Lung tumor suppressor GPRC5A binds EGFR and restrains its effector signaling

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Running title: Gprc5a interacts with and inhibits EGFR in lung epithelium

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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 Gprc5a−/− mice have established its role as a tumor suppressor function in this setting, but its the basis for its role have been obscure. Here we report that GPRC5A functions as a negative modulator of EGFR signaling. Mouse tracheal epithelial cells (MTEC) from Gprc5a−/− 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 EGRF inhibitory activity. Gprc5a−/− MTEC were much more susceptible to EGFR inhibitors than wile-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 Gprc5a−/− mouse lungs. Moreover, in these lungs EGFR inhibitor treatment inhibited EGRF and STAT3 activation along with cell proliferation. Lastly, 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.

Key words: EGFR, GPRC5A, lung cancer, mouse model, STAT3
Introduction

Epidermal growth factor receptor (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. EGF 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 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 pre-neoplastic changes, including bronchial preneoplasia (4), the indolent bronchioalveolar carcinoma (BAC) and non-small cell lung cancer (NSCLC) (4,5). These observations imply that a desensitization mechanism to restrain or terminate EGFR activation has been disrupted during lung tumorigenesis. 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), suggesting that it may be important for homeostasis of lung tissue. GPRC5A gene locus is at 12p13. LOH (loss of heterozygosity) of chromosome 12p was found to frequently occur in NSCLCs (10,11). And GPRC5A was significantly repressed in most of NSCLC (8,12) and all of chronic obstructive pulmonary disease (COPD) 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 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-κB (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 mouse model of lung inflammation and lung cancer (16), it raises several questions: 1) why EGFR activation is regulatable in normal lung tissue whereas it is dysregulatable in inflammatory and precancerous lung tissues? 2) what are the mechanisms involved in dysregulation of EGFR activation in these lungs? And 3) 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 deficiency leads to dysregulated EGFR signaling in promotion of lung tumorigenesis.

Materials and Methods

Cell Lines and Cell Culture

Epithelial cells were obtained from normal tracheal tissue of 3-week-old Gprc5a−/− and Gprc5a+/+ mice (C57 BL/6 × 129sv) as described (13,14). The epithelial cells were cultured in K-SFM supplemented with epidermal growth factor (EGF; 5 ng/ml) and bovine pituitary extract (50 μg/ml; Invitrogen). Human embryonic kidney cells HEK293T and NSCLC cells were
obtained from the American Type Culture Collection (ATCC) and were tested and authenticated by DNA typing at the Shanghai Jiao Tong University Analysis Core. The cells were cultured in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% fetal calf serum, at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂.

**Reagents and Antibodies**

Detailed information is provided in the *Extended Experimental Procedures*.

**Transfection and Luciferase Assay**

Transfections were performed with STAT3-driven-luciferase plasmid (STAT3-luc) as reporter, and pcDNA-EGFR, pcDNA-GPRC5A-myc, or GPRC5A mutant plasmids. Plasmid pRL-tk-dual (Promega) was used as internal control in all transfection assays. All transfections were done with Lipofectamine 2000 (Invitrogen) according to the instruction of manufacture (17). EGF treatment was 50 ng/ml for 18 hours or as indicated. Duplicate samples were assayed three times.

**Immunoprecipitation (IP) and Western Blotting**

Cells were lysed with RIPA lysis buffer (Cell Signaling Technology). IP was performed with 2 μg of antibody against myc, EGFR or normal IgG (N IgG) (as a negative control) in 1.0 mg whole cell lysate. Cell lysates and/or IP cellular proteins were separated by SDS/PAGE in a 10% acrylamide gel and transferred onto nitrocellulose membrane for immunoblot as described previously (18,19).
Plasmid Construction

STAT3-driven luciferase plasmid was as described previously (20). pcDNA-EGFR was as described (21). pcDNA3.1(+)−GPRC5A-Myc plasmid was as described previously (8,13). GPRC5A mutant plasmids were constructed with primers described in the Extended Experimental Procedures.

Immunofluorescence (IF)

HEK293T cells were seeded onto cover slips and incubated overnight, next day transfection was performed with plasmids encoding EGFR-HA plus GFP, or GPRC5A-GFP, or ∆TM-GFP. The procedure of IF was done as described previously (18,19). In each of them, 200 cells were analyzed in five different microscopic fields at 40× magnification using an Olympus confocal microscope. Images were captured with Nikon camera, and images of DAPI and FITC staining were superimposed digitally.

Cell Viability Assay

Cell proliferation and survival was assayed by cell counting kit-8 (CCK8) according the instruction of manufacturer. CCK8 was from Dojindo molecular technologies (Rockville, Maryland 20850 USA). Briefly, Gprc5a+/+ and/or Gprc5a−/− MTEC were seeded on a 96-well plate at 2000 cells in 100 μl/well. Three days later, cell cultures were changed with fresh medium, and treated with serial diluted reagents. Three more days later, plates were added CCK8 for assay of OD450 as measurement of cellular viability according to the instruction of the kit.

Tumorigenicity
Gprc5a-knockout (ko) mice (Gprc5a<sup>−/−</sup> mice) were generated in a mixed background of 129sv × C57BL/6 as described previously (8). Mice were maintained according to a protocol approved by Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee [experimental animal use permission No: SYXK (Shanghai) 2008-0050] in the specific pathogen-free animal facility in the university. Eight-week-old wild-type (Gprc5a<sup>+/+</sup>) and Gprc5a<sup>−/−</sup> mice were received 2 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 H&E staining analysis, the rest lung tissues were homogenated in liquid nitrogen for extraction of protein and RNA.

**Hematoxylin-Eosin (H&E) Staining and 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 Hematoxylin and Eosin (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).

**Immunohistochemistry**

The fixed tissue samples were also processed for immunohistochemistry (IHC). Detailed information is provided in the Extended Experimental Procedures.

**Erlotinib Studies in Mice**
Eight-week-old wild-type and Gprc5a<sup>−/−</sup> mice were treated twice weekly with i.p. injections of NNK (100 mg/kg of body weight). Nine and half months later (or at age of 11.5 month), mice were i.p. daily injected with Erlotinib (at 10 mg/kg body weight in 0.2 ml volume, Selleckchem, Houston, TX, USA) in 6% Captisol vehicle, total 14 injection in two weeks similarly as described (22); control mice were injected with vehicle in the same protocol. Mice were sacrificed at age of 12 month for analysis, one piece of lung was fixed in paraflin for H&E staining analysis, the rest lung tissues were homogenated 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 ± SEM. Results were compared using unpaired t-tests assuming unequal distribution. P < 0.05 was considered statistically significant.

**Results**

**EGFR-STAT3 signaling is dysregulated in Gprc5a<sup>−/−</sup> mouse tracheal epithelial cells (MTEC)**

To determine the role of Gprc5a on EGFR signaling, we examined EGFR signaling in Gprc5a<sup>−/−</sup> and Gprc5a<sup>+/+</sup> (wild-type) mouse tracheal epithelial cells (MTEC) by immunoblot analysis. We found that p-EGFR (Y1068) was increased in Gprc5a<sup>−/−</sup> MTEC compared with Gprc5a<sup>+/+</sup>MTEC after EGF exposure (50 ng/ml for 30 minutes) (Fig. 1A). In addition, p-STAT3 and cyclin D1 were also increased in Gprc5a<sup>−/−</sup> MTEC as indicated by Immunoblot (Fig. 1A). RT-PCR analysis showed that mRNA of EGFR was also increased in Gprc5a<sup>−/−</sup>MTEC (Fig. 1B). Kinetic analysis showed that EGF-induced activation of EGFR (p-EGFR) in Gprc5a<sup>−/−</sup>
MTEC began as early as 5 minutes, and persisted for at least 4 hour following EGF stimulation (Fig.1C). In addition, p-STAT3 was also increased in Gprc5a−/− MTEC compared to Gprc5a+/+ MTEC (Fig. 1C). Although EGF-induced ERK was increased in Gprc5a−/− MTEC at 5 minutes, it was fade 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, while EGFR expression rendered host cells to respond to EGF, co-expression of GPRC5A inhibited EGFR auto-phosphorylation (p-EGFR) (Fig.1D). The auto-phosphorylation residues Y1068 and Y1086 are the docking sites for STAT3 activation (23). Similarly, EGF-induced STAT3 phosphorylation (p-STAT3) was also inhibited by co-expression of GPRC5A (Fig.1D). The inhibitory effect of GPRC5A on EGFR and STAT3 activation was significant, began as early as 5 minutes, and persisted for at least 12 hour 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, co-expression 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 (7-TM) (Fig.2B), but only at the 7-TM domain deletion (Δ7TM) (Fig.2B). Consistently, while GPRC5A and all mutants inhibited p-EGFR and p-STAT3, only 7-TM deletion mutant (Δ7TM) failed to do so in immunoblot (Fig.2C). The protein levels of ΔC and 7-TM mutants appeared to be low in immunoblot (Fig.2C), but immunofluorescent (IF) analysis showed that their expression levels were quite similar to wild-type GPRC5A (Supplementary Fig.S1). The discrepancy may be due to the low solubility of ΔC and 7-TM mutants in cell lysates since 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 1-2 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 wild-type GPRC5A (Fig.2E). Of note, deletion of transmembrane domain 2-7 (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 contribute to the inhibitory activity of GPRC5A on EGFR signaling.

**GPRC5A physically interacts with EGFR via its 7-transmembrane domains**
Next, we asked if GPRC5A inhibits EGFR via physical interaction since both are membrane proteins. Following transfection with GPRC5A and EGFR, we performed a co-immunoprecipitation (IP) 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 since 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 while 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-2C).

To determine if EGFR and GPRC5A could interact with each other in intact cells, we examined the co-localization of these two proteins by immuno-fluorescent (IF) microscopy. GPRC5A-GFP (Green) was indeed co-localized with EGFR (Red), and remained co-localized for at least 60 minutes following EGF treatment (Fig.3C). In contrast, neither GFP (control), nor Δ7TM-GFP (Green) was co-localized with EGFR (Red) (Fig.3C), suggesting that the co-localization 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 if GPRC5A disrupts or attenuates EGF-induced EGFR dimerization. By IP-Western blot analysis, we examined the heterodimers of EGFR-HA and EGFR-Flag following co-transfection of cells with corresponding plasmids (Fig.3D). The results showed that anti-Flag pulled down more HA-tagged EGFR after a 15 min EGF treatment, suggesting that the heterodimer between HA-EGFR and Flag-EGFR were induced by EGF stimulation. However, the heterodimer of HA-EGFR and
Flag-EGFR was significantly reduced when GPRC5A was co-expressed (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.

**Gprc5a−/− MTEC were susceptible to EGFR inhibitors**

To characterize the biological consequences of *Gprc5a* deletion in MTEC, we compared the susceptibility of these cells to anoikis, programmed cell death induced by detaching cells from extracellular matrix (ECM). Cell culture on a Poly-Hema-coated plate, which prevents cell attachment, led 35% Gprc5a+/+ MTEC apoptotic (sub-G1 population) in 24 has measured by FACS analysis; in comparison, only 5% of Gprc5a−/− MTEC were apoptotic in the same condition (Fig.4A). This result suggests that Gprc5a−/− MTEC are more resistant to anoikis than Gprc5a+/+ MTEC.

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

**EGFR and STAT3 signaling were aberrantly activated in the lung tissues of Gprc5a−/− mice**

To determine if Gprc5a deletion leads to dysregulated EGFR signaling in lung *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-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 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 KO group was higher than WT group in each pair of all groups (*Fig.5C*). Noticeably, EGFR mRNA was significantly increased in KO-12m and KO-NNK-12m group (*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 immunohistochemical (IHC) staining. The result showed that EGFR positive cells were heterogeneously identified in the tumor cells from KO-NNK-12m lung.
tissues (Fig.5D, 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 staining is specific since 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 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 KO-2m group and all groups of WT mice (Fig.5E-5F). And there was no EGFR staining in alveolar epithelial cells in 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-5I) and p-STAT3 (Fig.5J-5L, Supplementary Fig.S6) were similar to that of EGFR (Fig.5D-F). They were heterogeneously 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 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 EGFR inhibitor Erlotinib in treatment of Gprc5a<sup>−/−</sup> mouse model. Gprc5a-ko-NNK mice (n=4) were treated with Erlotinib for two 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 HE staining analysis, the rest lobes of lung was 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-6F). EGFR, p-EGFR and p-STAT3 were positive in the tumor tissues (Fig.6A, 6C left and 6E left) and in the epithelium of S/TB (Fig.6B left, 6D left and 6F left) of mouse lungs from Gprc5a-ko-NNK mice with no treatment (C) (4/4), respectively. However, these proteins were significantly repressed in all of lung tissues from mice treated with Erlotinib(Er) (4/4) (Fig.6A right-6F 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(Er) (Fig.6G and 6H), 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<sup>−/−</sup> mouse lungs in vivo.

**Overexpression of GPRC5A suppressed EGFR signaling in human NSCLC cells**
Next, we examined EGFR and GPRC5A in ten human non-small cell lung cancer (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 if 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, while A549 cells express relatively high level of wild type 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 EGF-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 pathological model, normal and inflammatory lung tissues. This is because that pulmonary inflammation has been associated with lung tumorigenesis, while the pathological 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 7D). 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 inhibits 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 secretive factor (27). Both can induce EGFR ubiquitination and degradation. CBL is an SH2 domain adaptor protein which 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 over-activation 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 KO-NNK-12m group. This implies that additional factors, possibly growth factors and pro-inflammatory cytokines from the microenvironment, are required to provoke EGFR-STAT3 activation in vivo. Consistent with this contention, it has been reported that EGF and TNFα were frequently expressed in NSCLCs and form an autocrine loop resulting in EGFR hyper-activation(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 KO-NNK-12m group may regulate EGFR expression at post-transcriptional 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 (KO)-NNK-12m mice developed full penetrance of lung cancer, suggesting that chronic inflammation provides additional impetus for lung carcinogenesis. Thus, pro-inflammatory cytokines are likely the cofactors for EGFR activation in Gprc5a−/− mouse lungs. Previously, in vitro analysis indicated that leukemia inhibitory factor (lif) stimulated STAT3 signaling in Gprc5a−/− MTEC in an
autocrine fashion (14). The biological 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 IL-6 family cytokines in activation of STAT3 signaling during lung tumorigenesis in $Gprc5a^{-/-}$ 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 small and terminal bronchioles where are enriched of bronchioalveolar 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 fifty percent of NSCLCs, most commonly occurred in oncogenes (34-37). However, there are still more than forty percent of NSCLC cases with unidentified “driver mutations”(36). It raises question whether 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). And 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, 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 non-phosphorylated 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, it is probably not fully functional. On contrary, inflammation is a relatively simple pathological process, there are not much genetic and epigenetic events involved. Thus the effect of GPRC5A expression may easily be reflected. This assumption is supported by the fact that activated EGFR was strongly correlated repressed
GPCR5A in inflammatory lung tissues. Because pulmonary inflammation has been linked to lung tumorigenesis, there is a mechanistic link between these two different pathological processes. Thus, we expect that repression of GPCR5A may play an important role in the early stage, rather than later stage, of lung tumorigenesis.

Taken together, our study demonstrates that lung tumor suppressor GPCR5A restrains EGFR from overactivation in lung epithelial cells. Gprc5a-deficiency leads to dysregulated EGFR which may contribute to the initiation of lung tumorigenesis.

References:


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Author contributions

Conception and design: J.Deng, E.Y. Chin, and B.P. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.Xu, S.Zhong, Q.Li, and J. Zhang
Analysis and interpretations of data (e.g. statistical analysis, computational analysis): S.Zhong, H.Yin, Y.Liao, R.Yang, C.Ding,
Writing, review, and or revision of the manuscript: J.Deng, S.Zhong, E.Y. Chin, and B.P. Zhou
Study supervision: J.Deng, E.Y. Chin

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Additional information

Supplementary information accompanies this paper
Figure legend

Figure 1. Dysregulated EGFR-STAT3 signaling in Gprc5a−/− MTEC. A and C, immunoblot of cell lysates from Gprc5a−/− or Gprc5a+/+ mouse tracheal epithelial cells (MTEC) with antibodies as indicated. Cells were treated with EGF (50 ng/ml) for 5 minutes (min) (A) or various time as indicated (C). The bars (lower panel) indicate quantitation of p-EGFR (Y1068)/β-actin (upper panel). B, image of RT-PCR (upper) and quantitation of RT-PCR (lower) for EGFR mRNA in Gprc5a−/− or Gprc5a+/+ MTEC were as indicated. D-E, HEK293T cells were transfected with the plasmids encoding EGFR plus vector (V) or GPRC5A. Immunoblot of the cell lysates harvested at either 30 minutes (D) or various time as indicated following EGF treatment (E, upper panel). Quantitation of p-EGFR (Y1068)/β-actin by immunoblot analysis is shown in bars (E, lower panel). F, HEK293T cells were co-transfected with STAT3-luciferase reporter and other plasmids as indicated. Cell lysates were harvested for luciferase activity assay following EGF treatment.

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 of cell lysates from transfected cells as indicated (EGF for 30 min). D, schematic depiction of the domains of GPRC5A and another set of GPRC5A mutants used. E, luciferase assay was performed as described above.

Figure 3. GPRC5A interacts with EGFR via the 7-transmembrane domain. A-B, HEK293T cells were co-transfected with the plasmids encoding EGFR, plus: vector (V) or GPRC5A-myc
(5A), or the 7-transmembrane mutant-myc (7TM), or 7-transmembrane deletion mutant-myc (Δ7TM). Cell lysates were immunoprecipitated (IP) with antibodies to myc or EGFR, and the immunoprecipitates were analyzed by Western blot. C, cells were transfected as indicated. Immunofluorescent (IF) analysis was used to visualize the co-localization of EGFR-HA (Red) with either GFP (control), or GPRC5A-GFP or Δ7TM-GFP (all GFP Green). D, cells were co-transfected with the plasmids containing EGFR-HA and EGFR-Flag, plus either vector or GPRC5A-myc. The cells were treated with or without EGF for 15 minutes, and cell lysates were generated. Lysates were subjected to immunoprecipitation-Western blot as above.

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 hour later for analysis of cellular DNA content by fluorescence-activated cell sorting (FACS). The percentage of apoptotic cells (sub-G1 cell population) is indicated. B, immunoblot of cell lysates from Gprc5a−/− MTEC with antibodies as indicated. Cells were pretreated with erlotinib (20 μM), gefitinib (50 μM), AG1478 (20 μM), and LY294002 (20 μM) as indicated for 4 hours, then treated with EGF (50 ng/ml) for 30 minutes (min). 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, IC50 values of reagents on Gprc5a−/− vs Gprc5a+/+ MTEC are indicated. NA = not applicable.

Figure 5. Activated EGFR and STAT3 were identified in the normal epithelium of small and terminal bronchioles (S/TB) as well as the tumors from Gprc5a−/− mouse lungs. A, the
experimental scheme is presented; lung tissues were collected at the indicated time (mouse age) without (as control) or with NNK treatment (upper panel). Representative images of lung tissues stained with H&E is presented (lower panel). Black arrow indicates tumor. The scale bar represents 400 μm. B, tumor incidence in Gprc5a−/− and Gprc5a+/+ mice are presented. C, RT-PCR analysis for EGFR mRNA in the lung tissues of mouse group, representative image as indicated (upper). Quantification of RT-PCR analysis for EGFR mRNA from each group (n=4)(below). Representative images of IHC staining for EGFR (D), p-EGFR (Y1068) (G), p-STAT3 (Y705) (J) in the tumor tissues from Gprc5a−/− mice (KO-NNK-12m). The scale bars represent 100 μm. Representative images of IHC Staining with anti-EGFR (E), p-EGFR (Y1068) (H), p-STAT3 (Y705) (K) in the epithelium of small and terminal bronchioles (S/TB) in the lung of wild type and Gprc5a−/− mice from the indicated groups. The scale bar represents 50 μm. Quantification of the IHC staining is represented as IHC scores (intensity x percentage of the positive cells in the epithelium of S/TB) of EGFR (F), p-EGFR (Y1068) (I), p-STAT3 (Y705) (L) in the epithelium of S/TB in the lungs from indicated groups.

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 panel) or Erlotinib treatment (Er) (n=4) (right panel) 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 small and terminal bronchioles (S/TB) of the lung of KO-NNK-12m mice either with no treatment (left panel) or Erlotinib treatment (right panel in all above) are presented. The scale bar represents 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 (Er) treatment or without.

**Figure 7. Overexpression of GPRC5A suppressed EGFR signaling in human NSCLC cells.**

A and B, immunoblot of cell lysates from human NSCLC cell lines with antibodies were as indicated. Vector and GPRC5A stable transfectants of H1975 and A549 were either treated with or without EGF (50 ng/ml) for 30 minutes. C, representative images of IHC staining of ten human normal and ten inflammatory lung tissues for EGFR (upper) and GPRC5A (below). The scale bars represent 50 μm. D, quantification of the IHC staining is represented as percentage of the EGFR positive cells in the epithelium of S/TB (upper); GPRC5A IHC scores (intensity x percentage of the positive cells, below) in the lungs from indicated groups.
Figure 2

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![Graph showing Relative LUC Activity](image)

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![Graph showing Relative LUC Activity](image)
Figure 3

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Figure 4

A

![Graph showing apoptotic cells (%) for WT and KO MTEC at 0h and 24h](image)

B

![Western blot images for KO-MTEC with EGF, EGFR, p-EGFR, EGFR, p-STAT3, STAT3, p-AKT, AKT, and β-actin](image)

C

![Graphs showing OD450 for different nM concentrations of Erlotinib, Gefitinib, AG1478, and RO31-8220](image)

D

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Figure 7

A

B

C

D

Normal lungs Inflammatory lungs

EGFR

p-EGFR

EGFR

p-STAT3

STAT3

GPRC5A

p-AKT

p-ERK

b-actin

H1975

Vector

GPRC5A

A549

Vector

GPRC5A

p-EGFR

EGFR

p-STAT3

STAT3

GPRC5A

p-AKT

p-ERK

b-actin

Vector

GPRC5A

EGF

EGF

EGF

EGF

EGF

EGF
Lung tumor suppressor GPRC5A binds EGFR and restrains its effector signaling

Jiong Deng, Shuangshuang Zhong, Huijing Yin, et al.

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