiregulin and HB-EGF precursors while it activates metalloproteinases that cause their release from airway epithelial cells. These observations extend the range of biological effects of histamine usually associated with

allergic airway reactions to promoting a chronically elevated expression of epithelial EGFR ligands, with probable downstream consequences for airway remodeling.

It has been reported that ADAM17 is involved in the release mechanism of EGFR ligands, such as HB-EGF, amphiregulin, and TGF- α (28–31). ADAM9, 10, 12, 15 (32–36) and MMP2, 3, 7, 9 (25, 37–40) were also reported to play roles in EGFR ligand shedding. However, the extent of the differences of shedding mechanisms among EGFR ligands has not been determined. For example, ADAM12 is reported to be involved in shedding of HB-EGF but not amphiregulin, EGF, and TGF- α (41). In this study, both TAPI-1 and GM6001 inhibited the HB-EGF and amphiregulin release, but these data do not shed light on the involvement of any specific metalloproteinase. Further study will be necessary to characterize the difference of release mechanisms among the EGFR ligands.

Although a number of different forms of stimulation such as virus infection, cigarette smoke, and insulin have been reported to induce EGFR ligand release from airway epithelial cells, little is known about their release and the significance for asthma (8, 16, 18). As candidates of chronic epithelial stimulation that are reported to be involved in the pathogenesis of asthma, we stimulated BEAS-2B cells with Th2 and Th17 cytokines, IL-13 (42–44), IL-17A (13, 45, 46), and IL-22 (47, 48). However, none of them induced amphiregulin release (data not shown). Interestingly, another mast cell-derived mediator, LTD₄, has also been reported to induce HB-EGF release from airway epithelial cells (17), supporting the concept of activated mast cell involvement in the process of airway remodeling in asthma.

In this study, amphiregulin induced proliferation as much as HB-EGF in NHBE cells. In contrast, amphiregulin was less potent in inducing proliferation and migration in hASM cells. We reasoned that differences in the effects of the two ligands might relate to the pattern of receptor expression on the two cell types. Four receptor family members are known for EGFR (ErbB family: EGFR, ErbB2, ErbB3, ErbB4). They dimerize after ligand binding and phosphorylate each other (49). At the level of mRNA, NHBE cells have ErbB1 (EGFR), ErbB2, and ErbB3, while hASM cells have ErbB1 and ErbB2. Previously, several studies have shown that EGFR signaling may be triggered through ErbB3, which dimerized with EGFR (50–53), and cells expressing high levels of amphiregulin had more phosphorylation of ErbB3 (54). Others have shown that amphiregulin-bound EGFR is less degraded in lysosomes after internalization than other EGFR ligands, such as EGF and HB-EGF (55, 56), and the C-terminal domain of ErbB3 recruited more receptor recycling to the cell membrane than homodimers of EGFR (57). It is thus possible that a part of the EGFR signaling by amphiregulin in NHBE cells is caused by heterodimers of EGFR and ErbB3 prolonging and enhancing the signaling.

Previous studies have suggested a possible relationship between histamine and airway remodeling in asthma. Pretreatment with histamine increased IL-1 β -induced GM-CSM release and reduced TNF- α -induced RANTES release from airway smooth muscle cells (58). Histamine enhanced proliferation and induced mRNA and protein expression of connective tissue growth factor in lung fibroblasts (59). In our study, histamine provoked EGFR activation and promoted goblet cell differentiation of airway epithelial cells. It is well known that EGFR signaling is followed by the activation of the mitogen-activated protein kinase (MAPK) pathway and the Akt pathway (8). The MAPK pathway regulates cell proliferation and survival, and the Akt pathway plays a role in cell proliferation, apoptosis, and cell migration (60, 61), so that histamine may be involved in a range of processes that promote airway remodeling *via* activation of pathways downstream of EGFR. Previously, Panettieri *et al.* (5) showed that histamine triggered

proliferation of airway smooth muscle cells using canine trachea-derived smooth muscle cells. However, we did not observe a significant proliferative effect of histamine on hASM cells (Supplemental Fig. S4), likely a reflection of species differences, although, in addition, we employed 1/10 the concentration used by Panettieri and colleagues (100 μ M histamine). However, 100 μ M of histamine did not evoke any additional effect on the release of amphiregulin from airway bronchial epithelial cells (data not shown).

Immunohistochemistry of tissue sections from human subjects procured by bronchoscopic biopsy of proximal airways demonstrated that amphiregulin and HB-EGF were expressed in tissue sections from human healthy controls and subjects with asthma. We focused on these two ligands because they were demonstrated to be up-regulated at the mRNA level by histamine. Some but not all subjects with asthma had elevated expression of amphiregulin compared to controls, consistent with the current view of asthma as a syndrome comprising multiple phenotypes. HB-EGF has been previously reported by us to be up-regulated in patients with severe asthma (14). A limitation of our study is that we studied patients in a stable state, since bronchoscopic biopsies could not be readily obtained during exacerbations. However, it is also possible that patients in exacerbation may have even more pronounced changes in the expression of these ligands.

Clinically, the anti-IgE antibody omalizumab has been used for asthma therapy, reducing airway inflammation and the frequency of exacerbations (62, 63). Although it is not yet demonstrated that it can decrease or reverse the airway remodeling associated with asthma, some studies report that clinical improvements persist after treatment withdrawal (64). This finding suggests the possibility that mast cell degranulation and histamine release may contribute to the chronicity of disease through airway remodeling in patients with asthma.

In summary, our data demonstrate that histamine induces the proliferation and migration of airway smooth muscle cells and epithelial cells, which are key characteristics of airway remodeling of asthma, through EGFR ligand (amphiregulin and HB-EGF) release. Current therapeutics are focused on the attenuation of inflammation, but have uncertain capacity to decrease or reverse the ongoing remodeling process. Given the importance of airway remodeling for the symptoms and functional abnormalities associated with chronic asthma, histamine and mast cells may merit greater consideration as therapeutic targets to prevent airway remodeling. **FJ**

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Figure S1

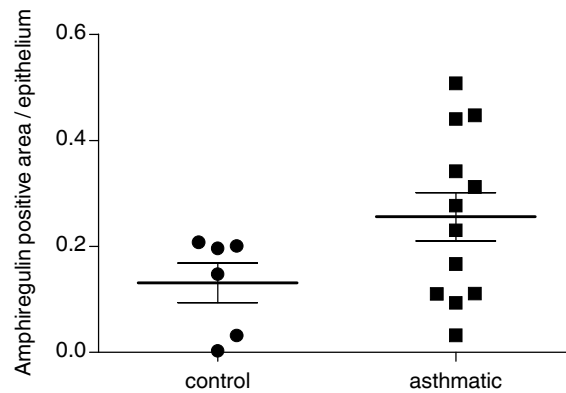


Figure S1. Morphometric analysis of the immunoreactivity of amphiregulin in the epithelium. A morphometric analysis of the immunoreactivity of amphiregulin in the epithelium was performed on tissue sections from control (n=6) and asthmatic subjects (n=12). The area of positive staining was normalized to the total area of epithelium.

Figure S2

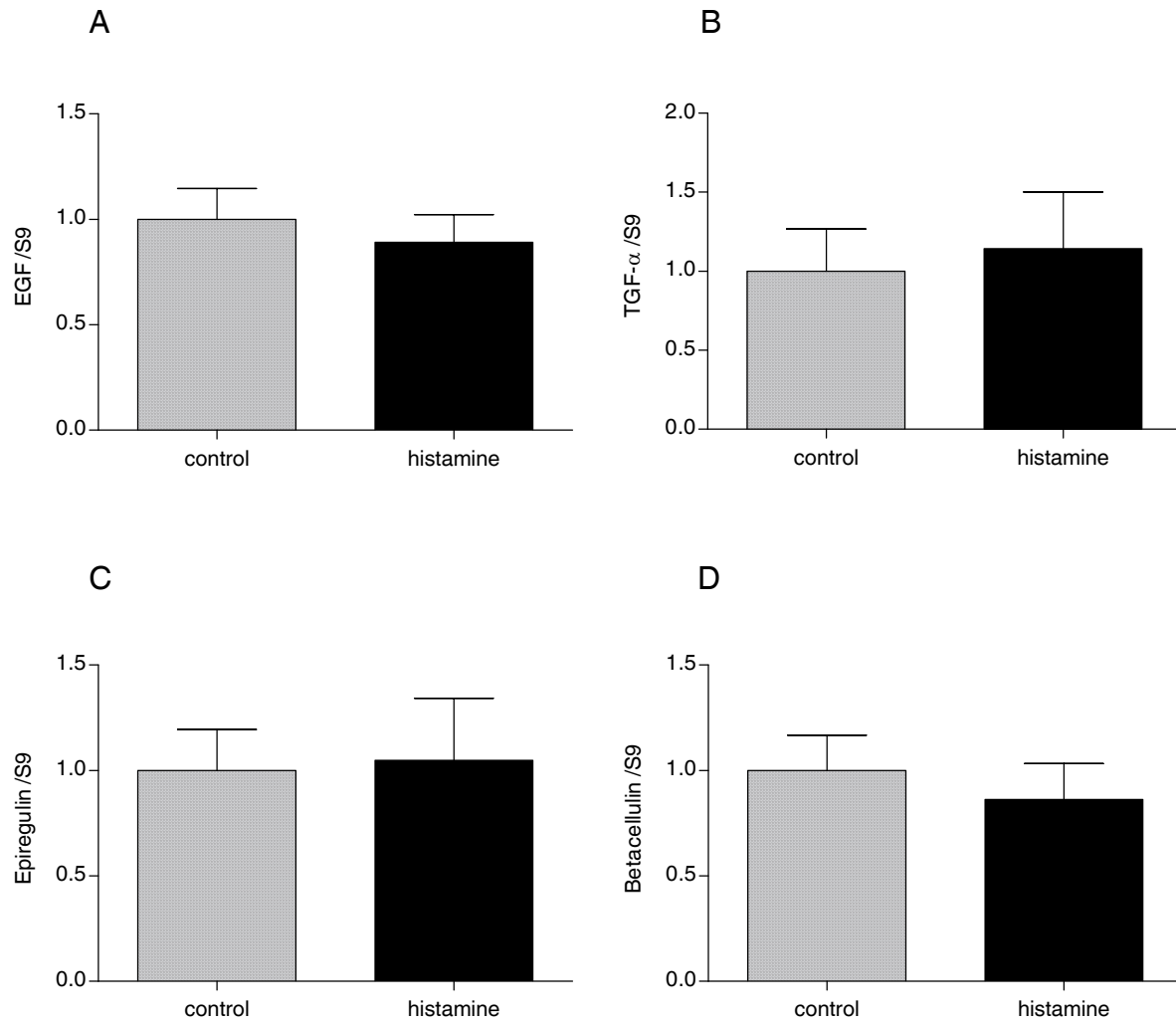


Figure S2. mRNA expression of other EGFR ligands in NHBE cells.

NHBE cells were incubated with histamine (10 μ M) for 4 hours. mRNA of EGF (A), TGF- α (B), epiregulin (C), and betacellulin (D) were measured by quantitative PCR. Data are represented as means + S.E. (n = 6).

Figure S3

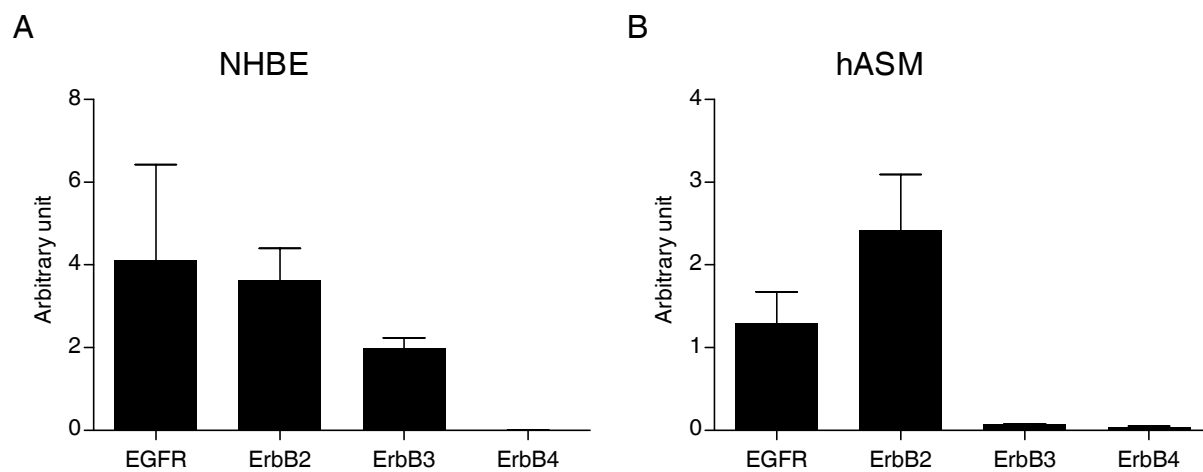


Figure S3. Expression profile of ErbB family receptors in NHBE cells and hASM cells. mRNA of EGFR (ErbB1) and ErbB2-4 was measured by real time quantitative PCR in NHBE cells (left) and hASM cells (right). Data are represented as means + S.E. (n = 3).

Figure S4

hASM

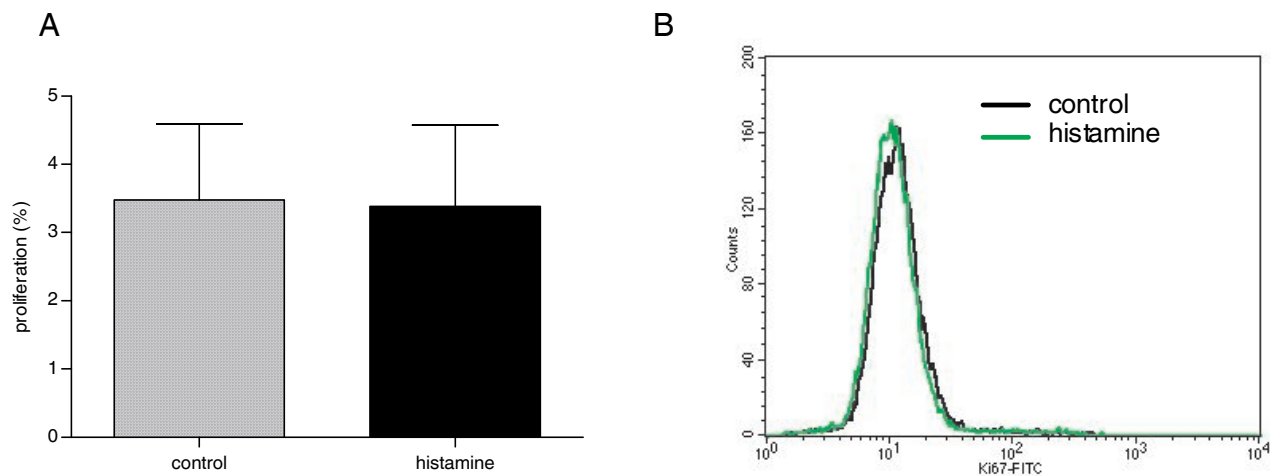


Figure S4. Histamine does not induce proliferation of hASM cells.

A, Cell proliferation of hASM cells with and without histamine (10 μ M) was measured by flow cytometric quantification of Ki67. Cells were incubated with and without histamine for 24 hours. Data are represented as means + S.E. (n = 4).

B, A representative result from the flow cytometric analysis of Ki-67 is shown. control (black), histamine (green).