Expression of G protein-coupled receptor 19 in human lung cancer cells is triggered by entry into S phase and supports G2/M cell cycle progression

Stefan Kastner¹, Tilman Voss¹, Simon Keuerleber², Christina Glöckel², Michael Freissmuth², and Wolfgang Sommergruber¹

¹ Boehringer Ingelheim RCV GmbH & Co KG, Department of Lead Discovery, Vienna, Austria
² Medical University of Vienna, Institute of Pharmacology, Center of Physiology and Pharmacology, Vienna, Austria

Running title  GPR19 in lung cancer and cell cycle
Key words  GPCR – GPR19 – cell cycle – lung cancer – E2F

Financial Support
Stefan Kastner, Tilman Voss and Wolfgang Sommergruber are supported by Boehringer Ingelheim.
Simon Keuerleber is supported by the FWF-funded doctoral program CCHD (Cell Communication in Health and Disease) and Christina Glöckel and Michael Freissmuth are supported by PLACEBO (Platform Austria for Chemical Biology) funded by the Austrian Gen-Au (Genome Research in Austria) program.

Corresponding Author
Wolfgang Sommergruber, Boehringer Ingelheim RCV GmbH & Co KG, Department of Lead Discovery, Dr. Boehringer-Gasse 5-11, 1121 Vienna, Austria. Phone: 0043-180105-2399; Fax: 0043-180105-32399; E-mail: wolfgang.sommergruber@boehringer-ingelheim.com

Disclosure of Potential Conflicts of Interest  No potential conflicts of interest were disclosed.

Note: This article contains supplementary data.
Abstract

It has long been known that G protein-coupled receptors (GPCRs) are subject to illegitimate expression in tumor cells. Presumably, hijacking the normal physiological functions of GPCRs contributes to all biological capabilities acquired during tumorigenesis. Here, we searched for GPCRs that were expressed in lung cancer: the mRNA encoding orphan G protein-coupled receptor 19 (GPR19) was found frequently overexpressed in tissue samples obtained from patients with small cell lung cancer (SCLC). Several observations indicate that overexpression of Gpr19 confers a specific advantage to lung cancer cells by accelerating transition through the cell cycle. (i) Knockdown of Gpr19 mRNA by RNA interference reduced cell growth of human lung cancer cell lines. (ii) Cell cycle progression through G2/M phase was impaired in cells transfected with siRNAs directed against Gpr19 and this was associated with increased protein levels of cyclin B1 and phosphorylated histone H3. (iii) The expression levels of Gpr19 mRNA varied along the cell cycle with a peak observed in S phase. (iv) The putative control of Gpr19 expression by E2F transcription factors was verified by chromatin immunoprecipitation: antibodies directed against E2F-1 to 4 allowed for the recovery of the Gpr19 promoter. (v) Removal of E2F binding sites in the Gpr19 promoter diminished the expression of a luciferase reporter. (vi) E2f and Gpr19 expression correlated in lung cancer patient samples. To the best of knowledge, this is the first example of a GPCR showing cell cycle-specific mRNA expression. Our data also validate GPR19 as a candidate target when overexpressed in lung cancer.
Introduction

Lung cancer is among the leading causes of cancer-related deaths worldwide and is strongly associated with smoking. Based on pathological features lung cancers are classified into two major groups. Small cell lung cancer (SCLC) accounts for 20% of all cases and displays characteristics of neuroendocrine tumors (1). Non-small cell lung cancer (NSCLC) is further subdivided into squamous cell carcinoma (30-40%), adenocarcinoma (30-40%) and large cell carcinoma (<10%). Both types of lung cancer not only differ in their cellular morphology but also in the available treatment and their prognosis – with SCLC exhibiting the most aggressive and metastatic phenotype (2).

The defining features of the family of G protein-coupled receptors (GPCRs) are the hydrophobic core of seven transmembrane-spanning α-helices and their eponymous action, i.e., the activation of their cognate heterotrimeric G protein(s). The human complement of GPCRs consists of more than 800 members (3). GPCRs are intimately involved in the control of virtually all cell types, their structure allows for binding of highly diverse ligands, thus they are considered the most druggable family of proteins (4). However, only about 60 GPCRs are currently targeted by drugs approved for clinical use (5).

Several arguments support the assumption that GPCRs may also be specifically targeted to limit the growth of cancer cells: signaling pathways controlled by GPCRs promote proliferation, survival, cell migration, angiogenesis, inflammation and subversion of the immune system (4, 6). Their uncontrolled exploitation – mostly seen by overexpression of GPCRs – could in turn contribute to the malignant transformation of cells (6, 7). It has long been known that illegitimate expression of GPCRs occurs in tumor cells (8) and an involvement in cell cycle control has been shown for several GPCRs and GPCR-associated proteins. For instance, H4 histamine receptors mediate a reversible cell cycle arrest in growth factor-induced hematopoietic progenitor cells (9) and GPCR kinase 5 was assigned an essential role in the regulation of prostate tumor growth by affecting cell cycle control (10).

In the present work, we searched for GPCRs that are aberrantly expressed in lung cancer with a particular focus on SCLC. The underlying rationale was the observation that GPR87 was found to be overexpressed in lung squamous cell carcinoma (11) and contributed to the viability of tumor cells.
Similarly, the chemokine receptor CXCR4 was proposed to contribute to lung cancer metastasis (13). In SCLC cells, it was suggested that an autocrine loop sustained RhoA activation via activation of a GPCR (14).

Our search identified G protein-coupled receptor 19 (GPR19), which was overexpressed predominantly in samples from patients with SCLC. Currently very little is known about the biological functions of GPR19 in normal and diseased tissues (15). RNA interference established a link to cell proliferation and transition through the cell cycle as progression through the G2/M phase was impaired in cells transfected with Gpr19 siRNAs. In fact, we observed that the levels of Gpr19 mRNA varied along the cell cycle with a peak in S phase. This might be accounted for by the binding of E2F transcription factors to the promoter region of Gpr19.
Materials and Methods

Cell culture and cell cycle arrest

Human lung-derived cell lines were cultured in normal growth medium as suggested by the provider (Supplementary Table S1) and maintained in logarithmic growth phase. They were authenticated using short tandem repeat analysis and tested for mycoplasma contamination on a regular basis. Cells were arrested at different stages of the cell cycle as follows: 100,000 cells per well were seeded in 6 well culture plates, allowed to adhere for 16 h in the respective growth medium and subsequently incubated in the presence of hydroxyurea (Sigma Aldrich; 1 mM) or aphidicolin (Sigma Aldrich; 3 µM). After 24 h the medium was exchanged for normal growth medium to remove the compounds and to thereby release the cells from the arrest.

RNA interference

siRNA oligonucleotides were purchased from Sigma Aldrich (GPR19 #1 (5'-CUGACCUUCUCAU CAGCGU-3'), GPR19 #2 (5'-CUCAGUCUACCACCAACUA-3'), CTL #2: (5'-CAGCAUCUUC UUUGGAUU-3'), polo-like kinase 1 (PLK1; 5'-GUCUCAAGGCCUCCUAUA-3')) and Thermo Fisher Scientific (CTL #1, pool of non-targeting si RNAs). An additional control siRNA (CTL #2) was used which had been designed against Gpr19 but did not cause Gpr19 mRNA downregulation. The final siRNA concentration was 20 nM and transfections were done using Lipofectamine® RNAiMAX (Life Technologies) following the manufacturer’s instructions (reverse transfection).

Cell proliferation

The effect of doxorubicin (1 µM) or siRNA (20 nM) on cell proliferation was determined by monitoring the number of viable cells using the alamarBlue® detection kit (Life Technologies). Experiments were done in 96 well culture plates. Fluorescence was recorded using a 2030 Multilabel Reader VICTOR™ X5 (PerkinElmer). In addition, proliferation of cells was assessed by determining cellular confluence using a CloneSelect™ Imager (Molecular Devices). The surface covered by cells
(96 well plate) was recorded on regular time points. Besides, phase contrast microscopy pictures of cells were recorded (20 x magnification).

Flow cytometry and cell cycle analysis

Cells were fixed with 70% ethanol (-20°C), washed with phosphate-buffered saline and subject to RNase A treatment (1 mg/ml; Life Technologies) for 5 min followed by propidium iodide DNA staining (50 µg/ml; Sigma Aldrich). Cellular fluorescence was quantified using a FACSCalibur™ (Becton Dickinson) flow cytometer. Fluorescence histograms were analyzed with FlowJo software 7.5 (Tree Star). The Watson curve fitting model was chosen for cell cycle analysis.

RNA isolation and cDNA synthesis

The ABI Prism® 6100 Nucleic Acid PrepStation (Life Technologies) was used to isolate RNA from cell lysates following the manufacturer’s instructions. Contaminating DNA was removed by DNase treatment. cDNA was synthesized in a GeneAmp® PCR System 9700 (Life Technologies) using the iScript™ cDNA Synthesis Kit (Bio-Rad) following the manufacturer’s protocol and diluted with nuclease-free water (1:5 to 1:8) for direct use in quantitative polymerase chain reaction (PCR) applications.

Quantification of gene expression by real-time PCR

Reverse transcription quantitative real-time PCR (RT-qPCR) was done on an Mx2005P qPCR machine (Stratagene) using the TaqMan® technology (Life Technologies). 10 µl of TaqMan® Gene Expression Master Mix (Life Technologies) were mixed with 1 µl of both FAM™- (gene of interest) and VIC™-labeled (reference gene) TaqMan® assays (Life Technologies; Supplementary Table S2) and 8 µl of diluted cDNA. PCR conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C (data collection).

The expression of genes of interest was normalized (geometric averaging) against multiple most stably expressed reference genes. Reference genes B2m, Actb, Cypa, Gapdh, Hprt1, Rplp0, Rnase P and Tfr were tested for stable expression as described (16). The final selection of reference genes was based on
this stable expression, a low difference in quantification cycles and a PCR efficiency comparable to
the ones for the genes of interest. Pairwise duplexing was done for all genes of interest against all
selected reference genes. Relative expression ratios were normalized against a control sample (17).
Assay controls (either devoid of template or lacking reverse transcriptase) were included in all
experiments and gave negative results.

**Immunofluorescence labeling**
Cells seeded in black poly-D-lysine 96 well culture plates were fixed with formaldehyde and blocked
with 1% goat serum (Dako) and 2% bovine serum albumin in phosphate-buffered saline. They were
immunostained for cyclin B1 (BD Biosciences) and phosphorylated histone H3 (Ser10; Upstate)
proteins. DNA was stained using Hoechst 33342 (Life Technologies).

**High-content screening**
Fluorescence-based cell detection was done with an ArrayScan® VTI HCS reader (Thermo Fisher
Scientific). Six wells per reaction condition and 36 fields (maximum) per well were recorded at 20 x
magnification. The total number of cells monitored per well was in the range of 300 to 1000
depending on siRNA treatment and time post siRNA transfection. For Plk1 siRNA-transfected
samples this was only valid on the second day after transfection due to the strong antimitotic effect of
the knockdown. The dataset was processed and visualized by Spotfire® DecisionSite® 9.1.2 (TIBCO)
and iView™ 1.0.182.1 (Thermo Fisher Scientific). Cells were detected on the basis of Hoechst 33342
dNA staining. Intensities of immunostaining for both, phosphorylated histone H3 and cyclin B1 were
examined by a Kolmogorov-Smirnov goodness-of-fit analysis as described (18).

**Transcription profiling and hierarchical clustering**
RNA from human NSCLC, SCLC and normal lung samples was purchased from Origene
Technologies. Detailed information on patient samples is listed in Supplementary Table S3.
Affymetrix microarray analysis was performed on the Human Exon 1.0 ST Array platform. Samples
were processed following the Affymetrix GeneChip® Whole Transcript Sense Target Labeling Assay
manual. Raw (CEL) files were Robust Multichip Average-normalized using the affy package in R/Bioconductor (19). Seven hundred seventy-three genes encoding GPCRs were selected on the basis of Gene Ontology number 0004930. Their expression values in NSCLC, SCLC and normal lung samples were extracted from the microarray data. Geometric mean values across all normal lung samples were used as reference for the values from NSCLC and SCLC patient samples. Hierarchical clustering (average linkage, Euclidean distance) of the log 2-transformed normalized expression data were performed as described (20). Gene expression levels of *Gpr19* (probe set 207183_at) in various human normal and cancerous tissues were investigated by employing the BioExpress® database (Gene Logic, 12).

Gene expression data of human lung-derived samples from Hou et al. (21) and Rohrbeck et al. (22) were extracted from the Oncomine™ data platform (Compendia Bioscience) and analyzed using Spotfire® DecisionSite® 9.1.2 (TIBCO).

**Chromatin immunoprecipitation (ChIP) and ChIP PCR**

ChIP analyses were performed with 5 µg of respective E2F antibodies as described (23). Rabbit polyclonal immunoglobulin G antibodies against E2F family members – E2F-1 (sc-193X), E2F-2 (sc-633X), E2F-3 (sc-878X; detects both isoforms), E2F-4 (sc-866X), E2F-5 (sc-999X), E2F-6 (sc-22823X), E2F-7 (sc-66870X) – were from Santa Cruz Biotechnology (24, 25, 26) and the isotype control was from Cell Signaling. DNA from the precipitate and the sheared chromatin input (1/100th the material used for ChIP) was recovered by phenol/chloroform/isoamyl alcohol extraction, precipitated using isopropanol/sodium acetate and resuspended in 100 µl of 10 mM Tris.

ChIP PCR was performed in a GeneAmp® PCR System 9700 (Life Technologies) using 2 µl of DNA solution and 200 nM of forward and reverse primer respectively in a JumpStart™ REDTaq® ReadyMix™ Reaction Mix (Sigma Aldrich). PCR conditions were 45 sec at 95°C followed by 32 cycles of 15 sec at 95°C, 15 sec at 59°C, 40 sec at 72°C and completed by 3 min at 72°C. Nuclease-free water instead of DNA was used as non-template control. PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.
Luciferase reporter assay

DMS 53, HEK-293, and NCI-H1703 cells were transfected by electroporation using the Amaxa™ Cell Line Nucleofector™ Kit V (Lonza; 3 µg of plasmid for DMS 53 and NCI-H1703, 5 µg of plasmid for HEK-293) and 10,000 cells were seeded in 96 well culture plates. At 14 h (DMS 53), 13 h (HEK-293), or 26 h (NCI-H1703) after transfection, cells were washed with phosphate-buffered saline, lysed using 100 µl of Beetle-Lysis-Juice® (PJK; shaking at 500 rpm for 5 min) and the light signal was measured in an EnVision™ 2101 Multilabel Reader (PerkinElmer; 2 sec) after 5 min.
Results

Gpr19 mRNA expression is high in SCLC patient samples and cell lines.

Many GPCRs control signaling pathways that regulate cell growth, proliferation and differentiation. It has long been known that tumor cells may express GPCRs in an aberrant fashion (8). In an unbiased approach we investigated gene expression levels of all known GPCRs in a series of human SCLC, NSCLC and normal lung RNA samples. Our survey also included odorant receptors, because these may be expressed in tissues other than the olfactory epithelium; in fact, they may occur in neoplasms such as prostate cancers (27). Hierarchical clustering identified Gpr19 among the GPCRs that showed a message overexpression mostly in SCLC but not in NSCLC samples relative to normal controls (Fig. 1A). High Gpr19 mRNA levels in SCLC samples were verified by a direct comparison with those seen in samples from NSCLC and normal lung (Fig. 1B). This was confirmed by RT-qPCR analysis (Fig. 1C).

We further quantified Gpr19 expression levels by RT-qPCR in a set of NSCLC, SCLC and normal lung-derived cell lines. The expression pattern of Gpr19 was recapitulated, i.e., cell lines derived from SCLC had higher Gpr19 expression than those derived from NSCLC and from normal lung (Fig. 1D). However, high levels of Gpr19 mRNA were also seen in a NSCLC cell line (NCI-H1703) and an immortalized lung epithelial cell line derived from a cystic fibrosis patient (IB3-1). This may be related to the fact that the IB3-1 cell line was immortalized by the large T antigen (28).

We utilized the BioExpress® database for examination of Gpr19 expression in various human normal and cancerous tissues. This search revealed high Gpr19 mRNA expression predominantly in the central nervous system (cerebral cortex, cerebellum, spinal cord; Fig. 1E) as described (29). In addition, Gpr19 mRNA levels were prominent in samples from SCLC and pancreas islet cell carcinoma.

RNA interference-mediated knockdown of Gpr19 reduces cell proliferation.

The prominent expression of Gpr19 preferentially in SCLC and in several lung cancer-derived cell lines suggested that this receptor may play a crucial role in cell proliferation and/or survival. We
investigated the effect of Gpr19 knockdown on proliferation in lung cancer cell lines NCI-H1703 (NSCLC) and DMS 53 (SCLC). These cell lines were chosen based on high Gpr19 mRNA expression and because they grow as monolayers. Most SCLC cell lines including COR-L88, NCI-H209, NCI-H345, NCI-H446 and SHP-77 failed to grow as monolayers but formed (floating) clusters, which rendered them less amenable to experimental manipulations. GPR19 is an orphan receptor, its physiological ligand is not known and there is no antagonist. Accordingly, we employed RNA interference to examine the role of this receptor in supporting cell growth. Cells were either treated with siRNAs targeting Gpr19 mRNA for destruction (GPR19 #1, GPR19 #2), control siRNA (CTL #1, CTL #2) or transfection reagent only (mock) or left untreated. Additional positive controls included the cytotoxic anthracycline doxorubicin and an siRNA directed against Plk1 (30). We monitored the extent of well surface covered by adherently-growing cells (confluence) to quantify cell proliferation. Images of wells covered by NCI-H1703 and DMS 53 cells were captured and analyzed at regular intervals after treatment using the CloneSelect™ Imager (Fig. 2A). Well thumbnails (pictures of the well bottom and cellular detection by the selected detection algorithm) were the basis for confluence calculation (Supplementary Fig. S1A). Measurements at regular intervals started 10 h after treatment and lasted until day 6 (see figure insets for data points from three experiments; Fig. 2A). At this time point, untreated, mock- and control siRNA-treated cells had already reached a high extent of confluence. Plk1 siRNA-transfected and doxorubicin-treated cells did not grow (absent gain in confluence over time). For cells transfected with either Gpr19-targeting siRNA #1 or #2 the cell growth was inhibited as gain in well confluence was modest.

We confirmed the growth inhibition by siRNAs directed against Gpr19 in an additional proliferation assay (alamarBlue®) which recorded the number of viable, i.e., metabolically active cells. Cells were transfected with siRNAs or treated with doxorubicin and the metabolic activity of cells was determined 3 and 6 days later (Fig. 2B). There was a clear-cut reduction in metabolically active cells transfected with siRNAs targeting Gpr19 compared to untreated, mock- and control siRNA-treated samples. In both cell lines the effect was more pronounced on day 6 than on day 3.

In order to directly visualize the effect on proliferation, we recorded pictures of cells from all different experimental conditions on days 3 and 6 after treatment using phase contrast microscopy.
Knockdown of Gpr19 induces G2/M arrest.

Knockdown of Gpr19 reduced cell proliferation in NCI-H1703 and DMS 53 cells. Hence, we examined which phase of the cell cycle was affected by the loss of Gpr19 mRNA. NCI-H1703 cells were either left untreated or transfected with control (CTL #1, CTL #2), Gpr19 (#1, #2) or Plk1 siRNAs. On days 2, 3 and 4 after transfection flow cytometry was done to analyze the distribution of cells in different stages of the cell cycle (Fig. 3A and B). Effective knockdown of Gpr19 mRNA was confirmed by RT-qPCR (Fig. 3C). The number of cells in G2/M phase increased over time when cells had been treated with Gpr19 siRNAs in comparison to untreated or control siRNA-treated cells. This increase in the G2/M population occurred at the expense of cells that were in S phase while the proportion of cells in G1 was less affected. As anticipated, knockdown of Plk1 caused a pronounced G2/M arrest (30). This experiment was also performed in DMS 53 cells using untreated cells or cells transfected with control siRNA CTL #1, Gpr19 siRNA GPR19 #2 (most effective depletion of Gpr19 mRNA) or Plk1 siRNA which yielded similar results (Supplementary Fig. S2A and B; knockdown of Gpr19 mRNA documented in Supplementary Fig. S2C).

We verified the observations obtained from flow cytometry by an independent approach that relied on high-content screening of cells stained for the G2/M phase marker cyclin B1 and the mitosis marker phosphorylated histone H3. This experiment was only performed with NCI-H1703 cells because DMS 53 cells were not suitable for imaging with the ArrayScan® VTI HCS reader. NCI-H1703 cells were transfected with control (CTL #1, CTL #2), Gpr19 (#1, #2) or Plk1 siRNAs or left untreated and analyzed on days 2, 3 and 4 after transfection. Knockdown of Gpr19 mRNA by Gpr19-targeting
siRNAs was effective as monitored by RT-qPCR (not shown). We used a Kolmogorov-Smirnov test to evaluate the distribution of cells that stained positive for phosphorylated histone H3 and cyclin B1 for each time point after siRNA transfection. This evaluation confirmed the increase in G2/M-arrested cells (Fig. 4A and B). Fig. 4C and D show representative pictures from the high-content screen based on Hoechst 33342 DNA staining and cells stained positive for phosphorylated histone H3 and cyclin B1, respectively, on day 3 after transfection. The level of both, immunoreactivity for phosphorylated histone H3 and cyclin B1 increased in cells transfected with the Gpr19 siRNA. This effect was most prominent on days 3 and 4 after transfection. The G2/M block induced by the Plk1 siRNA also resulted in increased levels of phosphorylated histone H3 and cyclin B1. In addition, high-content screening confirmed the G2/M arrest and the reduced proliferative capacity of cells treated with Gpr19 siRNA on the basis of Hoechst 33342 DNA staining (data not shown).

Manipulations that interfere with transition of cells through G2/M are predicted to result in aberrant cell division. Accordingly, we examined whether transfection with Gpr19 siRNAs elicited morphological changes that were consistent with impaired chromosomal segregation. This was indeed the case. High-content screening was indicative of abnormal DNA content in cells that had been treated with siRNAs directed against Gpr19. Visual inspection revealed an excess of polylobed and binucleated cells (Supplementary Fig. 3).

**Gpr19 mRNA expression is cell cycle-dependent and peaks during S phase.**

Observations from Gpr19 mRNA knockdown experiments are consistent with the hypothesis that this GPCR plays a role during the G2/M phase of the cell cycle. We therefore investigated whether Gpr19 mRNA was differentially expressed during the course of the cell cycle.

NCI-H1703 and DMS 53 cells were treated with hydroxyurea for 24 h to synchronize them at the G1-S transition. Samples were taken at various time points post release from cell cycle arrest for DNA content analysis by flow cytometry. Upon removal of hydroxyurea DMS 53 (Fig. 5A, left panel) and NCI-H1703 cells (Fig. 5B, left panel) moved subsequently through the cell cycle in an essentially synchronous fashion: the number of cells in S phase rapidly accumulated and the DNA content shifted from 2n to 4n within 8 h. After 12 h, the vast majority of cells had reached the G2/M phase of the cell
cycle. At later time points (24 h, 36 h and 48 h) the effect of hydroxyurea was lost and the cell cycle
distribution in the population approached that seen in an asynchronously growing culture. RNA was
also extracted at these time points and the message levels of several genes were determined by RT-
qPCR (Fig. 5A and B, right hand panels). The levels of mRNA encoding cyclin E1 were elevated
while cells were at the G1-S boundary and subsequently declined to reach a nadir after 12 h in both,
DMS 53 and NCI-H1703 cells. In contrast, a peak in cyclin B1 mRNA expression was seen after 12 h,
\textit{i.e.}, coincident with the accumulation of cells in the G2/M phase of the cell cycle. These results were
anticipated as cyclin B1 and cyclin E1 show a known cyclic pattern of mRNA expression over the
course of the cell cycle. Expression levels of cyclin E1 are highest prior to S-phase entry, fall during S
phase and remain low during G2/M phase whereas cyclin B1 message is low in G1 phase, rises during
S phase and peaks at G2/M phase (32). Both patterns were recapitulated in our experiments. The
mRNA of \textit{Rpl32} encodes a ribosomal protein of the 60S large ribosomal subunit and does not vary
through the cell cycle (32). Accordingly, we used \textit{Rpl32} as an internal control. In addition, we
examined the cell cycle-dependent changes in mRNAs encoding two different GPCRs, namely \textit{Lpar1}
for NCI-H1703 cells and \textit{Chrm3} for DMS 53 cells. These mRNAs remained essentially constant at the
time points examined. The mRNA encoding \textit{Gpr19}, however, was subject to cell cycle-dependent
regulation: \textit{Gpr19} mRNA levels rose when the synchronized cells moved through S phase, peaked
after 6 to 8 h and started to decline as cells reached G2/M phase, \textit{i.e.}, after 10 h (Fig. 5A and B, right
panels). At later time points (24 h, 36 h and 48 h) mRNA levels did not differ appreciably from those
seen in untreated control cells (open bar in the right hand panel of Fig. 5A and B), regardless of which
mRNA was examined. This is consistent with the fact that the cell population grew again
asynchronously at this stage.

We addressed the possibility that differential \textit{Gpr19} mRNA expression was due to an off-target effect
of hydroxyurea, \textit{i.e.}, unrelated to its effect on the cell cycle. For this reason we repeated the
synchronization study with DMS 53 cells using the DNA replication inhibitor aphidicolin. Cell cycle
profiles and the degree of synchronization were comparable to those of hydroxyurea-treated cells (Fig.
5C, left panel). Similarly, mRNA profiles were also comparable (Fig. 5C, right panel). Most
importantly, following aphidicolin-induced synchronization, the time-dependent changes in Gpr19 mRNA levels recapitulated those seen in hydroxyurea-treated cells. We explored the correlation between Gpr19 expression levels and the relative number of cells in G1, S and G2/M phase for both hydroxyurea-synchronized DMS 53 and NCI-H1703 cells (Fig. 6). The proportion of cells at different stages of the cell cycle was determined using the Watson curve fitting model from FlowJo. As hydroxyurea arrested cells at the G1-S transition, it was impossible to precisely discriminate between G1 and S phase populations at 0 h and 2 h after release of the hydroxyurea-induced block. These data points were therefore omitted. In both cell lines Gpr19 message levels correlated with the relative number of cells in S phase. Conversely, Gpr19 mRNA levels were inversely related to the fraction of G1 cells. In contrast, we did not observe any correlation between the amount of cells in G2/M phase and Gpr19 mRNA expression.

Transcription factors E2F-1, E2F-2, E2F-3 and E2F-4 are recruited to the Gpr19 promoter. The data in Fig. 5 and 6 suggested that accumulation of the mRNA encoding Gpr19 was initiated as soon as cells progressed from G1 to S phase. Members of the E2F family of transcription factors are known to boost transcription from E2F-targeted promoter sites during late G1 or early S phase (33), e.g., the component of the pre-replicative complex cell division cycle 6 (Cdc6; 34).

The Gpr19 promoter region (5 kb of the 5' upstream sequence ahead of the open reading frame) was examined for prospective E2F binding sites using ConSite and the University of California, Santa Cruz (UCSC) genome browser. Four potential E2F binding sites (-15, -185, -3184 and -3769 bp upstream of the Gpr19 open reading frame) were retrieved from the ConSite search. In contrast, only the one at -15 was identified by the UCSC genome browser search. We confirmed the presence of E2F binding sites in the Gpr19 promoter by chromatin immunoprecipitation. ChIP assays identified the presence of E2F-1, E2F-2, E2F-3 and E2F-4 in both NCI-H1703 and DMS 53 cells (Fig. 7). The promoters of Cdc6 and albumin served as positive and negative controls (34, 35), respectively. Chromatin fragmentation was achieved by sonication and shearing resulted in DNA fragments of 300 to 600 base pairs. It was therefore impossible to discriminate between recruitment of E2F-1, E2F-2, E2F-3 and E2F-4 to either binding sites -15 or -185 and/or to both of them. Primer pairs for PCR
amplification of all predicted E2F binding sites were chosen in a way that the PCR product did not contain any other site – except for primer pair GPR19 (-15; -185). With this primer pair the resulting PCR product covered both these predicted E2F binding sites of the Gpr19 promoter.

Several facts suggest that the binding of E2F-1, E2F-2, E2F-3 and E2F-4 to the promoter sites at position -15 and/or -185 of the Gpr19 gene were specific: (i) immunoprecipitation of these E2F-family members did neither pull down the coding sequence of Gpr19 nor (ii) the promoter region of albumin. (iii) Several E2F-family members with repressor activity (E2F-5, E2F-6, E2F-7; 36) did not pull down the Gpr19 promoter, either.

Both E2F binding sites at positions -15 and -185 of the Gpr19 promoter are crucial for luciferase reporter gene expression.

A fragment of the Gpr19 promoter (300 bp upstream of the Gpr19 open reading frame) was introduced upstream of a firefly luciferase reporter gene in order to evaluate its influence on gene expression (Fig. 8). In addition, the putative E2F transcription factor binding sites at positions -15 and/or -185 had been selectively removed. The removal of the putative E2F binding site at position -15 alone or in combination with the one at position -185 negatively affected reporter gene expression in HEK-293 and NCI-H1703 cells (Fig. 8, left and centerfold graphs). In contrast, the knockout of the putative E2F binding site at position -185 alone did not diminish luciferase expression. The opposite was true for DMS 53 cells: Removal of the E2F consensus site at position -185 from the Gpr19 promoter sequence diminished luciferase expression here (Fig. 8, right graph). This signal reduction was further augmented when the E2F consensus site at position -15 had also been erased. Hence, the consensus motif for the recruitment of E2F transcription factors to the promoter of Gpr19 at both positions -15 and -185 upstream of the Gpr19 open reading frame seemed to be important for luciferase reporter gene expression. The preferred usage of each position might be cell line-dependent.

E2F transcription factor gene expression correlates with Gpr19 expression in human lung cancer samples.
The recovery of the Gpr19 promoter in E2F ChIP assays and the results from Gpr19 promoter fragment-controlled luciferase reporter expression indicated a cause-and-effect relation between the action of E2F transcription factors and the expression of Gpr19. We addressed the question whether co-expression of E2F transcription factors and Gpr19 could be observed in patient samples as well. Indeed, based on two published studies (21, 22) and our in-house study described in Fig. 1 (panels A, B and C), a good correlation of the expression of Gpr19 and E2F transcription factors E2f-1 to E2f-3 could be detected (Fig. 9). The in-house study revealed a correlation coefficient of 0.863 for E2f-1, 0.682 for E2f-2, and 0.554 for E2f-3 whereas the correlation coefficient for E2f-4 with Gpr19 was 0.350. Similar correlations were seen in the other two studies. The study by Rohrbeck et al. and our in-house study identified high Gpr19 expression predominantly in SCLC patient samples (Fig. 9B and C). SCLC samples were not included in the study described by Hou et al. but high Gpr19 expression was observed in a subset of NSCLC samples here, namely large cell lung carcinoma (Fig. 9A). This was also true for a few lung cancer samples classified as adenocarcinoma or squamous cell carcinoma.
Discussion

The orphan receptor GPR19 was first identified in the central nervous system (29, 37); it is also found in ovary and testis (37) and abundantly expressed in human embryonic stem cells (38). The gene encoding human Gpr19 resides on the short arm of chromosome 12 in a region that is frequently rearranged in childhood leukemia and to a lesser extent in other neoplasms; accordingly, GPR19 has been postulated to play a role in cancer development (39). In fact, high levels of mRNA coding for GPR19 were found in metastatic melanoma (7, 40). Here we showed that GPR19 is associated with lung cancer. This conclusion is based on (i) an unbiased approach that surveyed GPCRs specifically for their overexpression in SCLC, (ii) data base mining and (iii) the presence of high Gpr19 mRNA levels in several human lung cancer-derived cell lines. High levels of Gpr19 mRNA were not only detected in patient samples from SCLC but also from pancreas islet cell cancer. Both these carcinoma types are often characterized by the presence of neuroendocrine markers (41, 42). This is also true for a subset of NSCLC called large cell neuroendocrine carcinoma (2) and approximately 30% of SCLC is associated with NSCLC elements (43). In fact, an increase in Gpr19 message was observed in large cell lung carcinoma relative to normal lung samples when utilizing the BioExpress® database (Fig. 1E). In addition, high levels of Gpr19 expression could also be attributed to some NSCLC patient samples (mainly large cell lung carcinoma) in the lung cancer gene expression study described by Hou and coworkers (21; Fig. 9A). These data sets did not discriminate between neuroendocrine and non-neuroendocrine large cell lung carcinoma. The NSCLC cell line NCI-H1703 also showed high levels of Gpr19 mRNA expression. It is not clear whether this reflects a neuroendocrine phenotype or de novo illegitimate expression. Thus, the discrimination between Gpr19 high and Gpr19 low expression lung cancers might not strictly follow histological boundaries. In addition, we confirmed the presence of high Gpr19 mRNA expression in melanoma (Fig. 1E).

We examined if expression of Gpr19 conferred a growth advantage in lung cancer cells. The results were unequivocal: regardless of the histological classification of the cell line (SCLC or NSCLC), knockdown of Gpr19 mRNA depressed cell proliferation. This finding is consistent with earlier observations that indicate a role of GPR19 during embryogenesis, in particular in the developing.
brain: high levels of Gpr19 mRNA are initially observed in germ cell layers of the embryo. Subsequently Gpr19 mRNA accumulates in the neural plate, the subventricular zone and other sites, from which neuronal cells emerge in the differentiating central nervous system (15).

GPCRs control signaling pathways that are typically associated with recruitment of quiescent cells into the cell cycle (G0/G1 transition) or with accelerated progression through G1 (4, 6). Surprisingly, knockdown of Gpr19 affected progression through the cell cycle at a later stage, namely in G2/M. This conclusion is based on two independent lines of evidence, namely the determination of DNA content by flow cytometry and the quantification of the G2/M marker cyclin B1 and the mitosis marker phosphorylated histone H3 by immunocytochemistry. Cyclin B1 exerts its function in conjunction with cyclin-dependent kinase 1 during late G2 phase and early mitosis (44). Histone H3 phosphorylation at Ser10 correlates with chromosome condensation during mitosis with phosphorylation levels starting to rise in late G2 phase (45). Thus, our observations are consistent with a model where GPR19 impinges on checkpoint controls that allow for transition through G2 and entry into mitosis or for initiation of the separation of daughter cells. Earlier findings also suggest that GPR19 may play a role in cell division: Gpr19 gene expression levels are high in murine spermatocytes undergoing meiotic cell division (46). In this context it is of interest that NCI-H1703 cell treated with siRNAs directed against Gpr19 exhibited a binuclear or polylabeled nuclear phenotype (Supplementary Fig. 3).

The mechanistic link between a GPCR and cell division during G2/M is not intuitively evident. It is, however, worth pointing out that heterotrimeric G proteins of the Gαi/Gαo-subfamily are involved in the control of the mitotic spindle; they are thought to regulate the mitotic force generator and thus to promote chromosomal segregation (47). The expression of a GPCR does not per se render a cell susceptible to regulation by the receptor – the ligand must also be present. Alternatively, the receptor has a high level of basal activity and engages its cognate G protein(s) in the absence of an agonist (48). The growth inhibitory action of Gpr19-directed siRNAs could also be accounted for by these two scenarios that are not necessarily mutually exclusive: (i) when overexpressed GPR19 displays strong constitutive activity. (ii) GPR19-expressing cells also synthesize and release the agonist; knockdown of the mRNA encoding the receptor disrupts the resulting autocrine loop. GPR19 was previously
proposed to couple to Gᵢ because it engaged a fusion protein comprised of Gᵢₐ and the last 5 amino acids of Gᵢₐ (49). Based on sequence alignments of the transmembrane core, GPR19 was classified as a class A (rhodopsin-like) receptor in spite of the absence of an NPxxY-motif in transmembrane helix seven; the cognate ligand was proposed to be a peptide (37). We attempted to verify Gᵢ-dependent inhibition of cAMP formation via GPR19. However, we failed to detect an effect of pertussis toxin on cAMP accumulation in forskolin-stimulated NCI-H1703 cells that highly express Gpr19 mRNA (data not shown). Because pertussis toxin-catalyzed ADP-ribosylation of Gᵢₐ/Gₒ blocks access of receptors to their C-terminus, this manipulation ought to have unmasked constitutive inhibition of cAMP accumulation. Similarly, we failed to find any effect of conditioned medium (supernatants from cell lines NCI-H1703 and NCI-H345 which highly express Gpr19 mRNA) on cAMP accumulation in HEK-293 cells that had been transfected with Gpr19 expression plasmids (data not shown). Thus, it appears unlikely that the action of GPR19 in the G2/M phase of the cell cycle arises from coupling to Gᵢ/Gₒ. Further progress in this area is contingent on the identification of the cognate agonist(s) of GPR19. Finally, a function other than direct signaling via G proteins might also be conceivable for GPR19, e.g., recruiting G protein-independent pathways via β-arrestins (50), via direct binding of tyrosine kinases (51), or by acting as a scaffolding protein (52).

Our experiments showed that the expression of Gpr19 was regulated during the cell cycle. This finding provides additional – albeit circumstantial – evidence for a role of GPR19 in the regulation of the cell cycle. The levels of mRNA encoding GPR19 peaked when most cells were in S phase. For soluble proteins, this typically implies that the proteins are required for S phase transition. GPR19, however, is a membrane protein, which must mature through the secretory pathway to reach its presumed site of action, the cell surface. While transcription and translation are rapid (less than 1 min for a protein of 400 amino acids), folding in the endoplasmic reticulum imposes a time lag of up to several hours (53). Besides, subsequent trafficking through and maturation steps in the secretory pathway result in an additional delay prior to insertion of the protein into the cell membrane. Pharmacochaperone-triggered folding of the A1-adenosine receptor suggests that more than 5 hours are required for the receptor to reach the cell surface (54). Thus, a delay of several hours is to be anticipated between induction of mRNA expression and accumulation of GPR19 at the cell surface.
Given this inherent delay, expression of \textit{Gpr19} is timely for a membrane protein required for the G2/M-phase.

\textit{GPR19} is not the only GPCR whose expression is cell cycle-dependent. In primary cultures of human microvascular endothelial cells (HMVECs), the protein levels of the chemokine receptor CXCR3 were found to be highest when cells exited S phase and entered G2/M (55). We stress that our data refer to mRNA levels of \textit{Gpr19} rather than accumulation of the protein at the cell surface. CXCR3 was also shown to be directly involved in the proliferation of endothelial cells: its ligands interferon-\(\gamma\)-inducible protein of 10 kDa (IP-10) and monokine induced by interferon-\(\gamma\) (Mig) stalled cell proliferation. This effect could be reversed by a CXCR3-specific antibody.

The promoter region of the gene encoding \textit{GPR19} contains several candidate binding sites for E2F transcription factors. We provide conclusive evidence that at least one of these was in fact occupied by E2F family members E2F-1, E2F-2, E2F-3 and E2F-4 in both DMS 53 and NCI-H1703 cells. E2F transcription factors are known to activate the expression of genes at the G1-S transition necessary for cell cycle progression (33, 35) including, \textit{e.g.}, \textit{Cdc6}, \textit{Cdc25} (34), \textit{thymidine kinase} or \textit{dihydrofolate reductase} (35). The family of E2F transcription factors has been subdivided into activators (E2F-1, E2F-2, E2F-3) and repressors (E2F-4, E2F-5, E2F-6, E2F-7, E2F-8) of gene transcription (33, 36).

Transactivation and repression result from interactions with different cofactors. E2F-4 and E2F-5 both prevent quiescent cells from entering G1 phase. Upon mitogenic stimulation, E2F-1, E2F-2 and E2F-3 accumulate in late G1 phase and cause a pattern of gene expression that drives cells into S phase. During late S phase this signal is attenuated by E2F-6, E2F-7 and E2F-8 (36). Classification of E2Fs into activators and repressors of gene transcription is an oversimplification, because some isoforms can mediate both transactivation and repression. However, we documented binding of three activating E2F isoforms to the \textit{Gpr19} promoter. Further, the cloning of \textit{Gpr19} promoter fragments of different length with and without E2F binding site integrity at positions -15 and/or -185 upstream of a luciferase reporter identified the importance of both sites for gene expression. It might be possible that both E2F binding sites are in use for the activation of \textit{Gpr19} gene expression in a cell-specific manner. In all three cell lines tested, the combined abolition (positions -15 and -185) led to the most pronounced reduction of luciferase reporter expression.
Besides, the expression of activator E2F transcription factors E2f-1, E2f-2 and E2f-3 could be correlated with the expression of Gpr19 in human lung cancer samples from independent lung cancer gene expression studies. Only a weak correlation was observed between Gpr19 and E2f-4. The observed Gpr19 promoter binding of the repressor E2F-4 might reflect its recruitment to the promoter during early G1 phase and subsequent displacement with activator E2Fs in late G1 and early S phase leading to Gpr19 transcription. Thus, it appears justified to conclude that there might be a cause-and-effect relation between E2F binding to the Gpr19 promoter and the observed accumulation of the mRNA coding for GPR19 during S phase. Our conclusion is further supported by the observation that Gpr19 mRNA was present at high levels in the immortalized cell line IB3-1. This cell line was derived from the lung epithelium of a patient suffering from cystic fibrosis with the help of a hybrid virus. Accordingly, IB3-1 cells contain high levels of SV40 large T antigen (28), which inactivates the retinoblastoma protein and hence derepresses E2F-dependent transcription.

When the Gpr19 message in Gpr19-expressing lung cancer-derived cell lines was attenuated by the use of RNA interference, proliferation of these cells was diminished (Fig. 2). Thus, GPR19 might be a vulnerable target, which – when overexpressed in lung cancer cells – is involved in cell proliferation. Transiently transfected GPR19 expression did not result in gain of proliferation in neither HEK-293 nor NCI-H1703 cells (data not shown). However, these studies have not been performed in non-malignant cells. Whether this indicates that GPR19 might lack the ability to transform cells into a malignant phenotype needs further investigation. Hence, the present data do not allow the categorization of Gpr19 as an oncogene (56). Furthermore, its overexpression particularly seen in lung cancer-derived cell lines and SCLC patient samples might be accounted for by the deregulated action of members from the E2F family of transcription factors, which is very often observed in lung cancer (57, 58).

Taken together, our observations highlight GPR19 as a candidate drug target for the treatment of a subset of lung cancers. To the best of our knowledge, GPR19 is the first GPCR whose mRNA has been shown to be expressed in a cell cycle-dependent manner and to impinge on G2/M transition. Retinoblastoma-and E2F-dependent transcription may not only regulate S phase-specific genes but also control the expression of genes that are required at later stages, e.g., in G2/M – particularly if...
these are membrane proteins subject to intracellular trafficking. Finally, we believe that the insights from our current work should also be of relevance to understand the physiological role of GPR19 during embryogenesis and brain development.

Acknowledgements

The authors thank Andreas Bernthaler and Andreas Wernitznig for excellent help with microarray gene expression analysis and Anja Ebert for kindly providing the ChIP assay protocol.

Grant Support

This work was supported by Boehringer Ingelheim, the FWF-funded doctoral program CCHD (Cell Communication in Health and Disease) and PLACEBO (Platform Austria for Chemical Biology) funded by the Austrian Gen-Au (Genome Research in Austria) program.
References


Giuliano KA, Chen YT, Taylor DL. High-content screening with siRNA optimizes a cell biological approach to drug discovery: defining the role of P53 activation in the cellular response to anticancer drugs. J Biomol Screen 2004;9:557-68.


Hebert DN, Molinari M. In and out of the ER: protein folding, quality control, degradation, and related human diseases. Physiol Rev 2007;87:1377-408.


**Figure legends**

**Figure 1. High Gpr19 expression in SCLC patient samples and cell lines.**

A, Heat map for differential expression of genes that encode for proteins with GPCR activity (Gene Ontology number 0004930) in human NSCLC and SCLC patient samples (Affymetrix Human Exon 1.0 ST Array). Cases are represented in columns and single genes in rows. Intensity values (pseudo color scale) are expression values normalized against normal lung samples and log 2-transformed. Enlargement shows the Gpr19-containing cluster. B, C, Results for Gpr19 gene expression from microarray (probe set NM_006143_at) and RT-qPCR analysis of human NSCLC, SCLC and normal lung samples combined per histological group (box (interquartile range) and whiskers (min-max) plot with median (central bar)). For each sample RT-qPCR was performed for Gpr19 normalized against reference genes Actb, Cypa, Hprt1 and Rplp0 and geometric mean values were calculated. Statistically significant differences were determined by a Kruskal-Wallis test followed by Dunn’s multiple comparison (**p<0.01; ***p<0.001). D, RT-qPCR results for Gpr19 gene expression (normalized against reference genes Actb, Cypa, Hprt1 and Rplp0; geometric mean) in various human NSCLC, SCLC and normal lung cell lines. E, Gpr19 gene expression determined by Affymetrix GeneChip analysis (chip sets HG-U133 A and HG-U133 B, probe set 207183_at) in human tissues (green = normal; red = cancer; yellow = metastasis), box (interquartile range) and whiskers (extend to 1.5 times the interquartile range) plot with median (central bar) and outliers (open circles). Sample numbers are indicated in brackets. Data were extracted from the BioExpress® database (Gene Logic).

**Figure 2. Inhibition of cell proliferation by siRNAs directed against Gpr19.**

A, Cell proliferation was determined by measuring confluence, i.e., % of total well area covered by cells: shown is the time course after siRNA transfection (20 nM) or addition of doxorubicin (1 µM); data are means ± SD of three wells per condition (96 well plate) from a representative experiment for untreated, mock- (transfection reagent only), siRNA- (CTL #1 and #2, Gpr19-targeting #1 and #2, Plkl-targeting) and doxorubicin (Dox)-treated NSCLC cell line NCI-H1703 and SCLC cell line DMS 53. Two additional experiments gave similar results. The figure insets illustrate the results from...
the last confluence measurement on day 6 post transfection from the three individual experiments (arithmetic means are indicated by the lines). B, Cell proliferation was assessed by measuring cell viability with alamarBlue® on day 3 and 6 after siRNA transfection and doxorubicin application for NCI-H1703 and DMS 53 cells in three independent experiments (done in triplicates); arithmetic means are indicated by the lines. Statistically significant differences across all experimental groups were determined by one way ANOVA followed by Tukey’s test. GPR19 #1 and GPR19 #2 p values refer to the least significant one from the comparison with CTL #1 and CTL #2 (*p<0.05; **p<0.01; ***p<0.001).

C, Relative Gpr19 mRNA levels on day 3 after siRNA transfection (normalized against reference genes Cypa and Hprt1, geometric mean of triplicates per condition; data are means + SD of three experiments).

Figure 3. Gpr19 siRNA-induced increase in relative number of cells with G2/M phase DNA content.

A, B, The cell cycle distribution (G1, S, G2/M phase) of NCI-H1703 cells (means + SD of three experiments) was determined staining the cellular DNA with propidium iodide and quantifying the distribution by flow cytometry on days 2, 3 and 4 after siRNA transfection (untreated cells; CTL #1 and #2, GPR19 #1 and #2, PLK1; 20 nM). Cellular debris was excluded in a forward versus side scatter dot plot. The profiles of DNA content were analyzed using the cell cycle tool (Watson model) of FlowJo 7.5 software (A); this model approximates G1 (green) and G2/M (blue) phase populations with Gaussian curves and calculates the S phase population (yellow) exactly based on the DNA content histogram (FL2-Area (FL2-A); purple line represents calculated sum of G1, S, G2/M cells based on the Watson model). For each day and cell cycle phase, differences across all experimental groups were tested for significance using one way ANOVA followed by Tukey’s test (B). GPR19 #1 and GPR19 #2 p values refer to the least significant one from the comparison with CTL #1 and CTL #2 (*p<0.05; **p<0.01).

C, Relative Gpr19 mRNA levels on day 2 after transfection (normalized against reference genes Cypa and Hprt1, geometric mean of triplicates per condition; data are means + SD of three experiments).
**Figure 4.** *Gpr19* siRNA-induced increase in immunoreactivity for phosphorylated histone H3 (pH3) and cyclin B1.

The levels of cyclin B1 (marker for G2/M) and of phosphorylated histone H3 (marker for mitosis) were determined by high-content screening after transfection with siRNA (untreated cells; CTL #1 and #2, GPR19 #1 and #2, PLK1; 20 nM). A, B, Kolmogorov-Smirnov (KS) statistic for pH3- (A) and cyclin B1-positive (B) cellular staining on days 2, 3 and 4 after siRNA transfection (means + SD of three experiments). For each day, differences across experimental groups – except for PLK1 (strong antimitotic effect of the mRNA knockdown) – were tested for significance using one way ANOVA followed by Tukey’s test. GPR19 #1 and GPR19 #2 p values refer to the least significant one from the comparison with CTL #1 and CTL #2 (*p<0.05; **p<0.01; ***p<0.001). C, D, Images of immunostaining for pH3 (C, yellow) and cyclin B1 (D, red) in one representative field of 36 (maximal) fields per well (96 well culture plate). Objects encircled in green represent cells detected based on Hoechst 33342 staining.

**Figure 5.** Differential expression of *Gpr19* mRNA over the course of the cell cycle.

DMS 53 cells (A, C) and NCI-H1703 (B) were treated with hydroxyurea (1 mM; A, B) or aphidicolin (3 µM; C) for 24 h. Following release of the block, DNA content was determined at regular time points by propidium iodide staining and flow cytometry (left hand panels) and the corresponding expression profiles of the indicated genes (encoding cyclin B1 and cyclin E1, the ribosomal protein RPL32 and the GPCRs GPR19, LPAR1 or CHRM3) by RT-qPCR (right hand panels). The gene of interest mRNA expression was normalized against four reference genes (*Actb*, *Cypa*, *Hprt1*, *Rplp0*). Gene expression plots show mRNA levels (geometric mean) relative to control samples (untreated (untr) cells) with error bars indicating 95% confidence intervals. *Gpr19* mRNA expression at each time point post release was compared to the mRNA expression of non-differentially expressed *Rpl32* using a t test with Welch’s correction (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Experiments were repeated yielding concurrent results.
**Figure 6. Correlation of Gpr19 mRNA levels with the amount of cells in S phase.**

Gpr19 expression levels were determined over the course of the cell cycle from hydroxyurea-arrested DMS 53 and NCI-H1703 cells (see Fig. 5) and plotted as a function of the relative number of cells in G1, S and G2/M phase. Shown is the result of linear regressions; squared correlation coefficient values (R^2) are given for each plot.

**Figure 7. Binding of E2F-1 to 4 to the promoter of Gpr19.**

Chromatin immunoprecipitation was performed with antibodies against E2F family members (E2F-1, 2, 3, 4, 5, 6, 7) and immunoglobulin G isotype control (IgG). As an additional negative control the immunoprecipitation was done in the absence of any antibody (no AB). DNA from the sheared chromatin input was serially diluted with water (undiluted, 1:5, 1:25, 1:125). Internal controls also included amplification of the promoter of a known E2F target gene (Cdc6) and of an E2F-independent gene (albumin) and to a region lying in the coding sequence (CDS) of Gpr19. Primer pair GPR19 (-15; -185) covered both predicted E2F binding sites of the Gpr19 promoter in the PCR product. Shown are the amplicons from a representative experiment, which was repeated twice with similar results. Full-length agarose gels for NCI-H1703 and PCR primer sequences are presented in Supplementary Fig. S4.

**Figure 8. The importance of both E2F binding sites in the Gpr19 promoter at positions -15 and -185 upstream of the Gpr19 open reading frame for luciferase reporter gene expression.**

HEK-293, NCI-H1703, and DMS 53 cells were transiently transfected with a control (pGL3-Basic) or a Gpr19 promoter fragment-containing luciferase reporter construct (pGL3-Basic-300GPR19; 300 bp of the Gpr19 promoter upstream of the Gpr19 open reading frame). Besides, cells were transfected with constructs in which the putative E2F transcription factor binding sites at position -15 and/or -185 upstream of the Gpr19 open reading frame had been eliminated (pGL3-Basic-300GPR19 (mut 15), pGL3-Basic-300GPR19 (mut 185), pGL3-Basic-300GPR19 (mut 15+185)). Data are means + standard deviation of six replicates per condition (96 well plate) from a representative experiment. Differences across all experimental groups were determined by one way ANOVA followed by
Tukey’s test. P values of GPR19-mutated constructs 15 and/or 185 refer to the comparison with the non-mutant promoter construct pGL3-Basic-300GPR19 (*p < 0.05; **p < 0.01; ***p < 0.001). The experiments were repeated yielding concurrent results.

**Figure 9. Co-expression of transcription factors E2f-1 to 4 and Gpr19 in three independent lung cancer patient sample sets.**

A, Study by Hou et al. (21): 19 large cell lung carcinoma, 65 normal lung (Lung), 45 lung adenocarcinoma, and 27 squamous cell carcinoma samples were compared. The study was performed on the U133 Plus 2 Array. B, Study by Rohrbeck et al. (22): 5 normal lung (Lung), 16 lung adenocarcinoma, 9 small cell lung carcinoma, and 15 squamous cell lung carcinoma samples were compared. The study was performed on the Human Genome Focus Array. C, In-house study (all patient samples had been ordered from Origene Technologies, details are described in Material and Methods and in Supplementary Table S3): 3 large cell lung carcinoma, 14 normal lung (Lung), 8 lung adenocarcinoma, 8 small cell lung carcinoma, and 5 squamous cell lung carcinoma samples were analyzed. The study was performed on the Human Exon 1.0 ST Array. For the generation of the row dendrogram, the following settings were used: Clustering method: Unweighted pair group method with arithmetic mean (UPGMA), distance measure: Euclidean, ordering weight: Average value, normalization: None, and empty value replacement: Constant value: 0. For the generation of the column dendrogram, the same settings were applied except for that correlation was used as the distance measure. Max, Average and Min refer to the expression level of the respective gene. The probe set for each gene used is indicated on the X-axis, the various tumor types are indicated by a color code in the column tissue type.
Figure 1

A

B

C

D

E

Expression value GPR19 probe set 207183_at

Microarray

RT-qPCR

GPR19 mRNA level

NSCLC SCLC Normal lung

Microarray GPR19 expression value

NSCLC SCLC Normal lung

Pancreas

Lung

CNS

Expression value GPR19 probe set 207183_at
Figure 2

A

NCI-H1703

DMS 53

Confluence [%]

Time post transfection [h]

Confluence [%]

Time post transfection [h]

B

alamarBlue day 3

alamarBlue day 6

C

Relative GPR19 mRNA level

Relative GPR19 mRNA level

DMS 53
**Figure 3**

A. 

B. 

C.
Figure 4

A. Day 2 post transfection

B. Day 3 post transfection (cyclin B1)

C. Day 3 post transfection (pH3)

D. Day 3 post transfection (cyclin B1)
Figure 5

A. DMS 53 Hydroxyurea arrest

B. NCI-H1703 Hydroxyurea arrest

C. DMS 53 Aphidicolin arrest
**Figure 6**

![Graphs showing the relationship between Relative GPR19 mRNA level and the percentage of cells in G1, S, and G2/M phases.](image)
Figure 7

**DMS 53**

- GPR19 (-15)
- GPR19 (-185)
- GPR19 (-15, -185)
- GPR19 (-3184)
- GPR19 (-3769)
- GPR19 (CDS)
- Albumin
- CD6

**NCI-H1703**

- GPR19 (-15)
- GPR19 (-185)
- GPR19 (-15, -185)
- GPR19 (-3184)
- GPR19 (-3769)
- GPR19 (CDS)
- Albumin
- CD6

Legend:
- IgG
- No AB
- E2F-1
- E2F-2
- E2F-3
- Input
- Input 1:125
- Input 1:5
- Input 1:25
- Water
- E2F-4
- E2F-5
- E2F-6
- E2F-7
Figure 8

The figure shows the relative light units for different cell lines (HEK-293, NCI-H1703, and DMS 53) and treatment conditions (pGL3-Basic, 300, 300 (mut 15), 300 (mut 185), 300 (mut 15+185)). The bars represent the mean ± SEM. Significant differences are indicated by asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 9
Expression of G protein-coupled receptor 19 in human lung cancer cells is triggered by entry into S phase and supports G2/M cell cycle progression.

Stefan Kastner, Tilman Voss, Simon Keuerleber, et al.

Mol Cancer Res  Published OnlineFirst August 21, 2012.

Access the most recent version of this article at:
do:10.1158/1541-7786.MCR-12-0139

Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2012/08/21/1541-7786.MCR-12-0139.DC2

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.