# Lung cancer suppressor gene GPRC5A mediates p53 activity in non-small cell lung cancer cells *in vitro*

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**Abstract.** Cellular tumor antigen p53 (p53) functions to maintain genomic stability and regulate cell apoptosis, while G protein-coupled receptor class C group 5 member A (GPCR5A) is a lung cancer suppressor gene whose expression is induced by retinoids. The present *in vitro* study assessed the effects of p53 on the regulation of GPRC5A expression and on non-small cell lung cancer (NSCLC) cells. Human NSCLC H1299 (p53-null) and A549 (wild-type p53) cell lines were subjected to p53 cDNA and small interfering (si)RNA transfection, respectively. GPRC5A expression was analyzed by reverse transcription-quantitative polymerase chain reaction and western blotting, and cell behavior was analyzed using cell viability and apoptosis assays. The results of the present study demonstrated that knockdown of GPRC5A expression markedly upregulated tumor cell viability and reduced tumor cell apoptosis, while p53 overexpression in H1299 cells significantly increased the expression level of GPRC5A. p53 overexpression and GPRC5A induction markedly inhibited tumor cell viability and induced apoptosis, while knockdown of p53 resulted in a decrease in GPRC5A expression, inhibited tumor cell apoptosis and increased tumor cell viability. In serum-free culture conditions, GPRC5A expression was decreased in the two cell lines; this decrease was less marked in p53 cDNA-transfected H1299 cells and more marked in p53 siRNA-transfected A549 cells. The results of the present study indicated that p53 antitumor activity may be mediated by GPRC5A in NSCLC cells.

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## Introduction

Lung cancer is the most significant health problem worldwide in males and females, accounting for >1.6 million new cases (~13% of total cancer diagnoses). Lung cancer was the leading cause of cancer mortality in males, and the second leading cause of cancer mortality in females, in 2008 (1). Histologically, lung cancer may be categorized as small cell lung cancer or non-small cell lung cancer (NSCLC), the latter accounting for ~85% of all lung cancer cases (1,2). The majority of lung cancer cases are diagnosed at the advanced stages of the disease, rendering curative surgery unavailable. However, current targeted therapies appear promising (2). Therefore, further research on the molecular mechanisms and pathogenesis of lung cancer may lead to novel strategies for the effective control of NSCLC.

In the present study, the effects of tumor suppressor genes, cellular tumor antigen p53 (p53) and G protein-coupled receptor class C group 5 member A (GPCR5A), on cell survival in NSCLC were investigated. Previous studies have demonstrated that p53 protein functions to maintain genomic stability and regulate cell apoptosis (3,4), and GPCR5A acts as a lung cancer suppressor gene whose expression is induced by retinoids (5-8). In addition, p53 mutation occurs in the majority of NSCLC tissues, and is associated with NSCLC tumorigenesis and poor survival in patients with NSCLC (9). Therefore, detailed investigation of p53 in NSCLC may aid the understanding of the association between genetic mutations and human carcinogenesis. A previous study demonstrated that GPRC5A may be a target gene of p53 in breast and ovarian cancers (10). GPRC5A is a retinoid-induced gene and is associated with human carcinogenesis. In NSCLC, GPRC5A was demonstrated to be a putative tumor suppressor gene (5,6,11-13). GPRC5A was demonstrated to be expressed

in fetal and adult lung tissues, although the expression was decreased or absent in NSCLC tissue specimens (14). A previous study in GPRC5A$^{-/-}$ mice demonstrated that 76% exhibited lung adenomas and 17% exhibited lung adenocarcinoma at 1-2 years of age, whereas only 11% of heterozygous mice and 10% of wild-type mice developed lung adenoma with no mice developing lung cancer (6). GPRC5A-knockout mice exhibited an increased lung cancer incidence following treatment with nicotine-derived nitrosamine ketone (12). Therefore, GPRC5A was considered to be a putative lung cancer suppressor gene. However, other studies have demonstrated that GPRC5A may act as an oncogene (10,15). In breast and ovarian cancers, GPRC5A expression was observed to be upregulated leading to promotion of tumor cell proliferation (10).

The present study investigated the role of p53 in NSCLC cell lines by manipulating p53 expression and measuring GPRC5A regulation, in addition to NSCLC cell behaviors, *in vitro*. The results of the present study provide an insight into the role of GPRC5A in NSCLC and the mediation of p53 antitumor activity, which may be useful for future treatments of NSCLC.

## Materials and methods

*Cell lines and culture*. Human NSCLC H1299 (p53-null) and A549 [wild-type (WT)-p53] cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin (100 U/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and streptomycin (100 g/ml; Sigma-Aldrich; Merck KGaA) in a humidified incubator with 5% $CO_2$ at 37˚C. H1299 cells were reported to be p53-null, whereas A549 cells express wild-type p53 (p53.free.fr/Database/Cancer_cell_lines/HB_cell_lines.html). For the serum-free culture experiment, cells were plated with complete medium containing 10% FBS overnight, and were then washed with serum-free medium twice and maintained in serum-free medium for the indicated time periods.

*p53 plasmid and gene transfection*. pcDNA-p53 plasmid carrying p53 cDNA was provided by Dr Xufeng Chen (University of California Los Angeles, CA, USA) (16). In order to overexpress p53 protein, H1299 cells were cultured and stably transfected with pcDNA-p53 plasmid or vector-only control pcDNA3.1 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Stable population transfection was obtained following selection with 1 $\mu$g/ml G418 (Amresco, LLC, Solon, OH, USA) for 2 weeks.

*p53 and GPRC5A small interfering (si)RNA and gene knockdown*. The siRNA duplexes targeting p53 and GPRC5A were obtained from Invitrogen; Thermo Fisher Scientific, Inc. In order to knockdown p53 or GPRC5A expression in A549 cells, sub-confluently cultured A549 cells were transfected with p53 siRNA, GPRC5A siRNA, or negative control siRNA using the RNAiMAX transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. p53 and GPRC5A expression was assessed 3 days post-transfection using western blotting. The p53 siRNA sequence was 5'-GACUCCAGUGGUAAUCUACTT-3'; the GPRC5A siRNA sequences was 5'-AGGCAGCAUUUUUCG CCUGTT-3' and the negative control (NC) siRNA sequence was 5'-GUAGAUUACCACUGGAGUCTT-3'. All siRNA oligos were from Invitrogen; Thermo Fisher Scientific, Inc.

*RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. Total RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the Thermoscript RT system (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. qPCR amplification was conducted in the ABI7000 sequence detector (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Expression levels of GPRC5A and p53 mRNA were expressed as a ratio compared with the expression level of GAPDH mRNA and measured using the $2^{-\Delta\Delta Cq}$ method (17). GPRC5A primer sequences were 5'-GCCTCACCTTCGCCT TCATC-3' and 5'-CAACTCGTTTCGATTTCTGACAA-3'; p53 primer sequences were 5'-CCTCAGCATCTTATCCGA GTGG-3' and 5'-TGGATGGTGGTACAGTCAGAGC-3'; and GAPDH primer sequences were 5'-GGCTGAGAACGGGAA GCTTGTCAT-3' and 5'-CAGCCTTCTCCATGGTGGTGA AGA-3'. PCR conditions were set to an initial denaturation at 95˚C for 5 min; and 40 cycles of 95˚C for 30 sec, 65˚C for 60 sec and 72˚C for 45 sec, using a mixture containing 2 $\mu$l cDNA, 1 $\mu$l primer, 5 $\mu$l SYBR-Green PCR Master mix and $ddH_2O$ to make a total of 20 $\mu$l.

*Protein extraction and western blotting*. Cells were washed with ice-cold PBS and lysed in lysis buffer (150 mM NaCl; 1% Triton X-100; 0.1% SDS; 50 mM Tris-HCl, pH 8.0; and protease inhibitor mixture), followed by centrifuged at 12,000 x g for 10 min at 4˚C. The supernatant was collected and assayed for protein concentration using the Bio-Rad DC Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and 50 $\mu$g of each protein sample was subjected to 12% SDS-PAGE gel followed by transfer onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) milk in TBS-Tween-20 (150 mM NaCl; 10 mM Tris-HCl, pH 7.5; and 0.1% Tween-20) for 1 h, and were then incubated overnight at 4˚C with the corresponding primary antibodies as follows: anti-p53 (sc-126; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-GAPDH (sc-47724; 1:5,000; Santa Cruz Biotechnology, Inc.) and anti-GPRC5A (10309-1-AP; 1:1,000; ProteinTech Group, Inc., Chicago, IL, USA). After three time washing with TBST, the membranes were incubated with secondary antibodies at room temperature for 1 h. Secondary antibodies (anti-rabbit IgG-HRP; 7074; 1:3,000 and anti-mouse IgG-HRP; 7076; 1:3,000) were from Cell Signaling Technology (Danvers, MA, USA). The specific bands were visualized using enhanced chemiluminescence (MultiSciences Biotech Co., Ltd., Hangzhou, China), and the protein levels were quantified by using NIH ImageJ version 1.6.0_24 (National Institutes of Health, Bethesda, MD, USA).

*Cell viability MTT assay*. Cells in the log growth phase were seeded in a 96-well plate at a density of $2x10^3$ cells/well and grown overnight, followed by transfection with p53, GPRC5A, or NC siRNA as described above. The cells were cultured for 2, 4 and 6 days. At the end of each time point, 20 $\mu$l 5 mg/ml MTT reagent (Sigma-Aldrich; Merck KGaA) was added to the culture and incubated for an additional 4 h. Subsequently, the cell culture medium in each well was replaced with 150 $\mu$l dimethyl sulfoxide and the plate was analyzed using a BioTek Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm. Cell viability was expressed as a % of the control. Experiments were performed in triplicate and repeated ≥3 times.

*Cellular apoptosis assay*. The cellular apoptosis rate was measured using a luminogenic caspase-3/7 substrate (Caspase-Glo 3/7), which detects cleaved caspase 3/7 in apoptotic cells as a luminescent signal. Cells were seeded in a 96-well plate at a density of $2x10^3$ cells/well and transfected with siRNA specific for p53, GPRC5A or NC siRNA as described above. A total of 6 days post-transfection, Caspase-Glo 3/7 reagent (Promega Corporation, Madison, WI, USA) was added into the cell culture and mixed using a plate shaker at 500 rpm for 30 sec, and cells were incubated for a further 1 h. The luminescence activity (relative caspase 3 and 7 activities) were measured using the BioTek Synergy 2 Microplate Reader. Experiments were performed in triplicate and repeated ≥3 times. The cellular apoptosis rate was expressed as a % of the control.

*Statistical analysis*. Experimental data are expressed as the mean ± standard error. A two-tailed Student's t-test was performed to compare the two different treatment groups. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA), GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) and Excel 2007 software (Microsoft Corporation, Redmond, WA, USA).

## Results

*Effects of GPRC5A knockdown on NSCLC cell viability*. In the present study, GPRC5A expression in NSCLC A549 and H1299 cell lines was knocked down using siRNA constructs, and the alterations in the mRNA and protein levels of GPRC5A were assessed using RT-qPCR and western blot analyses. It was observed that downregulation of GPRC5A expression decreased apoptosis and enhanced cell survival (Fig. 1).

*Overexpression of WT p53 protein increases GPRC5A expression in NSCLC cells*. p53-null NSCLC H1299 cells were stably transfected with WT-p53 cDNA, and it was observed that p53 overexpression in H1299 cells significantly increased the mRNA and protein expression of GPRC5A (Fig. 2A-C) compared with the vector-only controls ($P<0.01$). It was additionally observed that p53 overexpression markedly reduced tumor cell viability (Fig. 2D) and increased the activity of caspase 3/7, an apoptosis marker, in H1299 cells (Fig. 2E).

*Knockdown of p53 reduces GPRC5A expression in A549 cells*. In NSCLC A549 cells expressing WT-p53, it was demonstrated that knocking down p53 expression using p53 siRNA transfection led to downregulated expression of GPRC5A mRNA and protein (Fig. 3A-D). In addition, knocking down p53 expression enhanced cell survival (Fig. 3E) and decreased tumor cell apoptosis (Fig. 3F).

*Serum-free culture altered p53 and GPRC5A expression and cell survival in NSCLC cells*. In H1299 cells transfected with control empty vector plasmid, culturing cells in serum-free medium markedly reduced mRNA expression of GRPC5A in a time-dependent manner. When cells were engineered with p53 expression, a decreased level of GPRC5A mRNA was also identified in serum-free medium cultured cells. However, GPRC5A mRNA expression level in these cells were significantly higher than that in cells expressing no p53 ($P<0.05$). It is notable that culturing in serum-free condition medium did not affect the level of GPRC5A mRNA in A549 cells which expressed wild-type p53. In A549 cells that were transfected with p53-siRNA, GPRC5A mRNA level decreased in cells cultured with serum-free medium with statistical significance at indicated each time point ($P<0.05$; Fig. 4).

## Discussion

p53 is a well-studied protein in the literature. p53 has been described as the guardian of the human genome, as it conserves genome stability and integrity (4). p53 is able to activate DNA repair proteins following genomic DNA damage, and arrest cells at the G1/S phase of the cell cycle to fix DNA damage prior to entering cell cycle progression. When DNA damage is irreparable, p53 is able to induce cell apoptosis (18,19). Previous studies have demonstrated that numerous genes interact with p53 and are involved in p53 signaling pathways (19); however, there are multiple unknown factors which regulate the functions of p53 in human cells. Therefore, the present study investigated the interaction of p53 and GPRC5A as a mediator of p53 function in NSCLC cells *in vitro*. It was observed that p53 overexpression in NSCLC cells markedly increased p53 and GPRC5A levels, while p53 overexpression and GPRC5A induction reduced tumor cell viability and induced apoptosis. However, the knockdown of p53 expression additionally downregulated GPRC5A expression, reduced tumor cell apoptosis and increased tumor cell viability. The results of the present study indicate that p53 antitumor activity may be mediated by GPRC5A in NSCLC cells.

The precise mechanism of action of GPRC5A as a retinoid-induced gene remains unclear. However, it has been demonstrated that GPRC5A is able to mediate retinoid antitumor activity or cellular processes. Previous studies have demonstrated that the GPRC5A gene contains retinoic acid binding elements (RAREs), which facilitate the binding of all-trans retinoic acid and thereby induce GPRC5A expression (5-7). Cyclic adenosine 5'-phosphate has been reported to upregulate GPRC5A expression in human aortic smooth muscle cells (20). An additional study demonstrated that p53 was able to reduce GPRC5A expression in breast and ovarian

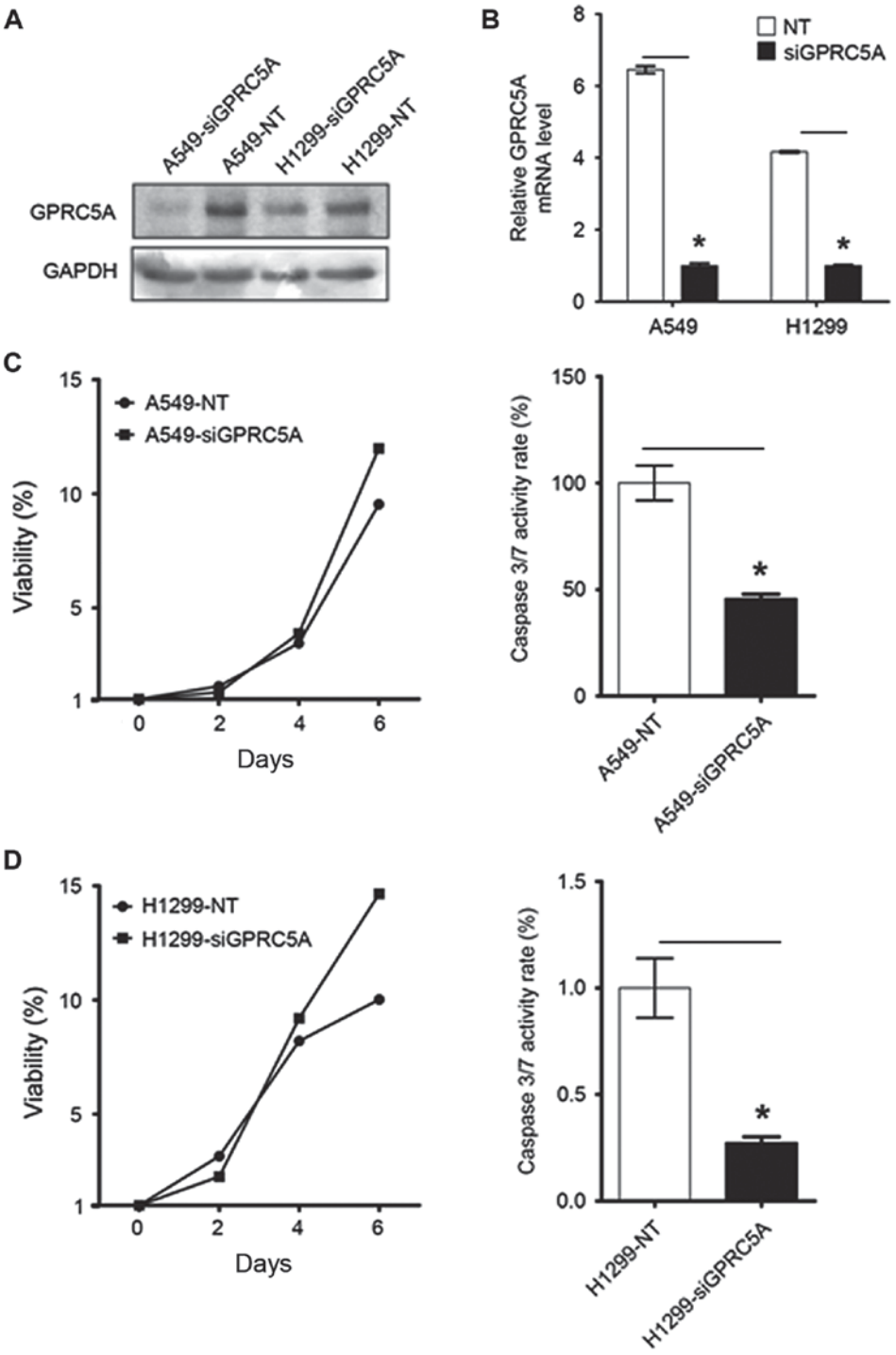


Figure 1. Effect of GPRC5A knockdown on regulation of NSCLC cell apoptosis and viability. (A) Western blot analysis. NSCLC cells were grown and transfected with GPRC5A or negative control siRNA and 48 h subsequently the cells were subjected to western blot analysis of GPRC5A expression. (B) RT-qPCR. Cells were subjected to RT-qPCR analysis of GPRC5A mRNA expression. (C) Cell viability and apoptosis Caspase-Glo 3/7 assays. A549 cells were seeded and transfected with GPRC5A siRNA for ≤6 days and subjected to cell MTT viability assay or Caspase-Glo 3/7apoptosis assay. (D) Cell MTT viability and Caspase-Glo apoptosis 3/7 assays. H1299 cells were seeded and transfected with or without GPRC5A siRNA for ≤6 days and subjected to cell viability MTT assay or Caspase-Glo 3/7 assay. *P<0.05 siRNA treatment vs. NT. GPRC5A, G protein-coupled receptor class C group 5 member A; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NT, controls of transfection with siRNA control for A549 cells or empty vector for H1299 cells.

cancer cell lines. The oncogenic function of GPRC5A in breast and ovarian cancer cells (10) is contrary to the role of GPRC5A in NSCLC demonstrated in the present study; therefore, GPRC5A may exhibit diverse functions in different types of cancer, or alterations in the GPRC5A signaling pathway may serve an important role in carcinogenesis. The present study investigated the role of p53 in regulating GPRC5A expression in NSCLC cell lines and demonstrated the opposing functions of p53, suggesting that p53 and GPRC5A exhibit anti-tumor functions in NSCLC. In addition, GPRC5A is a retinoid-induced gene (6), and culturing cells in serum-free conditions inhibited GPRC5A expression. The results of the

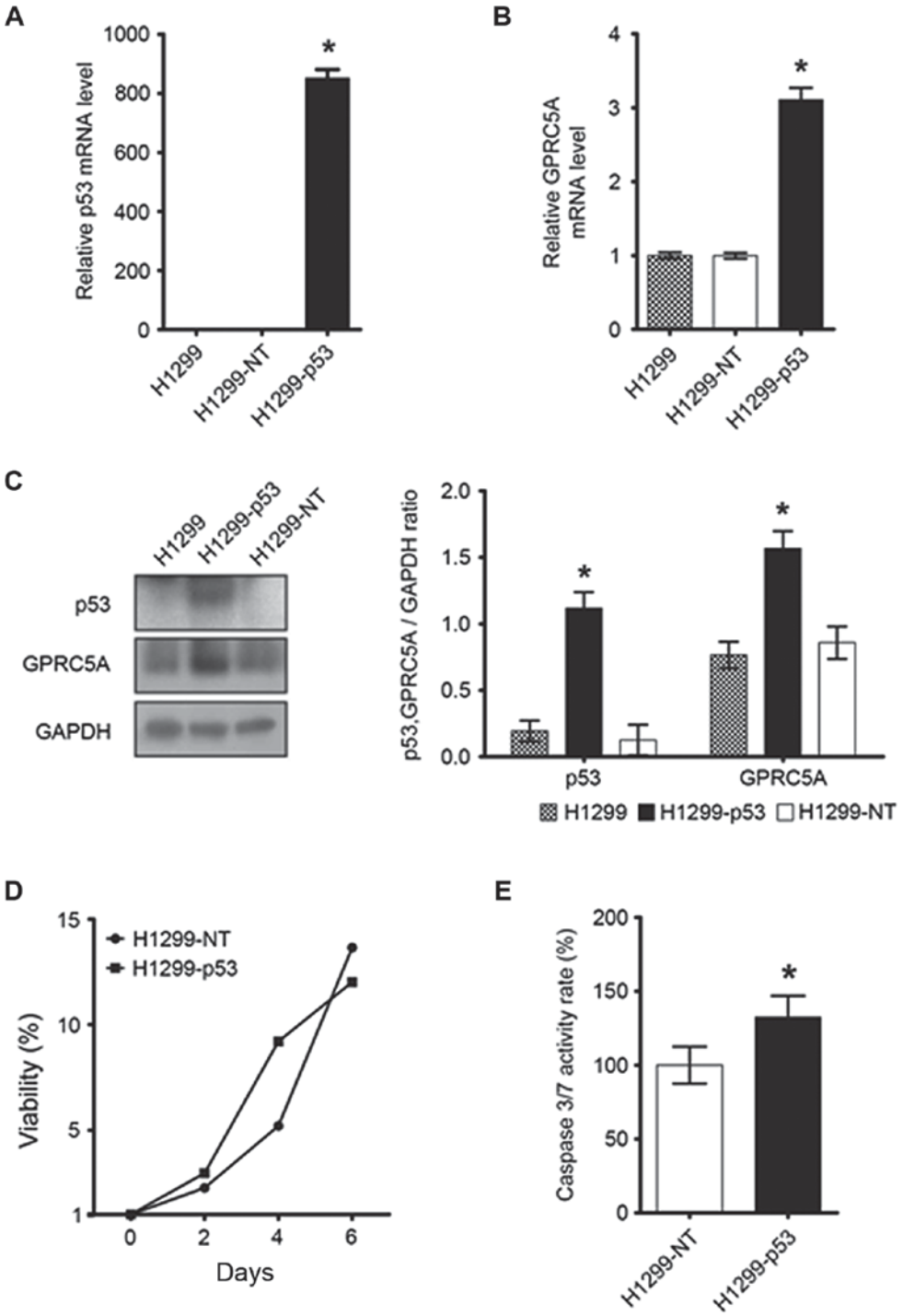


Figure 2. p53 induction of GPRC5A expression in H1299 cells by stable expression of wild-type p53 protein. (A) p53 mRNA level. Cells were grown and stably transfected with p53 cDNA or vector-only control and subjected to RT-qPCR analysis of p53 mRNA expression. (B) GPRC5A mRNA level. Cells were subjected to RT-qPCR analysis of GPRC5A mRNA levels. (C) Western blot analysis of p53 and GPRC5A. H1299 cells stably expressing p53 were subjected to western blot analysis of p53 protein expression. The graph presents the p53/GPRC5A ratio. (D) Cell viability assay. H1299 cells stably expressing p53 were subjected to a cell viability assay. (E) Apoptosis Caspase-Glo 3/7 assay. H1299 cells stably expressing p53 were subjected to an apoptosis assay using Caspase-Glo 3/7. *P<0.05 p53 expressed cells vs. NT or parental cells. GPRC5A, G protein-coupled receptor class C group 5 member A; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NT, empty vector transfection.

present study demonstrated that parental H1299 cells and p53 cDNA-transfected H1299 cells grown in serum-free medium exhibited decreased levels of GPRC5A expression. Increased expression of GPRC5A was observed in p53 cDNA-transfected H1299 cells compared with parental H1299 parental cells, and decreased expression of GPRC5A was observed in A549 cells when p53 was silenced. The results of the present study indicate that GPRC5A is able to mediate p53 antitumor activity in NSCLC.

GPRC5A acts as a tumor suppressor gene in NSCLC and is markedly downregulated in NSCLC tissue and cell lines (5,6). Although the mechanism underlying GPRC5A downregulation remains unknown, studies have demonstrated that a chromosome deletion at 12p12.3 was rare in NSCLC

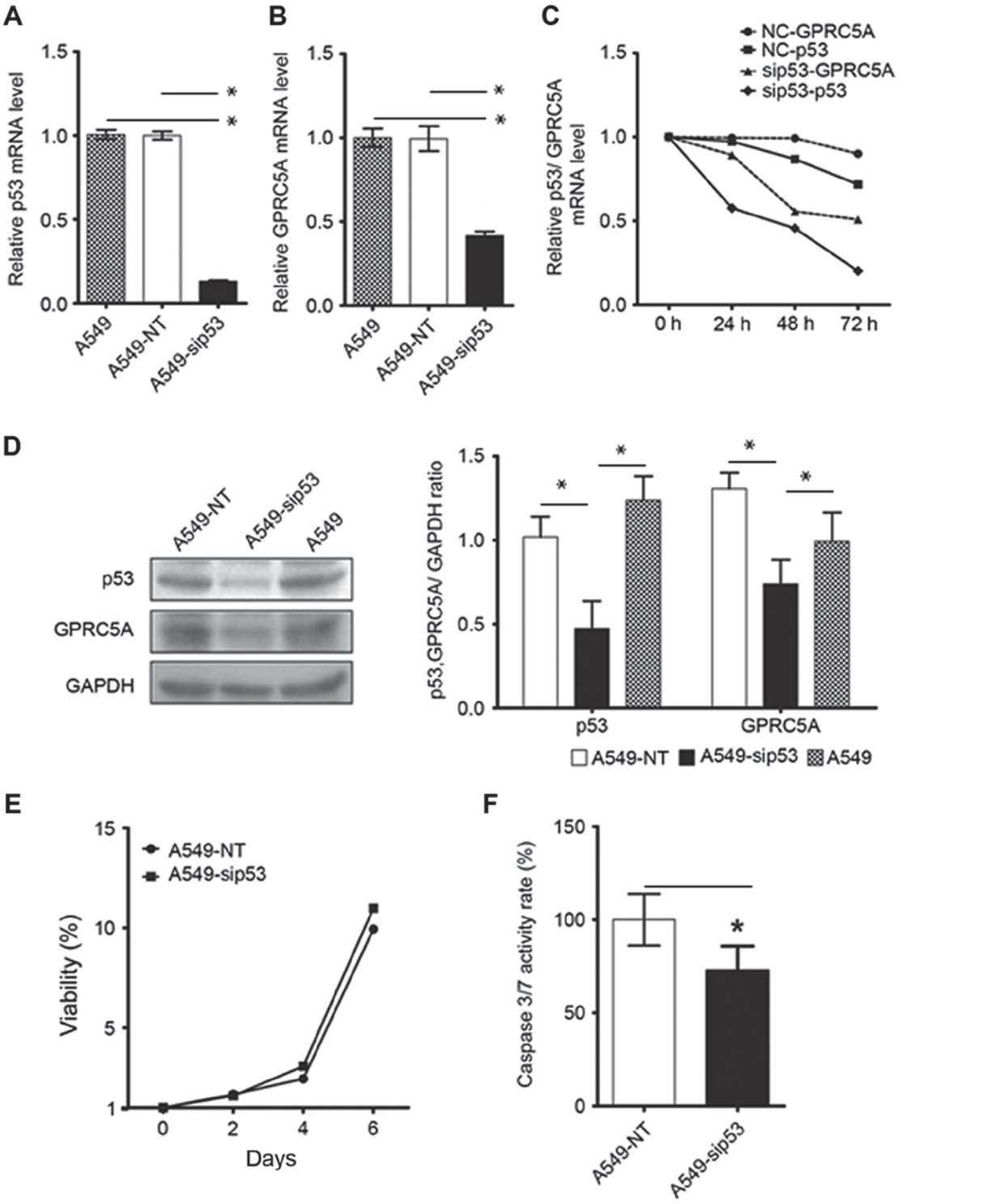


Figure 3. Effects of p53 siRNA on the regulation of GPRC5 expression and non-small cell lung cancer cell behavior. (A) p53 mRNA level. p53 siRNA-transfected A549 cells were subjected to RT-qPCR analysis of p53 mRNA expression. (B) GPRC5A mRNA level. p53 siRNA-transfected A549 cells were subjected to qRT-PCR analysis of GPRC5A mRNA expression. (C) Calculation of the ratio of p53 and GPRC5A. (D) Western blot analysis of p53 and GPRC5A. p53 siRNA-transfected A549 cells were subjected to western blot analysis. The graph presents the quantitative data from the western blotting. (E) Cell viability assay. p53 siRNA-transfected A549 cells were subjected to a cell viability assay. (F) Apoptosis Caspase-Glo 3/7 assay. p53 siRNA-transfected A549 cells were subjected to an apoptosis assay using Caspase-Glo 3/7. *P<0.05 siRNA treatment vs. NT. GPRC5A, G protein-coupled receptor class C group 5 member A; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NT, siRNA control transfection.

and that epigenetic alteration may contribute to GPRC5A downregulation (8). In the present study, it was demonstrated that p53 upregulated GPRC5A expression in NSCLC cells, and that downregulation of p53 decreased GPRC5A expression in NSCLC cells. The results of the present study indicate that the loss of p53 expression in NSCLC may be one of the mechanisms leading to the decreased in GPRC5A expression in NSCLC.

The results of the present study provide a proof-of-concept, and additional studies are required to further elucidate the role

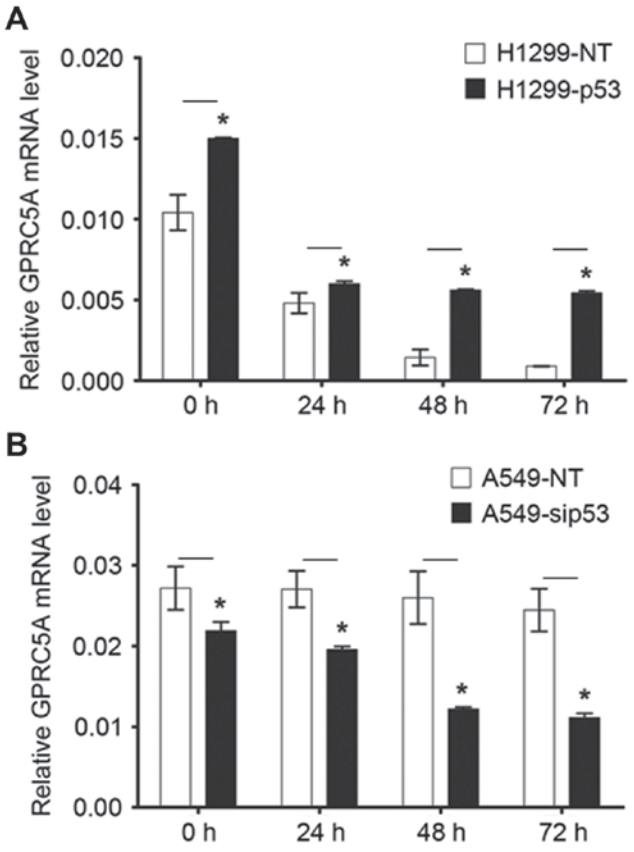


Figure 4. Effects of serum-free culture condition on GPRC5A expression. (A) GPRC5A mRNA level in p53-overexpressing cells. Relative GPRC5A mRNA levels in H1299 cells transfected with p53 cDNA or negative control cells were analyzed using RT-qPCR at 0, 24, 48 and 72 h. (B) GPRC5A mRNA level in p53-knockout cells. Relative GPRC5A mRNA levels in A549 cells transfected with p53 siRNA or negative control siRNA were analyzed using RT-qPCR at the 0, 24, 48 and 72 h. GAPDH served as a loading control. The results are presented as the mean ± standard error with the relative expression normalized to GAPDH. *P<0.05 vs. respective NT. GPRC5A, G protein-coupled receptor class C group 5 member A; siRNA, small interfering RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NT, siRNA control transfection.

of GPRC5A regulation and the association between GPRC5A and p53, in NSCLC.

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