Published in final edited form as:
Prostate. 2012 March ; 72(4): 399–409. doi:10.1002/pros.21442.

GPRC6A regulates prostate cancer progression

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Abstract

BACKGROUND—GPRC6A is a nutrient sensing GPCR that is activated *in vitro* by a variety of ligands, including amino acids, calcium, zinc, osteocalcin (OC) and testosterone. The association between nutritional factors and risk of prostate cancer, the finding of increased expression of OC in prostate cancer cells and the association between GPRC6A and risk of prostate cancer in Japanese men implicates a role of GPRC6A in prostate cancer.

METHODS—We examined if GPRC6A is expressed in human prostate cancer cell lines and used siRNA-mediated knockdown GPRC6A expression in prostate cancer cells to explore the function of GPRC6A *in vitro*. To assess the role GPRC6A in prostate cancer progression *in vivo* we intercrossed *Gprc6a*$^{-/-}$ mice onto the TRAMP mouse prostate cancer model.

RESULTS—*GPRC6A* transcripts were markedly increased in prostate cancer cell lines 22Rv1, PC-3 and LNCaP, compared to the normal prostate RWPE-1 cell line. In addition, a panel of GPRC6A ligands, including calcium, OC, and arginine, exhibited in prostate cancer cell lines a dose-dependent stimulation of ERK activity, cell proliferation, chemotaxis, and prostate specific antigen and *Runx 2* gene expression. These responses were inhibited by siRNA-mediated knockdown of GPRC6A. Finally, transfer of *Gprc6a* deficiency onto a TRAMP mouse model of prostate cancer significantly retarded prostate cancer progression and improved survival of compound *Gprc6a*$^{-/-}$/TRAMP mice.

CONCLUSIONS—GPRC6A is a novel molecular target for regulating prostate growth and cancer progression. Increments in GPRC6A may augment the ability of prostate cancer cells to proliferate in response to dietary and bone derived ligands.

Keywords

GPRC6A; GPCR; calcium; osteocalcin; siRNA; prostate cancer; cell proliferation; metastases

Introduction

Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of death from cancer in North American and European males (1). New therapeutic approaches are needed to prevent and treat advanced and metastatic prostate cancer. Nutritional factors, particularly high intake of protein and calcium, as well as metabolic syndrome, are known to modify prostate cancer risk and progression (2-6), but the molecular mechanisms linking nutrition to prostate cancer are unknown. There are also links between prostate cancedr and bone metabolism. Osteocalcin (OC), which encodes a vitamin-K dependent hormone predominantly produced by osteoblasts/osteocytes in bone, which

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functions to regulate energy metabolism, is also ectopically expressed by some prostate cancers that have a propensity to metastasize to bone. Polymorphisms in OC are also associated with prostate cancer progression (7-8). Recent evidence has also identified a correlation between the bone transcription factor Runx2 and advanced stages of prostate and breast cancer, as evidenced by the effects of depletion of *Runx2* by RNA interference to inhibit migration and invasive properties of the cells and prevent metastatic bone disease (9). It is possible that OC secreted by bone may directly target prostate cancer cells. Finally, androgen deprivation therapy is the principal medical therapy for prostate cancer (10), but androgen ablation often becomes ineffective in controlling prostate cancer progression and castration-resistant metastatic disease, particularly to bone, becomes incurable. There is growing evidence for the presence of a putative membrane androgen sensing receptor that mediates the rapid, non-genomic effects of androgens, which also might be involved in prostate cancer growth and metastasis (11). Regardless, clues to possible new molecular targets to regulate prostate cancer growth and progression may be discovered from a better understanding of the molecular mechanisms underlying nutritional risk factors, OC effects and androgen resistance in prostate cancer (12).

GPRC6A, a recently discovered member of family C G protein-coupled receptors (13-14), may provide a molecular mechanism to explain the link between prostate cancer progression and nutrition, OC responsiveness of prostate cancer cells, and continued androgen responsiveness in prostate cancer cells following inhibition of nuclear androgen receptor signaling (15). Recent work has shown that GPRC6A is capable of sensing extracellular calcium as well as amino acids and OC and to regulate a wide range of metabolic processes, suggesting that the physiological function of this G-protein coupled receptor is to link nutrient and OC signals to the regulation of energy metabolism (14, 16-17). In addition, ablation of this orphan G-protein coupled receptor leads to undermasculinization associated with decreased muscle mass, increased adiposity, and low circulating testosterone and elevated estradiol levels in male mice, suggesting that GPRC6A may also modulate sex steroid end organ responses (18). GPRC6A also mediates the non-genomic effects of testosterone *in vitro* and *in vivo* (19). Finally, GPRC6A is one of five novel genetic loci associated with prostate cancer in the Japanese population (20). Thus, GPRC6A is a candidate for the putative membrane androgen sensing receptor involved in prostate cancer progression as well as a nutrient and OC receptor regulation prostate cancer growth and progression.

In the current investigations, we examined the expression and function of GPRC6A in prostate cancer progression both *in vitro* and *in vivo* using a combination of molecular and mouse genetic approaches. We found that *GPRC6A* is expressed in normal prostate and is increased in cancer-derived prostate cell lines. Bothe BSA-coupled testosterone and OC stimulates ERK phosphorylation in HEK-293 cells transfected with GPRC6A and in 22RV1 and PC-3 human prostate cancer cell expressing endogenous GPRC6A. Moreover, RNAi-mediated knockdown of *GPRC6A* in prostate cancer cells inhibits ligand-stimulated proliferation and chemotaxis *in vitro*. Finally, deletion of *Gprc6a* in the TRAMP mouse model of prostate cancer significantly retarded prostate cancer progression and improved survival. Based on these findings we propose that GPRC6A may mediate the prostate response to nutrients, OC and non-genomic effects of androgens and that targeting this receptor with an antagonist may provide a complementary strategy to treat androgen resistant prostate cancer.

Materials and Methods

Reagents and Cell Culture

Testosterone 3-(*O*-carboxymethyl) oxime-BSA, L-Arginine, calcium chloride, and zinc chloride were obtained from Sigma-Aldrich. In the case of testosterone-BSA, the freshly purified testosterone was used in each experiment. Before each experiment, stock solutions of BSA conjugates were mixed with dextran (0.05 mg/ml) and charcoal (50 mg/ml) for 30 min, centrifuged at 3000 X *g* for 10 min, and passed through a 0.22-mm pore size filter to remove any potential contamination with free testosterone. Methytrienolone (R1881) was purchased from Perkin-Elmer. Osteocalcin (purified from bovine bone) was purchased from Biodesign International. Total human RNAs were obtained from Clontech.

The prostate cancer cell lines 22Rv1, PC-3 and LNcaP and the prostate cell lined RWPE-1 derived from normal prostate were obtained from the American Type Culture Collection (Manassas, VA). The prostate cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco Life Technologies, Inc.). Cells (10^3 cells/well) were cultured in triplicate in a 96-well flat-bottomed microculture dish using RPMI 1640 containing 10% CFBS in the presence and absence of various concentrations of GPRC6A ligands, including: calcium, amino acids, calcimimetic and OC for 72 h. Cell proliferation was determined by counting cells with a hemocytometer (21).

RT-PCR and Real-Time RT-PCR

Human tissue cDNAs were obtained from Clontech Laboratories, Inc. Total RNA from human prostate cancer cells was isolated with RNeasy Mini Kit (Qiagen Inc., Valencia, CA). RT-PCR was done using two-step RNA PCR (Perkin-Elmer). In separate reactions, 2.0 μg of DNase-treated total RNA was reverse-transcribed into cDNA with the respective reverse primers specified below and Moloney murine leukemia virus reverse transcriptase (Gibco Life Technologies, Inc.). Reactions were carried out at 42 °C for 60 min followed by 94 °C for 5 min and 5 °C for 5 min. The products of first-strand cDNA synthesis were directly amplified by PCR using AmpliTaq DNA polymerase (Perkin-Elmer). The primers for human GPRC6A are as follows: hGPRC6A.F203: caggagtgtgttggctttga and hGPRC6A.R630: atcaggtgagccattgcttt. For housekeeping gene control *G3PDH* gene, G3PDH.F143: gacccttcattgacctcaactaca and G3PDH.R1050: ggtcttactccttggaggccatgt. For quantitative real time RT-PCR assessment of *PSA and Runx 2II* genes expression we isolated and reverse transcribed total RNA isolated from 22Rv1 cells that stimulated with or without OC (80 ng/ml), arginine (30 mM) and R1881 (100 nM) as previously described using specific primer sets. The primers sequences were PSA.For: ttggaaatgaccaggccaag and PSA.Rev:agcaaccctggacctcacac; Runx 2.For: attcctgtagatccgagcacc and Runx 2.Rev: gctcacgtcgctcattttgc, and the threshold cycle (Ct) of tested-gene product from the indicated genotype was normalized to the Ct for cyclophilin A as we have previously described (18).

Measurement of Total and Phospho-ERK by Western-Blot Analysis

Briefly, human prostate cancer cells and the cells transfected with GPRC6A will be made quiescent by overnight incubation in serum-free DMEM/F12 containing 0.1% BSA and stimulated with various ligands at different doses. ERK activation will be assessed 5 to 30 minutes after treatment by immunoblotting using anti-phospho-ERK1/2 MAP kinase antibody (Cell Signaling Technology) corrected for the amount of ERK using an anti-ERK1/2 MAP Kinase antibody (Cell Signaling Technology) to measure ERK levels.

siRNA Suppression of GPRC6A Gene Expression—For *GPRC6A* knockdown experiments, two short interfering RNAs (siRNAs) (19 nucleotides each) have been designed from the hGPRC6A sequence (NM_148963). These are GPRC6A siRNA-202:

CCAGGAGTGTGTTGGCTTT and siRNA-514: GCCACAGGTGGGTTATGAA. Two siRNA hairpins were synthesized and cloned into a pSilencer™ 4.1-CMV neo vector (Ambion). A circular pSilencer™ 4.1-CMV neo vector that expresses a hairpin siRNA with limited homology to any known sequence was used as a negative control. The constructs of siRNA duplexes have been stably transfected into human prostate cancer cells using Lipofectamine™ (Invitrogen) and were selected by G418 (Invitrogen). Successful knock down of GPRC6A will be confirmed by assessing RT-PCR analysis of *GPRC6A* expression.

Chemotaxis Assay—The migration of 22Rv1 cells stably expressing negative control siRNA and human GPRC6A-specific siRNA using a previously described chemotaxis assay (22). The migration index was calculated, and was defined as number of cells crossing the filter toward calcium or OC (various concentrations)/number of cells migrating toward medium alone (control). Each experiment was performed at least three times, in duplicate.

Mouse models

Mice were maintained and used in accordance with recommendations as described (National Research Council. 1985; Guide for the Care and Use of Laboratory Animals DHHS Publication NIH 86-23, Institute on Laboratory Animal Resources, Rockville, MD) and guidelines established by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. The *Gprc6a*-deficient mouse model was created by replacing exon 2 of the *Gprc6a* gene with the hygromycin resistance gene (18). TRAMP transgenic mouse was purchased from The Jackson Laboratory (Stock #: 003135). Transfer of *Gprc6a* deficiency onto the TRAMP background, we first crossed male *Gprc6a*$^{-/-}$ *mice* with female TRAMP to generate *Gprc6a*$^{+/-}$/TRAMP mice. Then, we crossed male *Gprc6a*$^{+/-}$ mice with female *Gprc6a*$^{+/-}$/TRAMP mice. For genotyping Gprc6a deficiency mice, the PCR primers are Athx-1: gaataactagcaggaggggcgctggaaggag and Athx-2: cagagtggcagccattgctgctgtgacttcg (wild type pair); Athx-F: cacgagagatcgtggggtatcgacagag and Athx-R: ctacatggcgtgatttcatatgcgcgattgctg (knockout pair). For genotyping TRAMP transgenic mice, the PCR primers are oIMR0015: caaatgttgcttgtctggtg and oIMR0016: gtcagtcgagtgcacagttt (wild type pair); oIMR0068: cagagcagaattgtggagtgg and oIMR0069: ggacaaaccacaactagaatgcagtg (Transgene pair).

Statistics

We evaluated differences between groups by one-way analysis of variance. All values are expressed as means ± SEM. All computations were performed using the Statgraphic statistical graphics system (STSC Inc.).

Results

Detection of *GPRC6A* mRNA in Human Prostate Cancer Cell Line and Prostate Cancer Tissues

We performed RT-PCR with intron-spanning primers specific for human *GPRC6A* in human prostate tissue (Fig. 1A) and human prostate cell lines RWPE-1, 22Rv1, PC-3 and LNCaP (Fig. 1B). We amplified a product of the predicted size, 428 bp in all prostate cells and prostate tissue. We failed to amply GPRC6A from intestine, which was used as a negative control (13). The gene products from 22Rv1, PC-3 and LNCaP were identified as human *GPRC6A* by DNA sequence analysis. The level of *GPRC6A* expression was higher in prostate cancer cell lines, 22Rv1, PC-3 and LNCaP than in normal human prostate cell line RWPE-1 (Fig. 1B). To explore *GPRC6A* expression in prostate cancer tissues, we queried the Gene Expression Omnibus (GEO) database using the search terms GPRC6A and prostate (http://www.ncbi.nlm.nih.gov/sites/entrez?db=geo). We found that *GPRC6A* is up-regulated in primary prostate cancer (GEO accession GDS1439) (23). *GPRC6A* is also

highly expressed in other cancers, including higher proliferation index $CD133^+$ glioblastomas (GEO accession GDS2728) (24), and human myeloid leukemia cell lines (GEO accession GDS2251) (25). Together these data suggest a potential role of GPRC6A in malignant transformation of the prostate cancer and other cancers.

Effects of Calcium, OC, and Arginine on ERK Activity, Cell Number and Gene Expression in Human Prostate Cancer Cell Lines

Next, we confirmed that calcium (1-20 mM), zinc (0.2-0.8 mM), OC (5-60 ng/ml), arginine (5-30 mM), and testosterone-BSA (5-100 nM) are functional ligands for GPRC6A (16-17, 19). We found that calcium, zinc, OC, arginine, and testosterone resulted in a dose-dependent stimulation of ERK activity in HEK-293 cells overexpressing GPRC6A (Fig 2A), whereas non-transfected HEK-293 cells failed to respond to any of these ligands. We also examined the effects of these GPRC6A ligands on ERK activation in androgen receptor positive 22Rv1 and androgen receptor negative PC-3 cells (Fig. 2B). We found that calcium (10 mM), zinc (0.2 mM), OC (20 ng/ml), arginine (20 mM), and testosterone-BSA (60 nM) increased phospho-ERK in both of these cells. To determine if GPRC6A ligands enhance prostate cell growth, we assessed changes in cell numbers in 22Rv1 and PC-3 cells exposed to different concentrations of extracellular calcium or OC over a three-day culture period. Control 22Rv1 and PC-3 cells typically increased their cell number ~5-fold, respectively, in the absence of added ligands over this time period. The addition of calcium to the medium modestly increased the number of cells by ~30% (Fig. 3A), whereas OC resulted in a ~70% increase in cell number (Fig 3B). Finally, we examined if ligands of GPRC6A stimulated the expression of *PSA* and *Runx2* in 22Rv1 and PC-3 cells. After 8 hours stimulation by OC, arginine and R1881, we found that *PSA* expression was significantly increased (Fig. 4A and C). *Runx2* expression was also significantly increased in 22Rv1 and PC-3 cells in response to GPRC6A stimulation (Fig. 4B and D).

Human Prostate Cancer Cell Lines 22Rv1 and PC-3 Respond to Extracellular Calcium, OC and Arginine Through GPRC6A

To confirm the importance of GPRC6A signaling in prostate cancer cell line, we assess the response to GPRC6A ligands after knock-down of GPRC6A using siRNA. For these studies, we assessed ERK activity in 22Rv1 and PC-3 cells stably transfected with *GPRC6A* siRNA-202 and siRNA-514. These responses were compared control groups consisting of mock-transfected cells and transfected cells with a random negative control siRNA plasmid. We observed decreased levels of mRNA expression of *GPRC6A* in 22Rv1 or PC-3 cells transfected with interfering RNAs, *GPRC6A* siRNA-202 and siRNA-514, compared to controls (Fig. 5A). OC and testosterone stimulated phospho-ERK activity was significantly decreased in both 22Rv1 and PC-3 human prostate cancer cell transfected with *GPRC6A* siRNA-202 and siRNA-514 (Fig. 5B). In addition, in the current studies we found that extracellular calcium, testosterone, arginine and OC failed to stimulate ERK phosphorylation in 22Rv1 cells transfected with *GPRC6A* siRNA-202 (Fig. 5C).

GPRC6A Regulation of PSA and Runx2

To investigate whether the observed ligand-induced *PSA* and *Runx2* gene expression in the prostate cancer cells is due to simulation of GPRC6A, we examine the response to GPRC6A ligands after knock-down of GPRC6A. We assessed ERK activity in 22Rv1 stably transfected with *GPRC6A* siRNA-514 and compared this response to transfected cells with a random negative control siRNA plasmid, which were used as controls. We observed significantly decreased levels of mRNA expression of *PSA* and *Runx2* (Fig. 6A and B). These results suggest that endogenous *GPRC6A* accounts for the effects of calcium, OC and arginine to stimulate gene expression of *PSA* and *Runx2* in human prostate cancer cell lines.

GPRC6A-Mediated Human Prostate Cancer 22Rv1 Calcium- and OC-Induced Cell Chemotaxis

To assess whether GPRC6A mediates prostate cancer cells chemotaxis and metastases, we examined the ability of calcium and OC to evoke chemotaxis of human prostate cancer cell 22Rv1 expressing *GPRC6A*. As shown in Fig. 6C, calcium and OC induced the chemotaxis of 22Rv1 the prostate cancer cells (Fig. 6C). siRNA mediated inhibition of *GPRC6A* expression eliminated the ability of calcium and OC to evoke chemotaxis of the prostate cancer cells (Fig. 6C).

Effects of superimposed *Gprc6a* deficiency in the TRAMP mouse prostate cancer model

Finally, to examine the effects of GPRC6A on regulation of prostate cancer cell function *in vivo*, we intercrossed *Gprc6a*$^{-/-}$ mice onto the TRAMP mouse model of prostate cancer. Consistent with prior reports (26), 30 week-old TRAMP mice had evidence of intraepithelial hyperplasia in the ventral prostate (Fig. 7A, middle panel)), whereas, *Gprc6a*$^{-/-}$ mice had smaller but normal appearing prostate histology (Fig. 7A, left panel). Loss of *Gprc6a* in TRAMP mice resulted in markedly reduced intraepithelial hyperplasia (Fig. 7A, right middle and lower panels). In addition, a greater percentage of combined *Gprc6a*$^{-/-}$/TRAMP mice survived for 40 weeks compared to TRAMP mice with intact *Gprc6a* (75 vs 52%, respectively) (Fig. 7B).

Discussion

Links between environmental factors and prostate cancer risk and progress have been described, but the molecular targets mediating these effects are not known. The main objective of our study was to determine if GPRC6A, a recently characterized nutrient, OC and androgen-sensing GPCR, plays a role in the pathogenesis of prostate cancer. Consistent with an important role of GPRC6A in prostate cancer growth and progression, we found that *GPRC6A*, which is expressed in normal prostate tissue and cells at low levels, is markedly elevated in prostate cancer tissue and cells. *GPRC6A* was over-expressed in human prostate cancer cell lines, 22Rv1, LNCap and PC-3 cells, as well as in human prostate cancer tissues. We also demonstrated that the functional responses of prostate cancer cells to extracellular cations, OC and amino acids are mediated by GPRC6A, as evidenced by the ability of a wide range of agonists, including extracellular calcium, zinc, OC, testosterone and arginine, to stimulate GPRC6A-mediated ERK activation, cell proliferation, chemotaxis and gene expression in prostate cancer cells 22Rv1 and PC-3. In addition, we established that the responses to GPRC6A agonists in prostate cancer cells in vitro were blocked by transfection with siRNAs against *GPRC6A*. Finally, the *in vivo* relevance of GPRC6A-signaling in prostate cancer was demonstrated by the finding that ablation of *Gprc6a* in TRAMP mice improved survival and decreased prostate cell hyperplasia. Together these data suggest a potential role of GPRC6A in malignant transformation of the prostate.

GPRC6A represents a growing number of GPCRs that are upregulated in primary and metastatic cancers (27), where they promote tumor formation and cancer progression (28). Indeed, our data suggests that GPRC6A may provide a molecular mechanism to explain the associations between nutritional factors and prostate cancer risks (29-30). Activation of GPRC6A may also provide another mechanism to explain the effects of arginine deprivation therapy to affect cancer sensitivity (31-32). Another receptor closely related to GPRC6A, CASR, is also capable of sensing both amino acids and calcium, but not osteoclacin (17), and is associated with prostate cancer progression. In this regard, CASR is expressed in human-derived prostate cancer cell lines (33), its expression is associated with metastatic prostate cancer (34), extracellular calcium stimulates proliferation and PTHrp secretion in prostate cancer cell lines (35). However, calcium-mediated stimulation of PTHrp release

from prostate cancer cells was only partially inhibited by a dominant negative CASR construct, suggesting the possible presence of other mechanisms linking calcium and amino acids to prostate cancer growth.

GPRC6A's effect on prostate cell proliferation and migration may represent an accentuation in malignant cells of the physiological role of GPRC6A to integrate the response to nutrients and anabolic steroids with energy metabolism and responses of multiple tissues (18-19). GPRC6A potentially has both direct and indirect effects on prostate cancer. GPRC6A is a potent activator of ERK signaling (18) and is a possible downstream signaling pathway whereby this receptor directly regulate prostate cancer growth. Activation of ERK has a central role in prostate cancer cell proliferation (36-38). Indeed, i*n vitro* studies demonstrate that the growth-factor-induced proliferation of PC-3 cells requires ERK phosphorylation (36, 39) and treatment of PC-3 cells with PD98059, a chemical inhibitor of the ERK pathway, obliterates growth-factor-mediated cell proliferation. GPCR-mediated proliferation may be particularly relevant in androgen-independent prostate cancer, since ERK phosphorylation is noted during carbachol treatment of androgen-independent PC-3 and DU145 cells but not in androgen-dependent LNCaP cells (40), GPRC6A might also have indirect effects to regulate prostate cancer through its effects on sex steroid metabolism. In this regard, we have recently found that ablation of this orphan G-protein coupled receptor leads to undermasculinization associated with decreased muscle mass, increased adiposity, and low circulating testosterone and elevated estradiol levels in male mice, suggesting that GPRC6A may also modulate sex steroid end organ responses (18).

There are several implications of our findings. First, the increased expression of GPRC6A in prostate cancer supports the genome wide associative studies linking the GPRC6A locus to prostate cancer in Japanese males and may identify a new biological marker associated with worse outcomes. Further studies are needed to define how usefully GPRC6A might be as a marker for prostate cancer by establishing the relationship between GPRC6A expression, tumor grade and outcomes. Second, our finding that GPRC6A modulates prostate cancer progression raises the possibility that that disruption of GPRC6A will have a positive benefit to halt prostate cancer progression. If so, development of antagonists to GPRC6A could potentially lead to alternative strategies to treat prostate cancer. Moreover, GPRC6A may be a novel target that can be used for the selective elimination of possibly more aggressive prostate cancer cells independently of the functional status of the intracellular androgen receptor. Third, the ability of GPRC6A to mediate the non-genomic effects of testosterone (19) raises interesting questions about the inter-relationship between GPRC6A and nuclear androgen receptors in prostate cancers resistant to inhibition of AR. While current data support continued AR expression and function in castrate-resistant prostate cancer tumors (41), our data raise the possibility that GPRC6A might mediate some of the effects of androgens on prostate progression. Activation of GPRC6A stimulates PSA, which is also an AR target, and increases Rimx2, which is known to participate in epithelial-mesenchymal transition and prostate cancer metastasis (42). Additional studies will be needed to determine if GPRC6A represents the putative GPRC mediating the rapid response to androgens in prostate cancer cells and its relationship to the potential targeting of the nuclear AR to the plasma membrane (43).

Our results also provide a potential explanation for the propensity of prostate cancer to metastasize to bone via the effects of GPRC6A to sense OC, which in turn promotes the ability of prostate cancer cells to colonize, grow, and survive in the bone microenvironment (8, 44-45) Indeed, calcium and OC stimulation of chemotaxis in 22Rv1 human prostate cells *in vitro* and the fact that knockdown of the *GPRC6A* by siRNA inhibited extracellular calcium and OC-induced migration suggests that GPRC6A may function as a calcium and/or

OC-sensor that mediates prostate cancer cell migration toward the calcium and OC-rich bone microenvironment.

In conclusion, our data suggests that the integrative physiological function of GPRC6A to coordinate nutrient, OC and anabolic steroids actions on a variety of tissues is exploited in prostate cancer cells to regulate prostate cancer progression in vitro and in vivo. Increased expression of GPRC6A in prostate cancer cells may increase the susceptibility to develop prostate cancer and stimulate its progression by mediating the cell proliferation and migration to bone in response responses to a wide variety of ligands, including calcium, OC, and sex steroids. In addition, developing drugs to antagonize GPRC6A may provide novel strategies to prevent diagnose and treat prostate cancer. Regardless, the increased expression of GPRC6A in prostate cancer could potential be a diagnostic marker, a prognostics indicator and a potential therapeutic target. Thus, GPRC6A represents a new target in prostate cancer research. Further studies are needed to establish the role of GPRC6A in pathogenesis and treatment of human prostate cancer.

Acknowledgments

This work was supported by funds from NIH R01-AR37308 (to L. D. Q.).

Grant sponsor: NIH R01-AR37308.

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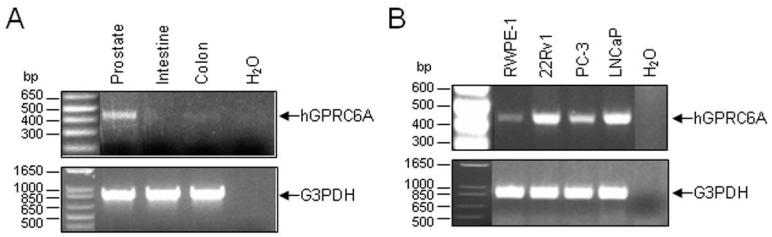

Figure 1. GPRC6A over-expression of human prostate cancer cell lines and human prostate cancer tissue

A). GPRC6A expression in normal human prostate gland. PCR products were amplified from normal human multiple tissue cDNAs: prostate, intestine and colon using human specific intron-spanning primers. B). GPRC6A over-expression of human prostate cancer cell lines by RT-PCR. RWPE-1 is human prostate epithelial cell. 22Rv1, PC-3 and LNCaP are human prostate cancer cell lines. The primers for GPRC6A application are described in Methods. We used the house-keeping control gene glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) for a positive control of RNA integrity.

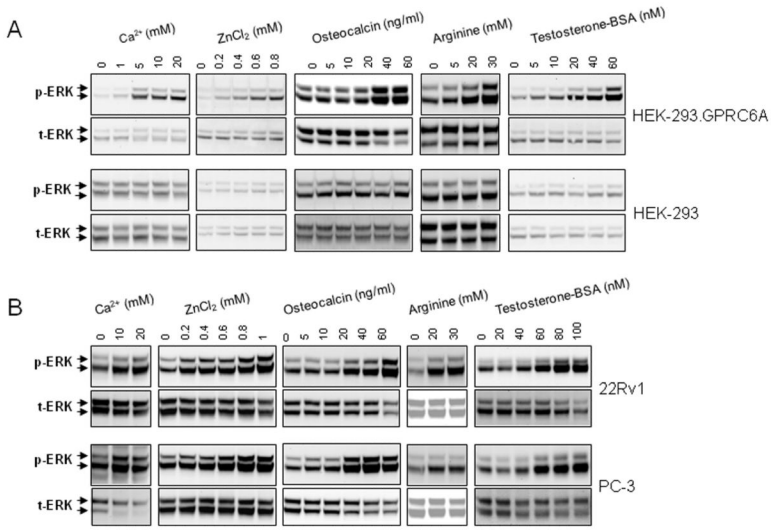

Figure 2. The ligands of GPRC6A stimulated ERK activation in human prostate cancer cell lines

A). Dose-dependent effects of extracellular calcium, zinc, OC, arginine, and testosterone-BSA on GPRC6A-mediated EKR activation in HEK cells transfected with GPRC6A. B). Dose-dependent effects of extracellular calcium, zinc, OC, arginine and testosterone on EKR activation in human prostate cancer cell lines 22Rv1 and PC-3. The HEK cells transfected with GRPC6A or without the plasmid cDNA of GPRC6A or 22Rv1 or PC-3 were incubated in Dulbecco's modified Eagle's medium /F-12 containing 0.1% bovine serum albumin quiescence media and exposed to the extracellular calcium, OC, arginine, or testosterone-BSA at indicated concentrations for 5 min, and ERK activation was determined as described under **Materials and methods**. Representative blots are shown, and the results were verified in at least three independent experiments.

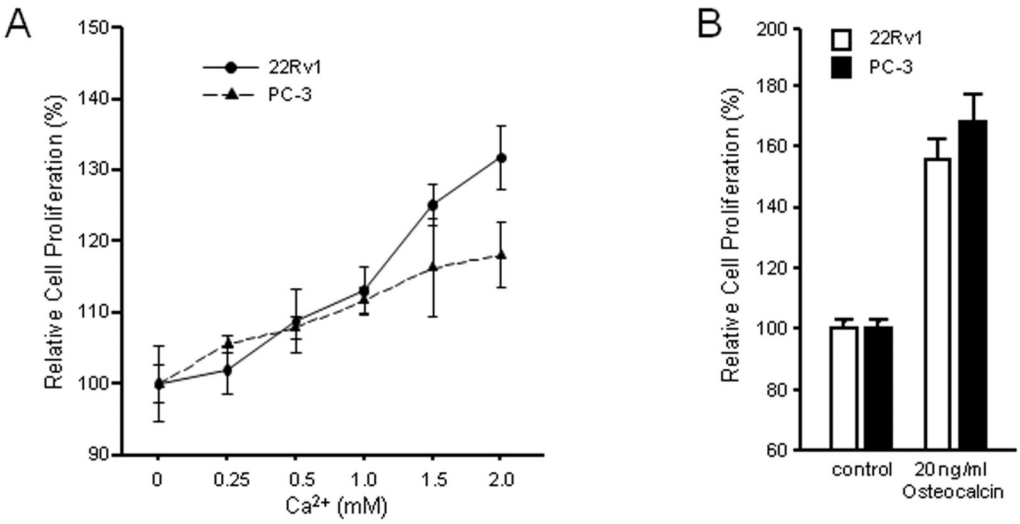

Figure 3. The ligands of GPRC6A stimulated human prostate cancer cell proliferation and gene expression

Human prostate cancer cells, 22Rv1 or PC-3 (10^3 cells/well) grown under subconfluent conditions were cultured in triplicate in a 96-well flat-bottomed microculture dish using RPMI 1640 containing 10% CFBS with various concentrations of GPRC6A ligands: calcium (A) and OC (B) for 72 h. Cell proliferation was determined by counting cells in a hemocytometer method as described in **Materials and methods**. In all of the above studies, values for relative cell proliferation (expressed as percent of control) represent the mean ± SEM of a minimum of three separate experiments. * indicates a significant difference from control and stimulation at $p<0.05$, respectively.

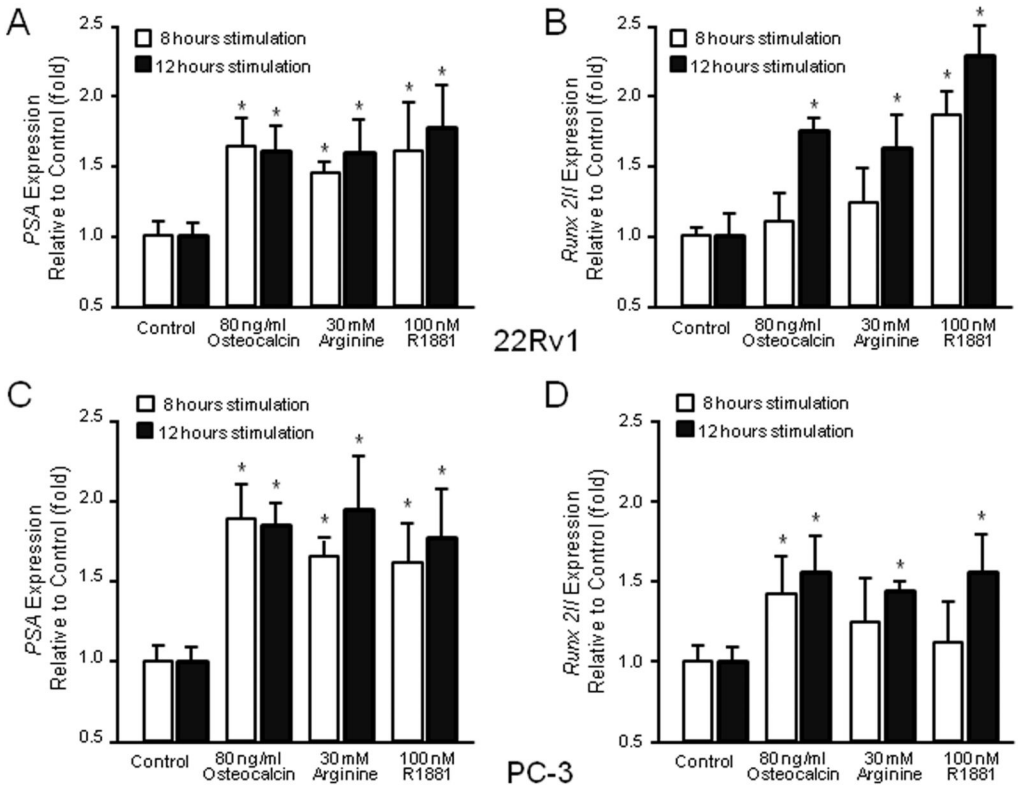

Figure 4. The ligands of GPRC6A stimulated human prostate cancer cells gene expression

OC, arginine and R1881 stimulated *PSA* and *Runx 2II* gene expression in human prostate cancer 22Rv1 (A and B) and PC-3 cells (C and D). * indicates a significant difference from control and stimulation at $p<0.05$, respectively.

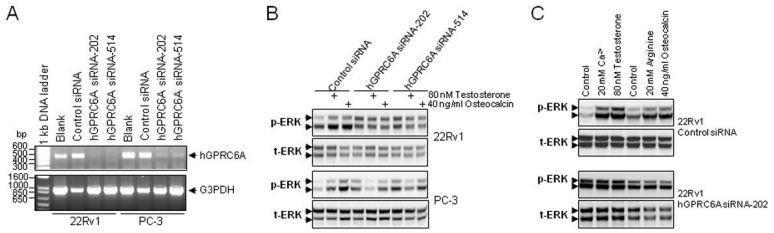

Figure 5. GPRC6A siRNAs inhibited GPRC6A-mediated activation of phosph-ERK in human prostate cancer cell lines

A). GPRC6A siRNAs, hGPRC6A siRNA-202 and siRNA-514 inhibited *GPRC6A* mRNA expression in 22Rv1 and PC-3 cells. B) GPRC6A-mediated OC and testosterone stimulated phospho-ERK activation blocked by transfecting hGPRC6A siRNA-202 and siRNA-514 in 22Rv1 and PC-3 cells. C). GPRC6A-mediated calcium, testosterone, arginine and OC stimulated phospho-ERK activation blocked by hGPRC6A siRNA-202 in 22Rv1 cells. Representative blots are shown, and the results were verified in at least three independent experiments.

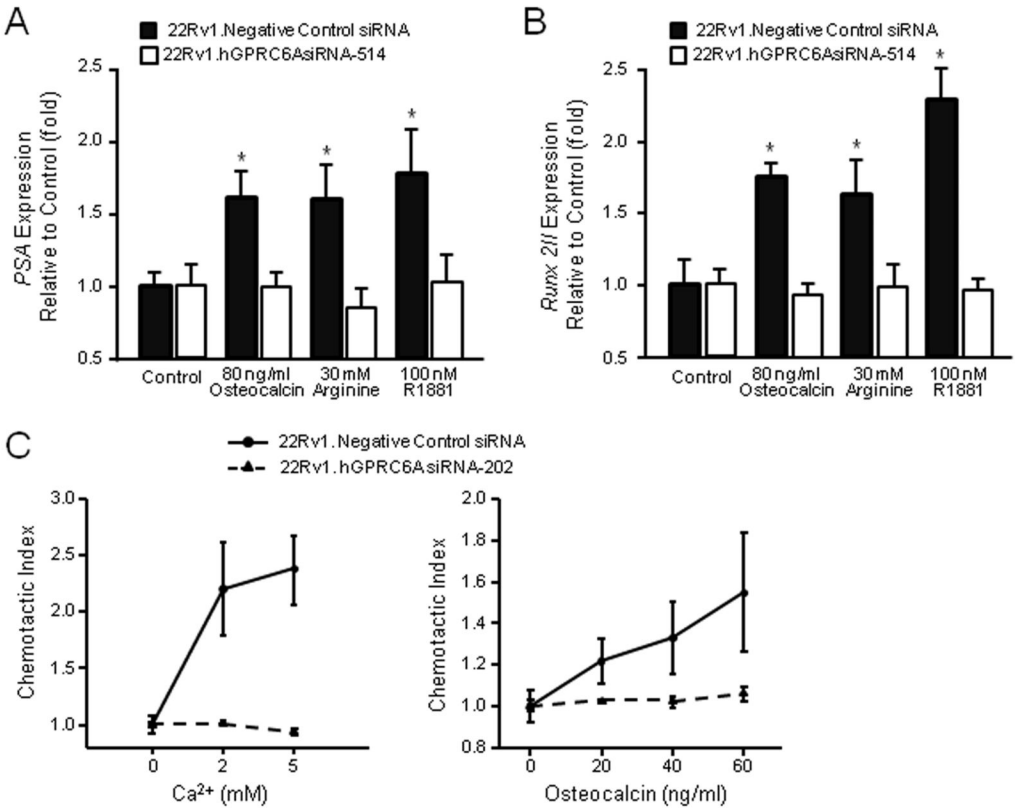

Figure 6. GPRC6A siRNAs inhibited GPRC6A-mediated stimulation gene expression of *PSA* and *Runx2* and activation of cell chemotaxis in human prostate cancer cell lines

The ligands of GPRC6A, OC, arginine and R1881 stimulated gene expression of *PSA* (A) and *Runx2* (B) were attenuated by GPRC6A siRNA in 22Rv1, human prostate cancer cells. C). Extracellular calcium and OC stimulated cell chemotaxis were inhibited by GPRC6A siRNA in 22Rv1, human prostate cancer cells. The experiments were described in **Materials and methods.** In all of the above studies, values for relative cell proliferation (expressed as percent of control) represent the mean ± SEM of a minimum of three separate experiments.

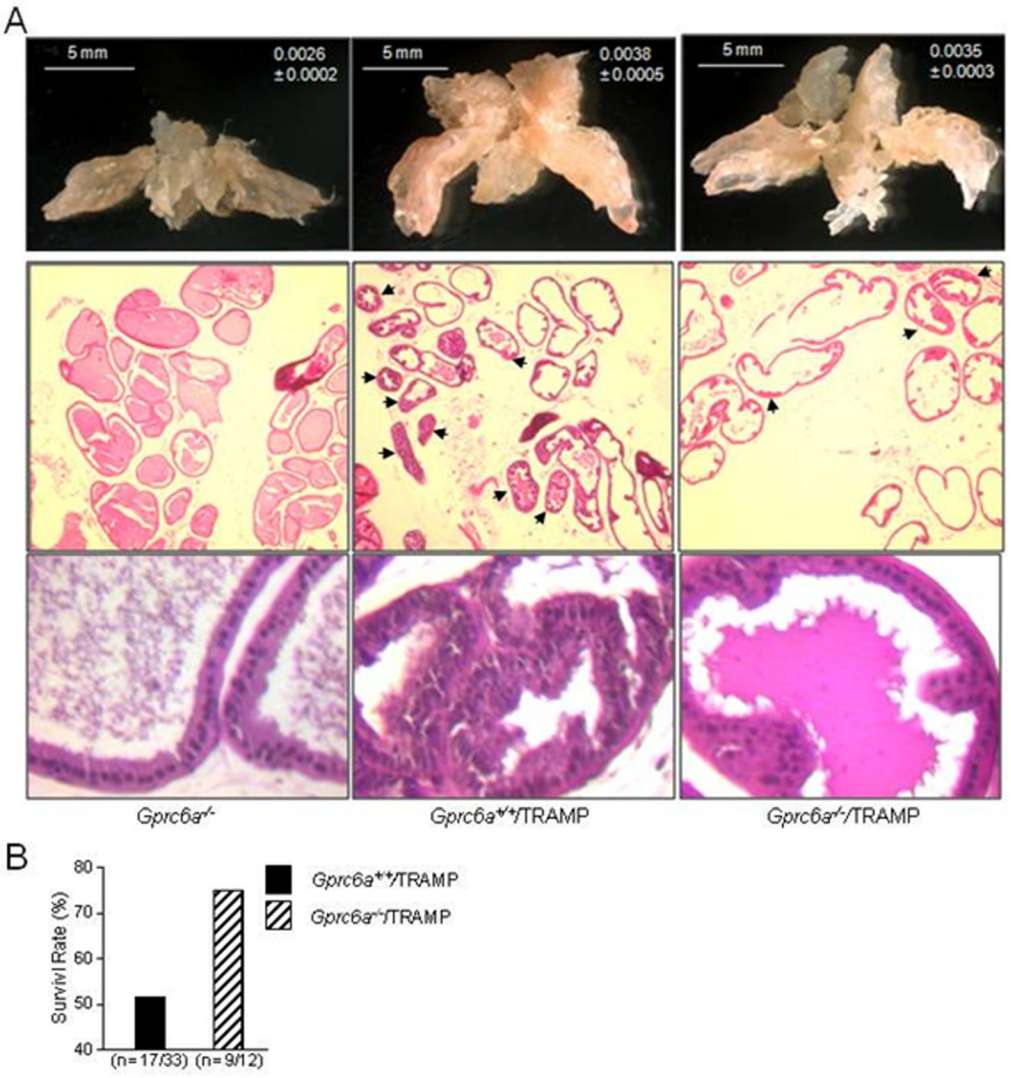

Figure 7. Effects of superimposed *Gprc6a* deficiency in the TRAMP mouse

(A) The gross appearance of whole prostatic glands (Upper panel) and hematoxylin/eosin stained histological sections of ventral prostate (Middle panel, X5 magnification; Lower panel, X20 magnification) from *Gprc6a*$^{-/-}$, TRAMP and *Gprc6a*$^{-/-}$/TRAMP mice at 30 weeks-of-age. Values (inset, upper panel) represent Mean±SEM of prostate gland weights/ body weights. Arrow (Lower panel) shows intraepithelial hyperplasia. (B) Comparison of the survival rates in TRAMP and compound *Gprc6a*$^{-/-}$/TRAMP mice.