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MUC5AC Expression through Bidirectional Communication of Notch and Epidermal Growth Factor Receptor Pathways

Jin Hyun Kang,* Eun Hee Lee, † Sung Woo Park, † and Il Yup Chung*

Hyperproduction of goblet cells and mucin in the airway epithelium is an important feature of airway inflammatory diseases. We investigated the involvement of Notch signaling in MUC5AC expression in NCI-H292 cells, a human lung carcinoma cell line. Epidermal growth factor (EGF) stimulated generation of the Notch intracellular domain (NICD) in a RBP-Jκ-dependent manner. Treatment with γ-secretase inhibitors L-685,458 or DAPT or introduction of small interfering RNA directed against Notch1 reduced EGF-induced MUC5AC expression. The inhibitory effect of L-685,458 on EGF-induced MUC5AC mRNA and protein expression was also observed in primary human bronchial epithelial cells. Blockage of Notch signaling with L-685,458 or Notch siRNA resulted in a decrease in EGF-induced phosphorylation of ERK. These results suggested that ERK activation is necessary for the regulation of EGF receptor (EGFR)–mediated MUC5AC expression by Notch signaling. Conversely, forced expression of NICD induced both EGFR and ERK phosphorylation with MUC5AC expression even in the absence of EGF. Treatment of the NICD-expressing cells with EGF further augmented ERK phosphorylation in an additive manner. The ERK phosphorylation induced by exogenous NICD was inhibited by treatment with an Ab that antagonizes EGFR activity as well as by inhibitors of EGFR and ERK, implying that Notch signaling induces MUC5AC expression by activating the EGFR pathway. Collectively, these results suggest that MUC5AC expression is regulated by a bidirectional circuit between Notch and EGFR signaling pathways.


Mucin hypersecretion with goblet cell hyperplasia/metaplasia is an important pathophysiologic feature of chronic airway inflammatory diseases such as severe asthma (1), chronic obstructive pulmonary disease (2), and cystic fibrosis (3). Normally, mucus secretion creates homeostasis for the conducting airways by forming a physical boundary that prevents entry of inhaled irritants, including microorganisms and particles. However, in many chronic inflammatory airway diseases, excess mucus is produced, leading to exacerbation of the disease. Airway mucous hypersecretion and plugging contribute significantly to the morbidity and mortality of chronic airway diseases. Among 12 mucin proteins known to be expressed in the respiratory tract, MUC5AC appears to be the most prominent, and also serves as the most specific marker of goblet cells (4–7). MUC5AC is produced in response to a wide variety of stimuli, including epidermal growth factor (EGF) receptor (EGFR) ligands, inflammatory cytokines, cholinergic agonists, neutrophil elastase, air pollutants, reactive oxygen species, and bacterial products (8–19). These stimuli induce MUC5AC expression through both distinct and shared signaling pathways. Among these, EGFR/EGF ligands have been extensively studied and shown to be expressed in airway epithelium (8, 20, 21). They are closely associated with proliferation, differentiation, and repair of airway epithelial cells in both normal and pathological conditions (22–25). The EGFR-mediated production of MUC5AC in airways requires, although not exclusively, activation of the ERK pathway, because pharmacologic inhibition of the ERK pathway shuts down MUC5AC mRNA expression and MUC5AC promoter activity (11). Further studies showed that ERK activity is required for binding of specificity protein 1 to its elements in the MUC5AC promoter and subsequent transcription activation (11, 26).

Notch plays an important role in the determination of cell fate in multiple organ systems (27). Four Notch receptors (Notch1–4) and five ligands (Jagged 1 and 2, and δ-like 1, 3, and 4) have been identified in mammals to date. Notch receptor–ligand interaction via cell–cell contact is followed by two successive proteolytic cleavages of Notch, one by a disintegrin and metalloprotease 10, and the other by a γ-secretase, leading to the liberation of Notch intracellular domain (NICD). The released NICD translocates to the nucleus, where it interacts with RBP-Jκ/CSL to activate expression of the primary target genes of Notch signaling such as hairy and enhancer of split-1 (HES-1). HES-1 is a basic helix-loop-helix transcriptional repressor and acts as a Notch effector by negatively regulating expression of downstream target genes such as tissue-specific transcription factors (28, 29). Although Notch signaling is primarily mediated by a canonical pathway through RBP-Jκ/CSL and subsequent trans activation of its downstream targets, Notch engages in crosstalk with other signaling pathways, especially the EGFR and MAPK pathways. Most notable is a dynamic interplay between the Notch and EGFR signaling pathways in the eye and wing development of fruit flies, in which the two pathways act in concert to direct one phenotype or to antagonize each other to produce another phenotype, depending on the developmental context (30–32).
Analysis of genetically modified animals with perturbed Notch signaling demonstrates that Notch signaling controls the fates of a wide variety of cell phenotypes in the lungs during embryonic development (33–37). Moreover, most of the Notch signaling components, including Notch receptors, Notch ligands, and their downstream effectors, are expressed in the airway epithelium of adult humans, and some of them exhibit altered expression in a diseased condition (38), suggesting active and dynamic roles of Notch signaling in adult lung tissues in addition to developing lung. However, the functional consequence of their expression remains poorly understood, especially in adult lungs. We previously demonstrated that inhibition of a γ-secretase inhibitor (GSI) attenuates the cardinal features of asthma, including goblet cell production, in an animal model of asthma (39). This observation suggests a positive correlation between Notch signaling and MUC5AC expression. In this study, we investigated the involvement of Notch signaling in MUC5AC expression, one of the major phenotypes that occurs upon provocation of airway epithelial cells, and explored the underlying mechanism using approaches that inhibit or activate Notch signaling.

Materials and Methods

Cell culture and inhibitor treatment

NCI-H292 cells, which are human pulmonary mucoepidermoid carcinoma cells, were cultured in RPMI 1640 medium (Welgene) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), HEPEs (25 mM), and sodium pyruvate (1 mM). At confluence, cells were starved in RPMI 1640 lacking FBS for 4 h, and incubated for 24 h in RPMI 1640 containing 1% FBS with 25 ng/ml recombinant human EGF (R&D Systems, Minneapolis, MN), γ-secretase inhibitors (GSI) L-685,458 (Calbiochem, La Jolla, CA) and DAPT (Calbiochem), EGFR inhibitor AG1478 (Calbiochem), MEK inhibitor U0126 (Calbiochem), p38 inhibitor SB203580 (Tocris, Ballwin, MO), and JNK inhibitor SP600125 (Tocris) were dissolved in DMSO. Cells were pretreated with inhibitors for 30 min prior to treatment with EGF.

Air–liquid interface cell culture for human bronchial epithelial cell

Primary human bronchial epithelial cells (HBEcs; lot 3F1191; Cambrex, Bio Science, Baltimore, MD) were prepared as previously described (40). Frozen passage-1 stock were thawed and cultured in T-flasks in bronchial epithelial cell growth medium (Cambrex Clonetics, Walkersville, MD) supplemented with bovine pituitary extract, hydrocortisone (0.5 μg/ml), recombinant human EGF (0.5 ng/ml), epithorine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), gentamicin sulfate (50 μg/ml), and amphotericin B (50 μg/ml). Medium was refreshed three times weekly. Cells were grown at 37°C in 5% CO2 in a humidified incubator and protected from light. At confluence, cells were starved in RPMI 1640 containing 1% FBS for 4 h, and incubated for 24 h in RPMI 1640 containing 1% FBS with 25 ng/ml recombinant human EGF (R&D Systems, Minneapolis, MN), γ-secretase inhibitors (GSI) L-685,458 (Calbiochem, La Jolla, CA) and DAPT (Calbiochem), EGFR inhibitor AG1478 (Calbiochem), MEK inhibitor U0126 (Calbiochem), p38 inhibitor SB203580 (Tocris, Ballwin, MO), and JNK inhibitor SP600125 (Tocris) were dissolved in DMSO. Cells were pretreated with inhibitors for 30 min prior to treatment with EGF.

Measurement of luciferase activity

For the reporter assay, NCI-H292 cells were transfected with RBP-jκ promoter–luciferase constructs (SABiosciences, Frederick, MD) using a Lipofectamine 2000-based gene transfer method (Invitrogen, Carlsbad, CA), according to the manufacturer’s specifications. In brief, cells were plated onto 12-well plates at 70–80% confluence. The cells were incubated at 37°C with a mixture of DNA constructs and Lipofectamine 2000 in Opti-MEM (Invitrogen) for 12 h. The transfected cells were treated with EGF in the presence or absence of GSI for 24 h. After 36 h, firefly and Renilla luciferase activities were measured sequentially from a single sample using the Dual-Luciferase Assay System kit (Promega, Madison, WI). For each transfection, relative firefly luciferase activity was normalized to Renilla luciferase activity.

Real-time PCR

Real-time quantitative PCR was performed in a 20 μl reaction with 0.5 μl cDNA, 0.8 μl of each primer (10 μM), and 10 μl SYBR Green Master Mix (Applied Biosystems, Foster City, CA), using the Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The following primers were used for amplification: MUC5AC, forward 5’-ACTGGG-GAGAAATATCGCATG-3’ and reverse 5’-CTGCTTCCTTCGTGTCCTGAG-3’. Amplification of cDNAs was performed in triplicate for 384-well plates. Amplification of GAPDH was performed to generate a standard curve to test assay sensitivity and for a quantification reference. The standard curve was derived from five points of a 2-fold cDNA dilution series.

Notch1 and HES-1 mRNA expression

Total mRNA was extracted from NCI-H292 cells using TRI reagent (Molecular Research Center, Cincinnati, OH). First-strand cDNA was synthesized from 2 μg total RNA using SuperScript II RNase reverse transcriptase (Invitrogen) in a 20 μl reaction, including the oligo(dT)18 primer, deoxynucleotide triphosphates (0.5 mM), MgCl2 (2.5 mM), and DTT (5 mM). Reverse transcription was performed at 42°C for 1 h and followed by heat inactivation at 70°C for 15 min. Synthesized cDNA was amplified for 30 cycles with Ex Taq DNA polymerase (TAKARA, Shiga, Japan). The following primers were used: Notch1, forward 5’-CGTC- ATCTCGCCGTCTCCTAC-3’ and reverse 5’-GGATCGAGGCTCTTGAG-3’. Primers for RT-PCR of HES-1 and GAPDH were ones that were also used for real-time PCR.

Western blot analysis

Cells were lysed by incubation in RIPA buffer (50 mM Tris-Cl [pH 7.4], 0.1% NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM Na2VO4, 1 mM NaF, and protease inhibitor mixture [Calbiochem]) for 30 min on ice. A total of 20 μg cell lysate resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk, probed with anti-NICD (Abcam, Cambridge, U.K.), anti-phospho-ERK1/2 (1:100 dilution; Cell Signaling Technology, Beverly, MA), anti-phospho-p38 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-phospho-EGFR (1:200; Santa Cruz Biotechnology), and treated with anti-rabbit HRP-conjugated Abs (Cell Signaling Technology). Anti-ERK2 (C-14; Santa Cruz Biotechnology), anti-p38 (Cell Signaling Technology), anti-EGFR (G38; Santa Cruz Biotechnology), and anti-GAPDH (Santa Cruz Biotechnology) Abs were used as loading controls. Immunostained proteins were detected with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Phospho-ERK levels in some results were analyzed by densitometry (Image acquisition and analysis software, UVU) and expressed as an intensity relative to phospho-ERK1/2.

Immunocytochemistry

HBEcs cultured at an air–liquid interface were stained for MUC5AC protein expression, as previously described (40). Cells were fixed in 4% paraformaldehyde and embedded in paraffin, which was sliced to 4 μm. Both HBEcs and NCI-H292 cells on slides were permeabilized in TBS containing 0.1% saponin for 15 min at room temperature. Slides were treated with 0.3% H2O2 for 10 min to block endogenous peroxidase, washed twice in TBS, treated with blocking Ab for 30 min at room temperature, and incubated at 4°C overnight with anti-MUC5AC Ab (Chemicon). After washing with TBS, slides were treated with biotinylated goat anti-mouse IgG for 30 min at room temperature, and then with avidin–biotin peroxidase complex (Vector Laboratories, Burlington, CA) for 30 min at room temperature. Color was developed by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dramatic Laboratories, Natrona, PA). The reaction was terminated by washing in running water until a uniform brown color became visible on the section. Negative control slides were incubated with isotype-matched Ab.
Expression of Notch 1 small interfering RNA

A mixture of dsRNA nucleotides targeting different regions of Notch1 mRNA and negative control small interfering (si)RNA (scrambled siRNA) was obtained from Dharmaco Research (Dharmacon, Chicago, IL). For transient expression, cells were transfected with Notch1 and scrambled siRNA oligonucleotides using DharmAfECT reagent, according to the manufacturer’s protocol.

NICD overexpression

The cDNA encoding the NICD from Notch1 was previously described (41). A Flag epitope sequence was introduced at the N terminus of NICD, and the recombinant NICD was subcloned into pcDNA3.1. The NICD construct was transfected into NCI-H292 cells using Lipofectamine 2000. At 36 h posttransfection, cells were cultured in serum-free medium for 2 h, pretreated with inhibitors or neutralizing Abs (anti-EGFR or anti–TGF-β) for 30 min, and treated with EGF for 15 min. Immunoblot analysis of cell lysates determined for ERK phosphorylation and NICD expression.

Statistical analysis

All data were analyzed using the SPSS program for independent t tests. Differences with a p value <0.05 were considered statistically significant. Results are expressed as mean ± SEM.

Results

Notch inhibitors block the expression of MUC5AC

We examined the effect of pharmacological inhibitors of Notch signaling on MUC5AC mRNA expression in NCI-H292 cells. Treatment with EGF (25 ng/ml) increased MUC5AC mRNA expression, as determined by real-time PCR. Two different GSIs, L-685,458 and DAPT, inhibited EGF-mediated MUC5AC mRNA expression in a dose-dependent manner (Fig. 1A). To address the involvement of Notch signaling in EGF-mediated MUC5AC expression, we determined NICD generation using an Ab specific for Notch1-derived NICD. EGF increased NICD generation (Fig. 1B), indicating that ligation of EGFR leads to activation of Notch signaling. L-685,458 inhibited EGF-induced NICD generation in a dose-dependent manner. Furthermore, L-685,458 inhibited the HES-1 expression that was elevated by EGF treatment (Fig. 1C). To test whether downregulation of MUC5AC by GSI paralleled a known phenotype of goblet cell hyperplasia/metaplasia, we examined FOXA2 expression. Targeted disruption of the FOXA2 transcription factor gene in bronchial epithelial cells causes mucus metaplasia and increases in MUC5AC protein expression. FOXA2 also inhibits activation of MUC5AC promoter (42). EGFR activation and FOXA2 expression have an inverse relationship (40). EGF treatment slightly reduced FOXA2 mRNA expression, and this was suppressed by L-685,458 (Fig. 1D), consistent with the finding that FOXA2 inhibits MUC5AC gene expression. To determine whether Notch signaling activated by EGF exerts its function via RBP-jk, we used a RBP-jk-Luc reporter plasmid. When NCI-H292 cells were transfected with the reporter and treated with EGF, promoter activity increased ~2.5-fold (Fig. 1E). GSI treatment reduced the reporter activity to basal levels, suggesting involvement of RBP-jk in EGF-activated Notch signaling. Next, we examined the effect of GSI on MUC5AC protein expression, determined by immunocytochemistry. The percentage of MUC5AC-positive cells increased from 4.1 ± 0.7% to 26 ± 2.8% following EGF stimulation. This was almost completely abolished by the EGFR inhibitor AG1478. Treatment with L-685,458 or DAPT (each at 10 μM) inhibited EGF-induced MUC5AC protein expression, similar to the inhibition by treatment with U0126, a specific ERK inhibitor (Fig. 2). The concentrations of GSI used did not affect the viability of the epithelial cells during culture (Fig. 2A and data not shown). To evaluate the involvement of Notch signaling in EGF-induced MUC5AC expression in a more physiological setting, we implemented an air–liquid interface culture for HBECs. Incubation of the air–liquid interface culture with medium containing a low dose of EGF (0.25 ng/ml) for 14 d resulted in little expression of MUC5AC mRNA. After the differentiated cells were extensively rinsed and exposed to high dose of EGF (40 ng/ml) for 36 h, MUC5AC mRNA levels were increased. Addition of GSI led to inhibition of MUC5AC mRNA expression with an increase in FOXA2 mRNA level (Fig. 3A). Immunocytochemistry analysis showed that GSI-induced MUC5AC protein expression was also inhibited by GSI treatment (Fig. 3B). Collectively, these results suggest that GSI induces MUC5AC expression at least in part through activation of Notch signaling.

Silencing of Notch1 reduces EGF-induced MUC5AC expression

To determine whether the selective reduction of endogenous Notch1 led to a decrease in MUC5AC expression, we used siRNA oligonucleotides directed against Notch1. EGF increased Notch1 and HES-1 mRNA synthesis. When NCI-H292 cells were transfected with a pool of four Notch1 siRNA oligonucleotides, EGF-mediated accumulation of NICD and mRNAs for Notch1 and HES-1 was considerably inhibited, whereas scrambled siRNA oligonucleotides had little effect (Fig. 4A, 4B). In line with these findings, Notch1 siRNA attenuated EGF-induced expression of...
were enumerated and expressed as percentages. Data are shown as means ± SEM of three independent experiments. **p < 0.01 compared with EGF-treated cells.

FIGURE 2. Blockade of Notch signaling reduces MUC5AC protein production. A, NCI-H292 cells were treated with AG1478, U0126, and two different GSIs, L-685,458 and DAPT, in the presence of EGF for 36 h. All inhibitors were used at a concentration of 10 μM. The cells were stained with anti-MUC5AC. These results are representative of three independent experiments. Original magnification ×200. B, MUC5AC-positive cells were enumerated and expressed as percentages. Data are shown as means ± SEM of three independent experiments. **p < 0.01 compared with EGF-treated cells.

MUC5AC mRNA and protein (Fig. 4C, 4D). These results show that silencing of Notch1 expression leads to downregulation of EGF-induced MUC5AC expression, supporting the involvement of Notch signaling in EGF-induced MUC5AC expression.

Notch1 silencing reduces EGF-mediated ERK phosphorylation

It has previously been shown that activation of ERK is essential for EGF-mediated expression of MUC5AC (11). Hence, we examined whether the inhibitory effect of GSI on EGF-mediated expression of MUC5AC occurred through alteration of ERK activities. As expected, EGF induced ERK phosphorylation, which was reduced by the MEK inhibitor U0126, but unaffected by p38 inhibitor SB203580 and JNK inhibitor SP600125 (Fig. 5A). GSI severely reduced ERK phosphorylation in a dose-dependent manner (Fig. 5B). EGF did not induce phosphorylation of p38 MAPK, and the basal level of active p38 was selectively reduced only by SB203580 (Fig. 5A). JNK phosphorylation was not detected in this cell type under conditions tested (data not shown). In parallel, we also tested the effects of these inhibitors on EGF-induced MUC5AC mRNA levels. Like AG1478 and U016, GSI inhibited EGF-induced MUC5AC mRNA expression, whereas inhibitors of p38 and JNK had no effect (Fig. 5C). This finding was consistent with their inhibitory effects on MUC5AC protein expression, as shown in Fig. 2. Introduction of Notch1 siRNA attenuated ERK phosphorylation in EGF-treated cells (Fig. 5D). It is noticed that Notch1 siRNA reduced the basal level of phospho-ERK in untreated cells. These data suggest that Notch signaling is involved in EGF-mediated MUC5AC expression through regulation of the ERK pathway.

Exogenous NICD expression induces ERK phosphorylation by activating EGFR

As blockage of Notch signaling disturbed EGF-induced ERK phosphorylation, we asked whether activation of Notch signaling itself elevated ERK phosphorylation without addition of EGF. Transfection with NICD, an active form of Notch1, clearly induced ERK phosphorylation in a dose-dependent manner, whereas an empty vector had no effect (Fig. 6A). Probing of the immunoblot with anti–phospho-EGFR revealed NICD-induced EGFR phosphorylation in a dose-dependent manner (Fig. 6A). NICD-induced ERK phosphorylation was completely inhibited by EGFR and ERK inhibitors (Fig. 6B). These data suggest that Notch signaling can induce ERK phosphorylation via the EGFR pathway. In contrast, GSI had no effect on the NICD-induced ERK phosphorylation, implying that the ERK phosphorylation results from an effect of the exogenous NICD. To examine the combined effect of Notch and EGFR signaling pathways, NCI-H292 cells were transfected with NICD, cultured for 36 h, pretreated with inhibitors for 30 min, and treated with EGF or left untreated for 15 min. EGF treatment induced a comparable level of ERK phosphorylation to that induced by NICD transfection (Fig. 6B, lane 6 versus lane 2). A combination of NICD expression and EGF treatment augmented ERK phosphorylation approximately twice as much as either alone (Fig. 6B, lane 7 versus lane 2 or lane 6). Inhibitors of both EGFR and ERK completely abolished NICD-induced ERK phosphorylation (Fig. 6B). Importantly, GSI inhibited ERK phosphorylation by only 50%, confirming that it inhibited EGFR-, but not NICD-mediated ERK phosphorylation as GSI itself did not inhibit the de novo synthesis of the ectopically expressed NICD (Fig. 6B, lane 8). This result suggests that Notch signaling is essentially involved in EGF-induced MUC5AC expression. When NICD-transfected cells were incubated with neutralizing Abs to EGFR, the NICD effect was completely nullified, whereas a control Ab had no effect (Fig. 6C, lane 4 versus lane 3), suggesting that activation of Notch signaling led to ERK phosphorylation.

FIGURE 3. GSI inhibits EGF-induced MUC5AC mRNA and protein expression in HBECs. HBECs cultured at air–liquid interface for 14 d were washed and treated with EGF (40 ng/ml) in the presence and absence of L-685,458 (10 μM) for an additional 36 h. A, Real-time PCR analysis for MUC5AC and FOXA2 mRNA. Data are shown as means ± SEM of eight independent experiments performed in triplicate. *p < 0.05, **p < 0.01 compared with EGF-treated cells. B, Immunocytochemistry for MUC5AC protein. MUC5AC-immunopositive cells were evident in EGF-treated cells and reduced by addition of L-685,458. Original magnification ×1000.
through EGFR activation. In contrast, anti–TGF-α slightly inhibited the NICD-mediated ERK phosphorylation (Fig. 6C, lane 5). The EGFR Ab overrode the ERK phosphorylation resulting from the additive effect of both NICD expression and EGF stimulation, although anti–TGF-α Ab had a minimal effect on ERK phosphorylation (Fig. 6C, lane 9 versus lane 10). Exogenous NICD augmented MUC5AC mRNA expression in a dose-dependent manner (Fig. 6D). These results suggest that activation of Notch signaling induces MUC5AC expression through activation of the ERK pathway without the aid of exogenous EGF.

**Discussion**

It is well known that the EGF/EGFR axis induces MUC5AC expression in lung epithelial cells (8, 11, 24, 26, 43), and that the Ras/Raf/ERK pathway is mainly, although not solely, responsible for the induction of MUC5AC by EGF/EGFR (11, 26). Notch signaling has been implicated in epithelial growth and differentiation in embryonic and adult lungs (35, 44), and a wide variety of Notch receptors and their related components are abundantly expressed in adult lungs (38). It has recently been shown that ectopic Notch signaling in the developing mouse lung results in more mucous cells (45); however, the functions of Notch in homeostatic and...
MUC5AC expression was induced even in the absence of exogenous EGFR ligand (Fig. 6). These findings suggest that Notch signaling directly modulates EGFR activity rather than synthesis of EGFR ligand. In addition, the fact that NICD, an intracellular domain of Notch, is able to induce EGFR phosphorylation suggests that EGFR activation is unlikely to occur through a direct molecular contact between Notch1 and EGFR on the cell surface, but rather might be resulted from a NICD-downstream effect(s). It has previously been suggested that both ligand-dependent and ligand-independent EGFR activation pathways lead to mucin synthesis (21). In ligand-dependent EGFR activation, a variety of stimuli of MUC5AC induces secretion of EGFR ligands, including cleavage of membrane-bound EGFR proligands, which in turn activates EGFR. In contrast, ligand-independent EGFR activation may occur in the absence of ligand binding by directly phosphorylating tyrosine residues in the intracellular domain in response to stimuli such as cigarette smoke and oxidative stress. Our data support ligand-independent EGFR activation, but how Notch signaling modulates EGFR activity in a more direct manner remains to be established.

There are a few reports that the EGF/Ras–MAPK pathway can activate Notch signaling. Oncogenic Ras increases levels and completely blocked by treatments that specifically interfered with EGFR activity (Fig. 6). Thus, it appears that Notch signaling induces MUC5AC expression through the following mechanism: Notch signaling activates the EGFR pathway, which in turn activates the ERK pathway, leading to MUC5AC expression. From the other angle, ligation of EGF to EGFR forms a feed-forward network for MUC5AC expression by activating the Notch pathway, whose signal is integrated into the EGFR–ERK pathway, leading to MUC5AC expression (Fig. 7).

A few reports have previously shown the cooperative relationship between Notch signaling and EGFR/Ras–MAPK pathways, especially in maintaining neoplastic phenotypes. Activated Notch1-mediated oncogenic transformation requires ERK activation (46). In lung cancer cells, expression of Notch3 is positively correlated with EGFR expression, and dominant-active Notch3 activates ERK phosphorylation, whereas dominant-negative Notch3 inhibits ERK phosphorylation. Furthermore, Notch3 siRNA or GSI inhibits ERK phosphorylation, and the effect of Notch signaling is impeded by both ERK and EGFR inhibitors (47–49). In addition, Notch1 upregulates EGFR expression through p53 (50). These results suggest that Notch signaling stimulates ERK activation at least in part through a mechanism involving the synthesis of EGFR. Our results showed that exogenous NICD expression elicited increases in both EGFR and ERK phosphorylations (Fig. 6A). The ERK phosphorylation was completely inhibited by blockage of EGFR activity, but only very weakly inhibited by blockage of synthesis of its ligand, TGF-α (Fig. 6C), providing evidence that activation of Notch signaling directly modulates EGFR activity rather than synthesis of EGFR ligand. In addition, the fact that NICD, an intracellular domain of Notch, is able to induce EGFR phosphorylation suggests that EGFR activation is unlikely to occur through a direct molecular contact between Notch1 and EGFR on the cell surface, but rather might be resulted from a NICD-downstream effect(s). It has previously been suggested that both ligand-dependent and ligand-independent EGFR activation pathways lead to mucin synthesis (21). In ligand-dependent EGFR activation, a variety of stimuli of MUC5AC induces secretion of EGFR ligands, including cleavage of membrane-bound EGFR proligands, which in turn activates EGFR. In contrast, ligand-independent EGFR activation may occur in the absence of ligand binding by directly phosphorylating tyrosine residues in the intracellular domain in response to stimuli such as cigarette smoke and oxidative stress. Our data support ligand-independent EGFR activation, but how Notch signaling modulates EGFR activity in a more direct manner remains to be established.

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activity of NICD derived from Notch1, and also upregulates Notch ligand Delta1 and presenilin1 through a p38-mediated pathway in human fibroblast and epithelial cells (51). Proinflammatory cytokines, including TNF, IL-1β, and IFN-γ, stimulate γ-secretase activity-dependent cleavage through a JNK-dependent pathway in human lung epithelial cells (52). In Drosophila, the EGFR pathway promotes the expression of a Notch ligand, which acts as an inducer for cone cells (31). Thus, EGFR/Ras–MAPK pathways can activate Notch signaling through the synthesis and elevated activity of molecules involved in Notch processing. Our results are consistent with these observations; in our study, EGF caused increased synthesis of Notch1 and the generation of NICD, and both GSI and Notch1 siRNA inhibited EGF-induced ERK phosphorylation. By the same token, however, an important question is raised: how might this relate to ERK activation through the classical EGFR–Ras pathway? The fact that GSI or Notch siRNA almost completely inhibited EGF-induced ERK phosphorylation implies that EGF-induced ERK phosphorylation may occur predominately through activation of Notch signaling. Thus, it is puzzling that binding of EGF to EGFR causes ERK phosphorylation through the somewhat indirect means of activation of Notch signaling, despite having a more straightforward pathway in EGFR-Ras-ERK. The reason for this is not clear, although this strategy might lead to enforcement of ERK activity through a feed-forward loop with sustained influence on downstream effectors. In this context, it is noteworthy that a recent report proposes a model in which TGF-α–induced EGFR activation occurs in two phases (53). TGF-α binds EGFR directly, inducing a first phase of EGFR phosphorylation that results in the activation of ERK and in production of MUC5AC and CCL20. CCL20 then binds to its receptor CCR6, which induces a second phase of EGFR phosphorylation by TNF-α converting enzyme–dependent cleavage of EGFR proligands. The second phase of EGFR activation leads to exaggerated mucin production independently of ERK. Investigating whether this applies to Notch signaling–dependent EGFR activation for MUC5AC would be worthwhile, although this pathway appears to involve ERK.

Our results show that GSI or Notch1 siRNA inhibited MUC5AC expression in lung epithelial cells. Although the effect of alteration of Notch signaling on MUC5AC expression of airway epithelial cells has not been tested, several reports show that Notch signaling is actively involved in goblet cell differentiation in intestinal epithelium. Oral administration of GSI to mice results in increased expression in lung epithelial cells. Although the effect of alteration of Notch signaling on MUC5AC expression of airway epithelial cells. It is identified as a primary route of Notch signaling also converges into the EGFR–ERK pathway leading to mucin synthesis and/or goblet cell metaplasia in response to a wide variety of stimuli, most of which culminate in upregulation of EGFR activity (21). Our results provide evidence that Notch signaling also converges into the EGFR–ERK pathway to induce MUC5AC expression. Therefore, Notch signaling itself may be a therapeutic focus, but, more importantly, the interplay between EGFR and Notch signaling for the expression of MUC5AC reiterates the importance of the EGFR–ERK pathway as a major therapeutic target for many chronic inflammatory diseases associated with excess mucin production. In addition, this study provides a cellular and biochemical basis for the in vivo observation that pharmacological blockade of Notch signaling reduces goblet cell production in an animal model of asthma (39).

Disclosures
The authors have no financial conflicts of interest.

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