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Epigenetic repression of regulator of G-protein signaling 2 promotes androgen-independent prostate cancer cell growth

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Abstract

G-protein coupled receptor (GPCR)-stimulated androgen-independent activation of androgen receptor (AR) contributes to acquisition of a hormone-refractory phenotype by prostate cancer. We previously reported that regulator of G-protein signaling (RGS) 2, an inhibitor of GPCRs, inhibits androgen-independent AR activation.¹ Here, we show reduced RGS2 protein expression in human prostate cancer specimens compared to adjacent normal or hyperplastic tissue. Methylation-specific PCR analysis and bisulfite sequencing indicated that methylation of the CpG island in the RGS2 gene promoter correlated with RGS2 down-regulation in prostate cancer. *In vitro* methylation of this promoter suppressed reporter gene expression in transient transfection studies, whereas reversal of this promoter methylation with 5-aza-2'-deoxycytidine (5-Aza-dC) induced RGS2 re-expression in androgen-independent prostate cancer cells and inhibited their growth under androgen-deficient conditions. Interestingly, the inhibitory effect of 5-Aza-dC was significantly reduced by an RGS2-targeted short hairpin RNA, indicating that re-expressed RGS2 contributed to this growth inhibition. Restoration of RGS2 levels by ectopic expression in androgen-independent prostate cancer cells suppressed growth of xenografts in castrated mice. Thus, RGS2 promoter hypermethylation represses its expression and unmasks a latent pathway for AR transactivation in prostate cancer cells. Targeting this reversible process may provide a new strategy for suppressing prostate cancer progression by reestablishing its androgen-sensitivity.

Keywords

Prostate cancer progression; DNA methylation; RGS2; G-protein coupled receptors; Androgen receptor

INTRODUCTION

Prostate cancer is the most common cancer in American men.² Androgen ablation therapies contribute significantly to disease control during the early stages of prostate cancer since

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prostate cancer growth depends on androgen activation of AR. However, the majority of prostate cancers eventually progress to a hormone-refractory phenotype against which current treatments are relatively ineffective.^{3,4}

Some hormone-refractory prostate cancers are caused by androgen-independent activation of AR.^{5,6} Early research to understand hormone-refractory prostate cancer focused on molecular alterations, such as AR over-expression or mutations, which could allow AR to respond to lower levels of androgens⁷ or be directly activated by other ligands.³ More recently, it has been suggested that AR can be indirectly activated by cell-surface receptors including HER2, interleukin-6 receptor and GPCRs.^{8,9}

GPCR-mediated signal transduction is crucial for important cellular responses.¹⁰ Excessive activation of GPCRs facilitates prostate cancer progression by causing androgen-independent AR activation.^{8,11-14} Thus, inhibition of the excessively activated GPCRs has potential therapeutic benefit for prostate cancer patients.^{15,16} We previously reported that RGS2, a member of the regulator of G-protein signaling (RGS) family, blocks androgen-independent AR activation in prostate cancer cells.¹ RGS proteins accelerate the termination of GPCR signaling,¹⁷ thus playing important roles in neuronal, cardiovascular, and lymphocytic activities,¹⁸⁻²⁰ and tumorigenesis.²¹ RGS2 dysregulation was implicated in solid tumor development²² and RGS2 over-expression inhibited transformation of myeloid cells.²³ We found that RGS2 was down-regulated in androgen-independent prostate cancer cells, and that silencing RGS2 expression in androgen-sensitive prostate cancer cells stimulates AR activation and cell growth in the absence of androgens.¹ This suggests that the loss of RGS2 may unmask a latent pathway for AR activation by various GPCRs in prostate cancer, thereby contributing to the development of a hormone-refractory phenotype. Interestingly, RGS2 down-regulation was also found in acute myeloid leukemia,²³ hot thyroid nodules,²⁴ and advanced colorectal cancers,²⁵ suggesting that RGS2 down-regulation may be involved in several types of neoplasia. However, the mechanism underlying this RGS2 down-regulation is unknown.

The purpose of the present studies was to determine molecular mechanisms contributing to the loss of RGS2 expression in prostate cancer. Our data indicated that the RGS2 gene promoter can be methylated. Aberrant methylation in gene promoters is a hallmark of human cancers, and acts as a powerful heritable suppressor of transcriptional activity.²⁶⁻²⁸ We found that the degree of RGS2 promoter methylation correlated with RGS2 down-regulation, and conclude that this epigenetic silencing of RGS2 facilitates prostate cancer progression. More importantly, our study suggests that targeting methylation-dependent RGS2 suppression may provide a new strategy for suppressing androgen-independent growth of prostate cancer.

MATERIALS AND METHODS

Cells and reagents

Prostate cancer CWR22Rv1 and LNCaP-C33 (passage<33) cells from American Type Culture Collection (ATCC) were maintained in RPMI-1640 medium with 5% fetal bovine serum (FBS). An androgen-independent LNCaP-C81 (passage>81) cell line was established by 12-months of continuous culture of LNCaP-C33 cells.²⁹ Normal human prostate epithelial PrEC cells were cultured in PrEGM (Cambrex).

Trizol and RPMI-1640 medium were from Invitrogen. FBS and charcoal-stripped serum (CSS) were from Hyclone. Anti- β -actin antibody was from Santa Cruz. Rabbit anti-RGS2 C-terminal peptide (KKPQITTEPHAT) antibody and RGS2 knock-out (KO) mice²⁰ were from Dr. David Siderovski (University of North Carolina). Adenovirus encoding RGS2 or

green fluorescent protein (GFP) was from Dr. Peter Chidiac (University of Western Ontario).³⁰ Propidium iodide (PI) and a fluorescein isothiocyanate (FITC)-labeled Annexin V Staining Kit were from Millipore. 5-Aza-dC and bisulfite were from Sigma-Aldrich.

Immunohistochemistry analysis of human prostate tissues

Immunohistochemistry was performed as previously described³¹ using the anti-RGS2 antibody (1 µg/ml). Formalin-fixed prostate tissues of RGS2 wild-type (WT) and KO mice were used to optimize immunohistochemistry.

Prostate tissue microarrays were from US Biomax (T195, PR8011 and PR807a) and Korea ISU-ABXIS Co. (A302IV and A718VIII). RGS2 staining intensity of microarrays was graded from 0 (low) to 3 (high) by two pathologists (C.D. and Z.G.). Ten archived, formalin-fixed, paraffin-embedded prostate cancer blocks from the Creighton University Medical Center were also immunohistochemically stained for RGS2 protein, approved by the Creighton University Institutional Review Board. RGS2 protein was measured on these stained tissue sections using the Automated Cellular Imaging System (ChromaVision). This allows objective quantification of staining intensity ranging from 0 (none) to 255 (maximum). The “histo-score” (H-score) is the percentage of positive cells times the average intensity^{31,32}. Average H-scores from 10 randomly chosen 200× fields were used to quantitatively grade RGS2 protein levels.

Protein extraction, electrophoresis and Western blot analysis

Protein was extracted from cells or mouse prostate tissues using 1× RIPA lysis buffer (Santa Cruz). Samples (40 µg) were electrophoresed and subjected to Western blot using antibodies of RGS2 and β-actin. Images were captured with a Li-Cor Odyssey (LI-COR Biosciences).³¹

5-Aza-dC treatment and cell growth assays

Every 24 h, fresh medium containing 5-Aza-dC (0-10 µM) was added to both LNCaP-C81 and CWR22Rv1 cell cultures. After 10 days of drug treatment, the cells were washed and then harvested for cell growth assays and RGS2 expression analysis.

Cells pre-treated without or with 10 µM 5-Aza-dC were seeded into 96-well plates (8×10³ cells/well), and allowed to recover for 24 h in RPMI 1640 phenol red-free medium supplemented with 5% CSS. Cells were then cultured for 48 h in medium supplemented with 5% FBS or 5% CSS with or without 5 nM dihydrotestosterone (DHT). The CellTiter 96 AQueous Cell Proliferation Assay kit (Promega) was used to determine the number of viable cells.¹⁴

To determine the effect of silencing endogenous RGS2 on the 5-Aza-dC-induced inhibition of androgen-independent growth of prostate cancer cells, LNCaP-C81 cells stably expressing RGS2 short hairpin RNA (shRNA) or control scramble shRNA¹ were treated with 10 µM 5-Aza-dC and then subjected to Western blot or *in vitro* cell growth assays. LNCaP-C81 and CWR22Rv1 cells were seeded into 24-well plates and cultured in 5% CSS in the presence or absence of 5 nM DHT and counted using a Coulter Counter ZM (Coulter Electronics).

Flow cytometric analysis of cell apoptosis

Cell apoptosis was quantified by measuring externalization of phosphatidylserine using an Annexin V Staining Kit. Labeled cells were analyzed with a FACSaria flow cytometer (Becton Dickinson) at the Creighton University Flow Cytometry Core Facility.

Adenoviral infection and subcutaneous injections of prostate cancer cells into nude mice

CWR22Rv1 cells were infected with adenovirus encoding either RGS2 or GFP for 24 h at a multiplicity of infection of 20. Infection efficiency, the percentage of cells expressing GFP, was about 90%. Cells (2.5×10^6 in 150 μ l of 50% Matrigel) were injected subcutaneously in the dorsal surface of 6-week-old castrated male nude mice (Charles River). Tumor sizes were measured twice a week and expressed in mm³ using the ellipsoid formula. The Creighton University Biosafety and Institutional Animal Care and Use Committees approved these experiments.

Conventional PCR and quantitative real-time PCR

Total RNA was isolated from cells treated with or without 5-Aza-dC using Trizol. The level of RGS2 expression was analyzed by conventional and quantitative real-time PCR as described¹ using the primers listed in Supplemental Table 1. β -Actin was an internal control gene. PCR products were confirmed by DNA sequencing.

Bisulfite modification of genomic DNA and methylation-specific PCR (MSP)

Prior to bisulfite modification, samples were analyzed for WT RGS2 locus using WT primers (Supplemental Table 1), which detects a 164-bp fragment of the RGS2 promoter. DNA was bisulfite modified using an EZ DNA Methylation Kit (Zymo Research). PCR products using probes for unmethylated or methylated RGS2 (Supplemental Table 1) were separated on agarose gels and photographed with a Fotodyne Gel imaging system.

Cloning and bisulfite sequencing

For bisulfite sequencing, a 618 bp fragment ($-595/+23$) containing the central region of the putative RGS2 CpG island was amplified by nested PCR using two sets of primer pairs specific for bisulfite-modified sequences (Supplemental Table 1). PCR products were cloned into pDrive Cloning vector (Qiagen) and transfected into DH5 α cells (Invitrogen). Transfected cells were plated on X-gal/IPTG LB agar plate containing 100 μ g/ml ampicillin and incubated overnight at 37°C. Ten clones per DNA specimen were picked randomly for dideoxy sequencing using T7 and SP6 primers in a 3130 Genetic Analyzer (Applied Biosystems).

Luciferase reporter constructs and DNA methylation of RGS2 promoter *in vitro*

RGS2 gene promoter was amplified from human prostate genomic DNA using primers shown in Supplemental Table 1. The short ($-757/-1$) and long fragment ($-1453/-1$) of the RGS2 promoter with cohesive 5' *Bgl*III and 3' *Hind*III termini were subcloned upstream of luciferase in the pGL3 vector (Promega). Constructs were verified by DNA sequencing.

RGS2-pGL3 (2 μ g) was incubated with 8 U SssI methylase and 1 $\times$ methyl donor S-adenosyl-L-methionine (New England Biolabs) for 2 h at 37°C and 30 min at 65°C. The methylation efficiency was determined by resistance of the DNA to digestion with methylation-sensitive restriction enzymes HpaII and HhaI (New England Biolabs).

Luciferase assays

Human embryonic kidney HEK293 cells (ATCC) seeded in 24-well plates were transfected with 500 ng of different pGL3 plasmids and 10 ng of a control plasmid having a Renilla luciferase sequence (pRL-tk) to normalize luciferase activity. Relative luciferase activities of each construct were measured using the dual luciferase assay kit (Promega). The results are presented as fold increase compared to pGL3-basic vector. A SV40 promoter-containing pGL3 vector was used as a positive control.

Statistical analysis

Tissue microarray scoring was analyzed with a Kruskal-Wallis test and Dunns post-test. Other results are mean $\pm$ S.E. of at least three determinations and statistical comparisons used the Student's *t* test. $P < 0.05$ was considered to be significant.

RESULTS

Decreased RGS2 protein expression in human prostate tumors

Commercial human microarrays of 184 prostate samples containing prostate carcinoma, benign hyperplasia or adjacent normal tissue were immunohistochemically stained for RGS2 protein. Our Western blot detection of 24 kD proteins in prostate tissues from RGS2 WT mice but not RGS2 KO mice confirmed specificity of anti-RGS2 antibody²⁰ (Fig. 1A). Fig. 1B and Fig. 1C show that RGS2 protein was expressed at high levels in luminal epithelial cells in adjacent normal or benign hyperplastic prostate, but was decreased in prostate cancer. Expression levels of RGS2 protein in all tissue microarray cases were graded from 0 – 3 based on overall staining intensity (Table 1). This analysis revealed no significant differences between normal and hyperplastic tissues with over 90% of specimens positive for RGS2-staining, but the decrease in RGS2 protein in prostate cancer tissue was statistically significant (average staining intensity for adjacent normal, 1.9 ± 0.1 ; hyperplasia, 2.1 ± 0.2 ; and cancer, 0.9 ± 0.1) with about one-third of the cases having negligible RGS2 staining. However, in the prostate cancer samples, there was no apparent correlation between RGS2 immunostaining and tumor stage or Gleason score. These data suggest that RGS2 expression is generally decreased early in prostate cancer.

In vitro methylation abolished RGS2 promoter activity

DNA methylation is a well-known epigenetic mechanism that causes transcriptional silencing of critical genes associated with prostate cancer pathogenesis.³³ To determine whether promoter methylation plays a role in RGS2 repression, the luciferase activities of pGL3 plasmids containing two different lengths of proximal human RGS2 gene promoter (Fig. 2A), L-RGS2 (–1453/–1) and RGS2 (–757/–1), were assayed and compared. As shown in Fig. 2B, similar luciferase activity was detected in cells transfected with L-RGS2-pGL3 or RGS2-pGL3, which was much higher when compared to cells transfected with SV40-pGL3, suggesting that the 757 bp fragment in the RGS2-pGL3 plasmid contains a RGS2 promoter that has stronger activity than the SV40 promoter. *In vitro* methylation of RGS2-pGL3 by *SssI* methylase inhibits the luciferase activity by over 95% (methyl-RGS2 vs. RGS2 in Fig. 2B), showing that the RGS2 promoter is highly sensitive to methylation.

Hypermethylation of the RGS2 gene promoter correlates with RGS2 down-regulation in human prostate cancer specimens and prostate cancer cell lines

Analysis of RGS2 genomic architecture showed a dense CpG island (Fig. 2A). Thus, we first characterized methylation status of CpG dinucleotides within the RGS2 promoter in ten archived human prostate cancer specimens and their adjacent histologically non-cancerous tissues. Methylation specific PCR (MSP) showed higher methylation of the RGS2 promoter in tumor tissues ($48 \pm 6\%$) compared with their adjacent non-cancerous tissues ($24 \pm 4\%$) (Fig. 2C, semi-quantified in Fig. 2D). RGS2 protein levels in these prostate specimens were immunohistochemically stained and quantitatively assessed using H-scores.³¹ Consistent with DNA methylation-mediated gene silencing, RGS2 protein levels measured as H-score, were significantly lower in these prostate tumors than in their paired non-cancerous tissues (31 ± 6 vs. 92 ± 7 , respectively, Fig. 2D). Moreover, the proportion of cells with positive RGS2 immunoreactivity in tumor tissues varied from 0.1 to 0.76 while the proportion of

RGS2 positive cells was significantly higher, ranging from 0.49 to 0.97, in matched non-cancerous tissues (Supplemental Table 2).

Sublines of human prostate cancer LNCaP, androgen-sensitive LNCaP-C33 and androgen-independent LNCaP-C81 cells,²⁹ closely resemble the stages before and after the acquisition of hormone-refractoriness during prostate cancer progression.³⁴⁻³⁶ We previously showed endogenous RGS2 in LNCaP-C33 cells but not in LNCaP-C81 cells.¹ Thus, we used these cell lines and normal prostate epithelial PrEC cells as a model to investigate whether DNA methylation contributes to RGS2 down-regulation in prostate cancer.

MSP analysis showed strong methylation of the RGS2 promoter in LNCaP-C81 cells (Fig. 3A), with much less methylation in LNCaP-C33 cells. In contrast, methylation of the RGS2 promoter was almost undetectable in PrEC cells. We subsequently used bisulfite sequencing to analyze methylation status of the entire RGS2 promoter. Data in Fig. 3B indicated a significantly higher level of DNA methylation in LNCaP-C81 (96.1%) when compared to LNCaP-C33 (25.6%) and PrEC cells (1.6%). These results are in excellent agreement with the data obtained by MSP analysis, indicating a progressive increase in methylation of the RGS2 promoter from normal prostate epithelial cells to androgen-independent prostate cancer cells. Moreover, the extent of methylation of the RGS2 promoter was inversely correlated with expression levels of RGS2 mRNA in these cell lines (Fig. 3A and 3B vs. 3C). Interestingly, bisulfite sequencing of the RGS2 promoter in one human prostate specimen revealed 60.2% methylation in the prostate tumor compared to 10.1% methylation in adjacent non-cancerous prostate tissue (Fig. 3B).

Demethylation of the RGS2 promoter by 5-Aza-dC induces RGS2 expression and suppresses androgen-independent growth of prostate cancer cells

Next, we tested whether the DNA methylation inhibitor 5-Aza-dC could induce RGS2 re-expression in androgen-independent prostate cancer cells. In Fig. 4A, a dose-dependent increase in RGS2 mRNA was observed in LNCaP-C81 cells after treatment with various concentrations of 5-Aza-dC. MSP analysis indicated that 10 μ M 5-Aza-dC caused significant demethylation of the RGS2 promoter (Fig. 4B). We further tested whether the restoration of RGS2 expression by 5-Aza-dC would affect androgen-independent cell proliferation. As shown in Fig. 4C, in steroid-reduced medium (5% CSS), LNCaP-C81 cells grow rapidly, independent of the androgen DHT (lane 1 vs. 2). There was no significant difference in cell proliferation between 5-Aza-dC treated and untreated LNCaP-C81 cells cultured in CSS+DHT (lane 2 vs. 5) or FBS (lane 3 vs. 6). However, under steroid-reduced medium, proliferation of LNCaP-C81 cells pre-treated with 5-Aza-dC was 45% lower than that of untreated cells (lane 4 vs. 1).

In 5-Aza-dC treated LNCaP-C81 cells, stable expression of RGS2-targeted shRNA reduced endogenous RGS2 protein to about 25% of that in cells expressing control shRNA (Fig. 4D). RGS2 shRNA had no effect on DHT-stimulated proliferation of 5-Aza-dC treated LNCaP-C81 cells (lane 2 vs. 4, Fig. 4E). However, androgen-sensitivity of 5-Aza-dC treated LNCaP-C81 cells shown in Fig. 4E (lane 1 vs. 2) was partially reversed by RGS2 shRNA (lane 3 vs. 4).

We also examined effects of 5-Aza-dC on RGS2 expression and growth of prostate cancer CWR22Rv1 cells that possess features of clinically advanced disease such as AR-positive and androgen-independent proliferation.^{37,38} Western blot assays showed that RGS2 protein was barely detectable in CWR22Rv1 cells,¹ but 5-Aza-dC treatment markedly increased RGS2 expression (Fig. 4F, inset). In steroid-reduced medium, 5 nM DHT only modestly stimulated control CWR22Rv1 cell growth (closed circle vs. open circle) (Fig. 4F). 5-Aza-dC decreased CWR22Rv1 cell growth by 60% in the absence of DHT (open triangle vs.

open circle) without significant effect on DHT-stimulated growth (closed triangle vs. closed circle).

Furthermore, compared to untreated control cells, 10 μ M 5-Aza-dC treatment only slightly increased the percent of apoptosis of LNCaP-C81 (10.9% vs. 9.5%) and CWR22Rv1 cells (8.9% vs. 7.0%) under steroid-reduced conditions (Fig. 4G). Thus, potent inhibition of androgen-independent cell growth by 5-Aza-dC was probably not due to non-specific toxic effects.

RGS2 suppressed growth of prostate cancer cells in castrated male nude mice

We further investigated the effect of ectopic expression of RGS2 on androgen-independent prostate cancer growth *in vivo*. LNCaP-C81 cells formed tumors in castrated nude mice in preliminary studies, but tumors grew slowly, making it difficult to evaluate the inhibitory effect of RGS2. Thus, CWR22Rv1 cells were selected because they grow rapidly in castrated nude mice.³⁹ Shown in Fig. 5A, CWR22Rv1 cell tumors grew progressively in castrated nude mice inoculated with either uninfected cells or GFP-adenovirus infected cells during the observation period of 21 days. In contrast, two of six mice inoculated with RGS2-adenovirus infected cells had no gross tumors (Fig. 5B) while those that did form tumors grew more slowly and had significantly smaller tumor volumes at day 21 compared to tumors derived from GFP-infected cells ($215 \pm 87 \text{ mm}^3$ (n=4) vs. $550 \pm 45 \text{ mm}^3$ (n=6), $p < 0.01$, Fig. 5A).

DISCUSSION

In this study, we assessed RGS2 expression in human prostate tissue microarrays and performed a parallel analysis in prostate cancer cell lines, primary tumors and paired non-cancerous tissue to investigate the expression profile and methylation status of the RGS2 gene in prostate cancer. To the best of our knowledge, this is the first study that reports a correlation between the amount of RGS2 promoter methylation and its expression level. More importantly, RGS2 down-regulation due to its gene promoter methylation correlates with androgen-independent growth of prostate cancer cells, and re-expression of RGS2 reduces androgen-independent prostate cancer growth in culture and in a castrated mouse xenograft model.

We previously reported that expression of RGS2, but not other members of the RGS family, suppressed androgen-independent growth of prostate cancer cells and restored their androgen-sensitivity *in vitro*. Conversely, siRNA knockdown of RGS2 in androgen-sensitive prostate cancer cells gave them a growth advantage in androgen-reduced conditions.¹ Thus, down-regulation of RGS2 may contribute to the progression of prostate cancer to a hormone-refractory phenotype. However, the expression profiles of RGS2 protein in human prostate cancer have not been examined. Our present studies show that RGS2 protein was significantly decreased in prostate cancer specimens when compared to adjacent normal or hyperplastic tissue irrespective of the level of cancer cell differentiation or tumor architecture. Down-regulation of RGS2 was also found in acute myeloid leukemia,²³ in hot thyroid nodules,²⁴ and advanced colorectal cancer,²⁵ suggesting that RGS2 down-regulation may be involved in several types of neoplasia. However, the mechanisms underlying RGS2 down-regulation have not been addressed.

CpG-rich regions, referred to as CpG islands, are located in proximal promoter regions, and hypermethylation of CpG islands is a major epigenetic modification that suppresses gene expression in malignancies.⁴⁰ Analysis of RGS2 genomic architecture showed a dense CpG island in a region containing both transcriptional and translational start sites that extends to the first RGS2 exon. This region has a TATA box and binding sites for several putative

transcriptional factors.⁴¹ Such molecular architecture is similar to other genes known to be targets for hypermethylation-dependent silencing during prostate cancer tumorigenesis and progression.⁴² We cloned this putative RGS2 promoter and found that the 757 bp region immediately upstream of the RGS2 translation start site has significant promoter activity, which could be obliterated by *in vitro* methylation. Interestingly, a reduced level of RGS2 expression and strong methylation of this RGS2 promoter were observed in all ten human prostate cancer specimens when compared to their adjacent non-cancerous tissues, showing a positive correlation between methylation and RGS2 repression. Furthermore, methylation of the CpG island in RGS2 promoter was also strongly correlated with reduced RGS2 expression in prostate cancer cell lines. As expected, treatment of prostate cancer cells with the DNA methylation inhibitor 5-Aza-dC caused a restoration of RGS2 expression in association with a reduction in promoter methylation. Based on these results, we conclude that the RGS2 gene is a target of epigenetic silencing in human prostate cancer.

Numerous genes involved in repair of DNA damage, cell cycle regulation, and cell signaling are hypermethylated during the development and progression of prostate cancer.⁴³⁻⁴⁵ Our comparison of LNCaP-C33 and -C81 cells shows that RGS2 promoter hypermethylation can occur during prostate cancer progression. Since RGS2 suppresses GPCR-dependent transactivation of AR in prostate cancer,¹ our present studies suggest that repression of RGS2 due to promoter methylation unmasks a latent pathway for AR activation by diverse GPCRs, contributing to prostate cancer growth during androgen-ablation therapy.

Intriguingly, DNA methylation is reversible since 5-Aza-dC treatment restores RGS2 expression via demethylation of the RGS2 promoter. While 5-Aza-dC induced DNA demethylation is non-selective, cell culture data showed that suppression of androgen-independent growth of prostate cancer LNCaP-C81 cells by 5-Aza-dC was due, in part, to re-expressed RGS2. This was supported by data in another AR-positive but androgen-independent prostate cancer cell line, CWR22Rv1, showing that 5-Aza-dC induced RGS2 expression and suppressed androgen-independent cell growth. More importantly, restoration of RGS2 levels by ectopic expression in CWR22Rv1 cells attenuated growth of xenografts in castrated male nude mice. Therefore, a targeting strategy that resulted in selective demethylation of the RGS2 promoter in prostate cancer would help restore androgen-sensitivity of prostate cancer, thereby potentiating androgen-ablation therapy.

While our data show that RGS2 promoter hypermethylation can occur in prostate cancer, the mechanisms causing this hypermethylation remain to be elucidated. DNA methylation in mammalian cells is established and maintained by DNA methyltransferases (DNMTs) and DNMT1 is increased in prostate cancer.⁴⁶ DNMT-methylated DNA also recruits other proteins such as histone methyltransferases that affect chromatin remodeling and alter gene expression.⁴⁷ Recent studies showed that histone methyltransferases dynamically regulate histone methylation patterns and may have prognostic value in prostate cancer.⁴⁸ Understanding the molecular mechanisms underlying methylation and repression of RGS2 gene could provide new insights into epigenetic gene silencing in general.

Recent studies suggested that expression and methylation status of several cancer-related genes may provide clinically useful markers to diagnose early prostate cancer and to assess disease severity and prognosis^{33,49,50}, but the relevant functional implication of these changes is less certain. That may not be the case for RGS2 since loss of RGS2 is permissive for aberrant signaling through GPCRs that can lead to AR activation in the absence of androgens.¹ RGS2 protein levels were similar between adjacent normal and hyperplastic tissue, but were significantly decreased in prostate cancer. RGS2 gene methylation was also less prevalent in non-cancerous prostate tissues than in paired prostate cancer samples. Thus, methylation and down-regulation of the RGS2 gene are presumably events that occurred at

an early stage of prostate cancer, and may be novel markers to diagnose early prostate cancer. Interestingly, detailed inspection of ten archived prostate cancer cases revealed that lower RGS2 staining intensity with more heterogeneous staining in prostate cancer tissues. This heterogeneous staining could be a manifestation of both tumors and individual cancer cells within tumors having different degrees of androgen independence. This raises the possibility that RGS2 staining could provide valuable prognostic information. Currently, a larger study of prostate tissue specimens with full patient histories is in progress.

The observation that RGS2 was down-regulated in most prostate cancer tissues suggests additional roles of RGS2 *in vivo*. Recently, we found that the wet weight of the prostate gland of RGS2 KO mice is approximately 50% more than WT littermates (Xie, unpublished), suggesting that RGS2 plays a role in regulation of prostate gland development. Currently, we are performing detailed morphological and histological analysis of prostate glands from RGS2 KO mice to determine if increased gland weight is a consequence of localized dysplasia. These studies are important for elucidation of physiological functions of RGS2.

In summary, our data indicate that the RGS2 gene is a target for promoter hypermethylation-dependent silencing in human prostate cancer. Given the important role of RGS2 in suppressing androgen-independent AR activation, this aberrant epigenetic event might be of great importance in assessing the degree to which prostate cancer cells are poised for castration-resistant growth and proliferation irrespective of their stages or Gleason score. Hypermethylation of the RGS2 promoter is readily detected by methylation specific PCR, raising the possibility of diagnostic testing. More importantly, epigenetic silencing is reversible, raising the possibility of a treatment that helps restore the androgen-sensitivity of otherwise medically incurable hormone-refractory prostate cancer.

Novelty

This is the first study showing that the RGS2 gene is a target for promoter hypermethylation-dependent silencing in human prostate cancer. Restoration of RGS2 reduces androgen-independent prostate cancer growth *in vitro* and *in vivo*.

Impact

Methylation and down-regulation of the RGS2 gene may be novel markers to diagnose early prostate cancer. Targeting this reversible process may provide a new strategy for suppressing prostate cancer progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GPCR	G-protein coupled receptor
RGS2	regulator of G-protein signaling 2

5-Aza-dC	5-aza-2'-deoxycytidine
AR	androgen receptor
FBS	fetal bovine serum
CSS	charcoal-stripped serum
DHT	dihydrotestosterone
shRNA	short hairpin RNA
MSP	Methylation specific PCR
DNMTs	DNA methyltransferases

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Figure 1. Reduced RGS2 protein expression in human prostate tumors
 (A) Western blot analysis of RGS2 protein in prostate tissue lysate of WT and RGS2 KO mice using a polyclonal anti-RGS2 C-terminal peptide antibody. Representative immunostaining of RGS2 protein in human prostate cancer specimen (B) or human prostate tissue microarrays (C) using this anti-RGS2 antibody.

Figure 2. The RGS2 gene promoter is hypermethylated in prostate cancer specimens
 (A) Schematic of human RGS2 gene promoter-associated CpG island (gray bar) identified by CpG Island Searcher (<http://cpgislands.usc.edu>). DNA sequence is numbered from the translational start site (+1). Gray and black arrows show locations of primers used for MSP and cloning of the RGS2 promoter, respectively. (B) Inhibition of RGS2 gene promoter activity by *in vitro* DNA methylation. RGS2 and L-RGS2 are pGL3 vectors containing the short fragment and long fragment of the proximal RGS2 promoter, respectively. Methyl-RGS2 represents the RGS2 plasmid methylated *in vitro*. Relative luciferase activities of each construct are fold increase compared to pGL3 vector. Columns, means; bar, SE (n=4); *p < 0.01 compared to RGS2 plasmid. (C) MSP analysis of RGS2 promoter methylation in ten

achieved human prostate tumors (T) and adjacent non-cancerous tissues (N) using primers for amplification of methylated (M) and unmethylated (U) CpG dinucleotides. **(D)** Left: The percentage of methylation for each sample was calculated by dividing the intensity of the methylated band by the sum of the methylated and unmethylated band intensities using ImagePro Plus. Right: RGS2 protein expression levels, quantified by H-score analysis of immunohistochemical staining shown in Supplemental Table 2. For each group, the longer horizontal lines are means with standard error bars (n=10). Statistical differences denoted by $p < 0.05$ or $p < 0.001$.

Figure 3. Hypermethylation of the RGS2 promoter correlates with RGS2 down-regulation in prostate cancer cells

(A) MSP analysis of methylated (M) and unmethylated (U) CpG dinucleotides of the RGS2 promoter in PrEC, LNCaP-C33 (C33) and LNCaP-C81 (C81) cells. Besides being used to amplify unmodified DNA, wild-type primers (W) were also used to verify that bisulfite modification was complete. (B) Bisulfite sequencing analysis of RGS2 promoter methylation in prostate cell lines, human prostate tumor specimen 318-T and matched non-cancerous tissue 318-N. Filled pie-shaped regions indicate the fraction of sequenced clones exhibiting methylation at each CpG dinucleotide, with the percentage of average methylation of CpG sites across this region shown on the right. (C) RGS2 mRNA levels in prostate cell lines, expressed as copies per 10^3 β -actin. Columns, means; bar, SE (n=3).

Figure 4. Effect of 5-Aza-dC treatment on expression and methylation of the RGS2 gene and proliferation of androgen-independent prostate cancer cells

LNCaP-C81 cells were treated with 5-Aza-dC for ten days and then subjected to RT-PCR analysis of RGS2 expression with LNCaP-C33 as a positive control (**A**) or MSP analysis of RGS2 promoter methylation (**B**). (**C**) 5-Aza-dC treatment suppresses androgen-independent growth of LNCaP-C81 cells, measured by CellTiter 96 AQueous Cell Proliferation Assay. The absorbance at 490 nm is directly proportional to the number of living cells. Absorbance before 48 h culture in 5% FBS or 5% CSS with or without 5 nM DHT was subtracted from the data. Data are means $\pm$ S.E. of triplicates, repeated at least four times. * $p < 0.01$ compared with control cells cultured in 5% CSS. (**D** and **E**) 10 μ M 5-Aza-dC-treated LNCaP-C81 cells stably expressing RGS2 shRNA or control shRNA were subjected to Western blot analysis (**D**) or *in vitro* cell growth assay (**E**) for 48 h in 5% CSS with or without 5 nM DHT. Results represent an average of four independent experiments

performed in triplicate. * $p < 0.01$ compared with cells expressing control shRNA in 5% CSS. (F) CWR22Rv1 cells treated without (control) or with 10 μM 5-Aza-dC were subjected to Western blot analysis (Inset) or *in vitro* cell growth assay in 5% CSS with or without 5 nM DHT. (G) Effects of 5-Aza-dC treatment on prostate cancer cell apoptosis evaluated by flow cytometric analysis of annexin V and PI double staining. Average percentage of apoptotic cells (early-apoptotic, annexin V positive, PI negative; late-apoptotic, annexin V, PI double positive) of three independent experiments is shown.

Figure 5. RGS2-adenovirus suppressed tumorigenicity of prostate cancer xenografts in castrated male nude mice
CWR22Rv1 cells were uninfected or infected with GFP- or RGS2-adenovirus and then injected into the dorsal surface of 6-week-old castrated male nude mice (six mice per group). (A) Tumor sizes were measured twice a week and expressed in mm³ using the ellipsoid formula. (B) Tumor incidence in each group (lower left panel). One representative mouse in each group was photographed and the tumor indicated by the blue circle.

Table 1
RGS2 expression by immunohistochemistry staining in prostate cancer specimens

Prostate Specimens	n	Staining Intensity				Average $\pm$ SE
		0	1	2	3	
Adjacent Normal	46	4	15	13	14	1.9 $\pm$ 0.1
Hyperplasia	37	2	8	11	16	2.1 $\pm$ 0.2
Cancer	101	32	48	20	1	0.9 $\pm$ 0.1 ^{*#}
Gleason Score						
≤ 6	33	11	14	7	1	0.9 $\pm$ 0.1
7	30	10	14	6	0	0.9 $\pm$ 0.1
8-10	38	11	20	7	0	0.9 $\pm$ 0.1
Stage						
I	6	2	3	1	0	0.8 $\pm$ 0.3
II	57	21	28	8	0	0.7 $\pm$ 0.1
III	14	3	8	3	0	1.0 $\pm$ 0.2
IV	24	4	12	8	0	1.2 $\pm$ 0.1

Statistical significance was determined using Kruskal-Wallis and Dunns post-tests.

* p<0.001 for cancer vs. adjacent normal.

p<0.001 for cancer vs. hyperplasia.