Lung Tumor Suppressor GPRC5A Binds EGFR and Restrains Its Effector Signaling

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Abstract

GPRC5A is a G-protein-coupled receptor expressed in lung tissue but repressed in most human lung cancers. Studies in Gprc5a1−/− mice have established its role as a tumor-suppressor function in this setting, but the basis for its role has been obscure. Here, we report that GPRC5A functions as a negative modulator of EGFR signaling. Mouse tracheal epithelial cells (MTEC) from Gprc5a1−/− mice exhibited a relative increase in EGFR and downstream STAT3 signaling, whereas GPRC5A expression inhibited EGFR and STAT3 signaling. GPRC5A physically interacted with EGFR through its transmembrane domain, which was required for its EGFR inhibitory activity. Gprc5a1−/− MTEC were more susceptible to EGFR inhibitors than wild-type MTEC, suggesting their dependence on EGFR signaling for proliferation and survival. Dysregulated EGFR and STAT3 were identified in the normal epithelia of small and terminal bronchioles as well as tumors of Gprc5a1−/− mouse lungs. Moreover, in these lungs EGFR inhibitor treatment inhibited EGFR and STAT3 activation along with cell proliferation. Finally, overexpression of ectopic GPRC5A in human non–small-cell lung carcinoma cells inhibited both EGF-induced and constitutively activated EGFR signaling. Taken together, our results show how GPRC5A deficiency leads to dysregulated EGFR and STAT3 signaling and lung tumorigenesis. Cancer Res; 75(9); 1801–14. ©2015 AACR.

Introduction

EGFR (also known as ERBB1 or HER1) belongs to the ERBB family of cell-surface receptor tyrosine kinases. Activation of EGFR in normal lung tissue is regulatable. EGFR binding to EGFR triggers homodimerization or heterodimerization of this receptor with other ERBB members, leading to receptor phosphorylation and activation of downstream effectors such as ERK–MAPK, PI3K–AKT, and STAT3. Activation of the EGFR pathway provides a robust signal for cell proliferation and survival in response to extracellular stimuli; such signal fades away after normal organogenesis and tissue injury/repair to maintain homeostasis (1–3). However, dysregulated EGFR activation was found in the lungs with neoplastic and preneoplastic changes, including bronchial preneoplasia (4), the indolent bronchioalveolar carcinoma (BAC) and non–small cell lung cancer (NSCLC; refs. 4, 5). These observations imply that a desensitization mechanism to restrain or terminate EGFR activation has been disrupted during lung tumorigenesis. The identification of these mechanisms is, therefore, important to understand lung tumorigenesis, and also to design novel and effective approaches for preventing lung cancer development.

G-protein–coupled receptor family C group 5 type A (GPRC5A), also known as RAIG1 or RA13, is a retinoic acid-inducible gene. GPRC5A is predominately expressed in lung tissue (6–9) and has been disrupted during lung tumorigenesis. The identifi-
tissues. Gprc5a gene knockout (ko; Gprc5a$^{−/−}$) mice appeared to have normal lung development (9). However, Gprc5a$^{−/−}$ mice developed spontaneous lung cancer, mostly adenoma and adenocarcinoma, in about 1.5 to 2 years (8), indicating that Gprc5a is a lung tumor suppressor. Lung tumorigenesis in Gprc5a$^{−/−}$ mice was often accompanied by symptoms of severe inflammation, which was associated with aberrantly activated NF-kB (13). In addition, increased STAT3 signaling was found in Gprc5a$^{−/−}$ mouse tracheal epithelial cells (MTEC) in vitro (14). Taken together, loss or repression of Gprc5a predispose host to lung tumorigenesis. However, the molecular mechanism underlying tumorigenesis in the Gprc5a$^{−/−}$ mouse remains elusive.

Because aberrantly activated EGFR signaling is strongly associated with bronchial preneoplasia, inflammatory lungs in human (15), and in a mouse model of lung inflammation and lung cancer (16), it raises several questions: (i) why EGFR activation is regulatable in normal lung tissue whereas it is dysregulatable in inflammatory and precancerous lungs? (ii) what are the mechanisms involved in dysregulation of EGFR activation in these lungs? In addition, (iii) could dysregulated EGFR signaling be resulted from deficiency of a negative regulator of EGFR? In this study, we provide credible evidence that GPRC5A functions as a negative regulator of EGFR signaling. We propose, Gprc5a is important for lung homeostasis by restraining EGFR from overactivation, whereas GPRC5A deactivates STAT3 signaling via the Akt-eNOS pathway (13). In addition, Gprc5a$^{−/−}$ mice were received two weekly i.p. injections of NNK (100 mg/kg of body weight; Midwest Research Institute, Kansas City, MO) dissolved in saline solution (0.9% NaCl) or saline alone ($n = 8$). Ten months later, mice were sacrificed, one lobe of lung was fixed in paraffin for hematoxylin and eosin staining, the rest lung tissues were homogenated in liquid nitrogen for extraction of protein and RNA.

Immunohistochemical analysis

The lungs of all mice were resected, and then fixed separately in neutral-buffered formalin and embedded in paraffin blocks. Formalin-fixed paraffin-embedded tissue samples were sectioned (5 μm) and stained with H&E and used for microscopic observation. Normal human lung and inflammatory lung tissue samples were obtained from Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). The lungs of all mice were resected, and then fixed separately in neutral-buffered formalin and embedded in paraffin blocks. Formalin-fixed paraffin-embedded tissue samples were sectioned (5 μm) and stained with H&E and used for microscopic observation. Normal human lung and inflammatory lung tissue samples were obtained from Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China).

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Immunohistochemistry
The fixed tissue samples were also processed for IHC. Detailed information is provided in the Supplementary Experimental Procedures.

Erlotinib studies in mice
Eight-week-old WT and Gprc5a−/− mice were treated twice weekly with i.p. injections of NNK (100 mg/kg of body weight). Nine and half months (or at age of 11.5 months), mice were i.p. daily injected with erlotinib (at 10 mg/kg body weight; Selleckchem) in 6% Captisol vehicle, total 14 injection in 2 weeks similarly as described previously (22); control mice were injected with vehicle in the same protocol. Mice were sacrificed at age of 12 months for analysis, one lobe of lung was fixed in paraffin for H&E staining analysis, the rest lung tissues were homogenized in liquid nitrogen for extraction of protein.

Statistical analyses
Data were analyzed using the IBM SPSS Statistics 19 software. Data are expressed as the means ±sd. Results were compared using unpaired t tests assuming unequal distribution. A P value of <0.05 was considered statistically significant.

Results
EGFR–STAT3 signaling is dysregulated in Gprc5a−/− mouse tracheal epithelial cells
To determine the role of Gprc5a on EGFR signaling, we examined EGFR signaling in Gprc5a−/− and Gprc5a+/+ (WT) MTEC by immunoblot analysis. We found that p-EGFR (Y1068) was increased in Gprc5a−/− MTEC compared with Gprc5a+/+ MTEC after EGF exposure (50 ng/ml for 30 minutes; Fig. 1A). In addition, p-STAT3 and cyclin D1 were also increased in Gprc5a−/− MTEC as indicated by immunoblot analysis (Fig. 1A). RT-PCR analysis showed that mRNA of EGFR was also increased in Gprc5a−/− MTEC (Fig. 1B). Kinetic analysis showed that EGF-induced activation of EGFR (p-EGFR) in Gprc5a−/− MTEC began as early as 5 minutes, and persisted for at least 4 hours following EGF stimulation (Fig. 1C). In addition, p-STAT3 was also increased in Gprc5a−/− MTEC compared with Gprc5a+/+ MTEC (Fig. 1C). Although EGF-induced ERK was increased in Gprc5a−/− MTEC at 5 minutes, it faded away quickly from 10 minutes (Fig. 1C). Taken together, Gprc5a deletion leads to dysregulation of EGFR–STAT3 signaling in MTEC.

GPRC5A expression inhibits EGF-induced EGFR–STAT3 signaling
Next, we examined the effect of overexpression of GPRC5A. By transient transfection and immunoblot analysis, we found that, although EGFR expression rendered host cells to respond to EGF, coexpression of GPRC5A inhibited EGF auto-phosphorylation (p-EGFR; Fig. 1D). The autophosphorylation residues Y1068 and Y1086 are the docking sites for STAT3 activation (23). Similarly, EGF-induced STAT3 phosphorylation (p-STAT3) was also inhibited by coexpression of GPRC5A (Fig. 1D). The inhibitory effect of GPRC5A on EGFR and STAT3 activation was significant, as early as 5 minutes, and persisted for at least 12 hours following EGF treatment (Fig. 1E). Other signaling molecules, such as p-ERK, p-JNK, were not significantly different between two groups. Thus, GPRC5A mainly affected EGFR–STAT3 signaling. Consistently, coexpression of EGFR with or without GPRC5A in context of STAT3 promoter-driven reporter (STAT3-luc), resulted in similar conclusion (Fig. 1F). Taken together, GPRC5A expression inhibits EGF-induced EGFR-STAT3 signaling.

The 7-transmembrane domains of GPRC5A are required for EGFR inhibition
To determine the functional domain(s) of GPRC5A for EGFR inhibition, we generated a series of GPRC5A deletion mutants (Fig. 2A), and examined the effects of these mutants on STAT3-luc activation (Fig. 2B). The results showed that the inhibitory activity of GPRC5A was not blocked by deletion neither at the N-terminal domain deletion (ΔN), nor the C-terminal domain deletion (ΔC), nor both the N- and C-terminal domains (Δ–ΔTM; Fig. 2B), but only at the 7-TM domain deletion (Δ7TM; Fig. 2B). Consistently, although GPRC5A and all mutants inhibited p-EGFR and p-STAT3, only 7-TM deletion mutant (Δ7TM) failed to do so in immunoblot analysis (Fig. 2C). The protein levels of AC and 7-TM mutants appeared to be low in immunoblot analysis (Fig. 2C). However, immunofluorescent analysis showed that their expression levels were quite similar to WT GPRC5A (Supplementary Fig. S1). The discrepancy may be due to the low solubility of AC and 7-TM mutants in cell lysates because the 7-TM domain is very hydrophobic (6). Taken together, these results indicate that the 7-TM domain of GPRC5A is required for inhibition of EGFR–STAT3 signaling.

To determine which individual transmembrane domains are responsible for GPRC5A-mediated inhibition of EGFR, we generated another set of GPRC5A mutants with deletion of one to two individual transmembrane domains (Fig. 2D). Interestingly, all mutants with single or double transmembrane deletions still inhibited STAT3-luc activities, although the inhibitory effects of these mutants showed a slightly decrease in activity when compared with that of WT GPRC5A (Fig. 2E). Of note, deletion of transmembrane domains 2 to 7 (ΔTM2–7) lost most of the inhibitory effect on STAT3-luc activity (Fig. 2E). These results indicate that none of particular individual transmembrane domain is responsible and sufficient for the inhibitory activity; rather, it is the sum of all 7-TM domains that contributes to the inhibitory activity of GPRC5A on EGFR signaling.

GPRC5A physically interacts with EGFR via its 7-transmembrane domains
Next, we asked whether GPRC5A inhibits EGFR via physical interaction because both are membrane proteins. Following transfection with GPRC5A and EGFR, we performed a coimmunoprecipitation experiment. We found that myc-precipitated GPRC5A (GPRC5A-myc) pulled down EGFR, and vice versa (Fig. 3A). The interaction between GPRC5A and EGFR is independent of EGF because the complex was formed in the absence of EGF stimulation (Fig. 3A). A parallel series of experiments were performed using GPRC5A mutants (7-TM-myc and Δ7TM-myc) and EGFR. Mutants with deletions of TM domain (Δ7TM) lost interaction with the EGFR whereas the 7-TM mutants retained the ability to interact with EGFR (Fig. 3B). This observation is consistent with the luciferase assay, in which the 7-TM of GPRC5A is required for inhibiting EGFR-mediated activation of STAT3 (Fig. 2B and C).

To determine whether EGFR and GPRC5A could interact with each other in intact cells, we examined the colocalization of these
Figure 1.
Dysregulated EGFR–STAT3 signaling in Gprc5a+/− MTEC. A and C, immunoblot analysis of cell lysates from Gprc5a+/− or Gprc5a+/+ MTEC with antibodies as indicated. Cells were treated with EGF (50 ng/mL) for 5 minutes (A) or various times as indicated (C). The bars (bottom) indicate quantitation of p-EGFR (Y1068)/β-actin (top). B, image of RT-PCR (top) and quantitation of RT-PCR (bottom) for EGFR mRNA in Gprc5a+/− or Gprc5a+/+ MTEC were as indicated. D and E, HEK293T cells were transfected with the plasmids encoding EGFR plus vector (V) or GPRC5A. Immunoblot analysis of the cell lysates harvested at either 30 minutes (D) or various times as indicated following EGF treatment (E, top). Quantitation of p-EGFR (Y1068)/β-actin by immunoblot analysis is shown in bars (E, bottom). F, HEK293T cells were cotransfected with STAT3-luciferase reporter and other plasmids as indicated. Cell lysates were harvested for luciferase activity assay following EGF treatment.
two proteins by immunofluorescent microscopy. GPRC5A-GFP (green) was indeed colocalized with EGFR (red), and remained colocalized for at least 60 minutes following EGF treatment (Fig. 3C). In contrast, neither GFP (control), nor Δ7TM-GFP (green) was colocalized with EGFR (red; Fig. 3C), suggesting that the colocalization of GPRC5A with EGFR is specific. Thus, GPRC5A interacts with EGFR in vivo, and the 7-TM domain of GPRC5A is required for the interaction.

It is known that EGFR undergoes dimerization after ligand stimulation. We then asked whether GPRC5A disrupts or attenuates EGF-induced EGFR dimerization. By immunoprecipitation–Western blot analysis, we examined the heterodimers of EGFR-HA and EGFR-Flag following cotransfection of cells with corresponding plasmids (Fig. 3D). The results showed that anti-Flag pulled down more HA-tagged EGFR after a 15 minutes EGF treatment, suggesting that the heterodimer between HA-EGFR and Flag-EGFR was induced by EGF stimulation. However, the heterodimer of HA-EGFR and Flag-EGFR was significantly reduced when GPRC5A was coexpressed (Fig. 3D), suggesting that GPRC5A inhibits EGF-induced EGFR dimerization. We conclude that the physical interaction between GPRC5A and EGFR attenuates the process of EGFR dimerization.

Figure 2.
The 7-transmembrane domains of GPRC5A are required for inhibition of EGFR signaling. A, schematic depiction of GPRC5A and GPRC5A mutants used in this study. B, HEK293T cells were transfected with plasmids encoding STAT3-luc and EGFR, plus one of the following: Vector or GPRC5A or GPRC5A mutants as indicated. Cell lysates were harvested for luciferase activity after EGF treatment. C, immunoblot analysis of cell lysates from transfected cells as indicated (EGF for 30 minutes). D, schematic depiction of the domains of GPRC5A and another set of GPRC5A mutants used. E, luciferase assay was performed as described above.
Gprc5a<sup>−/−</sup> MTEC were susceptible to EGFR inhibitors

To characterize the biologic consequences of Gprc5a deletion in MTEC, we compared the susceptibility of these cells with anoikis, programmed cell death induced by detaching cells from extracellular matrix. Cell culture on a Poly-Hema–coated plate, which prevents cell attachment, led to a 35% increase of Gprc5a<sup>+</sup>/MTEC apoptotic cells (sub-G<sub>1</sub> population) in 24 hours, as measured by FACS analysis; in comparison, only 5% of Gprc5a<sup>−</sup>/MTEC were apoptotic in the same condition (Fig. 4A). This result suggests that Gprc5a<sup>−</sup>/MTEC are more resistant to anoikis than Gprc5a<sup>+</sup>/MTEC.

We then examined the effects of the inhibitors of different signal pathways on growth of Gprc5a<sup>−</sup>/MTEC and Gprc5a<sup>+</sup>/MTEC. The EGFR inhibitors erlotinib, gefitinib, and AG1478, the STAT3 inhibitor AG490, and the PI3K inhibitor LY294002 (LY) were shown to be functional and specific on MTEC cells by immunoblot analysis (Fig. 4B). Impressively, we found that Gprc5a<sup>−/−</sup> MTEC were much more sensitive than Gprc5a<sup>+</sup> MTEC to gefitinib and erlotinib, two EGFR inhibitors in routine clinical use, and to AG1478, another EGFR tyrosine kinase inhibitor commonly used in experiments (Fig. 4C). In comparison, Gprc5a<sup>−/−</sup> MTEC were only slightly more sensitive than Gprc5a<sup>+</sup>/MTEC to AG490 (STAT3 inhibitor; 5-fold), LY294002 (PI3K inhibitor; 2.5-fold), and BAY 11-7082 (NF-κB inhibitor; 2-fold; Fig. 4D). In addition, Gprc5a<sup>−/−</sup> MTEC were not sensitive to other inhibitors, RO31-8220 (PKC inhibitor; <2-fold; Fig. 4C and D), SB203580 (p38 inhibitor; not sensitive), and PD98059 (ERK inhibitor; not sensitive; Fig. 4D). This suggests that Gprc5a gene deletion in MTEC mainly affects the EGFR signaling pathway. Thus, the growth and survival of Gprc5a<sup>−/−</sup> MTEC are EGFR signaling dependent.
A

![Graph A](image)

**Figure 4.**
The survival of Gprc5a^-/- MTEC is EGFR signaling dependent. A, Gprc5a^-/- and Gprc5a^+/+ MTEC were plated in a 6-well plate that was coated with Poly-HEMA. Cells were harvested 24 hours later for analysis of cellular DNA content by FACS. The percentage of apoptotic cells (sub-G_1 cell population) is indicated. B, immunoblot analysis of cell lysates from Gprc5a^-/- MTEC with antibodies as indicated. Cells were pretreated with erlotinib (20 µmol/L), gefitinib (50 µmol/L), AG1478 (20 µmol/L), and LY294002 (20 µmol/L) as indicated for 4 hours, then treated with EGF (50 ng/mL) for 30 minutes. C, Gprc5a^-/- and Gprc5a^+/+ MTEC (96-well plate) were treated with erlotinib, gefitinib, AG1478 (EGFR RTK inhibitor), or RO31-8220 (PKC inhibitor) as indicated. Cellular viability was assayed using the CCK8 kit. D, IC_{50} values of reagents on Gprc5a^-/- versus Gprc5a^+/+ MTEC are indicated; NA, not applicable.
Tumor Incidence

<table>
<thead>
<tr>
<th>Mice</th>
<th>2m</th>
<th>12m</th>
<th>NNK-12m</th>
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<tr>
<td>WT</td>
<td>0/8</td>
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<td>KO</td>
<td>0/8</td>
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**A**

Wild type (WT) and Gprc5a-ko (KO) mice were treated with i.p. NNK at 2m and 12m.

**B**

EGFR mRNA expression in Mouse lungs.

**C**

IHC: EGFR, Gprc5a, β-Actin

- WT: 0/8
- KO: 8/8

**D, E, F**

IHC: EGFR

**G, H, I**

IHC: p-EGFR Y1068

**J, K, L**

IHC: p-STAT3 Y705

Normal epithelium of S/TB

**Statistical Analysis**

- EGFR: P < 0.001
- p-EGFR: P = 0.037
- p-STAT3: P = 0.003

n.s. = not significant
EGFR and STAT3 signaling was aberrantly activated in the lung tissues of Gprc5a−/− mice.

To determine whether Gprc5a deletion leads to dysregulated EGFR signaling in vivo, we examined the lung tissue samples of Gprc5a+/+ (WT) and Gprc5a−/− (KO) mice in the following groups: (i) mice without treatment at age of 2 months (2m); (ii) mice without treatment at age of 12 months (12m); and (iii) mice that were i.p. injected with tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)1-butanone (NNK) treatment at age of 2 months and lung tissues were collected at age of 12 months (NNK-12m; Fig. 5A). H&E staining showed that only Gprc5a-ko-12m-NNK (KO-NNK-12m) mice developed lung tumors (8/8); whereas other groups did not (Fig. 5A and B). This is consistent with the previous work (8, 24). Immunoblot analysis showed that p-STAT3 (Y705) and p-EGFR (Y1068) was significantly increased in some area of the KO-NNK-12m group, but not other groups (Supplementary Fig. S2). This suggests that dysregulated EGFR and STAT3 signaling might be involved in tumor development in Gprc5a-ko mice.

We then examined EGFR mRNA in the lungs from different groups by RT-PCR analysis. We found that EGFR mRNA in the KO group was higher than the WT group in each pair of all groups (Fig. 5C). Noticeably, EGFR mRNA was significantly increased in KO-12m and KO-NNK-12m groups (Fig. 5C). This suggests that Gprc5a deficiency increased EGFR expression in mouse lungs, which was further enhanced with aging. To precisely locate the dysregulated EGFR, we examined the lung tissues by IHC staining. The result showed that EGFR-positive cells were heterogeneously identified in the tumor cells from KO-NNK-12m lung tissues (Fig. 5D and Supplementary Fig. S3). The intensities of EGFR staining varied greatly among tumors, some were relatively high, some were low, even in different tumors from the same lung (KO-NNK-12m; #282-1, #282-2; Supplementary Fig. S4). EGFR-positive stain is specific because the same protocol of IHC staining with normal IgG showed nothing (Supplementary Fig. S5).

Interestingly, activated EGFR was also identified in the epithelium of small and terminal bronchioles (S/TB) in KO-NNK-12m mice (Fig. 5E) and, to a lesser degree, in the KO-12m group (Fig. 5E). Noticeably, EGFR was enriched in the cytoplasmic, rather than membrane, region of the epithelium of S/TB (Fig. 5E). However, this was not seen in the KO-2m group and all groups of WT mice (Fig. 5E and F). In addition, there was no EGFR staining in alveolar epithelial cells in the KO-NNK-12m group. EGFR-positive cells were relatively enriched in the epithelium of S/TB, which may explain the low level of EGFR on immunoblots (Supplementary Fig. S2).

We also examined p-EGFR (Y1068) and p-STAT3 (Y705) in the lung samples by IHC staining. The expression patterns of p-EGFR (Fig. 5G–I) and p-STAT3 (Fig. 5J–L and Supplementary Fig. S6) were similar to that of EGFR (Fig. 5D–F). They were heterogeneous expressed in tumors, and enriched in the epithelium of S/TB. In addition, IHC staining Ki67 showed more intense staining in the tumor area than in adjacent normal tissues (Supplementary Fig. S7), suggesting that increased cell proliferation in tumor regions of Gprc5a-ko mouse lungs. Taken together, activated EGFR and STAT3 were identified in the epithelium of S/TB, as well as in the lung tumors in the Gprc5a−/− mouse model, which associates with increased proliferation and tumorigenesis.

EGFR inhibitor repressed EGFR–STAT3 signaling and cell proliferation in Gprc5a−/− mouse lungs in vivo

To determine the role of dysregulated EGFR in vivo, we examined the effects of the EGFR inhibitor erlotinib in treatment of the Gprc5a−/− mouse model. Gprc5a-ko-NNK mice (n = 4) were treated with erlotinib for 2 weeks before sacrifice and collection of the lung tissues at age of 12 months for characterization. One lobe (right) of lung was fixed for IHC and H&E staining analysis, the rest lobes of lung were collected for mRNA, protein, and other analysis. We found that erlotinib treatment dramatically repressed EGFR, p-EGFR, and p-STAT3 at protein level in tumors and the epithelium of S/TB as indicated by IHC analysis (Fig. 6A–F). EGFR, p-EGFR, and p-STAT3 were positive in the tumor tissues (Fig. 6A, left; C, left; and E, left) and in the epithelium of S/TB (Fig. 6B, left; D, left; and F, left) of mouse lungs from Gprc5a-ko-NNK mice with no treatment (4 of 4; Fig. 6C), respectively. However, these proteins were significantly repressed in all of lung tissues from mice treated with erlotinib (4/4; Fig. 6A, right; F, right). This indicates that erlotinib treatment blocked not only EGFR signaling but also STAT3 signaling in vivo. Consistently, Ki67 was also greatly repressed in the lungs from mice treated with erlotinib (Fig. 6G and H), suggesting that blockage of EGFR suppressed cell proliferation in Gprc5a-ko-NNK mouse lungs. RT-PCR analysis showed that erlotinib treatment slightly, but not significantly, reduced EGFR mRNA (Fig. 6I). This suggests that erlotinib-induced inhibition of EGFR did not occur at mRNA level. Taken together, EGFR inhibitor erlotinib treatment suppressed EGFR and STAT3 signaling in vivo, and also inhibited cell proliferation in this model. Thus, activated EGFR signaling is essential for STAT3 activation, and proliferation in Gprc5a−/− mouse lungs in vivo.

Overexpression of GPRC5A suppressed EGFR signaling in human NSCLC cells

Next, we examined EGFR and GPRC5A in 10 human NSCLC cell lines, H1792, H1299, HCC827, H446, H1975, H460, H661, Calu-1, and H292G. However, it appeared that there is no
relationship between these two proteins among these cell lines (Fig. 7A). We reasoned that multiple oncogenic alterations in NSCLCs may account for dysregulated expression of EGFR and GPRC5A. To determine whether overexpression of exogenous GPRC5A affects EGFR signaling in human lung cancer cells, we selected two cell lines, H1975 and A549, with low GPRC5A, for characterization. H1975 cells express constitutively activated EGFR mutant, whereas A549 cells express relatively high level of WT EGFR. We established stable GPRC5A transfectants from each cell line for characterization. Immunoblot analysis showed that overexpression of GPRC5A inhibits both constitutively activated EGFR (p-EGFR) in H1975 cells and EGFR-induced p-EGFR in A549 cells (Fig. 7B). Thus, GPRC5A inhibits EGFR signaling in human lung cancer cells.

We also examined EGFR and GPRC5A in NSCLC lung tissues by IHC analysis. However, similar to the observation in NSCLC cell lines, no relationship was found between EGFR and GPRC5A among lung cancer tissues (data not shown). We assumed that multiple genetic and epigenetic alterations in established tumor cells may involve in the dysregulation of the two proteins. Then, we examined EGFR and GPRC5A in a relatively simple pathologic status, normal and inflammatory lung tissues. This is because that pulmonary inflammation has been associated with lung tumorigenesis, whereas the pathologic process of inflammation should not involve in too many genetic or epigenetic alterations as in tumor progression. Indeed, we found that repressed GPRC5A is, without one exception, strongly associated with activated EGFR in inflammatory lung tissues (n = 10) in comparison with those in normal lung tissues (n = 10; Fig. 7C and D). This suggests that GPRC5A repression and EGFR activation may contribute to the early stage of lung carcinogenesis.

**Discussion**

In this study, we showed that GPRC5A interacts with and inhibit EGFR signaling via its 7 transmembrane. Gprc5a deficiency led to dysregulated EGFR–STAT3 signaling in the epithelium of bronchioles both in vitro and in vivo. Importantly, inhibition of EGFR by erlotinib suppressed EGFR and STAT3 signaling in vivo, which correlates with repressed proliferation in mouse lungs. Moreover, overexpression of exogenous GPRC5A inhibits EGFR activation in human NSCLC cells. Thus, GPRC5A acts as a negative regulator of EGFR in lung homeostasis.

EGFR undergoes dimerization and autophosphorylation after ligand binding. Several molecules, such as CBL, LRIG1, CCN2, Mig6, and Muc15, have been shown to suppress EGFR activation through various mechanisms (25–29). LRIG1 is a single transmembrane receptor with unknown ligand (26, 30) and CCN2 is secretory factor (27). Both can induce EGFR ubiquitination and degradation. CBL is an SH2 domain adaptor protein that can interact with Grb2 (25). Mig6 is another cytoplasmic protein known to inhibit the kinase activity of EGFR (28). MUC15, a member of high-molecular weight glycoprotein mucin family, was recently found to inhibit EGFR signaling via physical interaction in liver cancer (29). Unlike all these molecules, GPRC5A is a 7-transmembrane G-protein–coupled receptor expressed specifically in lung (6, 8). By using its 7-transmembrane domain, GPRC5A interacts with EGFR to desensitize or terminate EGFR–STAT3 signaling during normal tissue repair/remodeling. It is interesting that although the 7-transmembrane domain was found to be required for physical interaction and inhibition of EGFR, none of the individual transmembrane domains was critical for inhibition of EGFR signaling. We propose, the interaction between GPRC5A and EGFR impedes the dimerization process of EGFR and restrain EGFR from overactivation following ligand exposure; however, in absence of GPRC5A, the negative regulatory loop on EGFR signaling was disrupted, resulting in a persistent activation of EGFR–STAT3 signaling, particularly under chronic inflammation and NNK treatment.

Interestingly, EGFR and STAT3 were not activated in the lung tissues from Gprc5a−/− mice at age of 2 months, but activated in those from the KO-NNK-12m group. This implies that additional factors, possibly growth factors and proinflammatory cytokines from the microenvironment, are required to provoke EGFR–STAT3 activation in vivo. Consistent with this contention, it has been reported that EGFR and TNFα were frequently expressed in NSCLCs and form an autocrine loop, resulting in EGFR hyperactivation (31). Interestingly, EGFR mRNA levels of KO-12m and KO-NNK-12m groups via RT-PCR were not completely consistent with EGFR protein levels in those groups. This suggests that unknown factors in the microenvironment of the KO-NNK-12m group may regulate EGFR expression at posttranscriptional level.

Previously, we showed that lung tumor development in Gprc5a−/− mice was associated with chronic inflammation. Gprc5a−/− mouse lungs had an increased susceptibility to LPS-induced inflammation and NF-κB activation (13). Consistently, exposure to non-typeable *Haemophilus influenzae* (NTHi), which induce pulmonary inflammation, promotes lung tumorigenesis in Gprc5a−/− mice (24). In this study, only Gprc5a−/− (KO)-NNK-12m mice developed full penetration of lung cancer, suggesting that chronic inflammation provides additional impetus for lung carcinogenesis. Thus, proinflammatory cytokines are likely the cofactors for EGFR activation in Gprc5a−/− mouse lungs. Previously, in vitro analysis indicated that leukemia inhibitory factor stimulated STAT3 signaling in Gprc5a−/− MTEC in an autocrine fashion (14). The biologic role of the regulation in vivo has not been examined. The fact that erlotinib treatment inhibited both p-EGFR and p-STAT3 in mouse lungs suggests that activated EGFR is essential for STAT3 activation in vivo. The role and contribution of Il6 family cytokines in activation of STAT3 signaling during lung tumorigenesis in the Gprc5a−/− mouse model require further investigation.

Importantly, activated EGFR was found heterogeneously expressed in and among tumors. More importantly, activated EGFR–STAT3 signaling was identified in the epithelium of S/TB, which is enriched with bronchoalveolar stem cells and progenitor cells (32, 33). It is likely that EGFR is only activated at certain lineage of lung epithelial cells, such as stem cells or progenitors, during tissue repair and/or tumorigenesis. Because tumors from Gprc5a−/− mice expressed type II cells markers (8), it is possible that these tumor cells are originated from those EGFR–STAT3-positive type II progenitor or stem cells. Taken together, the results of this study showed that Gprc5a deficiency induces dysregulated EGFR in promotion of lung tumorigenesis.

NSCLC accounts for 85% of lung cancers. "Driver mutations” or other oncogenic driver alterations in tumorigenesis are promising targets for prevention and therapy. The identified "driver mutations” in NSCLC, including EGFR, HER2, KRAS, ALK, BRAF, PIK3CA, AKT1, ROS1, NRS, and MAP2K1, account for more than 50% of NSCLCs, most commonly occurred in oncogenes (34–37). However, there are still more than 40% of NSCLC cases with unidentified "driver mutations” (36). It raises question whether
Figure 6.
Erlotinib treatment suppressed EGFR and STAT3 signaling, and cell proliferation in Gprc5a−/− lung tissues in vivo. Representative images of IHC staining for EGFR (A), EGFR-p (Y1068) (C), STAT3-p (Y705) (E), and Ki67 (G) in the lung tumor tissues of Gprc5a-ko-NNK-12m mice either with no treatment (n = 4; left) or erlotinib (Er) treatment (n = 4; right) are presented. Representative pictures of IHC staining for EGFR (B), p-EGFR (Y1068) (D), p-STAT3 (Y705) (F), and Ki67 (H) in the epithelium of S/TB of the lung of KO-NNK-12m mice either with no treatment (left) or erlotinib treatment (right in all above) are presented; scale bar, 50 μm. I, representative image (left) and quantitation (right, n = 4) of RT-PCR analysis for EGFR mRNA of lung tissues from mice either with erlotinib treatment or without. n.s., nonsignificant.
loss of lung tumor-suppressor genes would contribute to the initiation of lung tumorigenesis. Understanding the underlying mechanisms of those NSCLCs with unknown ‘driver mutations’ may thus provide potential targets for prevention and therapy.

Previously, two groups had independently identified 12p as one of the hot spots that was frequently deleted in lung cancers (10, 11). In addition, both of these groups predicted that tumor-suppressor genes may associate with this region. Because GPRC5A gene locus is at 12p12-p13, within one of deletion hot spots in the tissue of NSCLC, it is likely that GPRC5A is a candidate tumor-suppressor gene in this region (Supplementary Fig. S8). In previous work, we found that 62% (28/44) of homozygous Gprc5a−/− mice develop lung tumor. In addition, 15% (14/93) of heterozygous Gprc5a+/− mice also develop lung tumors (8). This suggests that reduced Gprc5a expression is sufficient to confer the susceptibility of lung tumorigenesis. Thus, we assumed that both LOH of GPRC5A and repressed GPRC5A would contribute to the initiation and promotion of lung tumorigenesis in human.

Interestingly, analysis by IHC did not show any relationship between EGFR and GPRC5A among various NSCLC cell lines and lung cancer tissues. It is probably because that multiple genetic and epigenetic alterations are involved in developed lung tumors, which complicates the system. In fact, we recently demonstrated that constitutively activated EGFR can phosphorylate the Y317/Y320 and Y347/Y350 sites in the C-terminal tail of GPRC5A and inhibit its tumor-suppressive functions (38). The cross-regulation is supported by IHC analysis, in which lung tumor tissues express low level, but phosphorylated GPRC5A whereas paired adjacent normal lung tissues express high level, but nonphosphorylated GPRC5A (38). It is possible that similar cross-regulation may also be conferred by other receptor tyrosine kinase (RTK). Thus, although GPRC5A may be expressed in some lung cancer tissues,
Gprc5a Interacts with and Inhibits EGFR in Lung Epithelium

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