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Knockout of the Tumor suppressor gene *Gprc5a* in mice leads to NF-κB activation in airway epithelium and promotes lung inflammation and tumorigenesis

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Abstract

Mouse models can be useful for increasing the understanding of lung tumorigenesis and assessing the potential of chemopreventive agents. We explored the role of inflammation in lung tumor development in mice with knockout of the tumor suppressor *Gprc5a*. Examination of normal lung tissue and tumors from 51 $Gprc5a^{+/+}$ (adenoma incidence 9.8%; adenocarcinoma 0%) and 38 $Gprc5a^{-/-}$ mice (adenoma 63%, adenocarcinoma 21%) revealed macrophages infiltration into lungs of 45% of the $Gprc5a^{-/-}$ mice and 8% of $Gprc5a^{+/+}$ mice and the direct association of macrophages with 42% of adenomas and 88% of adenocarcinomas in the knockout mice. $Gprc5a^{-/-}$ mouse lungs contained higher constitutive levels of proinflammatory cytokines and chemokines and were more sensitive than lungs of $Gprc5a^{+/+}$ mice to stimulation of NF-κB activation by lipopolysaccharide (LPS) in vivo. Studies with epithelial cells cultured from tracheas of $Gprc5a^{-/-}$ and $Gprc5a^{+/+}$ mice revealed that *Gprc5a* loss is associated with increased cell proliferation, resistance to cell death in suspension, and increased basal, TNFα-induced, and LPS-induced NF-κB activation, which were reversed partially in $Gprc5a^{-/-}$ adenocarcinoma cells by re-expression of *Gprc5a*. Compared to $Gprc5a^{+/+}$ cells, the $Gprc5a^{-/-}$ cells produced higher levels of chemokines and cytokines and their conditioned medium induced more extensive macrophage migration. Silencing *Gprc5a* and the p65 subunit of NF-κB in $Gprc5a^{+/+}$ and $Gprc5a^{-/-}$ cells, respectively reversed these effects. Thus, $Gprc5a^{-/-}$ loss enhances NF-κB activation in lung epithelial cells leading to increased autocrine and paracrine interactions, cell autonomy and enhanced inflammation, which may synergize in the creation of a tumor promoting microenvironment.

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Keywords

Lung cancer; tumor suppressor; mouse model; Gprc5a; NF-κB; inflammation; macrophages

Introduction

Lung cancer, the leading cause of cancer-related deaths, is linked mainly to tobacco smoking (1). However, host factors, both genetic and epigenetic, also play important roles in lung carcinogenesis. For example, pulmonary inflammation has been implicated in the development of lung cancer in humans (2) and in mouse models (3, 4). Besides causing DNA damage and mutations, tobacco smoke also induces chronic pulmonary inflammation, which can promote carcinogenesis (5), especially in cases where it also leads to chronic obstructive pulmonary disease (COPD) (6, 7). Furthermore, the development of lung cancers is strongly correlated with pulmonary inflammation even in non-smokers (8). Interestingly, anti-inflammatory drugs can reduce lung tumorigenesis in mouse models (2, 4, 9, 10), and incidence of lung cancer, especially adenocarcinomas, in humans (11). Inflammatory cells, especially tumor-associated macrophages (TAM) and neutrophils, play important role in conditioning the microenvironment by releasing numerous inflammatory cytokines, chemokines, and growth factors that can act on epithelial cells and enhance transformation and progression into premalignant, malignant, and metastatic lesions (12, 13). Therefore, targeting inflammation is considered to be a promising approach to chemoprevention and treatment of lung cancer (14, 15).

Many effects of chronic inflammation are mediated by nuclear factor kappa B (NF-κB), a transcription factor that controls the expression of genes involved in inflammation, immune responses, cell cycle, apoptosis, and angiogenesis in a variety of cells including epithelial cells, stromal cells, and macrophages (16, 17). Thus, NF-κB activation and signaling pathway link inflammation and cancer.

Recently, we have shown that deletion of a retinoic acid-inducible orphan G-protein–coupled receptor, *Gprc5a* (18, 19), which is expressed preferentially in lung tissue, predisposes mice to develop spontaneous lung tumors indicating that it functions as a lung-specific tumor suppressor (20). The carcinogenesis process in the *Gprc5a* knockout (*Gprc5a*$^{-/-}$) mouse takes 1 to 2 years emulating the long latency typical for human lung cancer development (20). However, the events that presumably take place during this period in the *Gprc5a* knockout mice are not understood.

Here, we report that the loss of *Gprc5a* in mouse lung epithelial cells results in activation of NF-κB and expression of various cytokines and chemokines in vitro and in vivo. These factors increase the proliferation and survival of the epithelial cells as well as induce infiltration of macrophages into the mouse lungs leading to the development of acidophilic macrophage pneumonia (AMP) (21) and the ensuing inflammation. We propose that the increased epithelial cell proliferation and resistance to cell death and the development of an inflammatory microenvironment in the lungs of *Gprc5a* knockout mice act in concert to promote tumorigenesis.

Materials and Methods

Animals

We used (129sv × C57BL/6) F1 *Gprc5a* wildtype and *Gprc5a* knockout mice, which recently have been named *Gprc5a*^{tm1Rlo}/*Gprc5a*^{tm1Rlo} (Gprc5a, G protein coupled receptor, family c, group 5, member A; tm1, targeted mutation 1; Rlo, Reuben Lotan) by the Mouse Genome Informatics (MGI), The Jackson Laboratory (Bar Harbor, ME). In the current study, the wildtype and knockout mice are called *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice, respectively. The mice were generated and maintained according to a protocol approved by the MD Anderson Institutional Animal Care and Use Committee as described (20).

Analysis of mouse lungs for identification of tumors, macrophages, and microvessels by histology and immunohistochemistry

Mouse lungs were excised, fixed in formalin, and embedded in paraffin. Sections (5 μm thick) of lung tissue and tumor specimens were stained with Hematoxylin and Eosine (H&E) and observed under the microscope by a veterinary pathologist (C.S.V.P) for diagnosis of adenoma, adenocarcinoma, and acidophilic macrophage pneumonia (AMP) according to histological criteria described previously (21–23). The AMP was graded as follows: grade 1 (5–10% macrophages in a microscopic field); grade 2 (10–20%); grade 3 (20–50%), and grade 4 (>50%). Macrophages were also identified by subjecting lung tissue sections to an antigen retrieval procedure using proteinase K (DAKO, Carpinteria, CA) before incubation with rat antibodies against mouse F4/80 (AbD Serotec, Raleigh, NC). Subsequently, the sections were incubated with goat anti-rat secondary antibody (Vector, Burlingame, CA) followed by incubation with avidin-biotin-peroxidase complex (DAKO) and development with diaminobenzidine chromogen (DAKO) and counterstaining with hematoxylin. Immunohistochemical analyses of the staining reactivity were done in duplicate on inflammation areas and tumors. Microvessel density was determined by staining tissue sections with antibodies against the CD31 antigen (BD Pharmingen, San Jose, CA) using a similar procedure as described above. The staining intensities for each antigen were quantified by counting the number of cells exhibiting positive reactivity in two separate microscopic fields.

Analysis of cytokines and chemokines

Lungs from *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice (n=5; 4 months old) were excised, homogenized, and extracted for analysis by Proteome Profiler Mouse Cytokine Array, Panel A, which includes immobilized antibodies against 40 cytokines and chemokines (Cat. No. ARY006; R&D Systems, Minneapolis, MN) using the manufacturer's instructions.

Treatment of mice with lipopolysaccharide (LPS)

Five months old *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice were injected intraperitoneally with 0.2 ml of a solution of LPS 50 μg/mL in PBS (*Escherichia coli* strain O111:B4; Sigma Chemical Co., St. Louis, MO) or 0.2 mL PBS (control) and killed 4 hr later. Their lungs were excised and processed for: a) analysis of TNFα levels by Quantikine Immunoassay (R&D Systems, Minneapolis, MN), b) western blot analysis of Ym1 protein, c) preparation of nuclear extract

for NF-κB DNA-binding analysis by EMSA, and d) fixation in formalin for immunohistochemical analysis of tissue sections for localization of the NF-κB subunit p65 as described below.

Immunoblotting

The procedure was performed as described previously (24). Primary polyclonal rabbit antibodies against the following antigens were purchased from the following sources: Ym1 from StemCell Technologies (Vancouver, BC, Canada); I-κBα (C-21) and p65 (A), from Santa Cruz Biotechnology (Santa Cruz, CA); actin from Sigma-Aldrich (St. Louis, MO). Rabbit antibodies against the mouse Gprc5a C-terminal peptide were described (20). Mouse monoclonal antibodies against a Myc epitope peptide tag were from Upstate Biothechnology (Lake Placid, NY).

Detection of NF-κB by immunohistochemistry

Histological sections of formalin-fixed and paraffin-embedded lung tissue were incubated with Target Retrieval Solution (pH 6.0, DAKO) then subjected to sequential incubations with rabbit polyclonal antibody against NF-κB p65 (14-6731; eBioscience, San Diego, CA), peroxidase conjugated anti-rabbit antibody (EnVision$^+$ Systems, DAKO) and 3,3-diaminobenzidine.

Electrophoretic mobility shift assay (EMSA)

NF-κB DNA-binding activity in nuclear extracts prepared from lung tissues or cell lines (see below) was examined as described (24). The following oligonucleotides were used for the analysis: wildtype NF-κB binding oligonucleotide; 5'-CGGAAAGTCCCCAGCGGAAAGTCC CTGAT-3'; mutant NF-κB binding oligonucleotide; 5'-CGGAAAGTgagCAGCGGAAAGTGag TGAT-3'.

Isolation, characterization, and maintenance of mouse tracheal epithelial cells and mouse lung adenocarcinoma cells

Epithelial cells were isolated in our laboratory from tracheas dissected from 3-weeks old *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice. The tracheas were minced into 1-mm^3 pieces, which were incubated in a tissue dissociating solution ACCUMAX™ (Innovative Cell Technologies, San Diego, CA). The dissociated cells and tissue fragments were then transferred to PRIMARIA™ tissue culture dishes (BD Biosciences, San Jose, CA) and incubated in AmnioMAX™-C100 medium (Invitrogen, Carlsbad, CA), which facilitated epithelial cell growth. The epithelial cells that have grown in these dishes were detached by trypsin treatment then sub-cultured and grown in keratinocyte-serum-free medium (K-SFM) (GIBCO; Invitrogen, Grand Island, NY) and aliquots were frozen in liquid nitrogen. The epithelial cells were designated *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$, respectively. The cells were karyotyped by G banding in the MD Anderson Institutional Molecular Cytogenetics Facility and found to be of mouse origin. For analysis of mutations in Trp53 and Kras, DNA was purified from the cells using reagents from Qiagen Inc., (Valencia, CA). The analysis was performed by Genewiz Inc (South Plainfield, NJ). PCR primers designed by the company were used to amplify exons 1 and 2 of the mouse Kras gene and exson's 5, 6, 7, and 8 of the

mouse Trp53 for sequence comparisons between *Gprc5a*$^{-/-}$ cell and *Gprc5a*$^{+/+}$cell. Twenty-five nanograms of genomic DNA were suspended in 25 μl polymerase chain reaction (PCR) buffer, containing 25 mM $MgCl_2$, 5 μM specific primer pairs, 10 mM dNTPs, and 0.5 μl Taq polymerase (HotStar Taq: Qiagen). DNA was sequenced using an Applied Biosystems 3730×l DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The same primers used for PCR amplification were also used for sequencing. The mouse lung cell line 959(−/−) was derived from an adenocarcinoma tumor in the lung of a 2 years old *Gprc5a*$^{-/-}$ male mouse using methods described before (20).

Cell proliferation assay

Cells were seeded in replicate wells of 96-well plates and grown for 1 to 5 days. The final cell number was estimated using the colorimetric Thiazolyl Blue Tetrazolium Bromide (MTT) viability assay.

mRNA analysis and real-time PCR

RNA extracted from lung epithelial cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH) was reverse transcribed into cDNA by RETROscript first-strand synthesis Kit (Ambion, Austin, TX). The cDNAs were subjected to quantitative PCR (QPCR) using primers for QPCR and TaqMan Gene Expression Master Mix from A&B Applied Biosystems (Austin, TX). Mouse actin was used as an internal control gene. The expression data was analyzed and normalized to actin using the 7500 Fast System Software from Applied Biosystems. For Duplex RT-PCR, cDNA was amplified with the Gprc5a primers (20) plus β-actin competition primers from Ambion in high-fidelity PCR master mix (Roche, Indianapolis, IN).

Cell survival in suspension and fluorescence-activated cell sorting analyses

Lung epithelial cells were suspended in K-SFM medium and dispensed into 6-well plates coated with the non-adhesive polymer Poly-(2-hydroxyethylmethacrylate) [PolyHEMA; Sigma-Aldrich] to prevent cell attachment and induce anoikis (25). After 24 or 48 hours, cells were harvested, fixed, stained with propidium iodide, and subjected to fluorescence-activated cell sorter analysis (Coulter EPICS Profile II Flow Cytometer, Miami, FL). The Sub-G1 cell population was considered to represent dead cells.

Immunofluorescent staining for p65 localization

Lung epithelial cells were cultured on glass coverslips, fixed with formaldehyde, permeabilized and incubated with monoclonal antibody to p65 (eBioscience) followed by FITC-conjugated second antibody (Molecular Probes, Eugene, OR). Cells were analyzed using a fluorescence microscope and digital images of FITC staining were captured.

Macrophage migration analysis

For these studies, we used the mouse alveolar macrophage-like cell line MH-S, a continuously proliferating cell line of simian virus 40-transformed alveolar macrophages isolated by bronchoalveolar lavage from Balb/c mice (26). These cells have been chosen because they express many phenotypic and functional characteristics of freshly isolated

alveolar macrophages (26, 27) and have been used previously to assess macrophage migration (28).

The cells were propagated in RPMI 1640 with 10% fetal bovine serum but were starved for 24 hour in serum-free RPMI 1640 medium before the migration assay. For the assay, 6000 cells suspended in 150 μl serum-free RPMI 1640 medium were placed in the upper insert of a Boyden Chamber (BD-Falcon), which has at its bottom a polyethylene terephthalate membrane with 8 μm pores (Becton-Dickinson, Franklin Lakes, NJ) and the inserts were placed inside wells of a 24-well plate (BD-Falcon) containing serum-free conditioned media isolated from 48-hour cultures of mouse *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ lung epithelial cells. Twenty four or 48 hr later, the inserts were fixed in 90% ethanol and the MH-S cells remaining in the upper surface of the membrane were removed using a cotton tip swab and cells that had migrated to the underside of the membrane were stained with 1% crystal violet, and the membrane was observed under the microscope for detection, counting and photography of migrated cells.

In exploratory experiments, we found that the migration of the MH-S cells after 24 hours incubation with conditioned medium from *Gprc5a*$^{-/-}$ lung epithelial cells was low <40 cells/microscopic field but the number of migrating cells increased to about 400 cells/field after 48 hours. After determining that the serum-free conditioned media of either *Gprc5a*$^{+/+}$ cells or *Gprc5a*$^{-/-}$ cells did not enhance the growth of the MH-S cells during a 48-hour migration assay, we performed all migration experiments for 48 hours.

Silencing genes by transfection of cells with siRNAs

siRNAs against p65 (Rel A mouse ON-TargetPlus SMARTpool,; Catalog # L-040776-00), against Gprc5a (Catalog # 057339), or nonspecific siRNA (siR-NS) were from Dharmacon (Lafayette, CO). Transfection was performed using X-tremeGENE siRNA transfection reagent (Roche, Indianapolis, IN). The medium was then harvested and centrifuged and the supernatant was used for macrophage migration assay. Some replicate plates were used for harvesting and processing cells for immunoblotting, EMSA, and/or RT-PCR analysis of selected cytokines and chemokines.

Differential expression of NF-κB target genes

We used data on global gene expression obtained previously by Affymetrix GeneChip® Mouse Genome 430 2.0 array analysis of RNA isolated from *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ epithelial cells.[3] The differential expression of genes reported to be targets of NF-κB (http://bioinfo.lifl.fr/NF-κB/) was derived from the microarray data based on the p-value of a random variance two-sample t-test with permutation with estimation of the false discovery rate and a fold difference in expression. Only genes with *Gprc5a*$^{-/-}$/*Gprc5a*$^{+/+}$ fold change >2 were selected for presentation.

Statistical analysis

Differences in tumor incidence and inflammation between *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ were analyzed for statistical significance by a two-sided Fisher's exact test using the R 2.6.0 statistical package (http://www.r-project.org). Differences between expression of the

macrophage marker F4/80 and the endothelial cell marker CD31 in adenomas and adenocarcinomas as well as expression of chemokine and cytokine in *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells with or without transfection of SiRNA or *Gprc5a* and induction of macrophage migration by conditioned media were analyzed for statistical significance using the two-tail Student's *t* test. P-values <0.05 were considered to be statistically significant.

Results

Increased macrophage infiltration into lungs of *Gprc5a*$^{-/-}$ mice compared with *Gprc5a*$^{+/+}$ mice and its association with tumor incidence

Observation of H&E-stained histological sections of lung specimens from *Gprc5a*$^{+/+}$ (wildtype) mice and *Gprc5a*$^{-/-}$ (knockout) mice collected in our previous study (20) revealed the presence of acidophilic macrophage pneumonia (AMP) associated with lung tumors (both adenoma and adenocarcinomas) (Fig. 1A). To relate the presence of inflammatory cells to tumor development, we examined their incidence and association. Fig. 1B shows that 24/38 of the *Gprc5a*$^{-/-}$ mice developed adenomas and 8/38 developed adenocarcinomas, whereas only 5/51 wildtype mice developed adenomas and none (0/51) developed an adenocarcinoma ($p=10^{-6}$ and 0.0007, respectively when compared to *Gprc5a*$^{-/-}$ mice). The incidence of AMP was significantly (p=0.003) higher in the *Gprc5a*$^{-/-}$ (17/38, 45%) than in the *Gprc5a*$^{+/+}$ mouse lungs (4/51, 7.8%) (Fig.1B). Notably, in the *Gprc5a*$^{-/-}$ mice, AMP was associated with 10/24 (41.6%) adenomas and with 7/8 (87.5%) adenocarcinomas (Fig. 1B, right panel), whereas none of the adenomas in the *Gprc5a*$^{+/+}$ mice was associated with AMP (Fig. 1B left panel).

In the *Gprc5a*$^{+/+}$ mice, 6/51 had lymphoid proliferation compared with 2/38 in the knockout mice and there was no relationship between lymphoid proliferation and tumor development. Immunohistochemical analysis of sections of one lymphoid nodule that was available from a *Gprc5a*$^{-/-}$ mouse lung revealed that most of the lymphocytes (>80%) were positive for CD45R (commonly expressed on all B and subset of memory T lymphocytes) and 5 to 10% were positive for CD4 (expressed on T cells that bind epitopes in class I histocompatibility molecules) and CD8 (expressed on T cells that bind epitopes in class II histocompatibility molecules) (data not shown).

Further immunohistochemical analysis of specimens from *Gprc5a*$^{-/-}$ mice including 10 adenomas (from 10 mice) and 8 adenocarcinomas (from 7 mice) using antibodies against F4/80, a marker of mature tissue macrophages (29), revealed that all those specimens had large numbers of F4/80^{+} cells located in the periphery of tumors and fewer macrophages that had infiltrated into the center of the lesions (Fig. 1C, four panels on bottom left). There was no statistically significant difference between adenomas and adenocarcinomas in the prevalence of F4/80^{+} cells in the periphery or within the tumors (Fig. 1C, bar graphs on bottom right).

Because inflammation can affect angiogenesis, we compared microvessel density (MVD) by quantitation of CD31^{+} cells in histological sections of lung tumors. No statistically significant differences in MVD were found between 6 adenomas and 6 adenocarcinomas (Mean±SD; 26.08±5.08 and 30.71±4.83 vessels/microscope field, respectively) or between 6

tumors with no or low grade (0 and 1) AMP and 6 tumors with higher grade AMP (3 and 4) (30.50±8.61 and 26.29±5.70 vessels/field, respectively). However, MVD in 4 cases with the most severe AMP (grade 4) was significantly lower than in 8 cases with lower grade AMP ≤ 3 (18.50±3.58 and 33.34±3.77 vessels/field, respectively; p=0.032, unpaired t test).

An increased inflammatory status in *Gprc5a*$^{-/-}$ mouse lungs

To begin to understand the mechanism underlying the preferential infiltration of macrophages into *Gprc5a*$^{-/-}$ mouse lungs, we compared the levels of 40 pro-inflammatory chemokines and cytokines in normal lung tissues of 5-month-old *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ mice. This age has been chosen because it precedes the appearance of tumors by 6–7 months. The levels of 23 cytokines and chemokines were higher by 20% to 515% and one was lower by 36% in the lungs of *Gprc5a*$^{-/-}$ relative to lungs of Gprc5a$^{+/+}$ mice (Fig. 2A). With the exception of Il-5, all other 23 factors can be produced by monocytes/macrophages (http://www.copewithcytokines.de/cope.cgi?key=monocytes). Furthermore, 18 of these 24 factors (marked with an asterisk) are known targets of the transcription factor NF-κB, whereas 9 (marked with a pound sign) are NF-κB activators.

To determine whether *Gprc5a*$^{-/-}$ mice respond differently than *Gprc5a*$^{+/+}$ mice to inflammatory challenge, we treated both types of mouse with LPS, which stimulates the production of TNFα by macrophages. Although low, the constitutive TNFα level in lung tissue of *Gprc5a*$^{-/-}$ mice was 3-fold higher than in *Gprc5a*$^{+/+}$ mice. LPS treatment increased the level of TNFα in both mouse genotypes by about 3 fold (Fig. 2B). Moreover, LPS treatment increased the level of Ym1, a chitinase-like secretory protein that is induced in alternatively activated macrophages (M2) regulated by IL13 during inflammation, in the lungs of *Gprc5a*$^{-/-}$ mice but not in *Gprc5a*$^{+/+}$ mice as indicated by western blotting (Fig. 2B, bottom left).

To determine whether LPS treatment also activated NF-κB differentially in lungs of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice, we used EMSA to analyze nuclear extracts of total lung homogenates prepared 4 hours after intraperitoneal injection of LPS. Fig. 2C shows that LPS induced NF-κB binding to DNA consensus sequence in the lungs of *Gprc5a*$^{-/-}$ to a much higher level than in *Gprc5a*$^{+/+}$ mice. While the cell type(s) in which this increased NF-κB activation has taken place is not clear because we prepared nuclear extracts from whole lung homogenates, an immunohistochemical analysis of lung sections for p65 localization 4 hours after LPS treatment showed more extensive translocation of cytoplasmic p65 protein into the nuclei of bronchiolar epithelial cells in lungs of *Gprc5a*$^{-/-}$ mouse than in *Gprc5a*$^{+/+}$ mouse lungs (Fig. 2D). These results show that deletion of the *Gprc5a* gene enhances NF-κB activation by LPS in mouse lungs in vivo and that at least some of the activation occurs in lung epithelial cells.

Enhanced in vitro proliferation and survival of lung epithelial cells isolated from tracheas of *Gprc5a*$^{-/-}$ compared to *Gprc5a*$^{+/+}$ mouse lungs

Because the lungs contain multiple cell types, both epithelial and mesenchymal, that interact with each other through direct contact or via soluble secreted factors, we wondered whether the loss of *Gprc5a* in epithelial cells leads to aberrant NF-κB activation also when the cells

are in a homogeneous culture in vitro. Therefore, we established epithelial cell lines from lung tracheas. Both *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cultured cells exhibited an epithelial morphology (Fig. 3A), however their growth patterns were distinct. The *Gprc5a*$^{+/+}$ cells were contact inhibited after reaching a confluent state and most of the cultures had undergone senescence after 5–7 passages. In contrast, the *Gprc5a*$^{-/-}$ cells continued to proliferate post-confluence and reached high densities. The expected differential expression of *Gprc5a* mRNA and protein in the cultured cells was demonstrated by RT-PCR and immunoblotting (Fig. 3B). However, no differences were found between the two cell lines in the expression of the NF-κB subunit p65 or the NF-κB inhibitor IκBα (Fig. 3B).

The *Gprc5a*$^{+/+}$ cells required seeding at high initial density (e.g., 8,000 cells per well) in order to proliferate even if slowly (Fig. 3C). In contrast, the *Gprc5a*$^{-/-}$ cells were able to proliferate even when seeded at a lower density and then continued to grow indefinitely without undergoing senescence, thus behaving as spontaneously immortalized cells.

The above cells also exhibited differential sensitivity to induction of anoikis by denial of cell attachment. Specifically, 36% and 69% of *Gprc5a*$^{+/+}$ cells have died after incubation in PolyHEMA-coated tissue culture dishes for 24 and 48 hours, respectively compared to only 4.5% and 36% death, respectively in *Gprc5a*$^{-/-}$ cell cultures (Fig. 3C, bottom left). These results indicated that *Gprc5a*$^{-/-}$ cells possess a higher proliferative capacity and higher resistance to anoikis than *Gprc5a*$^{+/+}$ cells. However, despite their ability to survive for a limited time under anchorage-independent conditions in liquid medium, the *Gprc5a*$^{-/-}$ cells had failed to form colonies in semisolid agar or to form tumors when injected either subcutaneously or into the tail vein of syngeneic mice indicating that they are immortal but not transformed (data not shown). Indeed, no mutation were found in Trp53 (exons 5, 6, 7, or 8) or the Kras gene (exons 1, 2).

Global gene expression analysis of the *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ cells performed as a part of a separate study[3] provided an opportunity to mine the data for genes that are known targets of NF-κB. Fig. 3D shows that the expression of the NF-κB target genes Cxcl5, Ccl28, Csf1, C3, Ccnd1, Thbs1, Cxcl1, Lamb2, and Nfκbiz was 2- to 45-fold higher in the *Gprc5a*$^{-/-}$ cells than in the *Gprc5a*$^{+/+}$ cells. In contrast, the expression of markers of squamous cell differentiation including Krt1, Tgm1, lor and Ivl was reduced in the *Gprc5a*$^{-/-}$ cells compared with the *Gprc5a*$^{+/+}$ cells by 2.8- to 18-fold (Fig. 3D).

Increased NF-κB activation in cells cultured from *Gprc5a*$^{-/-}$ mouse lung

Immunofluorescent staining with anti-p65 antibodies (Fig. 4A) demonstrated that without any treatment, p65 was localized primarily in the cytoplasm of *Gprc5a*$^{+/+}$ cells (Fig. 4A, upper left panel), whereas most *Gprc5a*$^{-/-}$ cells showed both cytoplasmic and nuclear localization (Fig. 4A, upper right panel), Exposure to TNFα induced p65 translocation from the cytoplasm to the nucleus in *Gprc5a*$^{-/-}$ cells to a greater extent than in *Gprc5a*$^{+/+}$ cells (Fig. 4A, lower panels). TNFα treatment also exerted different effects on the sensitivity of the cells to anoikis. Whereas TNFα increased slightly the induction of anoikis in suspended *Gprc5a*$^{+/+}$ cells, the same treatment reduced the already lower anoikis in *Gprc5a*$^{-/-}$ cells (Fig. 4A; bottom left bar graph).

Examination by EMSA of nuclear extracts of untreated, TNFα-treated, and LPS-treated *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells revealed that the basal NF-κB DNA-binding activity was much higher in *Gprc5a*$^{-/-}$ than in *Gprc5a*$^{+/+}$ cells (Fig. 4B, lanes 4 and 1, respectively) and that TNFα increased NF-κB activation in *Gprc5a*$^{+/+}$ cells to a level comparable to the basal level in untreated *Gprc5a*$^{-/-}$ cells (Fig. 4A, lanes 2 and 4). A similar TNFα treatment of *Gprc5a*$^{-/-}$ cells increased NF-κB activation above the basal already level (Figure 4A, lanes 4 and 5); Furthermore, whereas LPS treatment failed to activate NF-κB in *Gprc5a*$^{+/+}$ cells, it increased NF-κB activity in *Gprc5a*$^{-/-}$ cells to a level similar to TNFα treatment (Fig. 4B, lanes 6 and 3, respectively). The specificity of the DNA-binding activity of NF-κB in the EMSA assay was confirmed by competition with access cold oligonucleotide and supershifting with anti-p65 antibodies (Fig. 4B, lanes 7–9).

To complement these loss of function data with gain of function results, we generated stable transfectants of 959(−/−) adenocarcinoma cell line using a Myc-tagged *Gprc5a* expression vector or a vector control (Fig. 4C) and compared NF-κB activation by TNFα in these cells by EMSA. Figure 4D shows that expression of *Gprc5a* suppressed the basal NF-κB activity (compare lanes 1 and 3) and inhibited NF-κB activation by TNFα (Fig. 4D, lanes 2 and 4).

Enhanced induction of macrophage migration by medium conditioned by *Gprc5a*$^{-/-}$ compared to *Gprc5a*$^{+/+}$ cells

Because many NF-κB target genes play essential roles in inflammation (16, 17) and some mediate macrophage recruitment, we analyzed by quantitative PCR the expression of 8 such genes including 4 (Ccl28, Csf1, Cxcl1, and Cxcl5) that have been found to be upregulated in *Gprc5a*$^{-/-}$ cells by the global gene expression analysis (Fig. 3D). Fig. 5A shows that the levels of the mRNAs of all 8 genes were elevated in the *Gprc5a*$^{-/-}$ compared to *Gprc5a*$^{+/+}$ epithelial cells by 3 to 70 fold. Because some of these cytokines (e.g., Ccl2, Ccl5, Csf1) are known to promote macrophage recruitment in vivo, we examined the effects of conditioned serum-free medium from cultures of epithelial cells on the migration of mouse alveolar macrophage-like cell line MH-S and found that the medium of *Gprc5a*$^{-/-}$ cells induced the migration of 4 times as many macrophages as the medium of *Gprc5a*$^{+/+}$ cells (Fig. 5B). To assess whether Gprc5a was responsible for the observed differential induction of macrophage migration, we silenced *Gprc5a* expression by siRNA. The conditioned medium of *Gprc5a*$^{+/+}$ cells in which the gene was silenced exhibited an increased ability to induce macrophage migration (Fig. 5C, 5D).

Silencing of NF-κB p65 in *Gprc5a*$^{-/-}$ cells partially reverses their phenotype

To determine whether the increased expression of macrophage chemotactic factors was actually mediated by NF-κB in *Gprc5a*$^{-/-}$ cells, we silenced p65 by transient transfection with specific siRNA (Fig. 6A, western blot). The suppression of NF-κB increased the sensitivity of the *Gprc5a*$^{-/-}$ cells to anoikis (Fig. 6A, bar graph) and also decreased the expression of several chemokines including Ccl2, Ccl28, Csf1, and Cxcl10 (Fig. 6B). Small but statistically insignificant decreases were also noted in the levels of Cxcl5 and Ccl5. Importantly, the silencing of p65 decreased by 3-fold the ability of the conditioned medium of *Gprc5a*$^{-/-}$ cells to induce macrophage migration (Fig. 6C).

Discussion

Recently, we demonstrated that *Gprc5a*$^{-/-}$ mice develop spontaneous lung tumors after a lag period lasting from 12 and 24 months (20). The reason for this prolonged time was not clear but we surmised that the loss of *Gprc5a* is not sufficient by itself for full transformation of the epithelial cell precursors of the tumors. However, the nature of the additional changes that presumably are needed for tumor development and what drives them during the latent period remained unknown. In this study, we found by retrospective analysis of histological tumor specimens from the above study that many of the tumors in the *Gprc5a*$^{-/-}$ mouse lungs, especially the adenocarcinomas, were surrounded and infiltrated by macrophages. Because the migration of macrophages into the microenvironment of tumors is a frequent event and tumor-associated macrophages are known to secrete inflammatory factors that can promote lung tumor development and progression (13), we thought that the loss of *Gprc5a* may be causally related to the attraction of macrophages into the lungs of knockout mice and that once there the macrophages create an inflammatory microenvironment providing the conditions for transformation and promotion of carcinogenesis (30).

By comparing and contrasting constitutive and TNFα- or LPS-induced inflammation related proteins and transcripts using tissues and epithelial cell lines derived from lung tissues of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice at an early age before tumors develop, as well as lung tumor specimens, we obtained data that support, at least partially, the model presented in Fig. 6D. According to this model, the loss of *Gprc5a* in lung epithelial cells triggers at least three processes, one intrinsic that leads to cell autonomy, and the other two, extrinsic that create an inflammatory, angiogenic and protumorigenic microenvironment. We further propose that these processes cooperate to promote oncogenesis. The schema in Fig. 6D is “idealized” in that it depicts a series of steps in the process (numbered in Fig. 6D) although specimens used to generate our in vivo data were collected at specific time points and do not represent the dynamics of the processes involved and many of the steps may be concurrent rather than sequential.

We propose that *Gprc5a* loss (Fig. 6D, step 1) leads to lung carcinogenesis by the following interrelated steps: NF-κB activation and increased expression of target genes in the lung epithelial cells (Fig. 6D, step 2) that enhance proliferation potential, survival, and immortalization of the epithelial cells (Fig. 6D, steps 3 and 4). The secretion of NF-κB-regulated chemokines and cytokines by the *Gprc5a*$^{-/-}$ lung cells can lead to recruitment of macrophages (Fig. 6D, step 5) and to induction of angiogenesis (Fig. 6D, step 6). The macrophages produce and secrete into the microenvironment a variety of cytokines, chemokines and growth factors (Fig. 6D steps 7–9) that can enhance angiogenesis (Fig. 6D, step 7) and also act directly on the lung epithelial cells to enhance the development of adenomas and their progression to adenocarcinomas (Fig. 6D, steps 9–11). The finding that silencing of p65 in *Gprc5a*$^{-/-}$ cells partially decreased their resistance to anoikis and their ability to stimulate macrophage migration indicated that NF-κB activation might mediate many of the phenotypic characteristics that distinguish the *Gprc5a*$^{-/-}$ cells from their wildtype counterparts. It is important to note that we cannot exclude the possibility that some of the phenotypic characteristics of the cultured *Gprc5a* lung cells may have been

acquired by unintended selection during the in vitro establishment of the cell line from primary tracheal cells.

It is noteworthy that Cxcl10 produced by the *Gprc5a*$^{-/-}$ lung cells can potentially inhibit angiogenesis. In addition, some of the cytokines identified in the lung homogenates of *Gprc5a*$^{-/-}$ mice can suppress tumorigenesis (indicated in Fig. 6D in blue fonts) while other factors can exert both pro- and anti-tumorigenic effects depending on their concentration. Many of these factors could have been produced by macrophages with distinct properties ranging from the classically activated M1 to the alternatively activated M2, which are known to produce factors with potentially opposing effects (31). The increase in the level of the M2 macrophage marker Ym1 (chitinase) protein observed after LPS treatment in the lungs of *Gprc5a*$^{-/-}$ but not of *Gprc5a*$^{+/+}$ mice indicates that more protumorigenic M2 macrophages can be attracted to lungs of LPS-treated *Gprc5a*$^{-/-}$ mice or that alveolar macrophages already present in the lungs of such mice are more readily differentiated into M2 macrophages. The presence of different cell types and cytokines with potentially opposing effects is quite common in tumor microenvironments and it is the spatiotemporal balance of the proangiogenic and antiangiogenic factors and the protumorigenic and antitumorigenic factors in specific sites that eventually determines whether tumors will develop or not (12).

The exact mechanisms by which the macrophages and the inflammatory microenvironment enhance carcinogenesis in our *Gprc5a* knockout mouse model are not clear. Tumor-associated macrophages are known to release a plethora of growth factors, cytokines, chemokines and enzymes and reactive oxygen intermediates that enhance epithelial cell growth as well as causing genetic instability by DNA damage, and increase angiogenesis and cell invasiveness (13, 17, 31–33).

Although inflammation is known to increase angiogenesis (32, 34), we found no enhancing effect of low and moderate grade of AMP on angiogenesis in adenomas and adenocarcinomas from *Gprc5a*$^{-/-}$ mice, whereas the high grade AMP actually inhibited angiogenesis. A likely explanation is that high levels of proangiogenic Cxcl5, Cxcl1, and Ccl2 released by the *Gprc5a*$^{-/-}$ epithelial cells are sufficient to induce and maintain angiogenesis despite the production by the same cells of lower levels of the angiostatic Cxcl10 and independently of any pro- or anti-angioghenic signals from macrophages. Notably, Cxcl5 is the most highly differentially expressed chemokine between *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ epithelial cells. This finding is interesting and relevant to human lung cancer because the human orthologous gene CXCL5 has been previously found to be upregulated in human NSCLC by Cox-2 via NF-κB activation (35) and has been implicated as an angiogenic factor in the development of NSCLCs and as a marker of poor prognosis in NSCLC patients (36).

Our emphasis on the possible role of inflammation stems from the finding that 7/8 adenocarcinomas and 10/24 adenomas were associated with AMP suggesting that the presence of macrophages in the microenvironment may be important for the development of adenomas and for their progression to adenocarcinoma. However, since we have also found that 14/24 adenomas and 1/8 adenocarcinomas had developed without associated

macrophages it appears that at least a subset of adenomas can develop independently of macrophages.

The enhancement of lung carcinogenesis by inflammation has been demonstrated in several mouse models mostly involving exposure to carcinogens (3, 4). The role of lung epithelial cells in the inflammation has not received much attention until recently. The use of targeted expression of mutant Kras in bronchiolar epithelial cells in mouse lungs was found to induce inflammatory response with extensive infiltration of macrophages and neutrophils (37). In this model, the bronchiolar epithelial cells produced some of the same cytokines that were identified in our study, notably Cxcl1 and Cxcl5. However, the early mortality (median survival of 8 weeks) resulting from the robust inflammatory response in the Kras model was not seen in our study and our mice survived for 12 to 24 months. The reason for this difference is not clear.

Our finding that the *Gprc5a*$^{-/-}$ lung epithelial cells have a constitutively active nuclear p65 and are more responsive to NF-κB activation by Tnfα and LPS relative to *Gprc5a*$^{+/+}$ cells is a novel result. The transcription of several NF-κB target genes was also elevated in the *Gprc5a*$^{-/-}$ cells including cyclin D1 that could mediate enhancement of cell proliferation and increased survival under proapoptotic stress. The increased expression of cyclin D1 has been associated with enhanced growth of mouse lung premalignant and tumor cells in vitro and in vivo and has been proposed as a target for chemoprevention (38, 39). Interestingly, targeted p65 overexpression in mouse lung epithelial cells in transgenic mice caused proliferation of these cells in embryo lungs and protected them against apoptosis as did LPS treatment of wild type mice through activation of NF-κB (40).

Not only immune cells but also normal lung airway epithelial cells and lung cancer cells can produce a variety of cytokines, many of which are regulated by NF-κB and contribute to the development of chronic lung inflammation (28, 37, 41, 42). For example, the state of NF-κB activation in bronchioalveolar epithelial cells in several mouse strains was the overriding factor in determining the link between susceptibility to inflammation and lung cancer (43). Direct evidence for the pivotal roles of NF-κB activation in airway epithelial cells in induction of an inflammatory response and in promoting lung tumorigenesis has been demonstrated only recently in an ethyl carbamate (urethane)-induced lung carcinogenesis model (44). Many of the cytokines and chemokines like Tnfα and IL-12 p70 identified in the lungs of our untreated *Gprc5a*$^{-/-}$ mice were also increased in the urethane-treated wild type mice (44). Targeted expression of a dominant inhibitor of NF-κB in the airway epithelial cells in vivo decreased urethane induced inflammation and tumor formation indicating the importance of early activation of NF-κB signaling in airway epithelial cells in urethane induced tumorigenesis (44). A similar approach of in vivo inhibition of NF-κB activation was used recently to demonstrate the importance of NF-κB signaling in a lung carcinogenesis model based on a combination of mutant Kras knock-in and Trp53 deletion without any carcinogen treatment (45). The latter study has shown that NF-κB activation in primary mouse embryo fibroblasts required a concomitant activation of mutant Kras(G12D) and a loss of Trp53 but did not occur in cells with only one of these changes. Restoration of wildtype Trp53 expression in mouse lung adenocarcinoma cells with oncogenic Kras and Trp53 deletion showed inhibition of nuclear localization of the NF-κB subunit p65. The

changes in NF-κB activation depending on Kras and Trp53 status were correlated with apoptosis in vitro and suppression of tumor development and growth in vivo (45). Our model is distinct from the above two in that we achieved NF-κB activation in the lung epithelial cells by deletion of the *Gprc5a* gene and such a deletion also enhanced LPS-induced activation of NF-κB in lung airway cells in vivo. We found no mutations in either Kras or Trp53 in the cultured *Gprc5a*$^{-/-}$ epithelial cells. Thus, it appears that wild type Trp53 does not inhibit NF-κB activation in these cells. However, we cannot exclude a role for aberrations in Trp53 signaling pathway downstream of Trp53 in NF-κB activation in the *Gprc5a*$^{-/-}$ cells because functional and pathway analyses (Ingenuity Pathway Analysis) of the differentially expressed gene features between *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ cells revealed a significant modulation of gene sets associated with Trp53 signaling (Kadara et al., submitted)[3].

The *Gprc5a*$^{-/-}$ mouse model exhibits similarities to several inflammation-related aspects of human lung carcinogenesis. For example, the activation of NF-κB observed in the *Gprc5a*$^{-/-}$ cells and the higher response to LPS in vivo are similar to the reports on increased numbers of cells with nuclear p65 staining in bronchial biopsies taken from smokers with normal lung function or from smokers with chronic obstructive pulmonary disease (COPD), an inflammation driven pathology that increases the risk to develop lung cancer, compared to biopsies from non-smokers (46). Moreover, nearly all of the cytokines and chemokines found to be upregulated in the *Gprc5a*$^{-/-}$ compared to *Gprc5a*$^{+/+}$ cells and the lung homogenates of *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ mice are increased in COPD patients' lungs compared to non-smokers' lungs (47). In addition, aberrant activation of NF-κB has been reported in the majority of human adenocarcinomas and precursor lesions including atypical adenomatous hyperplasia compared to normal epithelium (48).

The mechanism by which the loss of *Gprc5a* in the knockout mouse and in the isolated tracheal epithelial cells leads to activation of NF-κB signaling is not clear. Previously, other G protein coupled receptors (GPCRs) have been found to stimulate inflammation by serving as receptors for a variety of chemokines (49) or by enhancing NF-κB signaling through activation of signaling upstream of NF-κB (50). However, activation of the beta-adrenergic receptor by isoproterenol has been found to suppress NF-κB activation. The mechanism proposed for this effect was enhancement by the GPCR of the stabilizing interaction between β-arrestin2 and the endogenous NF-κB inhibitory protein I-κBα, which prevents the nuclear translocation of cytoplasmic p65 (51). It is possible that Gprc5a inhibits NF-κB activation by a similar mechanism.

In conclusion, we have demonstrated that the loss of the lung-specific tumor suppressor *Gprc5a* leads to activation of NF-κB in lung epithelial cells in vivo and in vitro leading to autonomous cell growth as well as enhancement of inflammatory microenvironment, which appear to contribute to the lung tumorigenesis process in this new model of lung cancer. The model should be useful for assessment of the chemopreventive potential of agents targeting the microenvironment including anti-inflammatory agents, inhibitors of macrophage function and NF-κB inhibitors (9, 10, 52–54).

[3]Kadara H et al., submitted for publication

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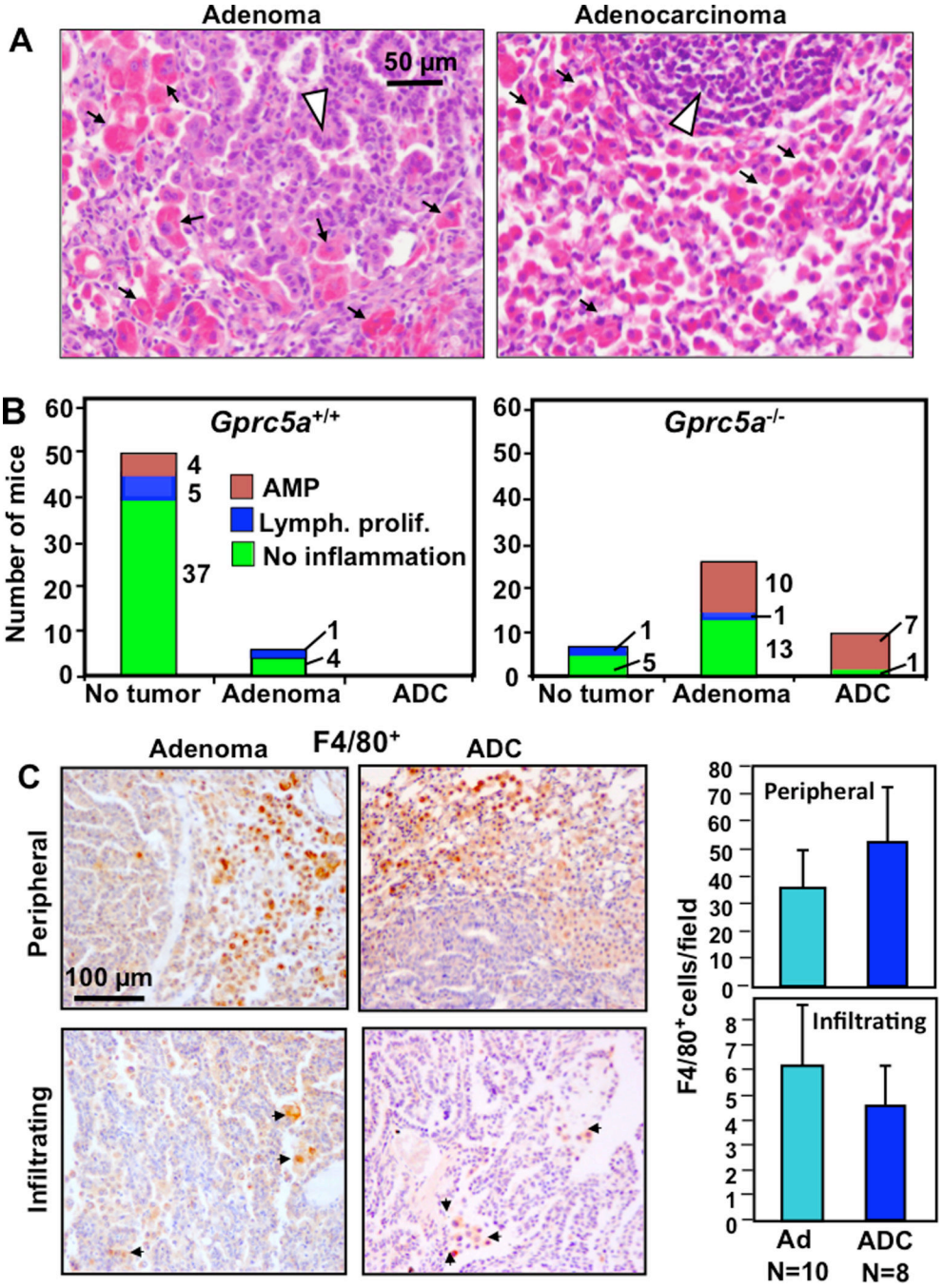


Fig. 1.

Relationships among macrophage pneumonia, lung tumors and angiogenesis. *A*, Photomicrographs of H&E stained sections of an adenoma and an adenocarcinoma showing association of AMP with the epithelial tumor cells. *B*, Quantitative analysis of the association of AMP with lung tumorigenesis. Tumor incidence is presented in relation to presence of inflammatory cells in histological sections of lungs of 51 *Gprc5a*$^{+/+}$ (left panel)) and 38 *Gprc5a*$^{-/-}$ mice (right panel). The numbers near each bar represent mice in the corresponding category. The differences between *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice were

statistically significant (Fisher exact test) for the following: incidence of adenoma and/or adenocarcinoma (ADC), p=1 E-6; adenoma incidence, p=1 E-7; adenocarcinoma incidence, p=0.0007; AMP incidence, p=8 E-5. *C*, Photomicrographs of histological sections of an adenoma and an adenocarcinoma from *Gprc5a*$^{-/-}$ mice stained with F4/80 antibodies for detection of mature macrophages in the periphery and within (infiltrating) tumors. The bar graphs (bottom, right) represent the mean number of F4/80^{+} cells in 10 adenomas (from 10 different mice) and 8 adenocarciomas (from 7 different mice). Note that the values on the Y-axis of the graph showing peripheral F4/80^{+} cells are much higher than in the graph showing data on infiltrating cells.

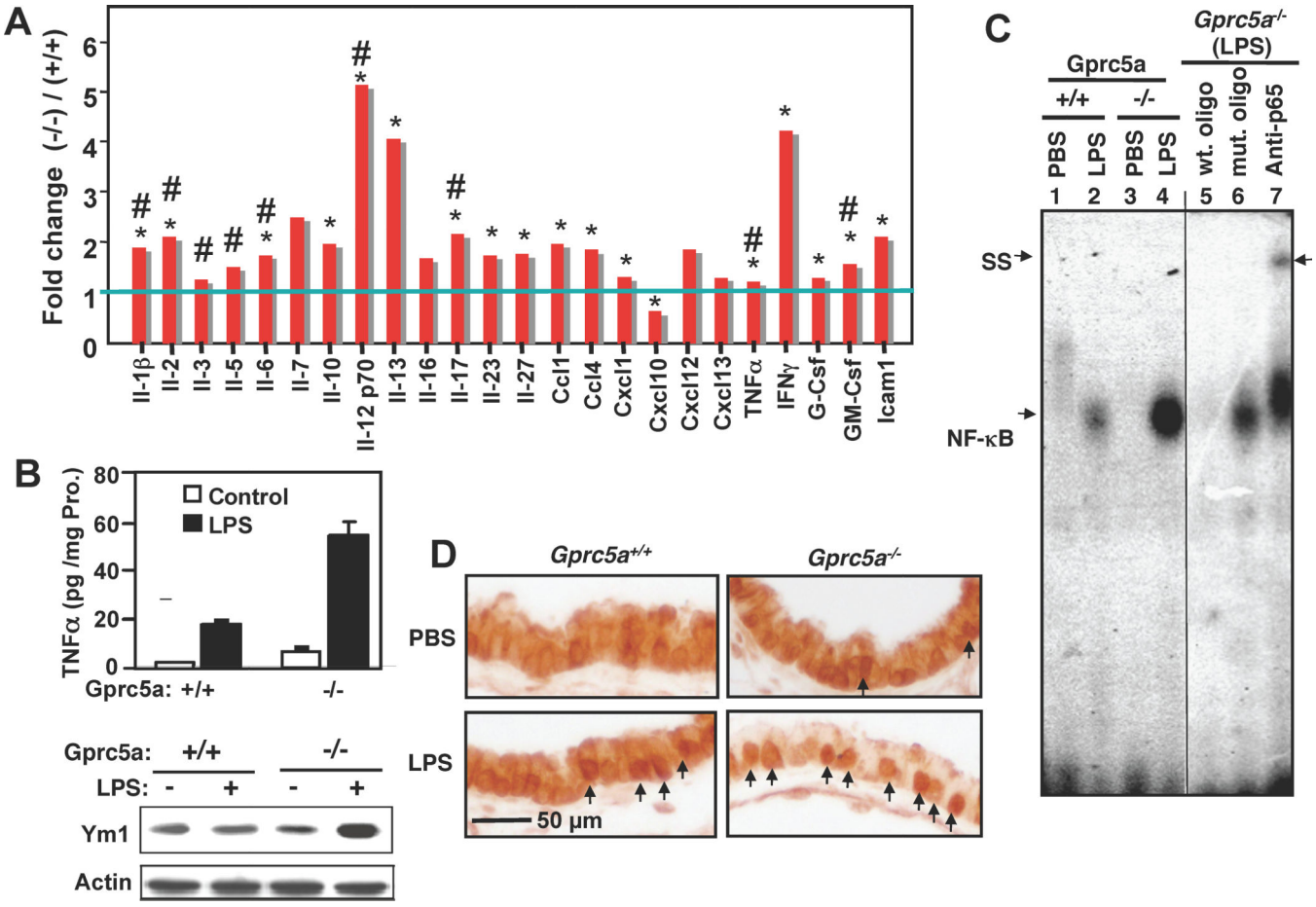


Fig. 2.

Expression of inflammatory cytokines in lung tissues from *Gprc5a*$^{-/-}$ and *Gprc5a*$^{+/+}$ mice and the effects of LPS treatment in vivo on NF-κB activation. *A*, Differential expression of cytokines and chemokines in extracts from *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mouse lung homogenates determined by an immobilized antibody array. The signs * and # above some of the bars represent NF-κB targets and NF-κB activators respectively. In addition to the indicated 24 analytes, the following 16 cytokines and chemokines showed <15% difference between the two samples and were not included in the graph (Il-1α, Il-1ra, Il-4, Ccl2, Ccl3, Ccl5, Ccl11, Ccl12, Ccl17, Cxcl9, Cxcl11, Kc. Timp-1, Term-1, M-Csf, C5a). *B*, TNFα levels in lung tissue homogenates of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice measured by ELISA using samples collected 4 hours after i.p. injection of LPS or PBS (Bar graph). Immunoblot of lung homogenates from control and LPS-treated *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice using antibodies against Ym1 and actin (bottom left). *C*, EMSA for detection of NF-κB DNA-binding proteins in nuclear extracts prepared from lung tissue homogenates of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice treated with PBS or LPS as above (lanes 1–4). Nuclear extracts from lungs of LPS-treated *Gprc5a*$^{-/-}$ mice were also used to analyze the specificity of the EMSA by interference with NF-κB binding to consensus binding site using either wildtype or mutant oligonucleotides as competitors (lanes 5 and 6, respectively). The presence of p65 in the shifted complex was analyzed by adding anti-p65 antibodies to the EMSA reaction to induce a supershifted complex (SS, lane 7). *D*, Photomicrographs of histological sections of

lung tissues collected from *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice 4 hours after i.p. injection of LPS or PBS and stained using antibodies against the p65 subunit of NF-κB to detect its translocation into the nucleus. Arrows point to positive nuclei.

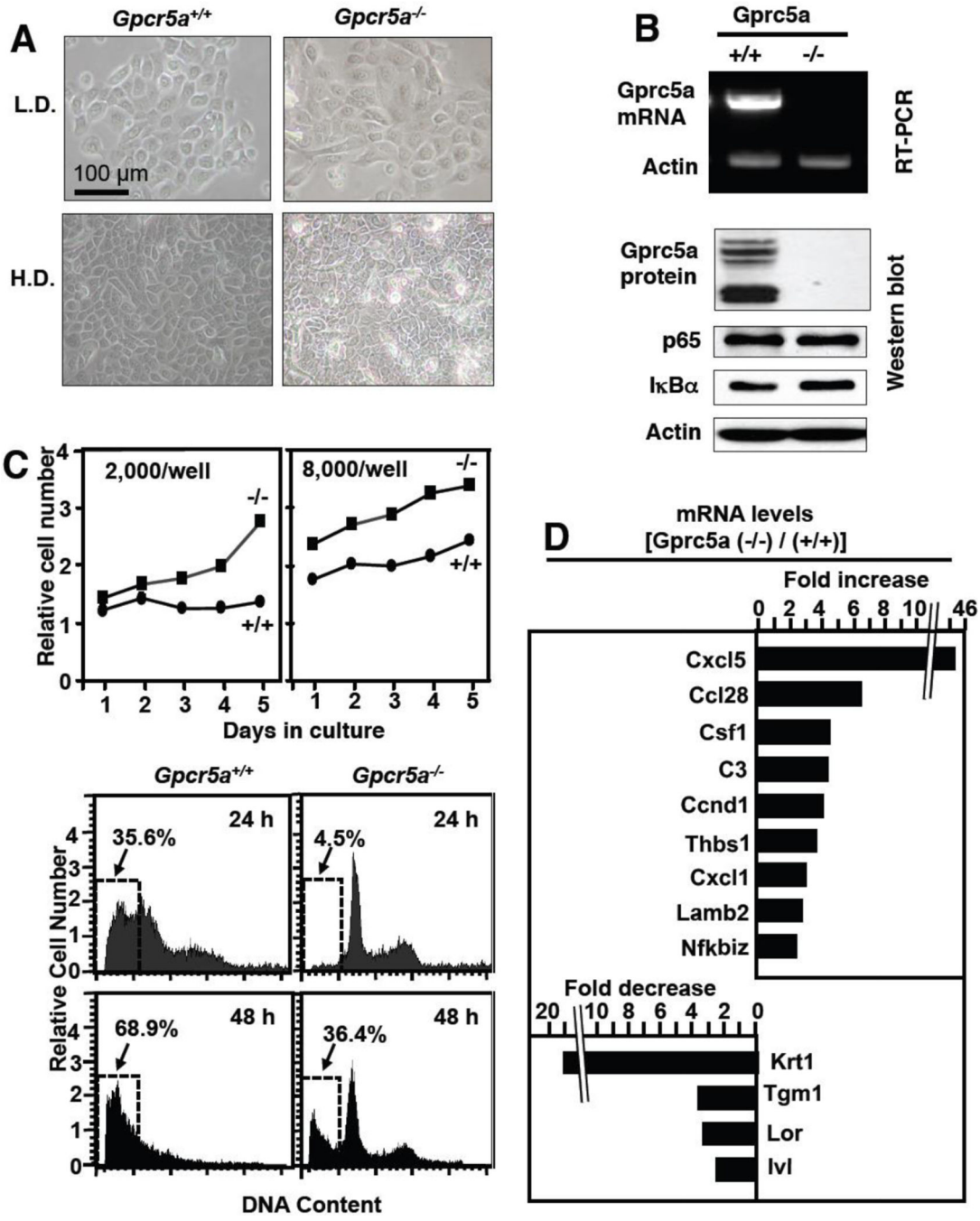


Fig. 3.

Characteristics of epithelial cells cultured from tracheas of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ mice. *A*, Photomicrographs of low density (L.D.) and high density (H.D.) cell cultures taken using a phase contrast microscope. *B*, Expression of *Gprc5a* mRNA (RT-PCR) and protein (Western blot) and NF-κB subunit p65, IκBα, and actin (Western blot) in the above cells. *C*, (line graph) Growth curves of *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells seeded in 96-well plates at 2 × 10^3 and 8 × 10^3 cells per-well and analyzed daily for 5 days using a MTT assay to estimate cell numbers. Each data point represents the mean±SD of triplicate cultures. *C* (Bottom,

left), FACS analysis of DNA content of cells stained with propidium iodide 24 and 48 hours after suspending them over PolyHEMA-coated tissue culture wells to induce anoikis. The sub-G1 cell population is indicated by dotted line boxes. The numbers above the boxes indicate the percentage of dead cells within the cell population. *D*, Differential expression of NF-κB target genes (upper panel) and squamous differentiation markers (lower panel) identified by mining our data on gene expression microarray analysis of mRNA from the above cells.[3] The full names of the genes and the p value for the significance of the difference in their expression level (univariate t-test with random variance) in *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells are as follows: Cxcl5, chemokine (C-X-C motif) ligand 5 ($p<10^{-7}$); Ccl28, chemokine (C-C motif) ligand 28 ($p<10^{-7}$); Csf1, colony stimulating factor 1 (macrophage) ($p<10^{-7}$); C3, complement component 3 ($p= 9\times10^{-7}$); Ccnd1, cyclin D1 ($p<10^{-7}$); Thbs1, thrombospondin 1 ($p= 6\times10^{-7}$); Cxcl1, chemokine (C-X-C motif) ligand 1 ($p=2.3\times10^{-6}$); Lamb2, laminin, beta 2 ($p=2.04\times10^{-5}$); Nfkbiz, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta ($p=4\times10^{-7}$); Krt1, keratin 1 ($p<10^{-7}$); Tgm1, Transglutaminase I ($p=9\times10^{-6}$), Lor, loricrin ($p=9\times10^{-7}$); Ivl, involucrin ($p=6\times10^{-7}$).

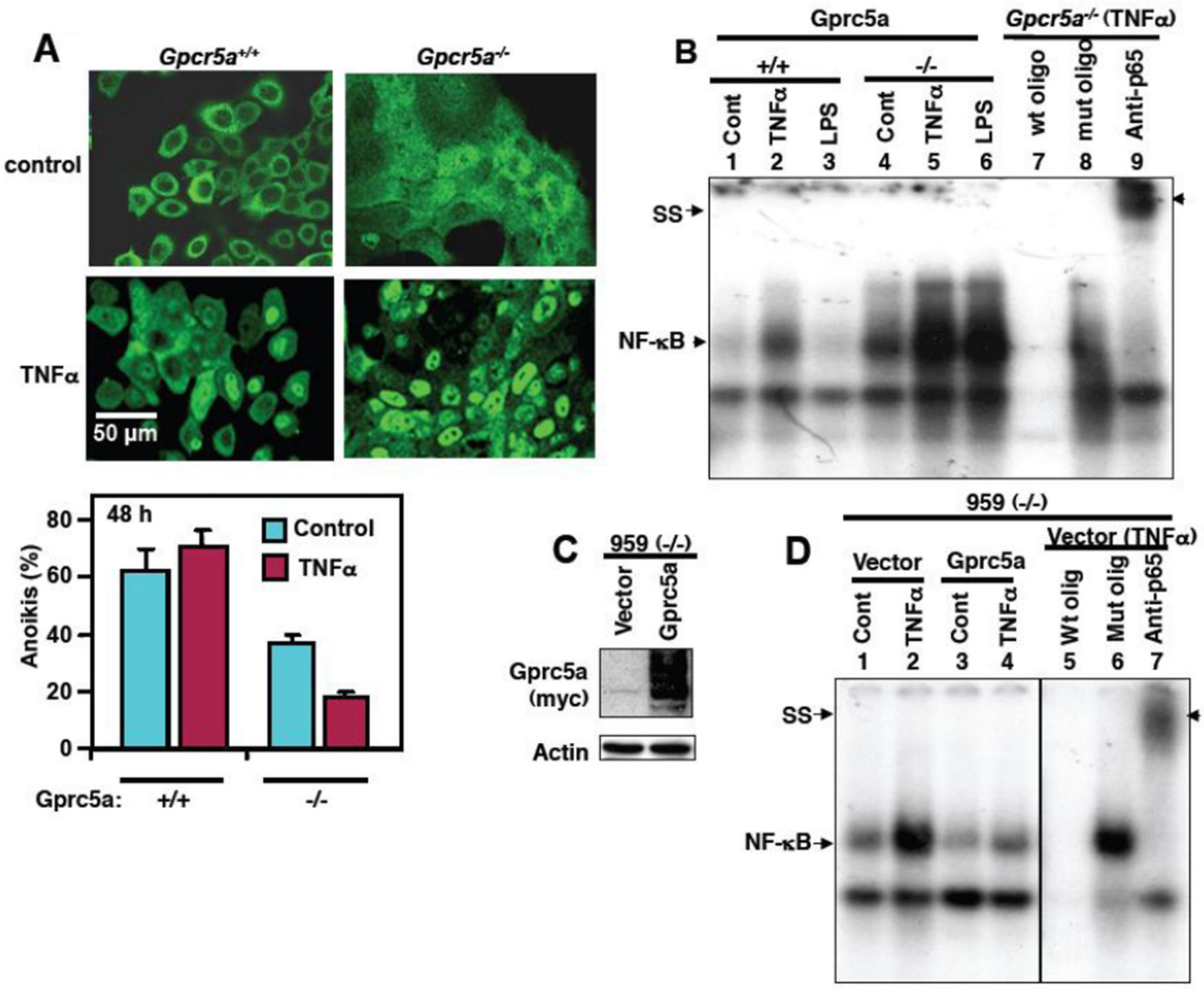


Fig. 4.

Differential activation of NF-κB in *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells. *A*, Localization of NF-κB subunit p65 in *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells after a 30-min exposure to TNFα (5 ng/ml) or control medium by immunofluorescent staining using anti-p65 primary antibodies and FITC-labeled secondary antibodies. *(Bottom left bar graph)*, Anoikis was analyzed as described in Fig. 3*C* above in *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells treated with control medium or medium with TNFα (5 ng/ml) for 48 hours in suspension over PolyHEMA-coated tissue culture wells. *B*, NF-κB activation in normal *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ lung cells detected by EMSA using nuclear extracts from cells harvested 30 minutes after treatment of with TNFα (5 ng/ml), LPS (1 μg/ml) or control medium (Cont). Competition for NF-κB DNA binding using cold wildtype (wt) or mutant (mut.) oligonucleotides (oligo) of the NF-κB-binding sequence (lanes 7 and 8). The ability of anti-NF-κB p65 antibodies to supershift (SS) the NF-κB complex is shown in lane 9. *C*, 959(−/−) adenocarcinoma cells were transfected with either vector only or a Myc-tagged *Gprc5a* expression vector and the expression of the protein was confirmed by western blotting. *D*, NF-κB DNA-binding

activities in transfected 959(−/−) cells detected by EMSA after treatment with TNFα or control medium as in *B* above.

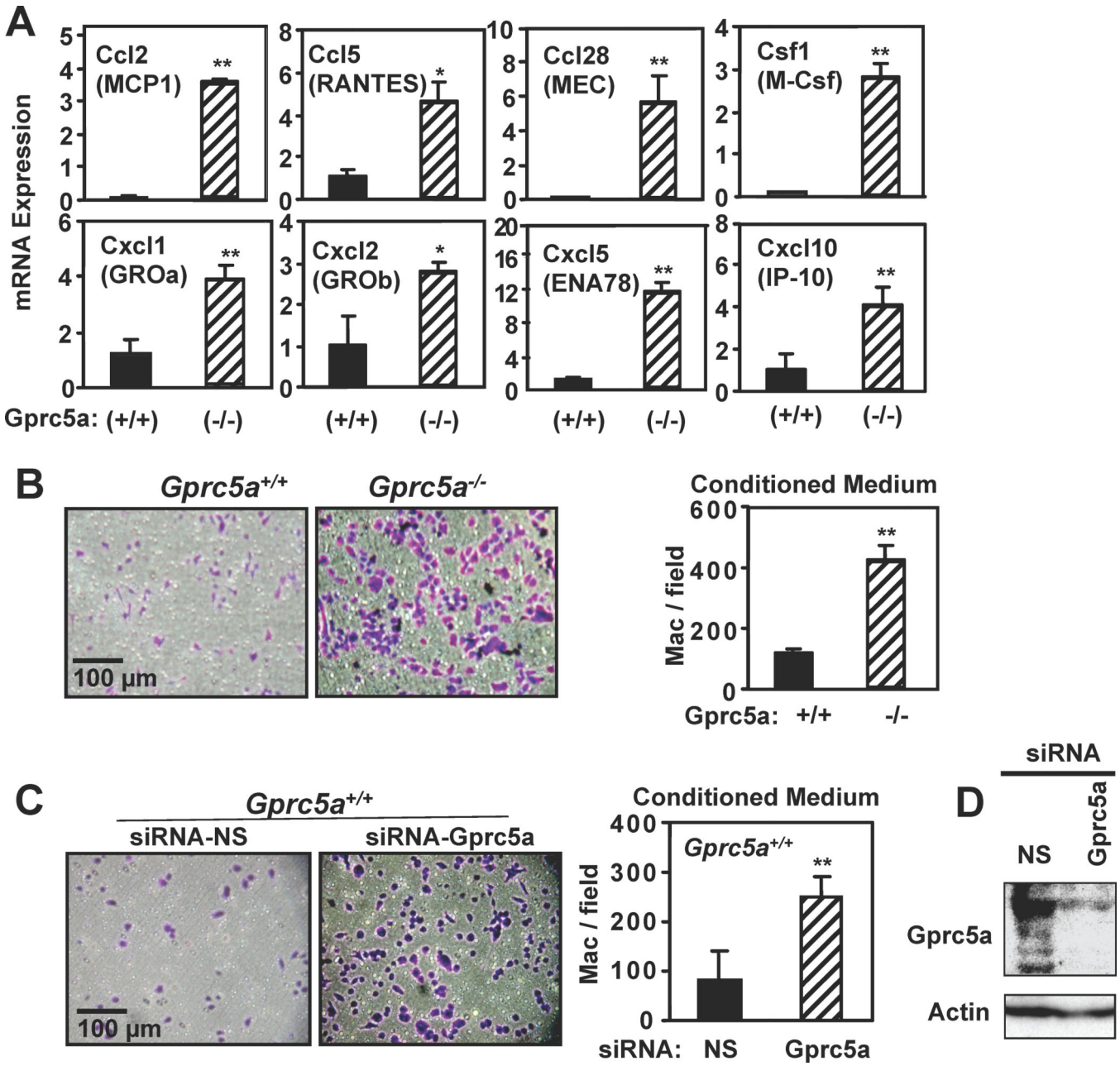
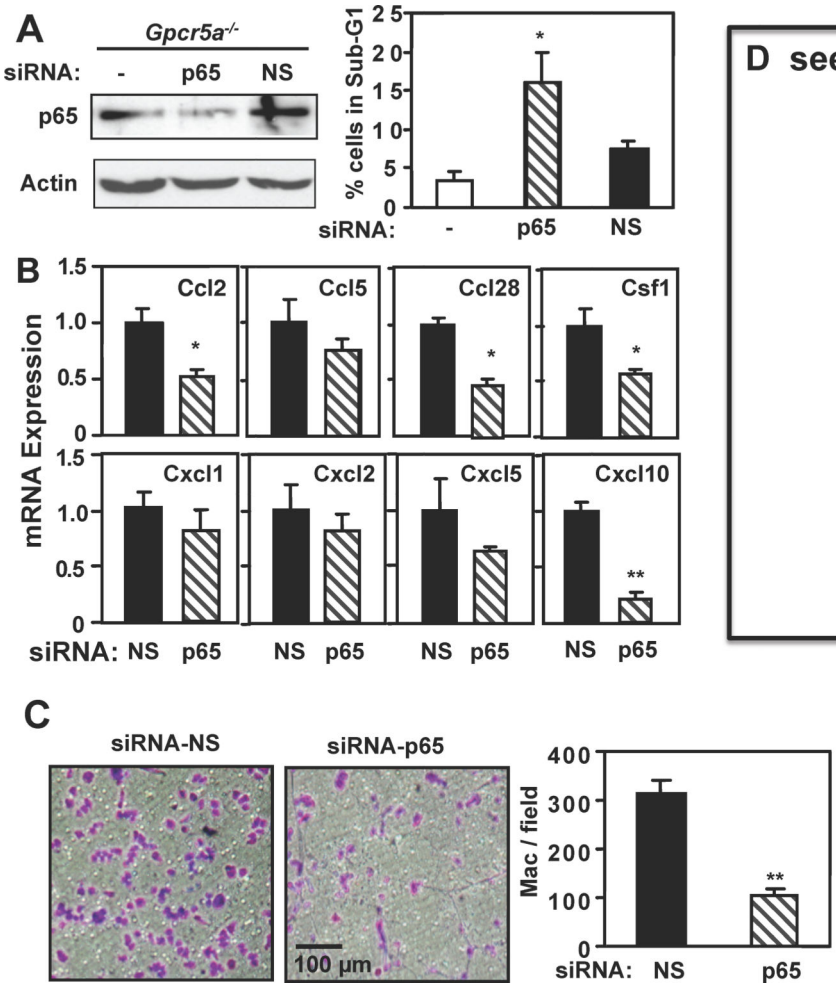


Fig. 5.

Differential expression of chemokines and cytokines in cultured *Gprc5a*$^{+/+}$ and *Gprc5a*$^{-/-}$ cells and the effects of conditioned media on macrophage migration. *A*, Real-time PCR analysis of the indicated chemokines and cytokines using mRNA from cultured lung epithelial cells. The data represent the mean±SE of triplicate measurements. *B*, Analysis of the ability of conditioned media of lung epithelial cells to promote macrophage migration. MH-S cells that had migrated to the membrane underside were stained with crystal violet and photographed under the microscope (left). The mean numbers of macrophages per field (±SD) were calculated after analyzing four fields (right). *C*, Analysis of the macrophage migration promoting activities of the conditioned media of *Gprc5a*$^{+/+}$ cells transiently

transfected with siRNA against *Gprc5a* or with a non-specific (NS) sequence. *D*, Confirmation of the silencing of Gprc5a by the specific siRNA using immunoblotting of protein prepared from $Gprc5a^{+/+}$ cells treated as in *C*. The significance of the differences between $Gprc5a^{-/-}$ and $Gprc5a^{+/+}$ in all above data is indicated by * ($P < 0.05$) and ** ($P < 0.001$) (Student's *t* test).



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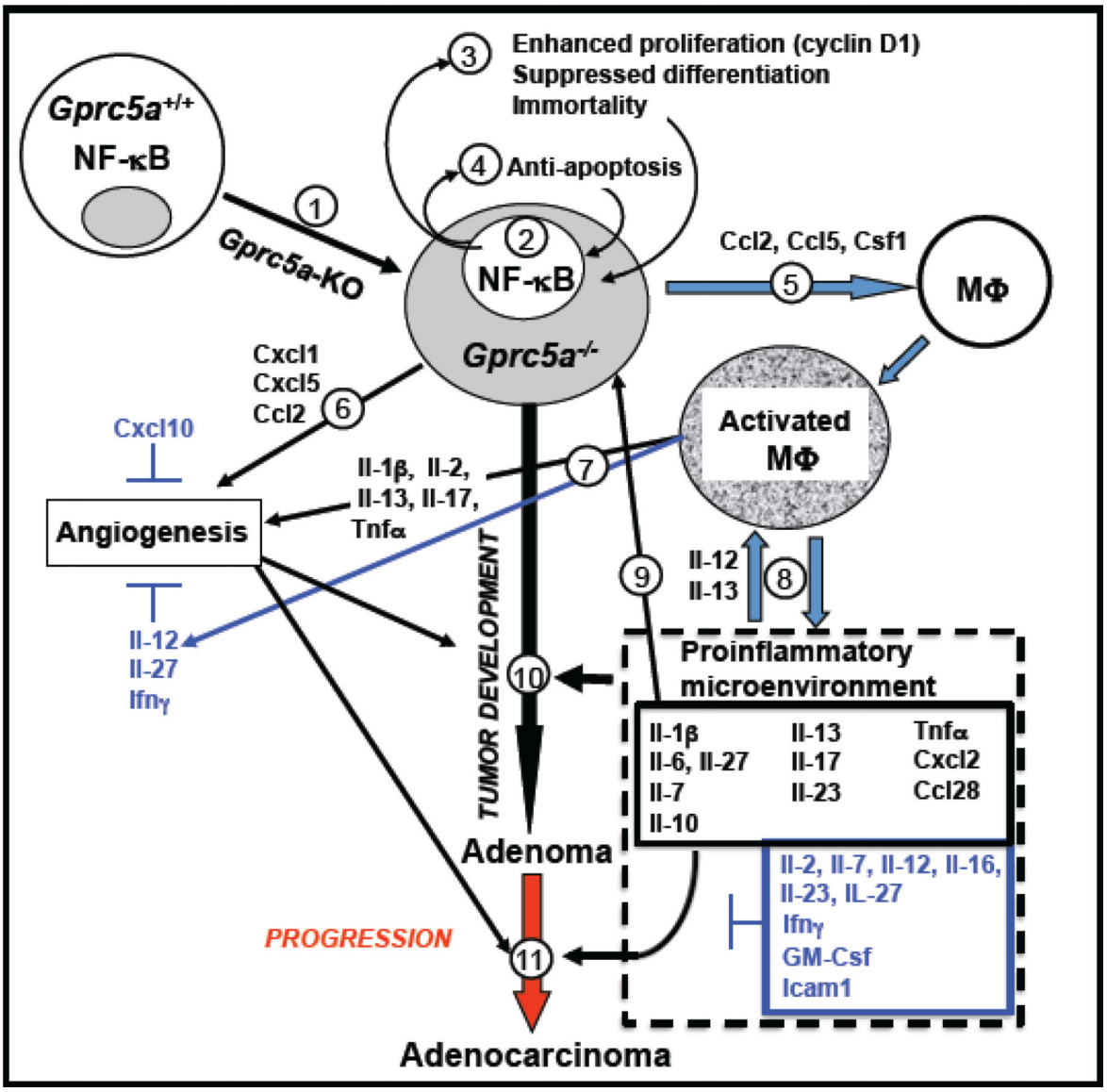

Fig. 6.

Implication of NF-κB signaling in the aberrant phenotype of *Gprc5a*$^{-/-}$ cells. *A (left)*, Silencing of p65 in *Gprc5a*$^{-/-}$ cells transfected transiently with siRNA against p65 but not in cells transfected with NS siRNA detected by immunoblotting. *A (right)*, The effects of transfecting *Gprc5a*$^{-/-}$ cells with siRNA against p65 or NS oligonucleotide on sub-G1 cell population representing dead cells) after a 24 hr suspension was determined as in Figure 3*D*. *B*, Real-time PCR analysis of the indicated chemokines and cytokines using total RNA from *Gprc5a*$^{-/-}$ cells treated with p65-specific or NS siRNA. Bars indicate mean (± S.D.) of triplicate measurements. *C*, analysis of the ability of conditioned media of *Gprc5a*$^{-/-}$ cells treated with siRNA to promote macrophage migration (left). The data are presented as the mean number of macrophages per field (±SD) in four fields (right). The statistical significance of the differences between *Gprc5a*$^{-/-}$ cells transfected with p65 specific siRNA or non-specific oligonucleotide is indicated by * ($P < 0.05$) and ** ($P < 0.001$) (Student's *t*

test). *D*, Schema of the proposed sequence of events (indicated by numbers) resulting from *Gprc5a* loss in lung airway epithelial cells in vivo. The cytokines presented in black fonts stimulate and those in blue fonts inhibit angiogenesis or tumor development as indicated.