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THE ROLE OF TYROSINE PHOSPHORYLATION IN THE FUNCTIONS OF THE TUMOR SUPPRESSOR GPRC5A

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THE ROLE OF TYROSINE PHOSPHORYLATION IN THE FUNCTIONS OF
THE TUMOR SUPPRESSOR GPRC5A

by

Xiaofeng Lin , BS. MS.

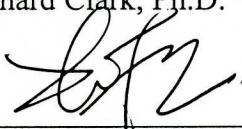
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Supervisory Professor: Reuben Lotan, Ph.D.



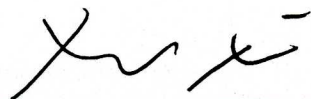
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**THE ROLE OF TYROSINE PHOSPHORYLATION IN THE FUNCTIONS OF
THE TUMOR SUPPRESSOR GPRC5A**

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Xiaofeng Lin, BS, MS.

Houston, Texas
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THE ROLE OF TYROSINE PHOSPHORYLATION IN THE FUNCTIONS OF THE TUMOR SUPPRESSOR GPRC5A

Publication No. _____

Xiaofeng Lin, B.M.

Supervisory Professor: Reuben Lotan, Ph.D.

The retinoic acid inducible G protein coupled receptor family C group 5 type A (GPRC5A) is expressed preferentially in normal lung tissue but its expression is suppressed in the majority of human non-small cell lung cancer cell lines and tissues. This differential expression has led to the idea that GPRC5A is a potential tumor suppressor. This notion was supported by the finding that mice with a deletion of the Gprc5a gene develop spontaneous lung tumors. However, there are various tumor cell lines and tissue samples, including lung, that exhibit higher GPRC5A expression than normal tissues and some reports by other groups that GPRC5A transfection increased cell growth and colony formation. Obviously, GPRC5A has failed to suppress the development of the tumors and the growth of the cell lines where its expression is not suppressed. Since no mutations were detected in the coding sequence of GPRC5A in 20 NSCLC cell lines, it's possible that GPRC5A acts as a tumor suppressor in the context of some cells but not in others. Alternatively, we raised the hypothesis that the GPRC5A protein may be inactivated by posttranslational modification(s) such as phosphorylation. It is well established that Serine/Threonine phosphorylation of G protein coupled receptors leads to their desensitization and in a few cases Tyrosine phosphorylation of GPCRs has been linked to internalization. Others reported that

GPRC5A can undergo tyrosine phosphorylation in the cytoplasmic domain after treatment of normal human mammary epithelial cells (HMECs) with epidermal growth factor (EGF) or Heregulin. This suggested that GPRC5A is a substrate of EGFR. Therefore, we hypothesized that tyrosine phosphorylation of GPRC5A by activation of EGFR signaling may lead to its inactivation. To test this hypothesis, we transfected human embryo kidney (HEK) 293 cells with GPRC5A and EGFR expression vectors and confirmed that GPRC5A can be tyrosine phosphorylated after activation of EGFR by EGF. Further, we found that EGFR and GPRC5A can interact either directly or through other proteins and that inhibition of the EGFR kinase activity decreased the phosphorylation of GPRC5A and the interaction between GPRC5A and EGFR. In c-terminal of GPRC5A, There are four tyrosine residues Y317, Y320, Y347, Y350. We prepared GPRC5A mutants in which all four tyrosine residues had been replaced by phenylalanine (mutant 4F) or each individual Tyr residue was replaced by Phe and found that Y317 is the major site for EGFR mediated phosphorylation in the HEK293T cell line. We also found that EGF can induce GPRC5A internalization both in H1792 transient and stable cell lines. EGF also partially inactivates the suppressive function of GPRC5A on cell invasion activity and anchorage-independent growth ability of H1792 stable cell lines. These finding support our hypothesis that GPRC5A may be inactivated by posttranslational modification- tyrosine phosphorylation.

TABLE OF CONTENTS	PAGE
ABSTRACT -----	v
TABLE OF CONTENTS -----	vii
FIGURES -----	x
CHAPTER 1 INTRODUCTION -----	1
1.1 GPRC5A background -----	1
1.2 Post-translational modification of GPCR -----	2
1.2.1 GPCR glycosylation -----	2
1.2.2 GPCR phosphorylation -----	3
1.3 The epidermal growth factor and its family -----	5
1.4 ErbB family and Lung Cancer-----	6
1.5 Dilemma-----	7
1.6 Hypothesis-----	9
CHAPTER 2 MATERIALS AND METHODS -----	10
2.1 Cell Culture and treatment -----	10
2.2 Cell Construction of GPRC5A wild-type and mutant plasmid -----	10
2.3 Transient and stable transfection of cells-----	11
2.4 Western Blotting-----	13
2.5 Immunoprecipitation (IP) assay-----	14
2.6 Immunofluorescence-----	14
2.7 Proliferation Assay as Measured by MTT Incorporation-----	15
2.8 Cell Migration Assay-----	16
2.9 Anchorage-Independent Colony Formation in Soft Agar Assay-----	16

2.10 Statistical analyses-----	17
CHAPTER 3 RESULTS-----	18
3.1 EGF-induced GPRC5A tyrosine phosphorylation depends on EGFR kinase activity-----	18
3.2 Interaction between GPRC5A and EGFR-----	18
3.3 Alignment of different species of GPRC5A C terminal -----	22
3.4 GPRC5A mutant (4F) cannot be phosphorylated by EGFR activation in HEK293 cells-----	24
3.5 Identification of the specific tyrosine residues in GPRC5A that undergo phosphorylation after EGFR activation-----	25
3.6 GPRC5A and EGFR family members expression in lung cancer cells-----	30
3.7 EGF-induced GPRC5A tyrosine phosphorylation in H292G lung cancer cells-----	30
3.8 Selection of H1792 NSCLC cells for over-expressing exogenous GPRC5A WT and 4F mutant for functional analyses -----	34
3.9 EGF induces tyrosine phosphorylation of GPRC5A in H1792 cells -----	34
3.10 GPRC5A 4F mutant cannot be phosphorylated by activated EGFR in H1792 cells-----	36
3.11 Lack of differences in the effects of WT GPRC5A and 4F mutant on cell proliferation -----	38
3.12 Effects of WT GPRC5A and 4F mutant on EGF-induced cell migration -	38
3.13 Effects of WT GPRC5A and 4F mutant on EGF-induced cell invasion ---	41

3.14 Effects of WT GPRC5A and 4F mutant on anchorage-independent colony formation-----	41
3.15 Differences in the EGF-induced internalization of WT GPRC5A and the 4F mutant in H1792 cells-----	44
CHAPTER 4 DISCUSSION -----	47
CHAPTER 5 REFERENCE -----	56
VITA -----	66

FIGURES	PAGE
Figure 1. GPRC5A protein expression levels in NSCLC cell lines-----	8
Figure 2 Demonstration that GPRC5A is a substrate for EGFR signaling -----	18
Figure 3 Demonstration of the interaction between EGFR and GPRC5A in HEK293T cells -----	20
Figure 4 Alignment of the C terminal domain of GPRC5A from different species ---	22
Figure 5 GPRC5A 4F mutant can not be phosphorylated by activated EGFR -----	26
Figure 6 The GPRC5A mutant Y317F / Y320F is phosphorylated less efficiently by activated EGFR than the mutant Y347F / Y350F or wild type GPRC5A ---- -----	27
Figure 7 Identification of residue Y317 of GPRC5A as the major EGFR phosphorylation target site in HEK 239T cells -----	28
Figure 8 Constitutive co-expression of GPRC5A , EGFR and Her-2 in certain NSCLC cell lines-----	30
Figure 9 Activation of EGFR by EGF induces GPRC5A tyrosine phosphorylation in H292G lung cancer cells-----	32
Figure 10 WT GPRC5A can be phosphorylated by activated EGFR in H1792 cells----	34
Figure 11 GPRC5A 4F mutant cannot be phosphorylated by activated EGFR in H1792 cells -----	36
Figure 12. Over-expression of GPRC5A WT or 4F does not affect the proliferation rate of the H1792 cells -----	38
Figure 13. GPRC5A suppresses the migration of H1792 cells independently of its tyrosine phosphorylation potential-----	39

Figure 14. Differential suppressive effects of WT GPRC5A and the 4F mutant on the invasion potential of H1792 cells-----	41
Figure 15. Differential effects of WT GPRC5A and the 4F mutant on anchorage-independent growth of H1792 cells -----	42
Figure 16. Differential EGF-induced internalization of WT GPRC5A and 4F mutant in H1792 cells transfected in a stable (A) or transient (B) fashion.-----	46

INTRODUCTION

1. GPRC5A background

The retinoic acid inducible G protein coupled receptor family C group 5 type A (GPRC5A) that is expressed preferentially in normal lung tissue (1) is thought to be a lung tumor suppressor (2). The reasons for this contention are: a) its expression is lower in lung cancer cell lines and tumor tissues than in normal lung cells and tissues, b) when over-expressed in lung tumor cells and also non lung tumor cells (e.g., HEK293T cells), GPRC5A inhibits colony formation in agarose, and c) mice with a deletion of both alleles of *Gprc5a* develop spontaneous tumors (2). The mechanism by which GPRC5A suppresses tumorigenesis is not yet clarified. In vivo studies with *Gprc5a* knockout mice as well as in vitro studies with lung cells derived from wild type and knockout mice suggested that *Gprc5a* inhibits the activation of the transcription factor nuclear factor kappa B (NF- κ B), which controls the expression of numerous genes involved in inflammation, proliferation, and survival (3-5). Specifically, lung epithelial cells isolated from *Gprc5a* deficient mice exhibit a constitutively activated NF- κ B compared to cells from wild type littermate mice with normal *Gprc5a* expression. This was evidenced by increased binding of the NF- κ B p65 subunit to the consensus DNA sequence from the I- κ B α promoter before and more so after treatment of the cells with tumor necrosis factor alpha (TNF α). Likewise, expression of exogenous human GPRC5A in human lung tumor cells with low endogenous GPRC5A level suppressed NF- κ B activation by TNF α (6).

Despite these findings, there are various tumor cell lines and tissue samples, including lung,

that exhibit higher GPRC5A expression than normal tissues and some reports that GPRC5A transfection increased cell growth and colony formation (2, 7-9). Obviously, GPRC5A has failed to suppress the development of the tumors and the growth of the cell lines where its expression is not suppressed. The reasons for this failure are not clear. Some tumor suppressor genes are inactivated by LOH and mutations. However, no mutations were detected in the coding sequence of GPRC5A in 20 NSCLC cell lines. It is possible that GPRC5A acts as a tumor suppressor in the context of some cells but not in others. **Alternatively, we raised the hypothesis that the GPRC5A protein may be inactivated by posttranslational modification(s) such as phosphorylation.**

2. Post-translational modification of GPCR

Most GPCR can be modified post-translationally by glycosylation, phosphorylation and palmitoylation and result in conformation change, different protein association, protein's subcellular localization and biological function changes (10-14).

2.1 GPCR glycosylation

Characteristically, GPCRs possess at least one site for N-glycosylation in the outer loop domain. When the glycosylated proteins are analyzed in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), they migrate with an apparent molecular weight higher than the protein molecular weight. In addition, the glycosylated protein might appear as a diffuse band on SDS-PAGE. When the protein is deglycosylated,

the migration band on SDS-PAGE gel will shift from a diffuse band to a well-focused band associated with a decrease in apparent molecular weight of the protein (15). In our system, GPRC5A also show diffuse band and higher molecular weight in SDS-PAGE gel.

2.2. GPCR phosphorylation

Many GPCRs can be phosphorylated on distinct sites including serine /threonine and tyrosine by a range of receptor kinases including protein kinase A, protein kinase C and G protein coupled receptor kinases (GRKs) (10). These post-translational modifications result in different signaling outcomes, such as inhibition or enhancement of the receptor's function. GPCRs are known to undergo phosphorylation as a part of their desensitization following agonist stimulation. This phosphorylation is directed to serine/threonine residues in the cytoplasmic tail and third cytoplasmic loop but rarely on tyrosine residues and the Ser/Thr phosphorylation leads to the internalization of GPCRs (11). One of the mechanisms associated with desensitization of GPCR signaling is initiated by serine/threonine kinases known as GPCR kinases (GRKs). The GRKs often phosphorylate Ser/Thr residues on the cytoplasmic domain of agonist bound GPCRs after dissociation of the interaction with G proteins and this enables the formation of a complex with the protein β -arrestin, which terminates G protein coupling (16).

Besides the well-established ser/thr phosphorylation, GPCRs can also undergo tyr phosphorylation on residues located in the cytoplasmic domain although the role of tyr phosphorylation in GPCR signaling is much less clear than ser/thr phosphorylation (10). It has been suggested that tyrosine phosphorylation of GPCR is required for src recruitment

and subsequent ser/thr phosphorylation by GRK (17). In some GPCRs, a tyrosine containing motif also linked to internalization has been identified in the cytoplasmic tail (7). For example, the cytokine-induced tyrosine phosphorylation of CXCR4, which reduces the expression of functional CXCR4 on the cell surface, contributes to GRK3 and β -arrestin2-mediated sequestration of this receptor in the cytoplasm (18).

It has been reported that GPRC5A is one of about 60 proteins that undergo tyrosine phosphorylation in human mammary epithelial cells (HMECs) or in HMEC overexpressing HER2 after treatment with either epidermal growth factor or heregulin (13). GPRC5A was also predicted to be the substrate of some oncogenic tyrosine kinase, such as Src and EGFR (14). These reports suggest the existence of a crosstalk between EGFR and GPCRs and that tyrosine phosphorylation of GPRC5A could be regulated by EGF receptor signaling.

Several studies used phosphoproteomic approaches to characterize tyrosine kinases and their substrates in normal and malignant cells from mammary and lung tissues. During some of those studies, several groups identified GPRC5A as a substrate for RTKs (19, 20). For example, Zhang et al and Wolf-Yadlin et al reported that GPRC5A can undergo tyrosine phosphorylation in normal HMEC cells treated with EGF or Heregulin (HRG). They identified tyrosine residue Y320 as the primary phosphorylated amino acid in cells treated with EGF and Y347 in the same cells treated with HRG suggesting that EGFR and HER2 can phosphorylate GPRC5A at different sites. These residues are located in the carboxy terminal region of the protein, which is in the cytoplasmic domain.

A recent global analysis of tyrosine kinases identified GPRC5A as a member of a group of substrates for tyrosine kinases in non-small cell lung cancer cell lines and tumor tissues expressing EGFR and Src kinases (21). Finally, a database of tyrosine phosphorylated sites (www.phosphosite.org) includes information on GPRC5A indicating that tyrosine residues at positions 317, 320, 347 and 350 are phosphorylated in various cells and tissues.

By aligning the C terminal sequences of GPRC5A from different species, we found that these tyrosines are conserved in evolution suggesting that they may play an important role in the function of GPRC5A. However, the effects of tyrosine phosphorylation on GPRC5A function were not explored in depth. In one study, it has been noted that phosphorylation of GPRC5A on Y347 induced in HMEC cells overexpressing transfected HER by treatment of with HRG was among the least correlated with effects of HRG on cell proliferation and migration suggesting that phosphorylated GPRC5A did not play a role in these cellular processes (22).

3. The epidermal growth factor and its family

The ErbB family is one of the major groups of receptor tyrosine kinases. It includes the Epidermal Growth Factor Receptor (EGFR/ErbB1), ErbB2 (HER2/Neu), ErbB3 (HER3), and ErbB4 (HER4). ErbB family members consist of an extracellular ligand-binding domain, a single transmembrane domain, an intracellular tyrosine-kinase domain and cytoplasmic tyrosine residues that serve as sites for receptor phosphorylation (23-25).

Many members of the EGF family of peptide hormones serve as ligands and bind to different ErbB receptors. For example, EGF, transforming growth factor- α , and

amphiregulin specifically bind to EGFR; β -cellulin, heparin-binding EGF (HB-EGF) and epiregulin, which show dual specificity, bind to both EGFR and ERBB4; Neuregulins (NRGs) bind to ERBB3 and ERBB4 or only ERBB4. However, none of the EGF family of peptides has been shown to bind to ERBB2 directly (26). Ligand binding enables receptor homo- or hetero-dimerization and results in tyrosine kinase activation and phosphorylation on tyrosine residues across receptor dimers. The phosphorylated tyrosine residues create docking sites for signaling molecules leading to the activation of pathways including Ras/MAPK, PI-3K/AKT, and STATs, and regulate multiple biological responses such as cell motility, invasion, adhesion, angiogenesis, apoptosis, and repair (27). It's interesting that ErbB2 does not require ligand binding for heterodimerization with other receptors and can form homodimers when the protein is overexpressed. ErbB2 is the preferred partner for heterodimerization with all the other ErbB members (26, 28). Unlike other ErbB members, ErbB3 does not have kinase activity although it has ligand. ErbB3 only becomes tyrosine phosphorylated and serves as a signaling factor when it is dimerized with another ErbB receptor (29, 30).

4. ErbB family and Lung Cancer

Lung cancer is the leading cause of cancer death in both man and woman. The major type of lung cancer is called non-small-cell lung cancer (NSCLC). It accounts for about 80% of all lung cancers and includes different subtypes such as adenocarcinoma, bronchioloalveolar, squamous, anaplastic and large-cell carcinomas (31). Most of lung cancers are caused due to smoking. However, the incidence of NSCLC among

non-smokers is significant. A variety of molecular changes, both genetic and epigenetic, have been identified in NSCLC. For example, EGFR is over-expressed in more than 60% of NSCLC cases (32). Moreover, the ligands of EGFR, such as EGF and TGF α , are also frequently expressed in NSCLCs and can form autocrine loops resulting in receptor hyperactivity (33). In addition to overexpression, ErbB mutations are also observed in lung cancer. For instance, the frequency of ErbB2 mutation was 4.2% in NSCLC and 9.8% in adenocarcinomas, and EGFR mutation in 2% of NSCLC and 4% in adenocarcinomas (34). Overexpression or mutation of ErbB family members may enhance receptor tyrosine kinase activity, which affects substrate specificity and downstream signaling pathway and contributes to early and late stages of tumorigenesis (35).

5. Dilemma:

Despite the findings supporting a tumor suppressor role for GPRC5A also in human lung, there are experimental findings that indicate that this function may not be extrapolated to all lung cancers. For example, GPRC5A mRNA expression is relatively high in some NSCLC cell lines and tumor tissues relative to normal lung cells and tissue, and 4 of 11 NSCLC cell lines still express GPRC5A protein at high levels (Fig. 1). Obviously, GPRC5A has failed to suppress the development of the tumors from which high-expressing cell lines were derived.

Fig. 1

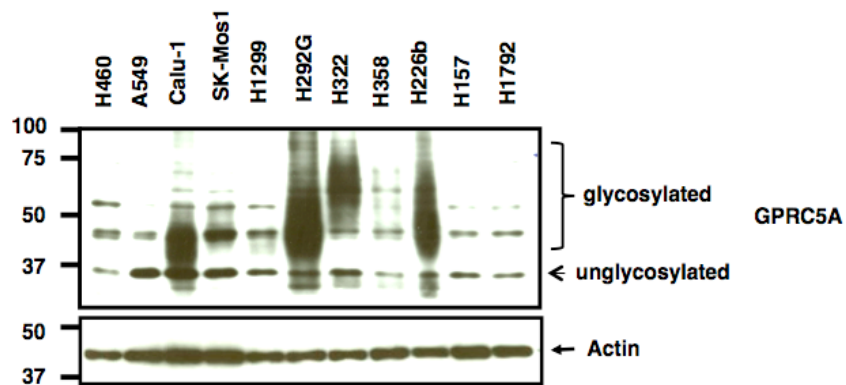


Figure 1. GPRC5A protein expression levels in NSCLC cell lines.

The NSCLC cell lines (indicated above each lane) were cultured in DMEM/F12 medium with 10% serum and subconfluent cultures were harvested, lysed and processed for SDS-polyacrylamide gel electrophoresis and immunoblotting using antibodies against GPRC5A and actin. The actin blotting was used to assess the loading of proteins in each lane.

6. Hypothesis:

Some tumor suppressor genes are inactivated by LOH and mutations. However, no mutations were detected in the coding sequence of GPRC5A in 20 NSCLC cell lines.

Therefore, we raised the hypothesis that GPRC5A may be inactivated either by epigenetic silencing of its transcription, or, for those cases that the protein is expressed in tumor cells, the protein may be inactivated by posttranslational modification(s) like tyrosine phosphorylation. This concept is supported by previous observations that the tumor suppressor Rb is inactivated by serine phosphorylation and the cell cycle regulating kinases CDK4 and CDK6 are inhibited by tyrosine phosphorylation (36-38).

MATERIALS AND METHODS

2.1 Cell Culture and treatment

Human non-small cell lung cancer cell lines (H460, A549, H226, H157, Calu-1, H226B, H322, H292G, H1792, H358 and Sk-Mes-1) were obtained from Adi Gazdar and John Minna (UT Southwestern, Dallas, TX). The HEK293T cells and human NSCLC cell lines were cultured in Dulbecco's modified Eagle's minimum essential medium mixed 1 : 1 with Ham's F12 medium. The medium was then supplemented with 10% fetal calf serum. Cells were cultured at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂.

2.2 Construction of GPRC5A wild-type and mutant plasmid

GPRC5A open reading frame was cloned by polymerase chain reaction (PCR), from cDNA of NSCLC cell line 292G using primers RAI3-F containing KpnI site (*Italic*) (GCAC *GGTACC* GCCACC ATG GCT ACA ACA GTC CCT GAT GGT TGC CGC AAT) and RAI3-R containing XbaI site (*Italic*) (GAGC *TCTAGA* CTA GCT GCC CTC TTT CTT TAC TTC ATA GTC TTT) and subcloned into pcDNA3.1(+) plasmid. To construct the pcDNA3.1(+)-GPRC5A-Myc plasmid, GPRC5A coding sequence with myc tag at its C-terminal was generated by PCR using primers RAI3-F and RAI3-Myc containing myc tag (underline) and XbaI site (*Italic*) (GAGC *TCTAGA* CTA CAG ATC CTC TTC AGA GAT GAG TTT CTG CTC GCT GCC CTC TTT CTT TAC TTC ATA GTC TTT) and subcloned into pcDNA3.1(+) plasmid. DNA sequencing was carried out by the Sequencing Core Facility at MD Anderson Cancer Center. Point Mutation of GPRC5A was performed

by Site-Directed Mutagenesis PCR, using PfuTurbo® DNA Polymerase (Stratagene, La Jolla, CA). Plasmid pcDNA3.1(+)-GPRC5A-Myc was used as a template. Primer Y317/320F-F (5'-GGT TTT GAA GAG ACC GGT GAC ACG CTC TTT GCC CCC TTT TCC ACA CAT TTT C-3') and primer Y317/320F-R (5'-GAA AAT GTG TGG AAA AGG GGG CAA AGA GCG TGT CAC CGG TCT CTT CAA AAC C-3') were used to generate plasmid Y317/320F-GPRC5A-Myc. Primer Y347/350F-F (5'-CCA CGC TTG GCC GAG CCC TTT TAA AGA CTT TGA AGT AAA GAA AGA GG-3') and primer Y347/350F-R (5'-CCT CTT TCT TTA CTT CAA AGT CTT TAA AAG GGC TCG GCC AAG CGT GG-3') were used to generate plasmid Y347/350F-GPRC5A-Myc. Cycling parameters were: 95°C, 1 min, 95°C, 50 sec, 60°C, 50 sec, 68°C, 8 min 30 sec, 18 cycles; 68°C for an additional 7 min after the final cycle. After PCR reaction, DpnI was used to digest the template plasmid, which includes the methylation modification. To generate the 4F mutant of GPRC5A, both Y317/320F-GPRC5A-Myc and Y347/350F-GPRC5A-Myc were digested with KpnI and EcoRI separately. Two specific digested fragments were observed in each sample. The shorter digested fragment from Y317/320F-GPRC5A-Myc sample and the longer digested fragment from Y347/350F-GPRC5A-Myc sample were ligated together to generate 4F mutant plasmid (Y317/320/347/350F-GPRC5A-Myc). The Nucleotide sequence of the GPRC5A point mutants were confirmed by DNA sequencing from the Sequencing Core Facility at MD Anderson Cancer Center.

2.3 Transient and stable transfection of cells.

H1792 cells, which are difficult to transfect, required the following procedure: cells were harvested by repeated pipetting after a brief treatment with trypsin/EDTA and counted.

Around 3×10^5 cells were mixed with 100 μ l of electroporation transfection solution (Solution V, Dharmacon, Lafayette, CO) wild-type GPRC5A-myc, or 4F mutant GPRC5A-myc with EGFR or vector were added to the cells at a final concentration of 4 μ g sample, and the mixtures were transferred to electroporation cuvettes and subjected to electroporation (Amaxa Biosystems, Cologne, Germany) according to the manufacturer's programs and instructions (program T-24 was used for H1792 cells). The transfected cell suspensions were immediately mixed with 500 μ l of pre-warmed DMEM/F-12 medium supplemented with 10% FBS. The cells were then transferred to six-well plates, incubated for 48 hrs, and treated with or without 100 ng/ml EGF in fresh medium after pre-starvation. Proteins were extracted from the treated cells for Western blot analysis and immunofluorescence assay.

HEK293T, which are transfected easily, were subjected to the following procedure: FuGENE6 transfection reagent (Roche Applied Sciences) for plasmid transfection was mixed with DNA at a ratio of 3:1 (μ l, for FuGENE6 Transfection Reagent, and μ g for DNA, respectively). The FuGENE6 Transfection Reagent was pipetted directly into the medium, without allowing contact with the wall of the plastic tube. The solution was mixed for one second using a Vortex mixer and incubated for 5 minutes at room temperature before adding the plasmid DNA into each of the tubes and mixing for one second. The transfection reagent: DNA complex was then incubated for 30 minutes at room temperature and the mixture was added into the culture dishes without changing the medium.

For generation of stable transfected cell lines, H1792 cells were transfected and incubated in medium, which including 500 μ g/ml Geneticin (G418) for two weeks. For each sample,

at least 48 single clones were isolated by trypsin/EDTA and cultured into individual plates. Positive clones were selected by immunofluorescence staining and western blotting as described below.

2.4 Western Blotting

Cells were lysed in 0.05M Tris-HCl buffer, pH 7.4, 0.15M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 1 mM Na_3VO_4 , 5 mM phosphatase inhibitor 4-nitrophenylphosphate (4-NPP), 5 mM beta-Glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 $\mu\text{g}/\text{mL}$ aprotinin, and 5 $\mu\text{g}/\text{mL}$ leupeptin. Lysates were incubated on ice for 15 minutes, and subjected to centrifugation at $13\,000 \times g$ for 30 minutes at 4°C to pellet cell debris. The supernatants were collected, and their protein concentrations were determined using a Protein Assay Kit (Bio-Rad, Hercules, CA). Samples containing 20 – 40 μg of protein were subjected to electrophoresis through 10% polyacrylamide slab gels in the presence of 0.1% SDS and transferred to nitrocellulose membranes (Bio-Rad) by electroblotting. After transfer, membranes were immersed in blocking solution (5% nonfat milk in PBS, 0.5% Tween-20) at room temperature for 1 hour and then incubated with mouse anti-myc tag monoclonal antibodies diluted 1 : 500 in blocking solution overnight at 4°C with shaking. Membranes were washed three times with PBS containing 0.5% Tween-20 and incubated with anti-mouse antibodies (horseradish peroxidase – conjugated) diluted at 1 : 8000 in blocking solution at room temperature for 1 hour. Membranes were then washed three more times with washing buffer, and antibody binding was detected using the enhanced chemiluminescence system (Amersham) and

Hyperfilm MP (Amersham). Four independent experiments were performed, each in duplicate.

2.5 Immunoprecipitation (IP) assay

HEK293T cells were grown in 10-cm diameter plates and when the cultures reached 80% confluency, cells were transiently transfected with 1 µg of pCDNA3.1(+)-GPCR5A-myc and 3 µg of pcDNA3-EGFR, HER-2 or empty vector. Cells were lysed 48 hours after transfection. Samples containing 1.0 mg protein were precleared with 2 µg of IgG antibody and 50 µl agarose beads (Roche Applied Science) for 1 hour at 4°C, non-specific protein conjugated with IgG and beads was removed by centrifugation at $13\,000 \times g$ for 1 minutes at 4 °C. The supernatants were collected, and incubated with 2 µg of primary antibody and 50 µl agarose beads overnight at 4°C. Beads were then washed five times with lysis buffer and the immunoprecipitated protein complexes were resolved by a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this IP-Western blotting assay, Immun-Blot PVDF membranes (Bio-Rad Lab, CA) was used to increase the transfer efficiency, and specific second antibody- TrueBlot mouse or rabbit IgG (eBioScience, San Diego, CA) were used to reduce the effect of immunoprecipitating heavy and light chains.

2.6 Immunofluorescence

NSCLC H1792 were seeded onto cover slips and incubated overnight, then they were transfected with GPCR5A wild-type or 4F mutant for 48 hours. The cells were then treated with EGF (100 ng/ml) or buffer control for 6 hours, rinsed with PBS, fixed in 3.7% formaldehyde for 30 minutes at 4°C, and permeabilized by incubation with 1% Triton

X-100 in PBS (PBST) for 5 minutes. After pre-blocking with 5% bovine serum albumin (Sigma) in PBS, cells were incubated overnight with mouse monoclonal myc-tag antibody (Upstate, 1 : 500 in PBST) at 4 °C, washed with PBS three times, and incubated with fluorescein isothiocyanate (FITC) – conjugated rabbit anti-mouse (Molecular Probes, Eugene, OR, diluted 1 : 1000 in PBST) for 1 hour at 37 °C. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) for 5 minutes at room temperature. Three independent experiments were performed. In each of them, 200 cells were analyzed in five different microscopic fields at 40× magnification using an Olympus confocal microscope. Images were captured with Nikon camera, and images of DAPI and FITC staining were overlaid digitally.

2.7 Proliferation Assay as Measured by MTT Incorporation

H1792 stable transfected cells were plated overnight in 96-well plates at 1×10^3 cells per well in growth medium with 10% FBS. After seeding, cells were maintained in growth media treated with 100 ng/ml EGF or buffer control for 24, 48, 72, 96 and 120 hours. At different time course 40 μ l of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) stock solution (5 mg/ml) was added to each wells for 4 hours at 37°C. Then MTT was removed and replaced by 200 μ l of DMSO for 10 min at 37°C until formazan crystals were dissolved. The absorbance was measured on SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA) at 550 nm with a reference at 690 nm. The values were calculated using the xl-fit for Windows software, version 4.0. Two independent experiments were each performed in triplicate.

2.8 Cell Migration and invasion Assay

For the migration assay, a trans-well assay was performed with an 8.0 μM pore size control cell culture inserts in 24 well plate (BD BioCoat #354578, San Jose, CA). The filter inserts were immersed in FBS free DMEM for 2 hours at room temperature. Cells were trypsinized, washed twice, re-suspended with FBS free DMEM, and seeded to the top of insert (4×10^4 cells in 0.6 ml DMEM). The same volume of DMEM (10% FBS) with or without 100 ng/ml EGF was loaded into the lower chamber and incubated overnight at 37°C in a 5% CO₂ incubator. The cells on the topside of the filter were scrubbed by cotton tipped swab moistened with serum-free medium, and the migrated cells, which attached to the lower side of the filter were fixed with 96% ethanol for 30 minutes and stained with 1.5% crystal violet for 10 minutes. The filters were washed by distilled water and air dried. The migrated cells were counted in five different microscopic fields under 10 \times magnifications using a Nikon fluorescence microscope.

For the invasion assay, the trans-well assay was performed with an 8.0 μM pore size control cell culture inserts coated with matrigel in 24 well plate (BD BioCoat #351150, San Jose, CA).

2.9 Anchorage-Independent Colony Formation in Soft Agar Assay

Equal volumes of 1.5% low-melting temperature agarose and 2X DMEM/F12 with 20% FBS were mixed and 0.3 ml of the mixture were added in each well of a 24-well plate to create a base semisolid layer. H1792 cells stable transfected with Vector, Wildtype or 4F

mutant of GPRC5A were suspended at 2000 cells / 0.3 ml in a mixture of 0.75% low-melting temperature agarose + 2X DMEM/F12 with 20% FBS + Matrigel (BD Biosciences #354234) diluted 15 : 15: 1 (vol/vol/vol). Cell suspensions were then placed on top of the previously cast semisolid layers in a 24-well plates. Colonies were allowed to form at 37°C in humidified incubator for 10 days. Aggregates of 50 or more cells were considered to be a colony. Colonies were counted in four different fields under a microscope at 4× magnification and photographed. The means and 95% confidence intervals (CIs) of the number of colonies in four microscopic fields were calculated. Two independent experiments were performed, each in triplicate.

2.10 Statistical analyses

All analyses were performed in triplicates and the significance of differences between groups was calculated using the student's t test. P value <0.05 was considered to be statistically significant.

RESULTS

Part I Characterization of GPRC5A tyrosine phosphorylation by EGFR in HEK293T cell lines

1. EGF-induced GPRC5A tyrosine phosphorylation depends on EGFR kinase activity

Western-blotting result shows that, at the presence of EGFR, the tyrosine phosphorylation of GPRC5A was strongly induced by EGF from 5 minutes and reaches the maximum level at 360 minutes, without affecting the total GPRC5A or EGFR protein expression levels (Fig. 2, left). However, in the absence of EGFR expression (Fig. 2, right), GPRC5A does not undergo any tyrosine phosphorylation even after treatment with EGF for 360 minutes. These results demonstrate that EGFR is required for the EGF-induced of tyrosine phosphorylation of GPRC5A in HEK293 cells. It is also apparent that the lower molecular weight species of GPRC5A (<37 KDa; the unglycosylated protein) is either poorly or not phosphorylated compared to the higher molecular weight GPRC5A bands (compare in Fig. 2 the bands within the green boxes in upper and lower panels on the left). Since the glycosylated GPRC5A molecules are mature they probably reside at the cell surface membrane where they may have an opportunity to interact with cell membrane EGFR.

2. Interaction between GPRC5A and EGFR

Because both GPRC5A and EGFR are located on the cell membrane, we examined the possibility that these proteins form a complex by applying co-immunoprecipitation analyses. Myc-tagged GPRC5A was co-expressed with EGFR in HEK293T cells, and the

Fig. 2

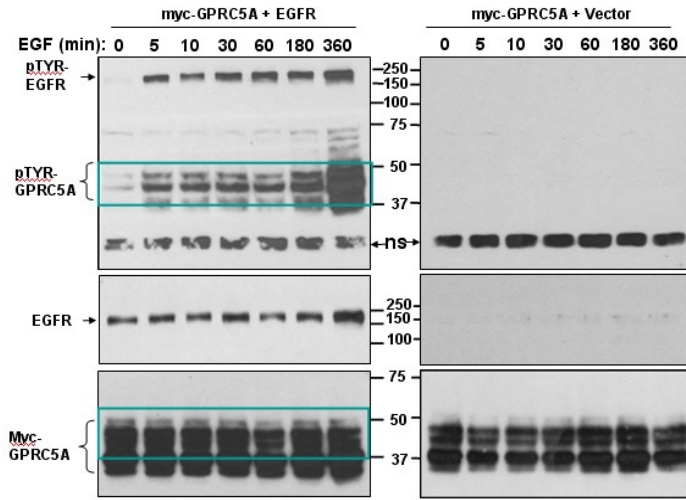


Fig. 2. Demonstration that GPRC5A is a substrate for EGFR signaling.

HEK293T cells were co-transfected with plasmids containing Myc-tagged GPRC5A and either EGFR or empty Vector. After 24 hours, the cells were starved in serum-free medium for another 24 hours and then the cells were treated with 100 ng/ml EGF for different times as indicated above each lane. Total cells' proteins were extracted and analyzed by western immunoblotting using antibodies py99 against phosphotyrosine (upper panels), or against EGFR (middle panels), or myc-tag (lower panels).

cells were treated with or without EGF and the EGFR inhibitor AG1478 to determine: a) whether GPRC5A and EGFR proteins interact with each other and b) whether the interaction between GPRC5A and EGFR is depended on EGFR tyrosine kinase activity. GPRC5A was immunoprecipitated from cell lysates with antibody against the Myc tag and the immunoprecipitated material was analyzed by western blotting for both Myc-tagged GPRC5A and EGFR.

As shown in Fig. 3A, EGFR was co-immunoprecipitated with GPRC5A. The interaction between GPRC5A and EGFR did not change in cells treated with EGF to activate the EGFR tyrosine activity, but less EGFR was co-precipitated in cells where EGFR activation by EGF was inhibited by AG1478. These results suggested that GPRC5A interacts with EGFR or that both proteins are present in the same complex and that the interaction between GPRC5A and EGFR may be influenced by EGFR tyrosine kinase activity.

Furthermore, Fig 3B shows, that GPRC5A was poorly phosphorylated in the absence of EGF but that the tyrosine phosphorylation was strongly induced by EGF treatment and reduced to below the basal level when the EGFR tyrosine activity was inhibited by AG1478. These results confirm that EGFR kinase activity is required for the EGF-induced tyrosine phosphorylation of GPRC5A.

Fig. 3C and 3D show that GPRC5A was co-immunoprecipitated with EGFR when the immunoprecipitation was performed using anti-EGFR antibody to pull down EGFR proteins from HEK 293T cells co-transfected with EGFR and Myc-GPRC5A, confirming

Fig. 3

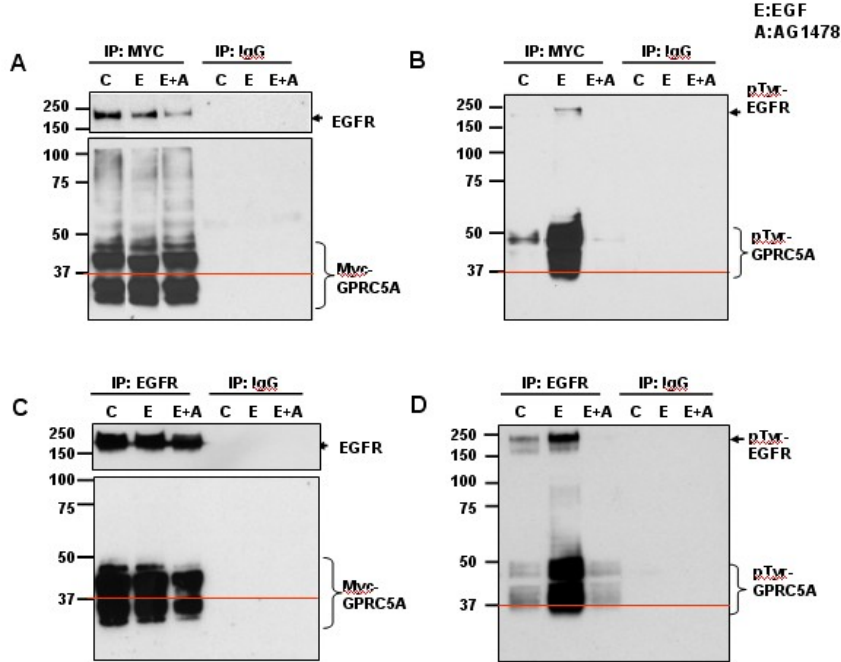


Fig 3. Demonstration of the interaction between EGFR and GPRC5A in HEK293T cells.

HEK293T cells were co-transfected with Myc-tagged GPRC5A and EGFR and 48 hours later, the cells were starved in serum-free medium for 24 hours and treated as indicated by the labels above each lane as follows: **C**: Control; **E**: EGF (100 ng/ml, 5 min); **E+A**: EGF+AG1478, cells were pretreated with 30 ng/ml AG1478 for 6 hours and afterwards treated with 100 ng/ml EGF for 5 min. Panels A and B: Anti-Myc antibody was used to immunoprecipitate the exogenous GPRC5A proteins from the cell lysates. The precipitates were then analyzed by western blotting using anti-EGFR antibodies or anti-Myc tag antibodies (panel A) or anti-phosphotyrosine (py99) antibodies (Panel B). In Panels C and D, anti-EGFR antibody was used to immunoprecipitate the EGFR proteins from the cell lysates. The precipitates were analyzed by western blotting as in panels A and B, respectively. IP with IgG served as control for specificity.

the interaction between the two membrane proteins. The level of interaction between GPRC5A and EGFR in the absence of EGF remained the same when the EGFR tyrosine activity was induced by EGF but the amount of GPRC5A in the complex was reduced after EGFR tyrosine activity was inhibited by AG1478 (Fig. 3 panel C lower left). Fig. 3D shows again that tyrosine phosphorylation of GPRC5A was strongly induced by EGF treatment and that inhibition of EGFR activation by AG1478 lowered dramatically the tyrosine phosphorylation of GPRC5A to basal level.

3. Alignment of different species of GPRC5A C terminal.

GPRC5A is a seven trans-membrane protein with a C-terminal cytoplasmic domain that includes several tyrosine residues. Several studies used global phosphoproteomic approaches to characterize receptor tyrosine kinases (RTKs) and their substrates in normal and malignant cells from mammary and lung tissues. During some of those studies, several groups identified GPRC5A as a substrate for RTKs. For example, Zhang et al (2005) and Wolf-Yadlin et al (2006) reported that GPRC5A can undergo tyrosine phosphorylation in normal HMEC cells treated with EGF or Heregulin (HRG). They identified tyrosine residue Y320 as the primary phosphorylated amino acid in cells treated with EGF and Y347 in the same cells treated with HRG suggesting that EGFR and HER2 can phosphorylate GPRC5A at different sites. These residues are located in the carboxy terminal region of the protein, which is in the cytoplasmic domain. Moreover, a database of tyrosine phosphorylated sites (www.phosphosite.org) includes information on GPRC5A indicating that tyrosine residues at positions 317, 320, 347 and 350 are phosphorylated in various cells and tissues. To determine whether these tyrosine residues

Fig. 4

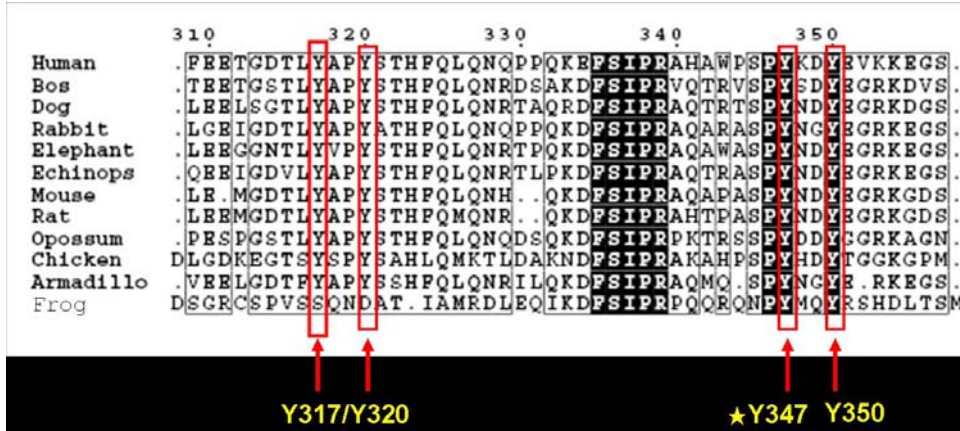


Fig. 4 Alignment of the C terminal domain of GPRC5A from different species

The sequence of c-terminal GPRC5A in different species were aligned by using the software from website “Multiple sequence alignment by Florence Corpet”.
(<http://bioinfo.genotoul.fr/multalin/multalin.html>) .

are conserved during evolution, we used the software “Multiple sequence alignment by Florence Corpet” (<http://bioinfo.genotoul.fr/multalin/multalin.html>) to align the sequence of GPRC5A in different species (Human, Bos, Dog, Rabbit, Elephant, Echinops, Mouse, Rat, Opossum, Chicken, Armadillo, Frog). As shown in Fig. 4 the four tyrosine residues at positions 317, 320, 347 and 350 are highly conserved suggesting that they may be important for GPRC5A’s functions.

4. GPRC5A mutant (4F) with four tyrosine residues at positions Y317, Y320, Y347 and Y350 replaced by phenylalanine cannot be phosphorylated by EGFR activation in HEK293 cells.

Besides the four tyrosine residues located in the C-terminus, there are other tyrosine residues in GPRC5A that could potentially undergo phosphorylation. To determine whether the major tyrosine phosphorylation sites of GPRC5A are located in the C-terminal, we constructed a mutant of GPRC5A, which we call 4F. In this mutant, the tyrosine residues Y317, Y320, Y347 and Y350 were replaced by phenylalanine (F) residues using oligonucleotide-directed mutagenesis, with Myc-tag in the C-terminal of the plasmid. We then compared the tyrosine phosphorylation level between GPRC5A wild-type and the 4F mutant after co-transfecting Myc-tagged GPRC5A wild-type or 4F mutant with EGFR expression plasmids into HEK293T cells. Fig. 5 (upper panel) shows that, in the presence of EGFR, the tyrosine phosphorylation of GPRC5A was strongly induced by EGF already after 5 minutes and reaches a high level after 360 minutes. In contrast, the 4F mutant of GPRC5A did not undergo any tyrosine phosphorylation even after the cells had been treated with EGF for 360 minutes (Fig. 5, lower panel). These results demonstrate that

activation of EGFR leads to the phosphorylation of one or more of the four tyrosine residues (Y317, Y320, Y347 and Y350) and that apparently none of the other tyrosine residue in GPRC5A can serve as substrate for EGFR, at least in HEK293 cells under the conditions of the assay.

5. Identification of the specific tyrosine residues in GPRC5A that undergo phosphorylation after EGFR activation.

To find out whether all four GPRC5A Tyr residues (Y317, Y320, Y347 and Y350) or only some of them are phosphorylated after EGFR activation, phenylalanine substitutions were made for each of these tyrosine sites individually as well as for combination of the adjacent pairs (Y317/320F or Y347/350F), which we called 2F mutants, using oligonucleotide-directed mutagenesis and the phosphorylation of the different mutants was compared after co-transfection with EGFR and treatment with EGF. Fig. 6 (left panel) shows that GPRC5A mutant Y317F/Y320F undergoes less phosphorylation compared to Y347/350F, suggesting that Y317 and Y320 (or one of them) are the preferred EGFR phosphorylation target sites in GPRC5A. Fig. 6 (right panel) indicates that no phosphorylation of GPRC5A wild type and 2F mutants occurs in the absence of EGFR. The lower phosphorylation of the two 2F mutants compared to the WT GPRC5A suggests that all 4 tyrosine residues may contribute to the optimal phosphorylation of GPRC5A.

We then checked the tyrosine phosphorylation level of single point mutants of GPRC5A and found that, none of the mutants, which contains the 317 phenylalanine-for-tyrosine

substitution (Y317F, and 4F) were phosphorylated by EGFR signaling in HEK293T cells (Fig. 7, lane 5, lane 13). In contrast, EGF did induce phosphorylation of GPRC5A wild-type and the mutants Y320F, Y347F and Y350F, these data demonstrates that residue Y317 of GPRC5A is the major EGFR phosphorylation target site in HEK 239T cells.

Fig 5

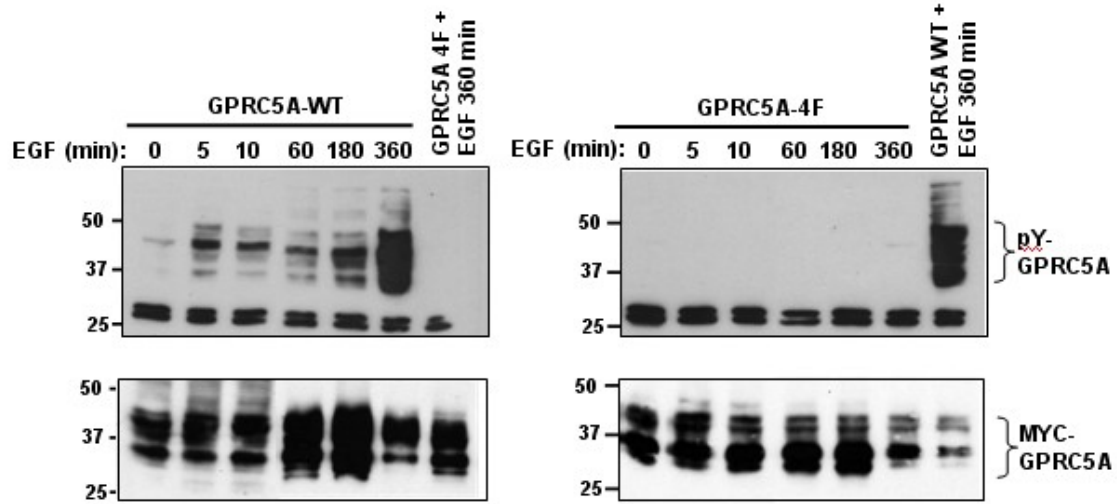


Fig 5. GPRC5A 4F mutant can not be phosphorylated by activated EGFR.

HEK293T cells were co-transfected with either Myc-tagged wildtype or Myc-tagged 4F mutant GPRC5A. Twenty four hours after transfection, cells were starved in serum-free medium for 24 hours and then treated with 100 ng/ml EGF for different times as indicated above each lane. Total cellular protein was extracted and analyzed by western blotting using anti-py99 anti-phosphotyrosine antibodies. The two of bands at the bottom of the gels are non-specific protein

Fig 6

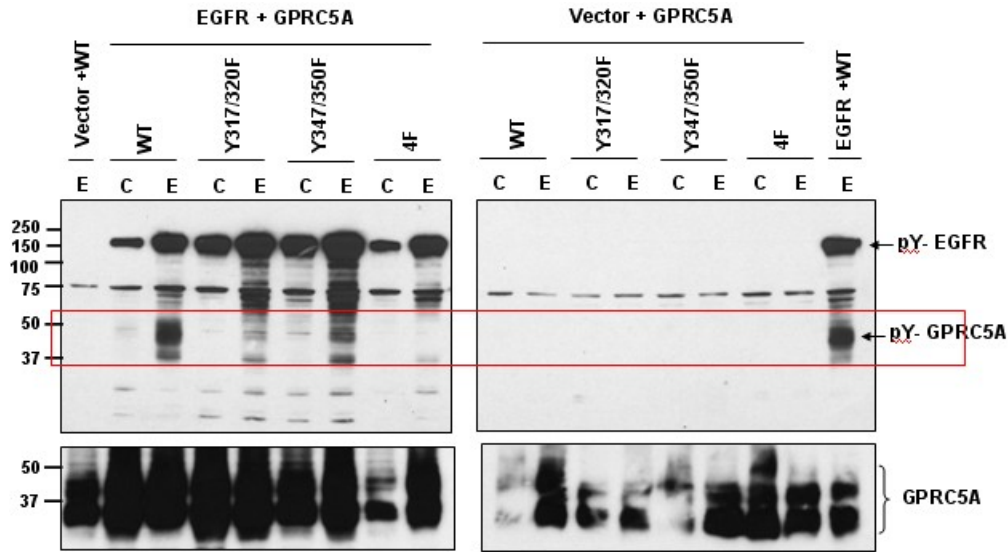


Fig. 6. The GPRC5A mutant Y317F / Y320F is phosphorylated less efficiently by activated EGFR than the mutant Y347F / Y350F or wild type GPRC5A.

Myc-tagged wildtype, mutant Y317/320F, Y347/350F and 4F mutant of GPRC5A were co-transfected with EGFR (Left panel) or Vector (right panel) plasmids into HEK293T cells. Twenty four hours after transfection, cells were starved in serum-free medium for 24 hours and then treated with 100 ng/ml EGF (E) or control buffer (C) for 5 min. Total cellular proteins were extracted and analyzed for by western blotting using anti-py99 anti-phosphotyrosine antibodies.

Transfected cDNA		WT	Y317F	Y320F	Y347F	Y350F	4F	Vec
Treatment	EGFR:	-	+	+	+	+	+	+
	EGF:	-	-	+	-	+	-	+

Western blot analysis of GPRC5A phosphorylation. The top panel shows pY (EGFR) short exp. with bands at 250, 150, and 100 kDa. The middle panel shows Anti-pY Ab with bands at 75, 50, and 37 kDa. The bottom panel shows Anti-myc Ab with bands at 50, 37, and 25 kDa. Red lines indicate the positions of pY (GPRC5A) and Myc- (GPRC5A).

Myc-tagged wildtype, single point mutant (Y317F, Y320F, Y347F and Y350F) and 4F mutant of GPRC5A were each co-expressed with EGFR or Vector plasmids in HEK293T cells. Twenty four hours after transfection, cells were starved in serum-free medium for 24 hours and then treated with 100 ng/ml EGF for 5 min. Total cellular proteins were extracted and analyzed by western blotting for phosphorylated EGFR (upper panel), phosphorylated GPRC5A (middle panel), and total GPRC5A (lower panel).

Part II. Characterization of GPRC5A tyrosine phosphorylation in lung cancer cell lines

6 . GPRC5A and EGFR family members expression in lung cancer cells

The studies in HEK 293 cells were based on co-expression of both GPRC5A and EGFR. We then wanted to analyze the potential interactions of constitutively expressed (endogenous) GPRC5A and EGFR. As already shown above in Fig. 1, the protein level of GPRC5A detected by antibodies prepared in our laboratory against the C-terminal peptide of human GPRC5A, was high in H292G, Calu-1, H322 and H226B cells (Fig. 8, panel A) . In those four cell lines, GPRC5A molecules migrated as bands with different molecular weights probably due to differential glycosylation. DNA sequence analyses of GPRC5A in these 11 NSCLC cell lines detected no mutations in the coding sequence (data not shown). Most of the NSCLC cell lines including those with high GPRC5A expressed EGFR and three also expressed HER2 (Fig. 8, panels B and C). GPRC5A, EGFR and Her-2 were all highly expressed in H292G cell. Therefore, we used the H292G for further studies.

7. EGF-induced GPRC5A tyrosine phosphorylation in H292G lung cancer cells

Since the H292G lung cancer cells express constitutively both EGFR and GPRC5A, we asked whether activation of EGFR signaling by EGF will increase tyrosine phosphorylation of GPRC5A in cells with “physiological levels” of EGFR and GPRC5A unmodified by transfection. Fig. 9A (left two lanes marked input) shows that EGF treatment increases the autophosphorylation of EGFR. Furthermore, the immunoprecipitation of GPRC5A shows that EGF treatment also increased the tyrosine phosphorylation of GPRC5A. The 22 KDA protein band at the bottom of the gel is a fragment of GPRC5A that includes about 50% of

Fig. 8.

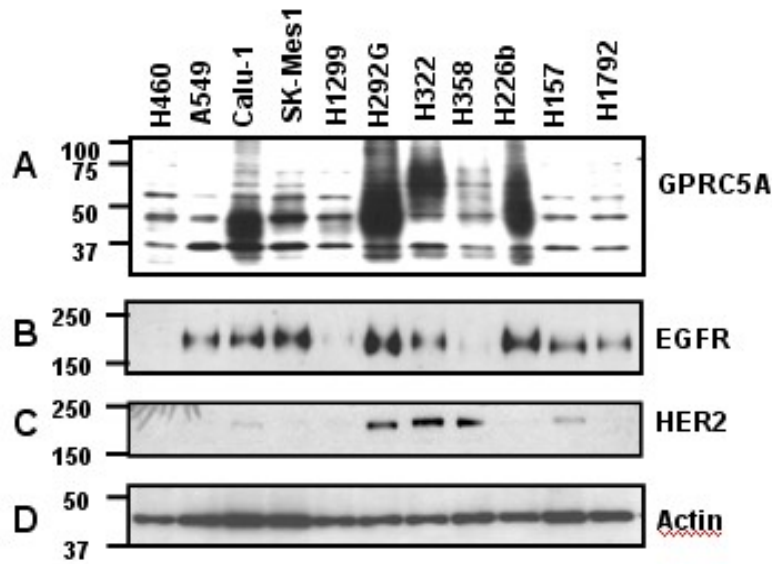


Fig. 8. Constitutive co-expression of GPRC5A , EGFR and Her-2 in certain NSCLC cell lines.

The eleven NSCLC cell lines named above each lane were grown in DMEM/F12 medium with 10% FBS and when the cultures reached about 80% confluency, the cells were the total length ending with the C-terminus (unpublished data). The tyrosine phosphorylation of this fragment is also increased by EGF. Fig. 9B shows that immunoblotting with anti-GPRC5A antibodies demonstrated that equal amounts of GPRC5A total protein and fragment were immunoprecipitated from untreated and harvested and their total proteins were extracted. The solubilized proteins were subjected to western blotting using antibodies against GPRC5A (A), EGFR (B), Her-2 (C), and actin (D).

EGF-treated cells. These findings show that EGFR activation leads to tyrosine phosphorylation of GPRC5A in H292G cells. However, the IP of GPRC5A did not co-IP EGFR, which is different from the findings in transfected HEK293 cells. The reason for this difference is not clear but it may be related to the high levels of expression of transfected EGFR and GPRC5A in the transfected HEK293 cells.

Fig. 9

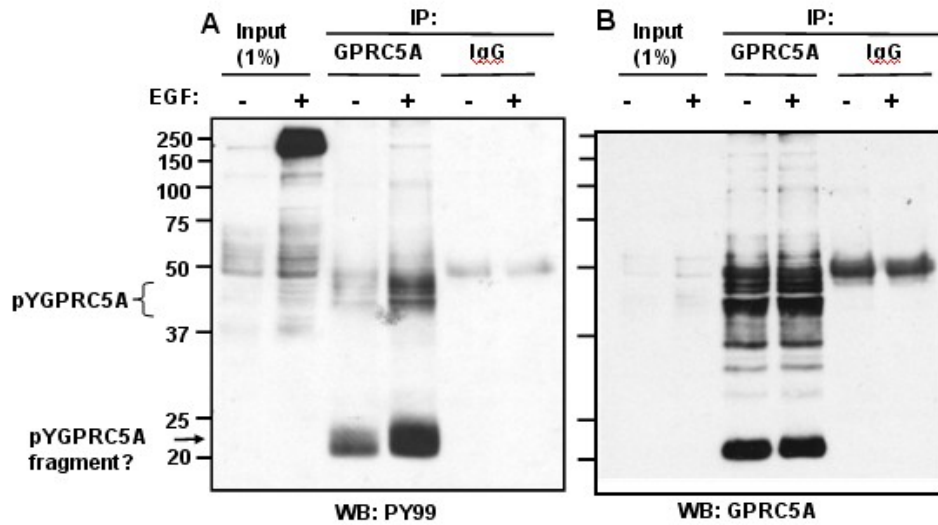


Fig. 9. Activation of EGFR by EGF induces GPRC5A tyrosine phosphorylation in H292G lung cancer cells.

Cells starved in serum-free medium for 24 hours were treated with EGF (100 ng/ml) or control buffer for 5 min. The cells were then harvested and lysed in immunoprecipitation buffer. A sample containing 1 mg of total protein extract was used for analysis as follows: 10 μ g of total protein was loaded on the gel as input. The remaining extract was used for immunoprecipitation with either 2 μ g of anti-GPRC5A antibody or IgG control. The immunoprecipitated material was then resuspended in SDS-PAGE buffer and analyzed by western blotting using either anti-phosphotyrosine PY-99 antibody (Panel A) or anti-GPRC5A (Panel B).

Part III. Comparison of the biological functions of wildtype GPRC5A and the non-phosphorylatable mutant 4F

8. Selection of H1792 NSCLC cells for over-expressing exogenous GPRC5A WT and 4F mutant for functional analyses.

We wished to compare the biological functions of WT GPRC5A and the non-phosphorylatable mutant 4F as a first approach to assess the role of tyrosine phosphorylation. For this study, we have chosen to use the H1792 NSCLC cell line to over-express exogenous GPRC5A and 4F mutant for three reasons: 1) these cells express a low level of endogenous GPRC5A (Fig. 1 and 8); 2) the cells express endogenous EGFR (Fig. 8), and 3) transfection of Myc-tagged WT GPRC5A into these cells inhibits their ability to form colonies in semisolid medium as we have shown previously (Tao et al., 2007). Therefore, we considered the H1792 cells as very suitable for examining the biological effects of wild-type and 4F mutant of GPRC5A on cells proliferation, migration ability, invasion ability, anchorage-independent growth ability.

9. EGF induces tyrosine phosphorylation of GPRC5A in H1792 cells

To determine whether the endogenous EGFR in H1792 cells can be activated and then phosphorylate GPRC5A, we treated WT-GPRC5A-Myc stable transfected H1792 cells with buffer control, EGF, or EGFR inhibitor followed by EGF and compared tyrosine phosphorylated proteins. Figure 10 shows that total protein extracts from EGF treated cells contained a phosphorylated protein migrating with a molecular weight expected from EGFR (lane 2 from left). This protein was not phosphorylated in control or in AG1478

Fig10.

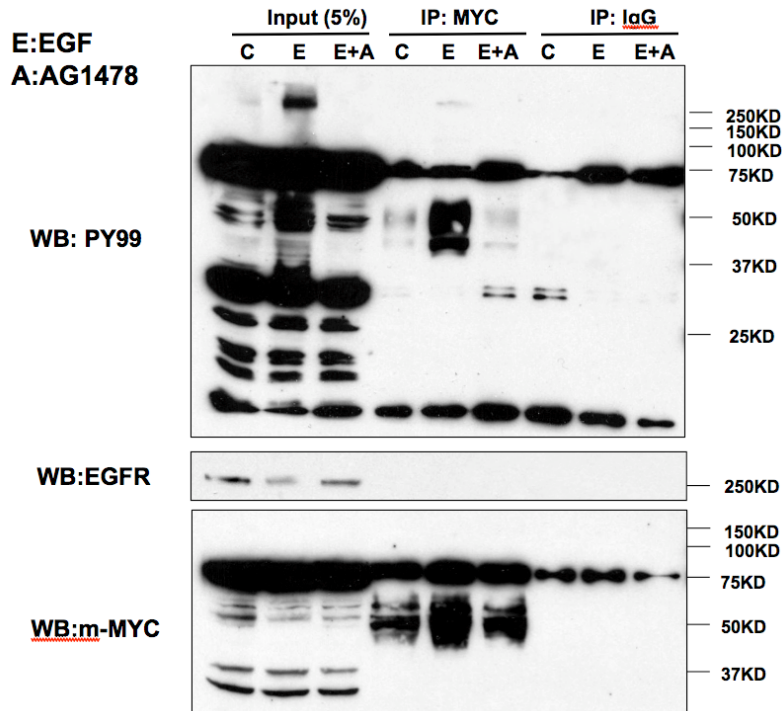


Fig 10. WT GPRC5A can be phosphorylated by activated EGFR in H1792 cells.

Stable transfected cells were starved in serum-free medium for 24 hours and then treated as indicated by the labels above each lane as follows: **C**: Control; **E**: EGF (100 ng/ml, 5 min); **E+A**: EGF+AG1478, cells were pretreated with 30 ng/ml AG1478 for 6 hours and afterwards treated with 100 ng/ml EGF for 5 min. Total protein extracts were prepared from the cells and samples representing 5% of the total protein were removed for further analysis before immunoprecipitating the exogenous GPRC5A proteins from the cell lysates using anti-Myc antibodies. The precipitates were then analyzed by western blotting using antibodies against phosphotyrosine (py99), EGFR, or Myc tag, and. IP with IgG served as control for specificity.

plus EGF treated cells supporting the conclusion that it is the endogenous EGFR. Immunoprecipitation of GPRC5A from these cell extracts using anti-Myc tag antibodies followed by immunoblotting of the precipitated material with anti-Myc antibodies or anti-phosphotyrosine antibodies (pY99) revealed that GPRC5A was phosphorylated in an EGF-dependent way (Fig. 10, upper panel, lane 5). A faint band migrating like the presumed EGFR was detected in the GPRC5A immunoprecipitate from EGF-treated cells (Fig. 10, lane 6, upper panel). Immunoblotting with anti-EGFR antibodies showed a band in the total extract (three left lanes in middle panel) but not in the immunoprecipitated material suggesting that if EGFR is co-immunoprecipitated with GPRC5A, its amount is very small.

10. GPRC5A 4F mutant cannot be phosphorylated by activated EGFR in H1792 cells

We compared the tyrosine phosphorylation level between the H1792 stable cell lines transfected with empty vector, WT GPRC5A, or 4F mutant of GPRC5A. Fig. 11 (upper panel) shows that, in the cell lines which over-expressing WT GPRC5A, the tyrosine phosphorylation of GPRC5A was strongly induced by EGF (100 ng/ml, 5 minutes). In contrast, the 4F mutant of GPRC5A did not undergo any tyrosine phosphorylation. These results demonstrate that activation of EGFR leads to the phosphorylation of one or more of the four tyrosine residues (Y317, Y320, Y347 and Y350) and that apparently none of the other tyrosine residue in GPRC5A can serve as substrate for EGFRH1792 cells under the conditions of the assay.

Fig 11

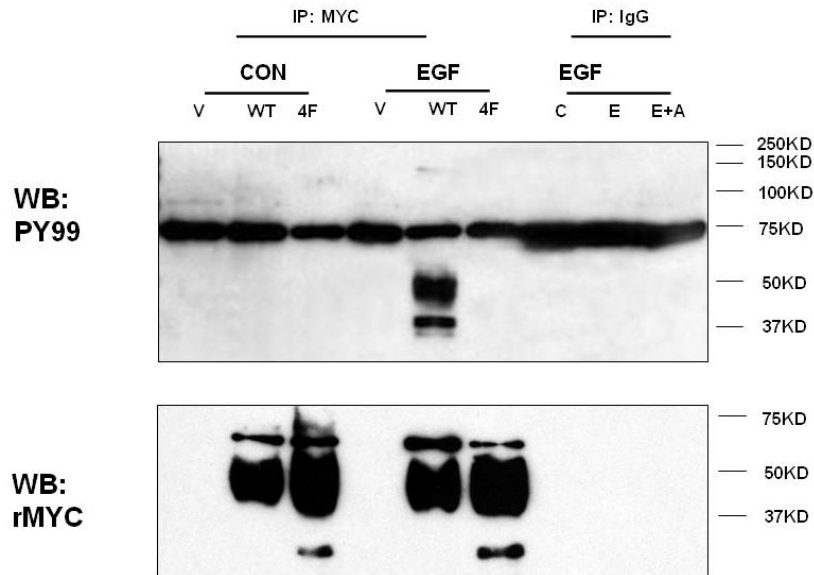


Fig 11. GPRC5A 4F mutant cannot be phosphorylated by activated EGFR in H1792 cells.

Stable transfected cells were starved in serum-free medium for 24 hours and then treated with or without EGF (100 ng/ml, 5 min); Anti-Myc antibody was used to immunoprecipitate the exogenous GPRC5A proteins from the cell lysates. The precipitates were then analyzed by western blotting using anti-Myc tag antibodies and anti-phosphotyrosine (py99) antibodies. IP with IgG served as control for specific

11. Lack of differences in the effects of WT GPRC5A and 4F mutant on cell proliferation

H1792 cells transfected in a stable manner with empty vector, WT GPRC5A, or 4F mutant of GPRC5A showed similar growth rates whether cultured with or without EGF (Fig. 12). This suggests that the over-expression of GPRC5A and its potential to undergo tyrosine phosphorylation do not affect the cell proliferation rate of the H1792 cells.

12. Effects of WT GPRC5A and 4F mutant on EGF-induced cell migration

We found that the H1792 cells exhibited a low level of migration in the absence of EGF but that EGF induced extensive migration of these cells (Fig. 13; left panels). Furthermore, both WT GPRC5A and the 4F mutant strongly suppress the spontaneous and the EGF-induced migration (Fig. 13; middle and right panels, respectively)). However, there was no significant difference between the effects of WT and the 4F mutant on cell migration without or with EGF treatment. This suggests that tyrosine phosphorylation potential of GPRC5A is not important for the suppressive effect of GPRC5A on the cell migration activity or that the level of expression of the WT and mutant GPRC5A was too high to detect differences in their efficacy.

Fig. 12

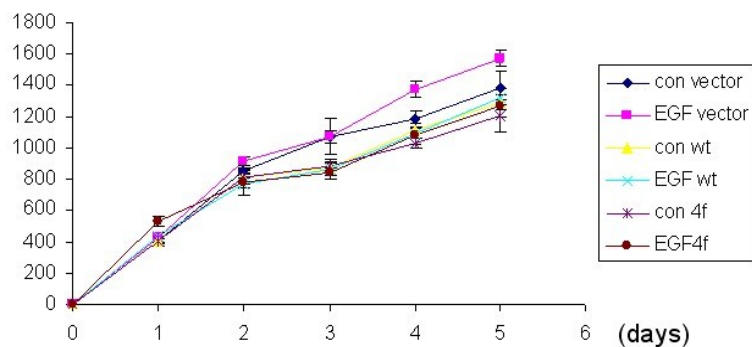


Fig. 12. Over-expression of GPRC5A WT or 4F does not affect the proliferation rate of the H1792 cells.

Cells transfected with vector only, WT GPRC5A, or 4F mutant were grown in 96-well plates in growth medium with 10% FBS with or without 100 ng/ml EGF for 24, 48, 72 , 96 and 120 hours. The relative number of cells in each well was measured at each of these time points by the MTT assay and the mean \pm SD of triplicate experiments is shown in the graph.

Fig. 13

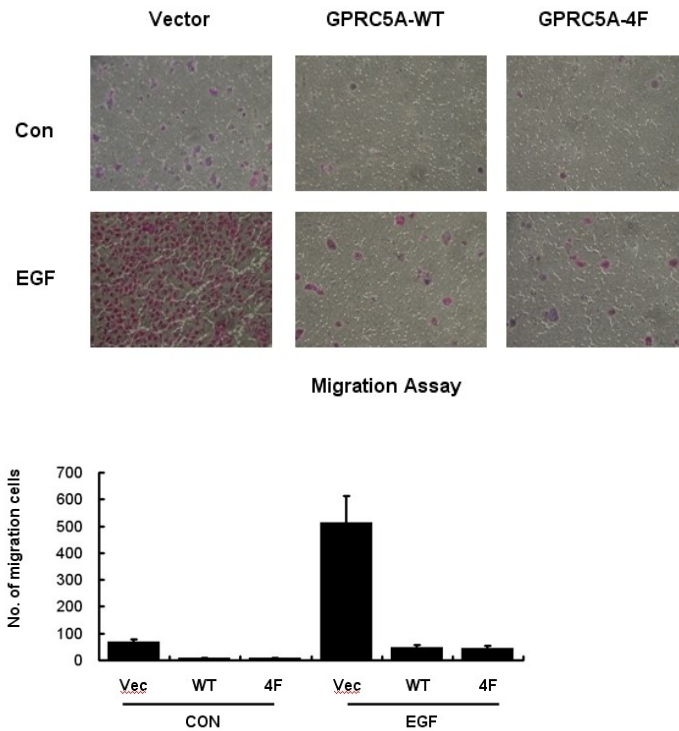


Fig. 13. GPRC5A suppresses the migration of H1792 cells independently of its tyrosine phosphorylation potential.

H1792 cells transfected in a stable manner with either vector, WT GPRC5A, or 4F mutant were incubated in cell culture inserts of a Boyden Chamber with 8- μ m diameter membranes pores in 24 well plate overnight without or with 100 ng/ml EGF and analyzed for migration ability as described in materials and methods. The photomicrographs of cells on the bottom side of the filter are shown in the upper 6 panels. The number of cells on the bottom side of the filter in four $\times 40$ microscopic fields was determined, and the data are presented in the bar graphs as the mean (and 95% confidence intervals) number of migration cells in one microscopic field, shown in the bar graph. The differences between the number of cells in WT and 4F mutant of GPRC5A-transfected cells treated with EGF were not statistically significant ($P > .005$; two-sided z test).

13. Effects of WT GPRC5A and 4F mutant on EGF-induced cell invasion

We then compared the invasion ability of the above three H1792 stably-transfected cells with and without EGF treatment (Fig. 14). We found that the basal invasion of vector-transfected cells was stimulated about 3-fold by EGF treatment. We also found that the invasion abilities of untreated H1792 cells transfected with WT GPRC5A or with the 4F mutant of GPRC5A were lower than that of cells transfected with vector only whether the cells were untreated or EGF-treated (Fig. 14). The suppression of invasion was higher in cells transfected with the 4F mutant than in cells transfected with the WT GPRC5A implying that tyrosine phosphorylation of GPRC5A may partially inactivate the suppressive function of GPRC5A on the cell invasion activity.

14. Effects of WT GPRC5A and 4F mutant on anchorage-independent colony formation

Previously, we have shown that H1792 cells transfected with WT GPRC5A form fewer colonies in semisolid medium than vector-transfected cells (Tao et al, 2007). Therefore, we compared the effects of WT and 4F mutant on anchorage-independent growth of cells with and without EGF treatment. We found that cells transfected with either WT or 4F formed fewer colonies than vector controls (Fig. 15). More interestingly, we observed that EGF stimulated colony formation by vector-transfected H1792 cells and that the cells transfected with the 4F mutant of GPRC5A showed more effective suppression of colony formation than WT GPRC5A transfected cells (Fig. 15). Because we found that the growth rate in monolayer cultures of the different transfectants was similar (Fig. 12), we excluded the possibility that the difference of colony formation are due to the differences in cell growth rate.

Fig. 14

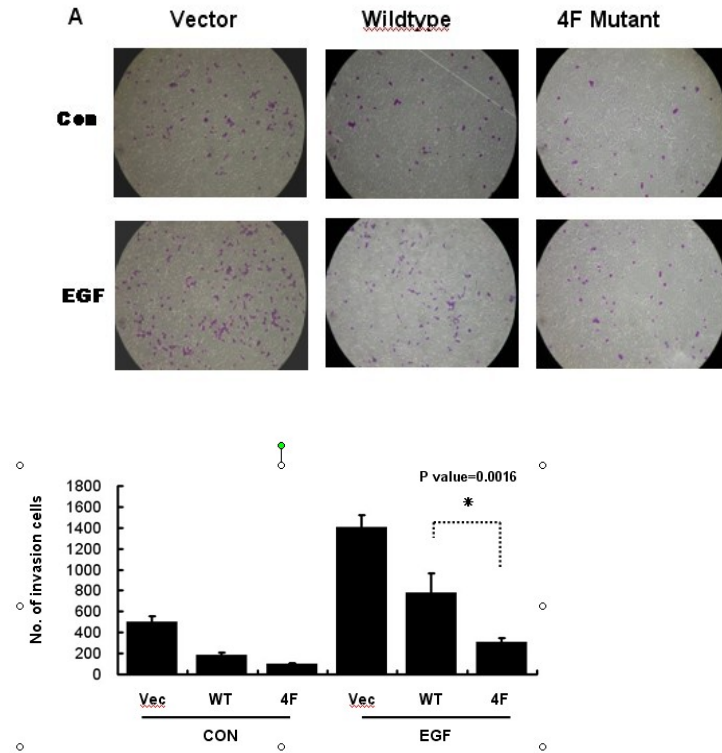


Fig.14. Differential suppressive effects of WT GPRC5A and the 4F mutant on the invasion potential of H1792 cells.

The experiment has been performed as described in the legend of Fig. 13 except that the membrane at the bottom of the inserts was coated with Matrigel to form a layer that the cells need to invade through in order to migrate to the underside of the membrane. After an overnight incubation, the number of cells on the bottom side of the filter in four $\times 40$ microscopic fields was determined, and the data are presented in the bar graphs as the mean (and 95% confidence intervals) number of invasion cells in one microscopic field. The differences between the number of colonies in WT and 4F mutant of GPRC5A-transfected cells treated with EGF were statistically significant ($P < .005$; two-sided z test) for all the cell lines.

Fig. 15

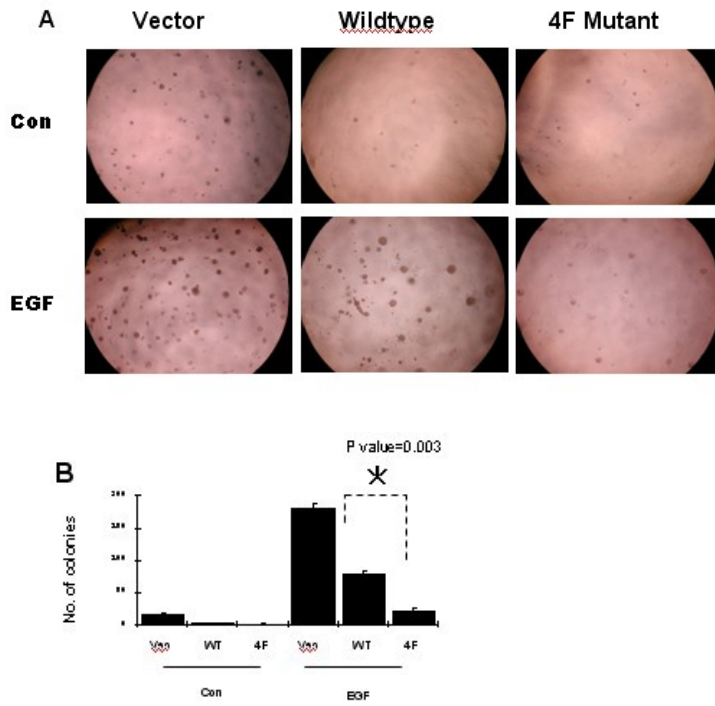


Fig.15. Differential effects of WT GPRC5A and the 4F mutant on anchorage-independent growth of H1792 cells.

Stable transfected cells were suspended in agarose/matrigel and analyzed for colony formation over 2 weeks. The photomicrographs of colonies at a $\times 40$ microscopic magnification are shown in (A). The number of colonies in four $\times 40$ microscopic fields was determined, and the data are presented in the bar graphs as the mean (and 95% confidence intervals) number of colonies in one microscopic field (B). The differences between the number of colonies in WT and 4F mutant of GPRC5A-transfected cells treated with EGF were statistically significant ($P = 0.003$; two-sided z test).

15. Differences in the EGF-induced internalization of WT GPRC5A and the 4F mutant in H1792 cells

Previously, we have shown that stably-expressed GPRC5A-myc is localized in perinuclear vesicles (probably Golgi) and the plasma membrane in H1792, HEK 293 and MDA 1478 cell lines. We found that EGF treatment induces internalization of WT GPRC5A-myc in H1792 transfected in a stable or transient manner (Fig. 16A and 16B, respectively). We then asked whether the internalization of the 4F mutant was different from that of WT GPRC5A. Fig. 16 (A and B) shows that in untreated cells (control for EGF), both WT and 4F mutant of GPRC5A were localized at the cell surface and perinuclear vesicles. The percentages of cells that exhibit cell surface location of GPRC5A are 75.6% and 78.1% respectively in WT and 4F mutant stably-transfected cell lines (n= 500). Similarly, in transiently-transfected cells, GPRC5A was located on the cell surface of WT and 4F mutant cells (n= 500) in 68.4% and 71.1%, respectively. However, in stable transfectants, treatment with EGF for 6 hours increased GPRC5A translocated from the surface to cytoplasm in 62.7% of WT transfectants compared to only 25.8% of 4F mutant cells (Fig. 16A). Similarly, in transient transfectants treated with EGF, GPRC5A translocated to cytoplasm in 82.6% of WT GPRC5A transfectants, while only 37.4% of 4F mutant cells showed such translocation (Fig. 16B). These data showed preferential retention of the 4F mutant at the cell surface, suggested that internalization of GPRC5A, which is enhanced by EGF treatment may be related to tyrosine phosphorylation of GPRC5A cytoplasmic domain. Further, the internalization may inactivate some of the effects that can be only achieved by cell surface membrane located GPRC5A.

Fig. 16A

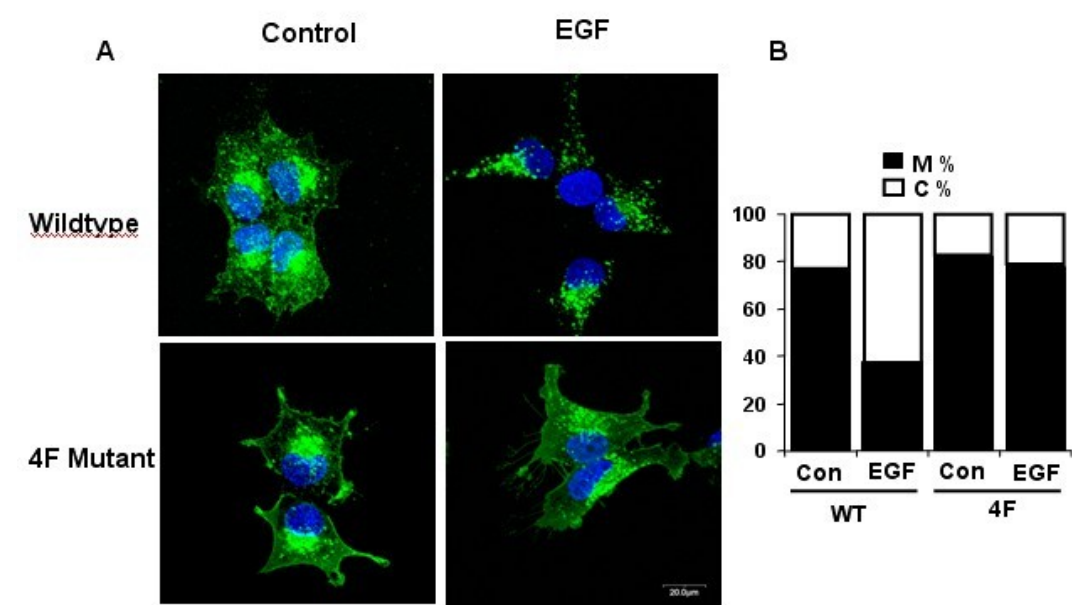


Fig. 16B

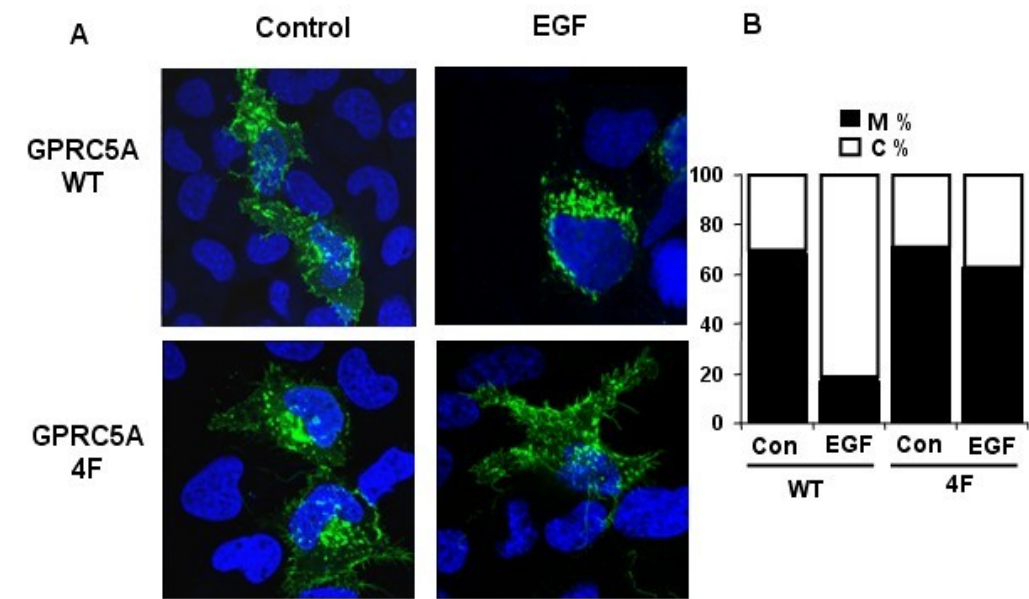


Fig 16. Differential EGF-induced internalization of WT GPRC5A and 4F mutant in H1792 cells transfected in a stable (A) or transient (B) fashion.

16A: Stably-transfected H1792 cells were starved in serum-free medium for 24 hours and treated with EGF (100 ng/ml) or control buffer for 6 hours. The cells were then fixed in 3.7% formaldehyde for 30 minutes. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and GPRC5A with anti-myc tag antibody followed by fluorescently-labeled secondary antibodies. An overlay of the GPRC5A and DAPI staining is shown in the photomicrographs. In panel B, 500 cells from five different microscopic fields at 40× were observed for localization of the fluorescence in the membrane (M) or cytoplasm (C) and the percentage of cells with each localization was calculated. **16B:** the experiment was performed as in 16A except that the cells were transfected transiently with WT and 4F mutant GPRC5A.

Discussion

G protein coupled receptors are classically activated by agonists, which upon binding to the receptor cause conformational changes that lead to dissociation of G proteins from the cytoplasmic domain(s) and activation of downstream signaling. In addition to this mechanism, GPCRs have been shown to interact with other membrane proteins to form homo- or hetero-dimeric complexes. The partners in the heterodimeric complexes can be other GPCRs or non-GPCR membrane proteins. Not all membrane proteins with seven transmembrane domains (7TM) are receptors and some may actually function without a ligand (agonist) by forming complexes and cross-talk with other membrane proteins. The cytoplasmic domain of most GPCRs undergo ser/thr phosphorylation by a GPCR-specific kinase (GRK) as a part of the desensitization mechanism. This leads to the formation of a complex with the cytoplasmic protein beta-arrestin and subsequent signaling through tyrosine kinases, NF-kappaB, MAPK and others. Several reports have indicated that GPCRs family member and receptor tyrosine kinase (RTK) can form physical associations with each other (47). It is now well known that some of the interactions between GPCRs and other membrane or intracellular proteins regulate receptor internalization, subcellular localization, trafficking and signaling. The associated proteins can interact directly or indirectly, via adaptor proteins, with intracellular receptor domains, such as the carboxyl-terminal tail (C-tail) to recruit associated proteins in large signaling networks (48, 49). Moreover, the interplay between different cellular membrane receptors, as well as drug-induced receptor crosstalk (50), may enable the normal or cancer cells to integrate a multitude of signals from its environment (51, 52).

In this study, we found that EGFR and GPRC5A can interact either directly or through other proteins and that GPRC5A can be phosphorylated after activation of EGFR by EGF. Inhibition of the EGFR kinase activity decreased the phosphorylation of GPRC5A and the interaction between GPRC5A and EGFR. The tyrosine phosphorylation appears to occur preferentially in glycosylated forms of GPRC5A. In c-terminal of GPRC5A, There are four tyrosine residues Y317, Y320, Y347, Y350. We found that among these four sites, Y317 is the preferred EGFR phosphorylation target sites in GPRC5A. However, these three other Y residues appear to contribute to the optimal phosphorylation of GPRC5A. We also found that EGF can induce GPRC5A internalization both in H1792 transient and stable cell lines. EGF also partially inactivates the suppressive function of GPRC5A on cell invasion activity and Anchorage-independent growth ability of H1792 stable cell lines. These finding support our hypothesis that GPRC5A may be inactivated by posttranslational modification- tyrosine phosphorylation.

Previous studies by other investigators have demonstrated that certain GPCRs transactivate EGFR via ligand-independent or ligand-dependent pathways. GPCRs can induce the Src-PDK1-ADAMs pathway (39-42) and then induce the cleavage of membrane-bound EGFR-ligand precursors (eg. AR, TGF-alpha, HB-EGF) (43). GPCRs can also directly activate the juxtamembrane tyrosine kinase domain of EGFR (44, 45). However, our study proposes a new model for the crosstalk between GPCR and EGFR signaling; in our model, a GPCR can interact with EGFR and serve as a substrate of EGFR kinase. This model is based on our finding that EGF-induces tyrosine phosphorylation of GRC5A by activation of EGFR signaling in HEK293T and H292G cell lines. Our results provide the first

experimental evidence for the prediction, which was made by others, that GPRC5A may be the substrate of some oncogenic tyrosine kinase, such as Src or EGFR. EGFR tyrosine kinase directly phosphorylates protein substrates (eg. GRB2, PI3K and Src), which possess an SH2 domain (46). However, since GPRC5A has no SH2 domain, it is not clear whether EGFR phosphorylates GPRC5A directly or indirectly.

The predicted molecular mass of the GPRC5A polypeptide is 40,251 daltons. However, in western blotting, it migrates as having a molecular weight of 37,000. In addition, we detected also several bands with higher molecular weights (37 to 50 kDa). Others in our group have shown that the 37 kDa band is the unglycosylated form of GPRC5A and the higher molecular weight bands are the glycosylated forms. The N-glycosylation (carbohydrates extending from asparagine residue) of membrane glycoproteins including GPCRs begins in the endoplasmic reticulum and continues in the Golgi so that the glycoproteins that reach the cell surface membrane are mature and fully glycosylated. Interestingly, our western blotting results shows that the higher molecular weight forms of GPRC5A (>37 kDa) were preferentially tyrosine phosphorylated suggesting that the phosphorylation of GPRC5A occurs after it reaches the cell surface membrane where it can form a complex with EGFR and undergo phosphorylation.

Our study provides the first evidence based on co-immunoprecipitation experiments that EGFR can form a complex with GPRC5A. We found that the interaction between EGFR and GPRC5A was partially dependent on the RTK kinase activity because less GPRC5A was co-precipitated when EGFR was pulled down from cells pretreated with the kinase

inhibitor (Fig. 2). These results suggest that the interaction of these two proteins may be stronger when the kinase is active or that tyrosine phosphorylated GPRC5A associates better with EGFR. In a study with another GPCR, the beta-2-adrenergic receptor (beta-2AR), Malbon and coworkers (53) have described a mechanism by which insulin can stimulate the sequestration of the beta-2AR by inducing the tyrosine phosphorylation, which mediated by insulin receptor of Y350 and Y364 in the C-terminal tail of the beta-2AR. The tyrosine phosphorylation Y350 and Y364 provides acceptor sites for beta-2AR to bind with some other proteins containing SH2 domain, such as Grb-2(54). Since Grb-2 can also bind to insulin receptor by SH2 domain, people speculate that two Grb-2 molecules might function together to associate with the insulin receptor and the beta-2AR as a tethered complex: RTK–Grb-2–Grb-2–GPCR and leads to internalization of beta-2AR (53). In our case, so far we only found the EGFR and GPRC5A can form complex together, we still don't know the interaction between these two proteins is directly or indirectly, whether there are some other partner protein, such as Src or Grb-2, associated with the EGFR-GPRC5A complex or not is still remain unknown. Further analysis is required to distinguish these possibilities.

Thirdly, we first use site-directed mutagenesis and identified that Y317, Y320, Y347 and Y350 are the predominant sites of tyrosine phosphorylation in the C-terminal domain of GPRC5A which be phosphorylated by EGFR activation in HEK293T cells. Several observations have been reported that GPRC5A could be tyrosine phosphorylated in the C-terminal domain, and different stimuli induce different tyrosine phosphorylation sites in different cell line and tissues. For example, Wolf-Yadlin and coworkers treat the 184A1

HMECs clone 24H cells, (which is overexpressing 30 fold Her-2 molecular), with HRG and found 322 phosphorylation sites can be tyrosine phosphorylated, one of these sites is GPRC5A Y347 (20) (Wolf-Yadlin et al, 2006); GPRC5A was also predicted to be the substrate of some oncogenic tyrosine kinase, such as Src and EGFR (21), the peptide which describe in this report are Y317, Y320, Y347 and Y350. All these observation are made by Mass Spectrometry (MS) approach, which is based on big screen system and not specific focus on GPRC5A. So far, no one else but us have characterized the tyrosine phosphorylation of GPRC5A or study the function in cultured cell lines. We also align the C-terminal sequence of GPRC5A in different species (Human, Bos, Dog, Rabbit, Elephant, Echinops, Mouse, Rat, Opossum, Chicken, Armadillo and Frog) and found that Y317, Y320, Y347 and Y350 are highly conserved. This finding gave us a hint that these four sites of tyrosine residues may represent some physiological significance.

When we first check the tyrosine phosphorylation of 4F mutant, we could not detect any phosphorylation level, suggesting that no any additional phosphorylation site(s) might exist in the GPRC5A, or in the other way, such a site(s) would be a very minor site of phosphorylation, and no evidence for its phosphorylation under physiological conditions was obtained. Later on, we compare tyrosine phosphorylation of the four different single point mutants and narrow down to the Y317 which is the predominant sites of tyrosine phosphorylation of GPRC5A in HEK293T cells. Our result is quite different with Wolf-Yadlin's finding, which indicate that Y347 is the most important residue for GPRC5A to be phosphorylated by EGFR and Her-2 signaling. This difference may due to the organ specificity or identical experiment condition. In our result, we also notice that the total

phosphorylation combine with the Y317/320F and Y347/350F mutant together is comparable lower than the WT GPRC5A, suggests that all these four tyrosine residues are together contribute to the optimal phosphorylation of GPRC5A. This is a very interesting phenomenon, because there are several evidences indicate that one single site phosphorylation can lead to protein conformation changes or partner protein interactions, which in turn to result in the regulation of the other residues phosphorylation within one protein. For example, CK1 induced-phosphorylation on Ser45 of beta-Catenin results in GSK-3 beta mediated phosphorylation on Ser33/37 and Thr41 and then followed by degradation of phosphorylated beta-catenin by the ubiquitin–proteasome system (55). Different phosphorylation site may also regulate different biological function of the protein, for example, Zhou BP and coworkers have shown that GSK-3 phosphorylates Snail at two motifs and regulate the function of Snail. Phosphorylation of the first motif regulates its degradation, whereas phosphorylation of the second motif controls its subcellular localization (56). Our results so far indicate that different phosphorylation motifs in the C-terminal of GPRC5A show different phosphorylation level and provide a model system in HEK293T cell line for the further study of the biological function of tyrosine phosphorylation of GPRC5A.

Last but not least, we characterized the biological function of GPRC5A tyrosine phosphorylation in lung cancer cell line. As we all know, phosphorylation can determine the protein's function by induce the conformation change and leads to different protein association, or change protein's subcellular localization and then result in biological function changes (11). Most of the GPCR internalization is mediated by serine and

threonine phosphorylation and arrestin binding (57-59). Very little has been reported on the tyrosine phosphorylation-mediated internalization until very recently, Paing MM and his co-workers demonstrated that the activated PAR1 is controlled a highly conserved tyrosine-based motif, YXXL (60). This study is the first one to describe a function for a tyrosine phosphorylation in GPCR internalization and reveal some complexities in the regulation of GPCR trafficking. In our study, we also found that the 4F mutant of GPRC5A in which tyrosine 317, 320, 347 and 350 were replaced with phenylalanine (F) was significantly impaired in EGF-induced internalization compared with wild-type GPRC5A in both transient and stable H1792 cells, demonstrating that tyrosine phosphorylation can regulate the internalization and subcellular localization of GPRC5A. Our result helps to expand the diversity of mechanisms which control the trafficking of GPCRs.

These internalization results gave us a hint that the tyrosine phosphorylation of GPRC5A may affect the GPRC5A's migration ability. However, when we check our stable cell lines, we found that both wildtype and 4F mutant can strongly suppress the EGF-induced migration ability of H1792 stable cells, we also found that there is no significant difference of cell proliferation among the cell lines which expressing Vector, wildtype and 4F of GPRC5A. Previously, Kumar N et al (20) modeled HER2 effects on cell behavior from MS phosphotyrosine data and predicted that EGF-induced tyrosine phosphorylation of GPRC5A at Y347 residue is one of 20 tyrosine residues which are the least co-related with the cell proliferation or migration signaling. Therefore, our results are consistence with Kumar's finding and got the same conclusion that tyrosine phosphorylation of GPRC5A

show no effect on cell proliferation and migration ability.

Interestingly, when we compare the invasion ability of the H1792 stable cell lines, we found that 4F mutant of GPRC5A show more effective suppression than wild-type GPRC5A on the EGF-induced invasion ability of H1792 cells. These results gave us a hint that the tyrosine phosphorylation of GPRC5A may inactive the GPRC5A's tumor suppressor function by affecting the tumor genesis such as invasion signaling or anchorage-independent growth ability instead of proliferation or motion ability.

Previously, Tao et al (2) showed that when GPRC5A over expressed in lung tumor cells (e.g. H1792 and MDA1478) and also non lung tumor cells (e.g., HEK293T cells), GPRC5A inhibits colony formation in agarose. In this study, when we checked the anchorage-independent growth ability of the H1792 stable cell lines, we found that 4F mutant of GPRC5A show more effective suppression than wild-type GPRC5A on the EGF-induced colony formation in agarose. This data strongly support our hypothesis and demonstrate that tyrosine phosphorylation of GPRC5A inactive the GPRC5A's tumor suppressor function on the anchorage-independent ability in H1792 cell lines.

Although there is a wealth of literature or some unpublished data from company data base which report the tyrosine phosphorylation residue in the C-terminal of GPRC5A, it essentially comes down to the MS based prediction areas: no one else has study the detail function of GPRC5A tyrosine phosphorylation. In this study, we demonstrated that the tyrosine phosphorylation of GPRC5A is regulated by EGFR kinase activity, and one or all

of the four tyrosine phosphorylation motifs (Y317, Y320, Y347 and Y350) within GPRC5A regulate the function of this protein. Most importantly, the tumor suppressive function inactivated by tyrosine phosphorylation may represent one of the molecular mechanisms and help us to understand why GPRC5A is a tumor suppressor but still show high protein level in some none-small cell lung cancers.

With this in mind, the next major challenges in the study of GPRC5A signaling will be defining the signaling complexes which are assembled in response to tyrosine phosphorylation of GPRC5A, the subcellular localization of these complexes and the biochemical and biological consequence occurred by these complexes. Phosphorylation-defective mutants of GPRC5A (4F or Y317F mutant), which have already been shown to function as dominant-negative mutants, should greatly help the discovery of these complex signaling events and will improve people's prospects for developing effective cancer treatments. For example, 4F mutant of GPRC5A may be a candidate gene therapy tool for people to combine with EGFR inhibitor in the clinical trial.

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