Tobacco chemical-induced mouse lung adenocarcinoma cell lines pin the prolactin orthologue proliferin as a lung tumour promoter

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Supplementary Material

Supplementary Materials and methods

Mouse models of endogenous lung adenocarcinoma

FVB (#001800), *Balb/c* (#001026), *FVB*-Tg(CAG-luc,-GFP)L2G85Chco/J (called CAG.Luc.eGFP; #008450; (1), B6.129S4-Krastm4Tyj/J (called *KRAS*^{G12D}; #008179; (2), and *C57BL/6* (#000664) mice were obtained from Jackson Laboratories (Bar Harbor, MN) and were bred on the corresponding background at the University of Patras Center for Animal Models of Disease. Experimental mice were sex-, weight (20-25 g)-, and age (6-12 week)-matched.

For chemically induced lung carcinogenesis, *FVB*, and *Balb/c* mice received the tobacco carcinogens urethane (Sigma Aldrich, U2500) intraperitoneally (1g/Kg in 100 μl phosphate-buffered saline) or diethylnitrosamine (200 mg/kg) (Sigma Aldrich, N0756) and were sacrificed after ten months (3,4). For mutant *KRAS*-driven lung tumorigenesis, *C57BL/6* mice heterozygous for the loxP-STOP-loxP.*KRAS*^{G12D} transgene (*KRAS*^{G12D} mice), which express mutant *KRAS* in any somatic cell upon CRE-mediated recombination, received 5 x10^8 intratracheal plaque-forming units of adenovirus encoding CRE recombinase (Ad-*Cre*; Baylor College of Medicine, Houston, TX) and were killed after four months (2).

Mouse models of transplantable lung adenocarcinoma

For heterotopic lung adenocarcinoma (LADC) development, mice were anesthetized using isoflurane inhalation and received s.c. injections of 100 μL phosphate-buffered saline (PBS) containing 1 x 10^6 mouse cancer cells. Three vertical tumor dimensions (δ1, δ2, and δ3) were monitored longitudinally and tumor volume was calculated

using the formula π * δ1 * δ2 * δ3 / 6 as described elsewhere (5-7). Mice were sacrificed after 3-4 weeks. For forced lung metastasis induction, mice were anesthetized using isoflurane inhalation and received i.v. injections of 100 µL phosphate-buffered saline (PBS) containing 0.25 x 10^6 murine cells. Mice were sacrificed after two weeks. Lung tumors were counted and sized using a Stemi DV4 stereoscope (Zeiss, Jena, Germany) in transillumination mode to visualize both superficial and deep lung metastases. For malignant pleural effusion precipitation, syngeneic mice received 150,000 murine cancer cells intrapleurally and mice were sacrificed after two weeks. Pleural effusions and tumors were evaluated as described elsewhere (5,6).

Isolation and culture of mouse cell types and lines

Mouse airway epithelial cells were cultured from the stripped tracheal epithelia of eight-week-old *FVB* and *Balb/c* mice as described previously (8). Bone marrow-derived macrophages were generated after one-week culture of whole bone marrow cells flushed from the four long bones (two tibias and two femurs) of *FVB* mice with 100 ng/mL recombinant murine (rm) macrophage colony-stimulating factor, thereby passaging the adherent cells, as described elsewhere (9). Bone marrow-derived mast cells were generated after four-week culture of whole bone marrow cells flushed from the four long bones (two tibias and two femurs) of *FVB* mice with 100 ng/mL rm interleukin-3 with or without 100 ng/mL rm KIT-ligand, thereby passaging the non-adherent cells (7).

Cytology, cytometry, and histology

For May-Grünwald Giemsa stain, cells were fixed with methanol for 2 min, were stained with May-Grünwald stain in 1 mM Na_2HPO_4, 2.5 mM KH_2PO_4, pH = 6.4 for 6 min, and subsequently with Giemsa stain in 2 mM Na_2HPO4, 5 mM KH_2PO_4, pH = 6.4 for 40 min, were washed with H_2O, and were dried. Slides were mounted with Entellan (Merck Millipore, Darmstadt, Germany), coverslipped, and analyzed. For flow cytometry and fluorescence-activated cell sorting (FACS), 10^6 cells suspended in 50 ml FACS buffer (PBS supplemented with 2% FBS and 0,1% NaN_3) were stained with the indicated antibodies according to the manufacturer's instructions and recommended dilutions (Supplementary table ST1) for 20 min in the dark, were washed with FACS buffer from excess antibody, and were resuspended in 1ml FACS buffer for further analysis. For histology, murine lungs were inflated, fixed in 4% paraformaldehyde overnight, embedded in paraffin or OCT and were stored at room temperature or -80^oC, respectively. Five-µm paraffin or 10-µm-cryosections were mounted on glass slides. Sections were labeled using the indicated antibodies (Supplementary table ST1), counterstained with Envision (Dako, Carpinteria, CA) or Hoechst 33258 (Sigma), and mounted with Entellan new (Merck Millipore) or Mowiol 4-88 (Calbiochem, Gibbstown, NJ). For isotype control, primary antibody was omitted. Immunoreactivity was quantified as described previously (10). For hematoxylin-eosin staining, slides were incubated with deionized H_2O for 2 minutes at room temperature and transferred to hematoxylin solution (Papanicolaou's solution 1b hematoxylin solution S; Merck Millipore) for 30 seconds at room temperature (RT). Then slides were washed up with tap water, incubated for 1 second in 1% acid alcohol at RT, incubated with lithium solution for 4 seconds at RT and washed with tap water again. Subsequently slides were incubated in eosin solution (Eosin Y solution 0.5% alcoholic; Merck Millipore) for 1 minute at RT and

washed with tap water. PAS-D staining was done as described elsewhere (11). Finally slides were dehydrated, coverslipped, and analyzed. Bright-field and fluorescent microscopy were carried out on AxioLab.A1 (Zeiss), AxioObserver.D1 (Zeiss) or TCS SP5 (Leica) microscopes. Digital images were processed with Fiji academic software (12) .

Cell proliferation and tumor-sphere assays

In vitro cancer cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (10). Tumor-sphere generation assay was performed as described elsewhere (13)

Bioluminescent imaging

Mice were imaged on a Xenogen Lumina II after i.v. delivery of 1 mg D-luciferin (Gold Biotechnology). Data were analyzed on Living Image v.4.2 (Perkin-Elmer, Waltham, MA) (6).

RNA Extraction, Sanger sequencing, and gene expression analyses

Cellular RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA) followed by RNAeasy purification and genomic DNA removal (Qiagen, Hilden, Germany). For lung tissue RNA, tissues were passed through 70 µm strainers (BD Biosciences, San Jose, CA) and 10^7 cells were subjected to RNA extraction. One µg RNA was reverse-transcribed using Oligo$(dT)_{18}$ and Superscript III (Thermo Fisher). *Kras*, and *Nras* cDNAs were amplified using specific primers (Supplementary table ST2) and Phusion Hot Start Flex polymerase (New England Biolabs, Ipswich, MA). DNA fragments were run on 2% agarose gels and were purified with NucleoSpin gel

and PCR clean-up columns (Macherey-Nagel, Düren, Germany) and were sequenced using the appropriate primers by VBC Biotech (Vienna, Austria). qPCR was performed using specific primers (Supplementary table ST2) and SYBR FAST qPCR Kit (Kapa Biosystems, Wilmington, MA) in a StepOne cycler (Applied Biosystems, Carlsbad, CA). Ct values from triplicate reactions were analyzed with the $2^{-\Delta CT}$ method (2). mRNA abundance was determined relative to β-glycuronidase (*Gusb*) and is given as $2^{-\Delta CT} = {}^{2\,-(\text{Ct of transcript})-(\text{Ct of Gusb})}$. Microarrays were done as described elsewhere (10).

Flow cytometry and Immunoblotting

Cell cytometry and data analysis were performed on a CyFlow ML instrument using FloMax Software (Partec GmbH, Münster, Germany). A CyFlow ML instrument with FloMax Software (Partec, Munster, Germany) was used for cell cytometry, sorting, and data analysis. Total protein extracts from cultured cells were prepared using Mg^{2+} lysis/wash buffer [25 mM HEPES (pH=7.5), 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol]. Proteins were separated by 8-15% Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and were electroblotted to PVDF membranes (Merck Millipore). Membranes were probed with primary antibodies followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Supplementary table ST1) and were visualized with enhanced chemiluminescence (Merck Millipore).

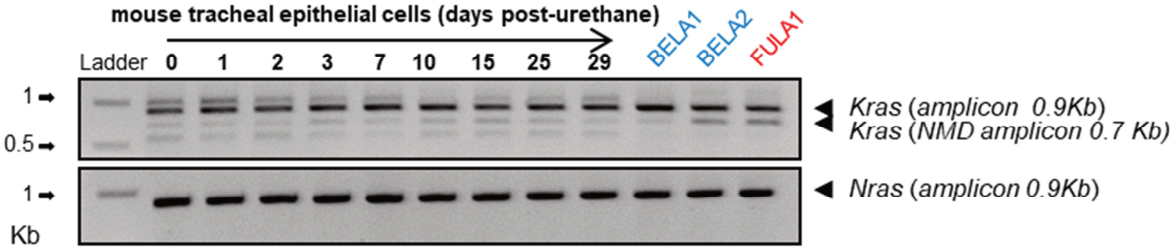
Statistics

Sample size for *in vivo* experiments was calculated using G*power [(http://www.gpower.hhu.de/) assuming $\alpha = 0.05$, $\beta = 0.8$, and effect size $d = 1.5$. No

data were excluded. Data acquisition was blinded on samples previously coded by a non-blinded investigator. All data were examined for normality by Kolmogorov-Smirnov test. Values are given as mean ± SD or median(interquartile range, IQR), as appropriate and indicated. Sample size (*n*) refers to biological replicates. Differences in means were examined by t-test or one- or two-way ANOVA with Bonferroni post-hoc tests. *P* values are two-tailed and $P < .05$ was considered significant. Analyses and plots were done on Prism v5.0 (GraphPad, La Jolla, CA).

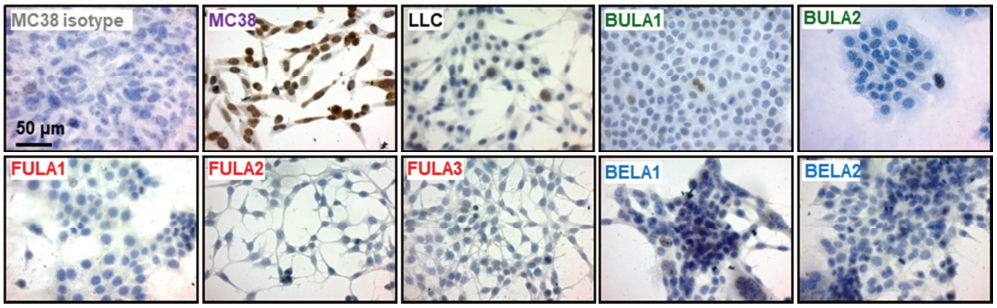
Supplementary Figures

Supplementary Figure SF1



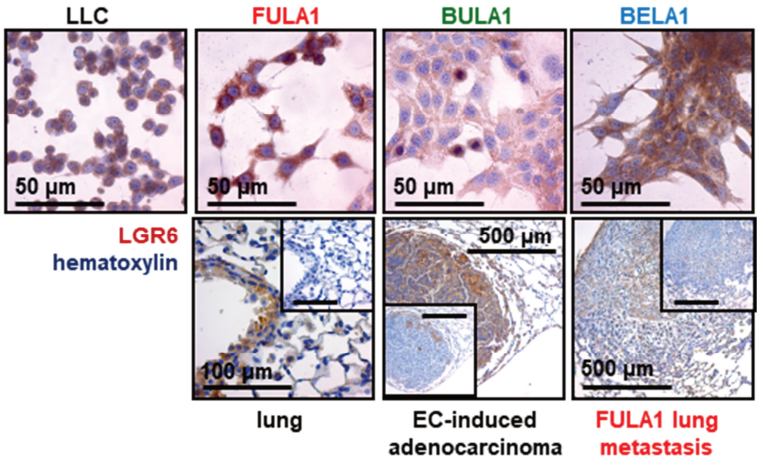
Kras and *Nras* mRNA expression by RT-PCR of mouse tracheal epithelial cells (mTECs) cultured from the lungs of urethane-exposed mice at various time-points post-injection and of select chemical-induced lung adenocarcinoma cell lines.

Supplementary Figure SF2



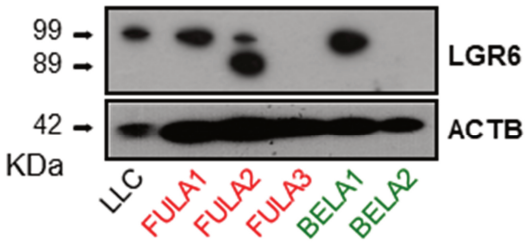
Immunoreactivity (brown color) of LLC, MC38, and chemical-induced LADC cell lines for TRP53 protein by immunocytochemistry. Note blue nuclear hematoxylin staining and the strong nuclear TRP53 immunoreactivity of MC38 cells.

Supplementary Figure SF3



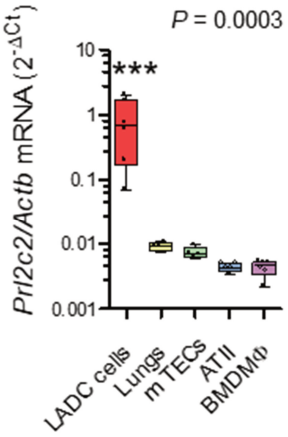
Immunoreactivity of LLC and urethane (EC)-induced LADC cell lines, naïve lungs, chemical-induced LADCs, and lung metastases induced by LADC cell lines for LGR6 (brown color). Blue color indicates hematoxylin counterstaining.

Supplementary Figure SF4



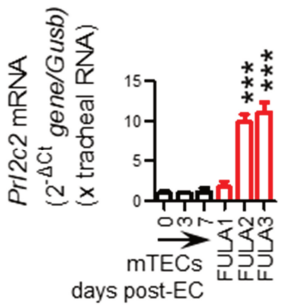
LGR6 and ACTB protein expression of LLC and select chemical-induced LADC cells by Western immunoblot.

Supplementary Figure SF5



Proliferin (PRL) transcript Prl2c2 normalized to β-actin transcript (Actb) expression of chemical-induced lung adenocarcinoma (LADC) cell lines, total mouse lung RNA, mouse tracheal epithelial cells (mTECs) alveolar type II cells and bone marrow-derived macrophages by microarray (GEO Datasets accession IDs are given in Figure 4). Data are presented as median with Tukey's whiskers (boxes: interquartile range; bars: 50% extreme quartiles) and raw data points (dots) (n = 4-6/group). P denotes overall one-way ANOVA probability and *** denote P < 0.001 for comparison of LADC cells with any other group by Bonferroni post-tests.

Supplementary Figure SF6



Prl2c2 mRNA expression by qPCR of mouse tracheal epithelial cells (mTECs) cultured from the lungs of urethane-exposed mice at various time-points post-injection and of urethane-induced LADC cell lines relative to Gusb. Data are presented as mean±SD (n = 3/group). *** denotes $P < 0.001$ for comparison with mTECs by one-way ANOVA with Bonferroni post-tests.

Supplementary Tables

Supplementary Table ST1
Antibodies

Antibody	Company	Product number
Proliferin Antibody (E-10)	Santa Cruz Biotechnology	sc-271891
p53 Antibody (FL-393)	Santa Cruz Biotechnology	sc-6243
K-Ras Antibody (F234)	Santa Cruz Biotechnology	sc-30
N-Ras Antibody (F155)	Santa Cruz Biotechnology	sc-31
β-Actin Antibody (C4)	Santa Cruz Biotechnology	sc-47778
Anti-Integrin beta 3 antibody [EPR2417Y]	Abcam	ab75872
Anti-EGFR antibody [EP38Y]	Abcam	ab52894
Anti-GPCR LGR6 antibody [EPR6874]	Abcam	ab126747
CD44 Monoclonal Antibody (IM7), PE-Cyanine5, eBioscience (Flow Cytometry)	Thermo Fisher Scientific	15-0441-82
Anti/TTF1 antibody	Thermo Fisher Scientific	MA5-16406

Supplementary Table ST2

PCR primers

Method[a]	Primer[b]	Sequence	Amplicon length
qPCR	Trp53F	CGCCGACCTATCCTTACCAT	bp 120
qPCR	Trp53R	TTCTTCTTCTGTACGGCGGT	
qPCR	EgfrF(1)	ATCAAAGTTCTGGGTTCGGG	bp 156
qPCR	EgfrR(1)	CATCACATAGGCTTCGTCAAGG	
qPCR	EgfrF(2)	AACTGTACCTATGGATGTGCTG	bp 154
qPCR	EgfrR(2)	GGATTTGGAAGAAACTGGAAGG	
qPCR	ProliferinF	CATCTCCAAAGCCACAGACAT	bp 145
qPCR	ProliferinR	GCGAGCATCTTCATTGTCAG	
qPCR	Itgb2F	GAATGCCTACTATAAACTCTCCTC	bp 117
qPCR	Itgb2R	GATTTGCCTATACTCGATGCT	
qPCR	Lgr6F	ATGACCTTGGCTCTCAACCA	bp 100
qPCR	Lgr6R	GCTGGATGCGGTTGTTATGT	
qPCR	GusbF	TTACTTTAAGACGCTGATCACC	bp 165
qPCR	GusbR	ACCTCCAAATGCCCATAGTC	
Sanger Seq	KrasF	CCATTTCGGACCCGGAG	bp 905
Sanger Seq	KrasR	CTTTAGTCTCTTCCACAGGCA	
Sanger Seq	NrasF	GCGCCTAGTGATTACGTAGC	bp 905
Sanger Seq	NrasR	TGAAGAGGTCTCAGGTTAGATGG	
RT-PCR	Trp53F	GTAGCTTCAGTTCATTGGGA	bp 1450
RT-PCR	Trp53R	TGAAGTCATAAGACAGCAAGGA	bp 1450

[a] Application: qPCR: quantitative (real-time) PCR, Sanger Seq: Sanger Sequencing, RT-PCR: Reverse transcription PCR [b] F: Forward, R Reverse

Supplementary Table ST3

Oligonucleotides

Oligonucleotide	Company	Product number
Integrin β3 shRNA (m) Lentiviral Particles is a pool of 3 different shRNA plasmids: sc-35677-VA Hairpin sequence: GATCCGCTACAGTATGTGATGAAATTCAAG AGATTTCATCACATACTGTAGCTTTTT sc-35677-VB Hairpin sequence: GATCCCATCCCATTTGCTAGTGTTTTCAAG AGAAACACTAGCAAATGGGATGTTTTT sc-35677-VC Hairpin sequence: GATCCGTCAGTATGTGGGAATGTATTCAAG AGATACATTCCCACATACTGACTTTTT	Santa Cruz Biotechnology	sc-35677-V
Proliferin-1 shRNA (m) Lentiviral Particles is a pool of 3 different shRNA plasmids: sc-61412-VA Hairpin sequence: GATCCGCTTCAGAATGGAGATGAATTCAAG AGATTCATCTCCATTCTGAAGCTTTTT sc-61412-VB Hairpin sequence: GATCCCCTGAAGTGTTACATGTTATTCAAG AGATAACATGTAACACTTCAGGTTTTT sc-61412-VC Hairpin sequence: GATCCCTCTGCTTCTGAAATATCATTCAAG AGATGATATTTCAGAAGCAGAGTTTTT	Santa Cruz Biotechnology	sc-61412-V
K-Ras shRNA (m) Lentiviral Particles is a pool of 3 different shRNA plasmids: sc-43876-VA Hairpin sequence: GATCCCTACAGGAAACAAGTAGTATTCAAG AGATACTACTTGTTTCCTGTAGTTTTT sc-43876-VB Hairpin sequence: GATCCGAACAGTAGACACGAAACATTCAAG AGATGTTTCGTGTCTACTGTTCTTTTT sc-43876-VC Hairpin sequence: GATCCCCATTCAGTTTCCATGTTATTCAAG AGATAACATGGAAACTGAATGGTTTTT	Santa Cruz Biotechnology	sc-43876-V
Mutant Kras plasmid eGFP.KRASG12C-2B.retro.puro	Addgene	64372

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