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Novel reciprocal regulation of cAMP signaling and apoptosis by orphan G-protein-coupled receptor GPRC5A gene expression ☆

Minoru Hirano, Liqing Zang, Takehiko Oka, Yoshiyuki Ito, Yasuhito Shimada, Yuhei Nishimura, Toshio Tanaka *

Department of Molecular and Cellular Pharmacology, Pharmacogenomics and Pharmacoinformatics, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu Mei 514-8507, Japan

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Abstract

GPRC5A is a member of G-protein-coupled receptors, which was originally identified as an all-*trans*-retinoic acid-induced gene. Although recent studies reported that this gene was highly expressed in the cancer cell lines and that GPRC5A might positively regulate cell proliferation, its mechanism remains unknown. We investigated the upstream and downstream signaling of GPRC5A and its biological function, and found that cAMP signaling is the novel GPRC5A induction pathway. When GPRC5A gene was overexpressed, intracellular cAMP concentration was decreased, and Gsα gene expression was downregulated. On the other hand, RNA interference of GPRC5A increased mRNA levels of Gsα and intracellular cAMP, reduced cell number, and induced apoptosis. Conversely, cell number was increased by GPRC5A overexpression. We first report the novel negative feedback model of cAMP signaling through GPRC5A gene expression. This evidence explains one of the mechanisms of the GPRC5A-regulated cell growth in some cancer cell lines.

Keywords: GPRC5A; GPCR; Orphan GPCR; cAMP; Gsα; Apoptosis; Cancer; Negative feedback

The G-protein-coupled receptors (GPCRs) constitute one of the largest superfamilies of receptors, which are characterized by seven transmembrane helices, three intracellular loops, three extracellular loops, an extracellular N-terminus, and an intracellular C-terminus. They respond to a wide variety of ligands, including hormones, odorants, light, taste, peptides, glycoproteins, neurotransmitters, and nucleotides. Upon binding of agonists, GPCRs catalyze the exchange of GDP bound to the Gα subunits for GTP. GTP-bound Gα protein subunits dissociate from Gβγ dimers, and both of them activate or inactivate downstream effectors such as adenylyl cyclase, phospholipase A2/Cβ or ion channels. Moreover, many GPCRs have constitutive activity without the ligand [1]. On the one hand, the down stream molecules of GPCR themselves have constitutive activity such as Gsα [2]. Therefore, the expressional regulation of the GPCRs and the effectors would be significant for some pathophysiological conditions [1]. However, there have been no study reports on linkage between the expressional regulation of GPCRs and the downstream effector molecules and their cellular function. GPRC5A, otherwise called RAIG1 or RAI3, is originally identified as one of the all-*trans*-retinoic acid (atRA)-induced genes by a differential display technique in a squamous carcinoma cell line UMSCC-22B [3]. According to the deduced amino acid sequence, this gene product is thought to be one of the orphan GPCRs that belong to the RAIG1 subfamily of the family C GPCRs. The RAIG1 family consists of GPRC5A, GPRC5B, GPRC5C, and GPRC5D [4,5]. Recently, it was reported that GPRC5A was highly expressed in some mutant p53-expressing tumor cell lines [6] and breast cancer specimens [7]. Moreover, siRNA against GPRC5A suppressed the cell growth of HEK 293, MCF7, and T47D [7]. This evidence shows that

☆ GPRC5A regulates cAMP signaling and apoptosis.

* Corresponding author. Fax: +81 59 232 1765.
E-mail address: tanaka@doc.medic.mie-u.ac.jp (T. Tanaka).

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GPRC5A might be a therapeutic target for some kinds of tumor. In spite of these observations, its downstream signaling and the mechanism of the positive regulation of cell growth have never been identified. In this study, we first identified GPRC5A as one of the significantly upregulated genes with cAMP elevating agent. Therefore, we first focus on the cAMP-inducibility of GPRC5A gene expression; next, we investigate cAMP regulation by modulating GPRC5A gene expression. Finally, we inquire into biological function of GPRC5A by overexpression and RNA interference techniques.

Materials and methods

Oligonucleotide. All oligonucleotides used in this study were purchased from Sigma–Aldrich and they are summarized in supplemental material.

Plasmid. The five promoter regions (from −5000, −3000, −1000, and −100 to +100 bp. of the transcription initiation site) of the human GPRC5A gene were obtained by PCR cloning with the *Kpn*I or *Mlu*I restriction enzyme recognition site-attached primers. cAMP-responsive element (CRE) mutagenesis in GPRC5A promoters was conducted using the PCR-based Quickchange Site-Directed Mutagenesis Kit (Stratagene). These promoter inserts were ligated into the pGL3 basic vector (Promega), which was digested with *Kpn*I and *Mlu*I. The GPRC5A expression vector (pIRES-GPRC5A) was gained by PCR cloning with *Eco*RV or *Not*I restriction enzyme recognition site-attached primers. GPRC5A-ORF was ligated into the pIRESneo2 vector, which was digested with *Eco*RV and *Not*I.

siRNA construction. Small interfering RNA (siRNA) was synthesized by a Silencer siRNA Construction Kit (Cat #1620, Ambion). The target sequence of GPRC5A mRNA was the same oligonucleotide as previously reported [7] (5′-AAC AGG CGA AAA ATG CTG CCT-3′).

Cell culture. Human aortic smooth muscle cells (HASMC; CCS-2571) were obtained from IWAKI. Human thyroid follicular epithelial cells (Nthy-ori 3-1, abbreviation; Nthy) were obtained from the European Collection of Cell Cultures. Human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco), 10% FCS, and 1% penicillin/streptomycin sulfate at 37 °C, 5% CO_2. HASMC was used between passage 6 and 9 for the experiments. For chemical treatment experiments, cells were grown until subconfluence (70–80%) and then incubated in serum-free DMEM for 24 h prior to each treatment.

RNA collection. Cells were fixed with the RLT buffer of the RNA purification kit (RNeasy, Qiagen) at the indicated time course. When pretreatment with RNA synthesis inhibitor actinomycin D (ActD) was conducted, ActD (final 2 μM) was added into the culture medium 1 h before each chemical treatment.

Real-time PCR quantification using SYBR Green I. The cDNA was synthesized from 0.5 μg of total RNA using Superscript II reverse transcriptase, then diluted fivefold with TE. The quantification of mRNA for each sample was conducted by the standard SYBR Green I system. Each sample value was divided by that of housekeeping gene actin-β. The data were represented by the relative expression to control. Therefore, control sample is always set to 1. The average of the relative expression was obtained from the three different experimental sets.

Promoter assay. HASMC was transfected with the ECM630 electroporation system (BTX). Then, 2×10^7 HASMCs were suspended in 200 μl DNA–Hepes-buffered saline (HeBS; 1× PBS, 10 mM Hepes, pH 7.2) solutions that contained 10 μg promoter vector and 1 μg pRL sv40 vector (internal control, Promega). Cell suspension was transferred to a BTX Disposable Cuvettes Model #640 (4-mm gap), and cells were then subjected to electroporation (200 V, 150 μF, Resistance; set to none). After electroporation, the cells were placed on ice for 10 min and then transferred to three wells of the culture medium. Twenty-four hours after electroporation, the cells were treated with each chemical, and after an additional 24 h, the cells were fixed with a passive lysis buffer (E1941, Promega). Dual-luciferase assay was conducted according to the manufacturer's instructions (Promega).

Cell counting by WST-8. For the proliferation assay and cAMP assay data correction, the cell number was quantified by Cell Counting Kit-8 (Dojindo). In order to quantify the cell number precisely, twofold serial dilution standard cells were prepared 6 h before assay. From the regression curve gained by the standard, the cell number of the unknown sample was calculated.

Measurement of Intracellular cAMP. For the overexpression experiment, 1.5×10^4 Nthy cells were seeded into each well of a 96-well plate in 10% FBS–DMEM. Twenty-four hours after seeding, transfection solution (6 μl DMEM, 0.4 μg no-insert vector or GPRC5A expression vector, and 0.2 μl Lipofectamine 2000) was added into each well. For the siRNA experiment, 6×10^3 cells were seeded. Twenty-four hours after seeding, transfection solution (20 μl DMEM, final 50 nM siRNA, and 0.1 μl Lipofectamine 2000) was added into each well. Forty-eight hours after transfection, cAMP assay was performed by cAMP EIA (RPN 225, Amersham) in accordance with the instruction manual. The cAMP concentrations per cell were corrected with the mean value of the cell number in each condition. Relative intracellular cAMP concentration to control was calculated by dividing the value of the test group by that of the control group.

Quantification of Gsα mRNA expression. First, 7.5×10^4 Nthy cells were seeded into a 24-well plate in 10% FBS–DMEM. Twenty-four hours after seeding, transfection solution (30 μl DMEM, 2 μg no-insert vector or GPRC5A expression vector, and 1 μl Lipofectamine 2000) was added into each well. Forty-eight hours after transfection, RNA was collected by RLT buffer (Qiagen). Reverse transcription and real-time PCR were conducted as mentioned above.

Apoptosis assay. 1.0×10^4 Nthy cells were seeded into a 96-well plate in 10% FBS–DMEM. Twenty-four hours after seeding, final 2 nM siRNA was transfected by Lipofectamine 2000. At the indicated time point, caspase 3/7 activity was measured by Caspase-Glo 3/7 Assay (Promega), and relative caspase activity was corrected by cell number with the WST-8 assay as described above.

Proliferation assay. 1×10^6 Nthy cells were resuspended in 200 μl HeBS that contained 20 μg expression vector (pIRESneo2 or pIRES-GPRC5A). This cell suspension was electroporated through the 300-V, 150-μF, and no-resistance setting of the ECM630 electroporation system. After electroporation, the cell suspension was spun down and the supernatant was eliminated. These cells were resuspended in 20 ml of 10% FBS–DMEM, and 100 μl cell suspension was transferred into each well of the 96-well plate. WST-8 assays were conducted at the indicated time and cell number measured 24 h after electroporation was set to baseline (=1.0).

Results

GPRC5A is transcriptionally induced by cAMP-elevating agents

We found GPRC5A to be one of the cAMP elevating agent-induced genes in human aortic smooth muscle cells (HASMCs) by custom-made cDNA microarray analysis (data not shown). When we conducted quantitative real-time PCR, forskolin increased the GPRC5A expression (Fig. 1A). Since cAMP elevating agent forskolin induced the GPRC5A mRNA level, it was assumed that GPRC5A is induced via cAMP signaling. Fig. 1A shows the time course of the GPRC5A expression level in 10 μM forskolin-treated HASMC. The mRNA level of GPRC5A was up-regulated about twofold as early as 1 h after forskolin treatment, and peaked 3 h after treatment (10-fold

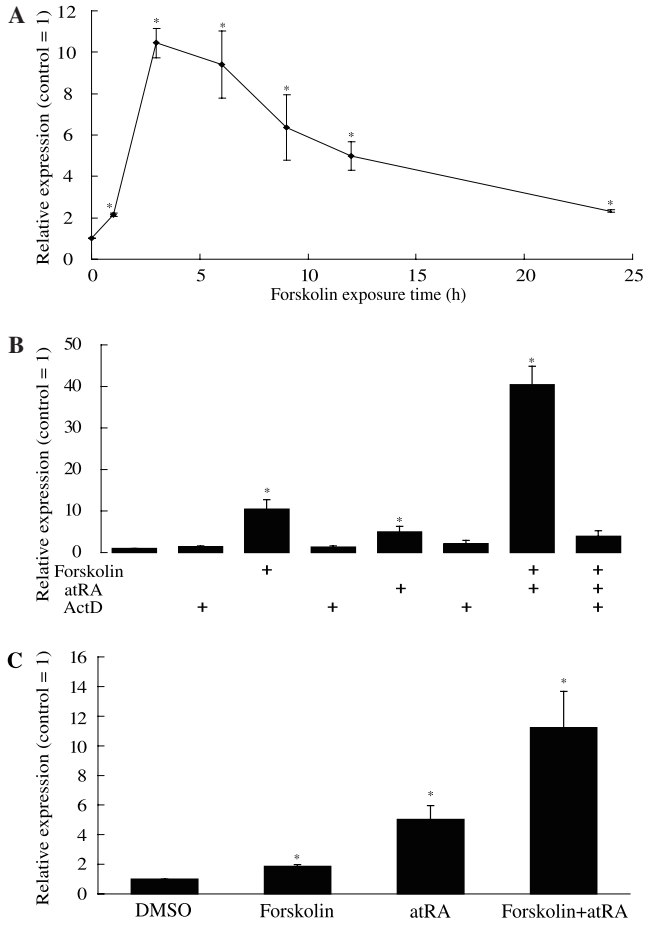

Fig. 1. (A) Time course of GPRC5A induction by 10 μM forskolin. HASMC was treated with DMSO (vehicle) or 10 μM forskolin. RNA was collected 1, 3, 6, 9, 12, or 24 h after treatment in three different experimental sets. (B,C) Synergistic induction of GPRC5A by forskolin and atRA. HASMC (B) or HEK293 cell (C) was treated with 10 μM forskolin, 10 μM atRA, or a combination for 3 h, with or without pre-treatment with RNA synthesis inhibitor actinomycin D (ActD) 1 h before inducer treatment. The values represent the average ± standard error of the three different experiments. The quantification of the GPRC5A expression level was conducted by SYBR Green I Real-time PCR. The relative expression of GPRC5A was calculated using the following formula: $GPRC5A_{(forskolin)}/ACTB_{(forskolin)}/GPRC5A_{(vehicle)}/ACTB_{(vehicle)}$. * $p < 0.05$ compared with control (two-tailed Student's t test).

induction), then gradually returned to baseline, but retained a twofold higher level than that of the control 24 h after treatment. The cAMP-inducibility of the GPRC5A expression was seen in some cell lines other than HASMC (data not shown).

GPRC5A is synergistically induced by cAMP and atRA signaling

Although GPRC5A was originally identified as a retinoic acid-inducible gene 1 (RAIG1) in the squamous carcinoma cell line [3], it is not known whether this gene is induced by cAMP and retinoids cooperatively. We treated HASMC with 10 μM forskolin and 10 μM all-*trans*-retinoic acid (atRA) for 6 h, then confirmed the GPRC5A expression level by quantitative real-time PCR. Thus, GPRC5A was synergistically induced by a combination of forskolin and atRA, and its induction was as high as a 40-fold increase in HASMC (Fig. 1B). Since pre-treatment with RNA synthesis inhibitor actinomycin D inhibited the single and combinatorial treatment of forskolin and atRA almost entirely, the increase in the GPRC5A mRNA level by cAMP-atRA signaling was presumed to be dependent on the de novo RNA synthesis. This cAMP-atRA synergistic effect on GPRC5A transcriptional induction was recapitulated in HEK 293 cells, and it was induced by an approximately 10-fold increase in the forskolin- and atRA-treated cells (Fig. 1C).

GPRC5A induction by cAMP signaling is mediated by the CRE motif close to the transcription initiation site

In order to clarify the critical major motif of GPRC5A induction by cAMP signaling, five different length-GPRC5A promoter-luciferase vectors were introduced into HASMC, and then luciferase activities were measured. Forskolin induced luciferase activities in all wild-type GPRC5A promoters (−5 to −0.1 kbp) (Fig. 2), and it was assumed that at least one of the functional cAMP-responsive elements existed −0.1 kb downstream of the transcription initiation site. When we introduced mutation into the CRE motif of −50 to −43 bp upstream from the transcription initiation site, increase in luciferase activity by forskolin was drastically inhibited in all promoter-luciferase reporter vectors. Moreover, the CRE-mutant promoter activities of the vehicle treatment were lower than those of the wild type. Taken together, the cAMP-inducibility and basal expression of GPRC5A transcription were likely mediated by this CRE motif.

GPRC5A expression is negatively correlated with Gsα gene expression and with intracellular cAMP

Searching large-scale cross-tissue microarray database GNF SymAtlas (http://symatlas.gnf.org/SymAtlas/) [8], it was likely that GPRC5A gene is expressed in limited tissues such as lung, thyroid or pancreas. For the GPRC5A functional analysis, we used the normal thyroid follicular epithelial cell line Nthy-ori 3-1 (Nthy), since this cell line is highly susceptible to transfection. First, we examine the cAMP regulatory effect by GPRC5A that was regulated by cAMP in itself. Nthy cells were transfected with no-insert vector or GPRC5A expression vector. Forty-eight hours after transfection, the intracellular cAMP level was measured. As a result, the intracellular cAMP level was significantly reduced in GPRC5A expression vector-transfected cells (Fig. 3A). Since Giα inhibitor pertussis toxin failed to inhibit cAMP reduction (data not shown), the other mechanism seemed to contribute to cAMP reduction. It is known that the expression level of Gsα affects both GPCR-adenylyl cyclase (AC)-cAMP cascade and constitutive AC pathways without GPCR ligand [2]. In fact, the

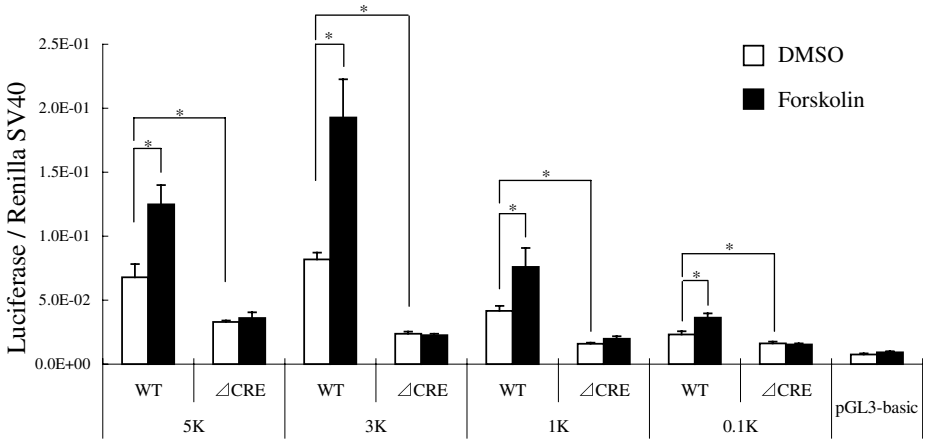

Fig. 2. Promoter analysis of GPRC5A. Five wild-type promoters (WT; from 5, 3, 1, 0.1 kbp to −100 bp of transcription initiation site) were inserted upstream of the luciferase-coding region. Mutagenesis was introduced into their prospective CRE motifs (−50 to −43). These eight promoters and non-insert Luciferase reporter vector (pGL3 basic) were electroporated into HASMC. Twenty-four hours after electroporation, the cells were treated with DMSO (vehicle) or 10 μM forskolin. After an additional 24 h, luciferase activity was measured. * $p < 0.05$ (two-tailed Student's t test).

Gsα mRNA level of 48-h GPRC5A-transfected cells was significantly reduced in parallel with the cAMP reduction (Fig. 3B). Therefore, decrease in intracellular cAMP concentration by GPRC5A expression is likely due to the downregulation of Gsα expression. To validate this hypothesis, we next used small interfering RNA (siRNA) targeted to GPRC5A mRNA (siGPRC5A). Synthesized siGPRC5A effectively suppressed GPRC5A mRNA levels (about 50%) in Nthy cells (Fig. 3C). In addition, the intracellullar cAMP level was increased by about 40% compared to the negative control siRNA-transfected group (Fig. 3D). Moreover, the increase in intracellular cAMP was paralleled by upregulation of Gsα gene expression levels (Fig. 3E). These results indicate that GPRC5A expression represses Gsα mRNA and consequently decreases intracellular cAMP levels.

GPRC5A expression controls cell number and apoptosis

In order to find out the biological significance of GPRC5A, we next examine whether GPRC5A expression affects cell proliferation. Nthy cells were transfected with siGPRC5A (12.5, 25, and 50 nM). Forty-eight hours after transfection, the cell number was quantified with WST-8 assay. siGPRC5A reduced the cell number of Nthy cells compared to negative control siRNA-transfected group (about 70% inhibition, 50 nM siGPRC5A, Fig. 4A). Decrease in cell number by siGPRC5A could result from apoptosis. Caspase 3/7 activities were measured in siGPRC5A transfected cells at 24, 48, and 72 h after siRNA transfection and it was elevated at all time points (Fig. 4B). Since several cAMP-elevating agents suppressed the proliferation of Nthy cells similarly (data not shown), one of the cell number reduction mechanisms by siGPRC5A might be via intracellular cAMP elevation. On the other hand, the overexpression of GPRC5A slightly increased the cell proliferation 96 and 144 h after transfection (Fig. 4C).

Discussion

In this study, we found that GPRC5A is transcriptionally induced by cAMP signaling in human aortic smooth muscle cells (HASMCs). GPRC5A was originally identified as a retinoic acid-induced gene in the squamous carcinoma cell line [3]. Since cAMP-elevating agent forskolin-induced GPRC5A mRNA expression level, it was assumed that the upregulation of GPRC5A expression was dependent on cAMP signaling. Moreover, GPRC5A upregulation by cAMP and atRA was synergistic in nature. Since an increase in the GPRC5A expression level was inhibited by RNA synthesis inhibitor actinomycin D, this induction was considered to depend on de novo RNA synthesis. Furthermore, when we introduced mutation to cAMP-responsive element (CRE) −50 bp upstream of the transcription initiation site, cAMP-inducibility by forskolin was almost entirely reduced. Moreover, basal expression activities were also reduced in the promoters whose CRE were mutated. Such evidence indicates that cAMP signaling is the novel pathway for the transcriptional induction and the basal expression of GPRC5A.

As expected by the deduced amino acid sequence, GPRC5A is considered to be a G-protein-coupled receptor [3]. Unfortunately, the endogenous ligand and the downstream signaling of this receptor have not been identified to date. In order to estimate whether cAMP-induced gene GPRC5A contributes to cAMP signaling downstream, human thyroid epithelial cell line Nthy was transfected with GPRC5A expression vector, and intracellular cAMP was measured. As a result, intracellular cAMP concentration was decreased in GPRC5A-expressing cells. Since Giα inhibitor pertussis toxin could not reverse the cAMP

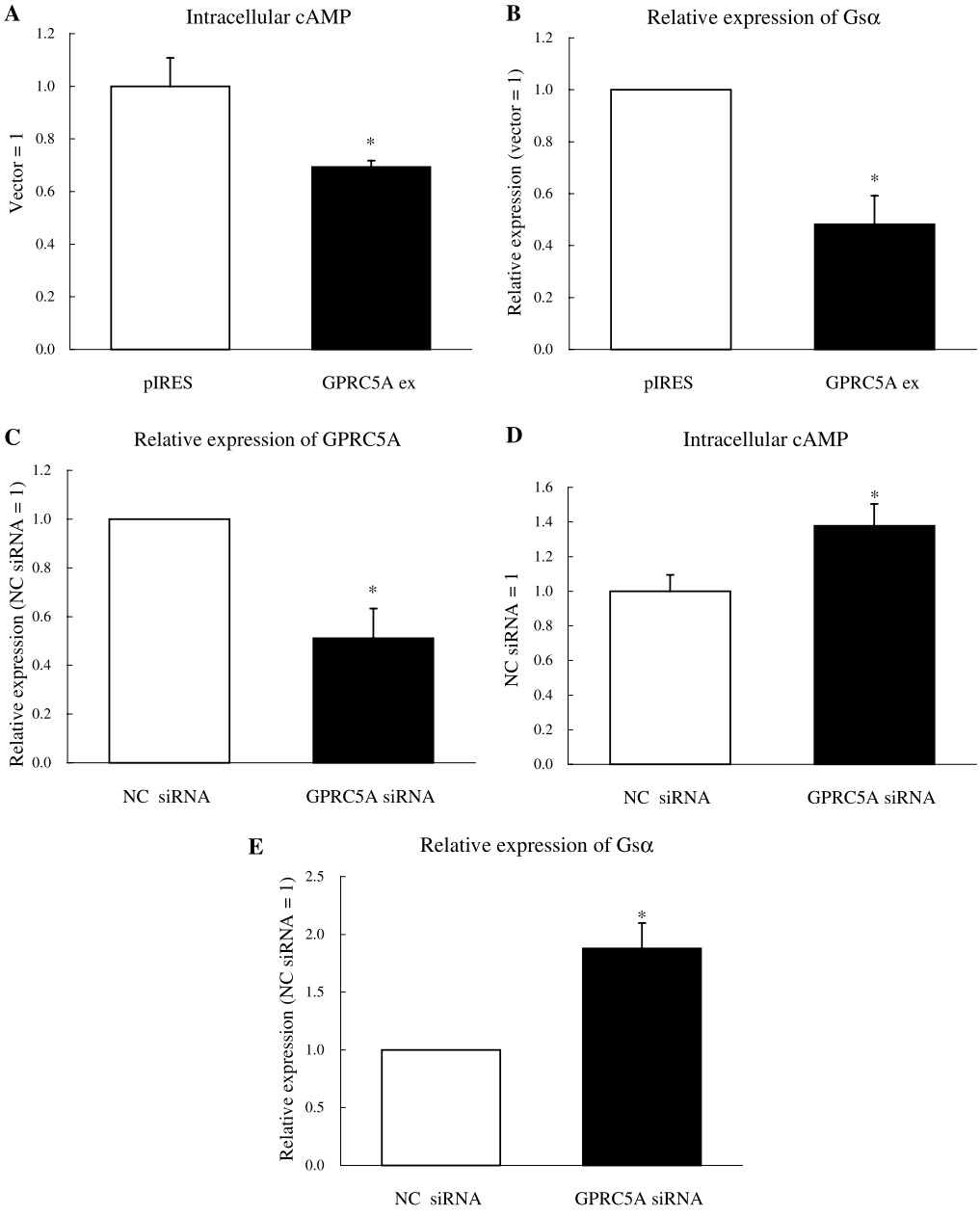

Fig. 3. (A,D) Intracellular cAMP measurement was performed 48 h after transfection of expression vector (A) or siRNA (D). The cAMP amount per well was divided by the mean cell number in each condition. The values were expressed as ratios to the cAMP amount per cell of the control groups. (B, C, and E) Gsα (B,E) or GPRC5A (GPRC5A) mRNA levels were quantified by SYBR Green I Real-time PCR. Nthy cells were transfected with expression vector (B) or siRNA (C,E), and then RNA was collected 24 (C,E) or 48 h (B) after transfection. The relative expression of objective gene was calculated using the following formula: Object-$Gene_{(test)}$/$ACTB_{(control)}$/Object-$Gene_{(test)}$/$ACTB_{(control)}$. pIRES, no-insert expression vector; GPRC5A ex, GPRC5A expression vector; NC siRNA, negative control siRNA. * $p < 0.05$ compared with control (two-tailed Student's t test).

reduction (data not shown), it was thought that the Giα did not become involved in the cAMP reduction. Therefore, we explored the other mechanism that reduces intracellular cAMP levels. We next focused attention on the Gsα expression level. As was expected from the hypothesis, Gsα mRNA was negatively correlated when GPRC5A expression was overexpressed or repressed. As indicated in the study by Yang et al. [2], the Gs expression level directly modulates intracellular cAMP concentration. Accordingly, the cAMP regulatory mechanism of GPRC5A expression level is partially explained by the decrease in Gsα expression level. This is the first report of the novel negative feedback mechanism for cAMP signaling; that is, cAMP-responsive GPCR itself could regulate cAMP signaling by modulating Gsα expression levels.

Recent studies have shown that GPRC5A was highly expressed in tumor cell lines [6], and breast cancer specimens [7] and the expression of GPRC5A in HEK 293 cells promoted anchorage-independent growth, and moreover, siRNA against GPRC5A suppressed cell growth [7], but its molecular mechanism has not been identified yet. Indeed, in our study, the overexpression of GPRC5A could

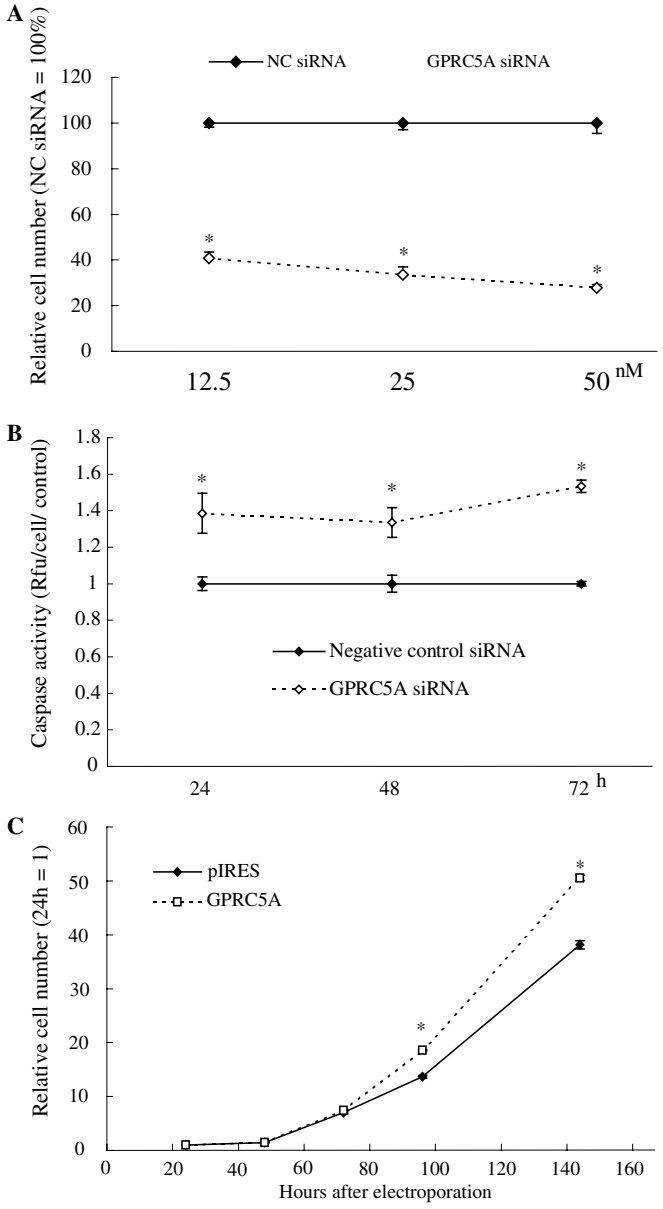

Fig. 4. (A) Nthy cells were transfected with negative control siRNA (NC siRNA) or GPRC5A siRNA. Forty-eight hours after transfection, the cell number was quantified with a WST-8 assay. The cell number was determined by the regression curve calculated from the standard serial dilutions. (B) Caspase 3/7 activities of Nthy cells that were transfected with 2 nM NC siRNA or GPRC5A siRNA were measured 24, 48, or 72 h after transfection. The values were expressed as relative caspase 3/7 activity normalized by control caspase 3/7 activity per cell. (C) No-insert vector (pIRES) or GPRC5A expression vector was electroporated into Nthy cells. At the indicated time after electroporation, the cell number was quantified with a WST-8 assay. * $p < 0.05$ compared with control (two-tailed Student's t test).

enhance the cell proliferation of Nthy cells. Moreover, siRNA targeted to GPRC5A (siGPRC5A) reduced the cell number in parallel with increased caspase 3/7 activity. Since cAMP promotes apoptosis in several cell types [9–11] and cAMP-elevating agents inhibit cell proliferation and induce caspase 3/7 activity in this cell line (data not shown), the anti-mitogenic and pro-apoptotic effect of siGRC5A was associated with cAMP elevation via the upregulation of Gsα gene expression. It is noteworthy that patients with some types of cancers who have reducing genotype of Gsα expression are at high risk for progression, metastasis, and tumor-related death [12–14]. These results suggest that GPRC5A is a novel cAMP-targeted gene and that it might induce cell proliferation through the downregulation of Gsα gene expression and cAMP signaling. This evidence supports the fact that GPRC5A is highly expressed in some cancers and indicates that GPRC5A may be a therapeutic target in some tumors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.10.016.

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