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RasGRP3 contributes to formation and maintenance of the prostate cancer phenotype

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Abstract

RasGRP3 mediates the activation of the Ras signaling pathway that is present in many human cancers. Here we explored the involvement of RasGRP3 in the formation and maintenance of the prostate cancer phenotype. RasGRP3 expression was elevated in multiple human prostate tumor tissue samples and in the human androgen-independent prostate cancer cell lines PC-3 and DU 145 compared to the androgen-dependent prostate cancer cell line LNCaP. Down regulation of endogenous RasGRP3 in PC-3 and DU145 cells reduced Ras-GTP formation, inhibited cell proliferation, impeded cell migration and induced apoptosis. Anchorage independent growth of the PC-3 cells and tumor formation in mouse xenografts of both cell lines was likewise inhibited. Inhibition of RasGRP3 expression reduced AKT and ERK1/2 phosphorylation and sensitized the cells to killing by carboplatin. Conversely, exogenous RasGRP3 elevated Ras-GTP, stimulated proliferation, and provided resistance to PMA-induced apoptosis in LNCaP cells. RasGRP3 overexpressing LNCaP cells displayed a markedly enhanced rate of xenograft tumor formation in both male and female mice compared to the parental line. Suppression of RasGRP3 expression in these cells inhibited downstream RasGRP3 responses, caused the cells to resume the LNCaP morphology, and suppressed growth, confirming the functional role of RasGRP3 in the altered behavior of these cells. We conclude that RasGRP3 contributes to the malignant phenotype of the prostate cancer cells and may constitute a novel therapeutic target for human prostate cancer.

Keywords

prostate cancer; Ras; phorbol ester; guanine nucleotide exchange factor; Ras activator; C1 domain

Introduction

Recent investigations have highlighted the important role of Ras activity for cell growth and survival in prostate cancer cells. Blocking Ras activation results in growth arrest and cell death (1,2). In the androgen-dependent prostate cancer cell line LNCaP, stable expression of an activated Ras mutant shifted the cells toward a more malignant phenotype with reduced

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androgen dependence; tumor formation was enhanced and the tumors failed to regress after androgen depletion (3). Conversely, expression of dominant negative Ras restored androgen dependence to C4-2 cells (a hormone-refractory derivative of LNCaP cells) both for growth *in vitro* and *in vivo* (4).

The Ras guanine-nucleotide-exchange factors (RasGEFs) are immediate upstream activators of Ras (5). Genetic loss of RasGEF function has biological effects similar to loss of the Ras proteins themselves (6,7). Conversely, members of the Ras guanine nucleotide releasing protein (RasGRP) family of RasGEFs (8) are among the cancer genes emerging from a screen using retroviral insertional mutagenesis to induce murine myeloid leukemia and B- and T-cell lymphoma (9).

RasGRP was initially identified from screens of genes whose overexpression induced transformation of fibroblasts and its transforming activity was shown to depend on its ability to activate Ras (10,11). RasGRP1 and RasGRP3 are co-regulated both by directly binding diacylglycerol through their C1 domains as well as by phosphorylated by protein kinase C (PKC), which itself is also activated by diacylglycerol (12–15). Thus, RasGRPs act as mediators for the many G-protein coupled receptors and receptor tyrosine kinases that activate phospholipase C, generating diacylglycerol.

RasGRP3 is one of four members of the RasGRP family of RasGEFs (10,16). RasGRP family members differ in their selectivities for activation of individual Ras family members; RasGRP3 activates H-Ras, R-Ras and Rap1 (17). Its expression has been described in human B cells, T cells and endothelial cells of embryonic blood vessels, as well as in mouse brain, spleen, and kidney (18–20). Additionally, RasGRP3 is highly expressed in human Burkitt's lymphoma, human pre–B-cell leukemia and human natural killer(NK)–like T-cell leukemia (18).

In the present study, we find that RasGRP3 transcripts are elevated in a subset of human prostate tumors. We also describe the expression of RasGRP3 in two androgen-independent human prostate cancer cell lines – PC-3 and DU 145. Using several conditional expression approaches, we demonstrate that RasGRP3 contributes to proliferation, anchorage independent growth, and tumor growth in mouse xenografts both in these tumor cell lines as well as in a line derived from the androgen-dependent LNCaP prostate tumor cells in which it was overexpressed. Considering that its C1 domain is a known molecular target for a number of natural products, RasGRP3 could be a novel target for prostate cancer therapy.

Materials and Methods

Cell line, reagents and antibodies

LNCaP clone FGC, PC-3, 22Rv1 and DU 145 cell lines were obtained from ATCC (Manassas, VA). The ERK1/2, p-ERK1/2, Snail and Slug antibodies were obtained from Cell Signaling (Beverly, MA). Ras antibody was purchased from Upstate (Lake Placid, NY). Anti-V5 antibody, the Virapower Lentiviral expression system, CyQuant NF cell proliferation assay kit, YO-PRO-1, 7-AAD, Stealth RNAi duplexes and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). Antibodies for androgen receptor (AR), p-AKT1/2/3, AKT1/2/3, vimentin, E-cadherin, Twist, and PSA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human HGF, VEGF, EGF and TNF-alpha were from R&D Systems (Abingdon, United Kingdom). The carboplatin was purchased from Sigma (St. Louis, MO). The AKT inhibitor V was from EMD (San Diego, CA).

siRNA transfection

The sequences of RasGRP3 siRNAs are provided in supplementary Table 1. The Stealth RNAi Negative Control Duplex pool (Invitrogen, Carlsbad, CA) and an ON-TARGETplus siCONTROL Non-targeting Pool (Dharmacon RNA Technologies, Lafayette, CO) were used as negative controls. Lipofectamine™ 2000 and 80 nM siRNA were applied. The cells were subjected to assays at times between 24 and 120 h after transfection as indicated.

Generation of tetracycline inducible H1 lentiviral shRNA constructs

The sequences encoding the shRNAs for this study are listed in Supplementary Table 2. The pLenti4/BLOCK-iT™-DEST vectors containing specific shRNA were constructed according to the manufacturer's instructions. All the constructs were verified by DNA sequencing. The lentiviral constructs were then produced and titered.

Establishment of tetracycline-regulated shRNA expressing stable cell lines and the cell lines stably overexpressing wild type RasGRP3 or its mutant

For detail experimental procedures, see Supplementary Materials and Methods.

Cell proliferation assay

Cell proliferation was measured using the CyQuant NF cell proliferation assay as described by the manufacturer (Invitrogen, Carlsbad, CA). This assay quantifies binding of a fluorescent dye to cellular DNA.

Detection of apoptotic cells

Cells were harvested and incubated with 1 μM YO-PRO-1 in DPBS for 20 min at 4° C in the dark. 7-AAD was then added at a 5 μg/ml final concentration 10 minutes before analysis by the FACSCalibur system (Becton Dickinson, Mountain View, CA). Data were analyzed with FlowJo 7 software (Tree Star, Inc Ashland, OR).

RT-PCR and Quantitative Real-Time PCR (Q-PCR)

Relative RasGRP3 mRNA levels were determined by RT-PCR and QPCR in human prostate cancer cell lines, normal prostate samples, prostate tumor samples and prostate cDNA arrays (TissueScan prostate qPCR Array Panel I and II, Origene, Rockville, MD). For detailed experimental procedures, see Supplementary Materials and Methods.

Analysis of Ras activation using the Raf1-RBD-GST pull-down assay

Activation of Ras was evaluated as described by Lorenzo and coworkers (13).

Western blot assay

The samples containing 20 μg total protein were separated by electrophoresis and transferred onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). After the membranes were blocked and labeled with the appropriate primary and secondary antibodies, the signal was developed by ECL (Amersham, Piscataway, NJ) and imaged on BioMax XAR or MR films (Kodak, Rochester, NJ).

Growth of cell lines in mouse xenograft system

NOD.SCID/NCr male mice (NIH, Frederick, MD) were injected subcutaneously in the flanks with 5×10^6 or 1×10^7 cells/injection. In the case of those cell lines containing tetracycline inducible expression systems, on the eighth day after injection half the animals in each treatment group were shifted to food containing deoxytetracycline. The animals were

sacrificed after 5 weeks for mice injected with PC-3 cells, RasGRP3-LNCaP cells or the LNCaP control cells, and after 8 weeks for mice injected with DU 145 cells.

Anchorage-independent growth assay in soft agar

The assay was performed as described previously (27) with minor modifications. For details see supplementary material.

Scratch wound assay

The cells plated at confluent density (~1×10^5 cells/well) on 24-well Essen ImageLock™ plates were transiently transfected with siRNAs the following day. After 12 hours, a single wound per well was created using the Essen WoundMaker™. The plates were scanned in the IncuCyte™ at two hour intervals for 24 hours. The data were analyzed by the IncuCyte™ scratch wound assay software.

Growth inhibition by chemotherapeutic drugs

After cells (1×10^4 cells/well) were seeded and cultured for 24 hours with or without 1 μg/ml tetracycline in 24 well plates, chemotherapeutic drugs were added. After further incubation for the indicated times, cells were analyzed by cell proliferation assay.

Statistical Analysis

The statistical analysis is described in Supplementary Materials and Methods.

Results

RasGRP3 is expressed in human prostate carcinoma cell lines

Both Yu et al. (21) and Tomlins et al. (22) described that RasGRP3, a Ras activator, was overexpressed in metastatic prostate cancer. Using Q-PCR, we examined 5 normal human prostate samples and 7 human prostate tumor samples for RasGRP3 expression. The levels of RasGRP3 in the tumors were significantly higher than in the controls (p=0.0303, Mann-Whitney test) (Figure 1A). Additionally, we have determined RasGRP3 mRNA expression by Q-PCR in commercially available cDNAs prepared from normal (n=15) and tumor prostate tissue (Stage I-IV, n=65). Using outlier robust t-statistics (23), we observed that 26 % of tumor samples (n=17), identified as outliers for RasGRP3 over-expression, expressed significantly higher RasGRP3 levels compared to normal prostate tissue (p=0.0226) (Figure 1B). No outliers expressing significantly lower RasGRP3 levels were identified (p=0.5420).

Gene expression array analysis of the NCI 60 cell line panel likewise indicated the expression of RasGRP3 in the PC-3 and DU 145 prostate cancer cell lines (http://dtp.nci.nih.gov/mtweb/hugosearch?genecard=RASGRP3). We confirmed by RT-PCR (Figure 1C) that RasGRP3 was expressed in these two androgen independent human prostate carcinoma cell lines, with a barely detectable level of expression in the androgen-dependent LNCaP cells and the 22Rv1 cells. The phenotype of LNCaP clone FGC, PC-3, 22Rv1 and DU 145 cell lines were well described previously (24–26). PC-3 harbors mutations in PTEN and P53; DU 145 has mutations in RB, P53, CDKN2A, and STK11 (30). For comparison, we show expression of RasGRP3 in the Ramos B cell leukemia cell line. The RT-PCR results were confirmed by Q-PCR (Figure 1C).

Effects of suppressing the level of endogenous RasGRP3 in PC-3 and DU 145 cells on Ras activation, apoptosis, and morphology

The biological function of RasGRP3 was investigated by knocking down endogenous RasGRP3 expression in the PC-3 and DU 145 cells. In preliminary studies, we tested the

ability of multiple RasGRP3 siRNAs to knockdown the exogenous RasGRP3-V5 in RasGRP3-LNCaP cells (Supplementary Figure 1A). To minimize possible off-target effects of the siRNAs, we also created a RasGRP3-siRNA pool containing equal amounts of RasGRP3-siRNA 126, 774 and 1260.

Both the RasGRP3-siRNA pool (Figure 2A) and the individual RasGRP3-siRNAs (Supplementary Figure 1C) inhibited the endogenous RasGRP3 expression in both the PC-3 and DU 145 cells. Inhibition of RasGRP3 expression was accompanied by a reduced level of Ras-GTP in both cell lines (Figure 2A), showing that the RasGRP3 was indeed an important contributor to the state of Ras activation in these cells.

Inhibition of RasGRP3 expression caused marked apoptosis both in the PC-3 and DU 145 cells (Figure 2B and Supplementary Figure 2A). Likewise, the morphology of the two cell lines (Supplementary Figure 2B) was shifted from a flattened morphology to a more dendritic or spindle shaped one (resembling LNCaP), with many rounded cells.

Inhibition of endogenous RasGRP3 expression inhibited migration of the PC-3 and DU 145 cells

Transient down regulation of endogenous RasGRP3 inhibited cell migration, as detected by the wound scratch assay, in both the PC-3 and DU 145 cell lines (Figure 2C and Supplementary Figure 3 for images).

Down regulation of RasGRP3 suppressed proliferation of the PC-3 and DU 145 cells

To achieve long term suppression of RasGRP3 expression, we established cell lines derived from PC-3 or DU 145 expressing tet-on inducible RasGRP3 shRNA. Because of promoter leakage with the tet-on system, the level of endogenous RasGRP3 expression in the absence of tetracycline was already somewhat reduced in the PC-3 derivative sh854 cell line and in both of the DU 145 derivative cell lines, sh236 and sh854 (Figure 3A). To confirm the specificity of the effects of the RasGRP3 shRNAs, we created a mutated variant of RasGRP3 retaining the amino acid sequence of RasGRP3 but altered in its coding sequence to diminish the homology with shRNA854. Stable cell lines were prepared by introducing this RasGRP3 mutant into the PC-3 derivative sh854 and DU 145 derivative sh854 cell lines. RT-PCR results showed that the RasGRP3 mutant was resistant to the shRNA854 (Figure 3A).

To examine the effect of suppressing endogenous RasGRP3 expression on growth of the PC-3 and DU 145 derivative lines, the cells were cultured with or without 1 μg/ml tetracycline for 120 hours. RasGRP3 expression was detected by RT-PCR (Figure 3A). Inhibition of cell proliferation mirrored the suppression of RasGRP3 expression (Figure 3B). A reduction in cell growth was detected in the cells that already expressed a reduced amount of RasGRP3 as a result of promoter leakage of the shRNA, as compared with the cell lines expressing a normal level of RasGRP3 (Figure 3A and B). A further reduction in cell proliferation accompanied the further reduction in RasGRP3 expression upon induction of the RasGRP3 shRNAs by tetracycline treatment. Induction of the control shRNAs by tetracycline had little effect (Figure 3A and B). As a control for shRNA specificity, expression of the shRNA854 resistant RasGRP3 mutant maintained RasGRP3 expression and rendered the cell growth rate insensitive to expression of the shRNA854. Using the siRNA pool to transiently down regulate endogenous RasGRP3, we likewise could show inhibition of cell proliferation of the PC-3 and DU 145 cells (Figure 2D).

Inhibition of endogenous RasGRP3 expression inhibited anchorage independent growth of the PC-3 cells

For PC-3 cells, the induction of the RasGRP3 shRNA expression with tetracycline treatment decreased both the total number and size of colonies in soft agar (Figure 3D and Supplementary Figure 4). No inhibition was observed for either of the scrambled shRNA controls. The inhibition of the growth in soft agar caused by the sh854 RNA was blocked by introducing the shRNA854 resistant mutant RasGRP3 back into the sh854 derivative PC-3 cell line. Because of the very weak growth of DU 145 cells in soft agar (27), the corresponding studies could not be done reliably in that cell line.

Down regulation of endogenous RasGRP3 suppressed xenograft tumor formation by the PC-3 and DU 145 cells

The effect of endogenous expression of RasGRP3 on tumor formation by the PC-3 and DU 145 cells was assessed in a mouse xenograft model, using the cell lines expressing either control shRNA, the shRNA854, or the shRNA854m cells that further expressed the mutated RasGRP3 resistant to shRNA854 (Figure 3C). Expression of the shRNA854 caused a marked reduction in the size and the number of tumors for both of the PC-3 and DU 145 cell lines (Table 1). In the animals treated with deoxytetracycline, a further reduction was observed, leading to no detectable tumors at the time when the experiment was terminated. In the case of control cells expressing scrambled shRNA, robust growth was observed and deoxytetracycline had no effect. Both for the PC-3 and DU 145 derived cells, expression of the mutated RasGRP3 resulted in resistance to the effect of shRNA854 on tumor growth and, in the case of DU 145, fully restored growth to the level of the cells expressing the scrambled control.

Endogenous RasGRP3 contributes to AKT and ERK1/2 activation in both DU 145 and PC-3 cells

Akt and ERK1/2 represent primary downstream effectors of Ras signaling. Modulation of RasGRP3, upstream of Ras, would therefore be expected to contribute to Akt and ERK1/2 activation. HGF (hepatocyte growth factor) represents an important signaling molecule in prostate cancer (28,29), leading to activation of phospholipase C (30), an upstream effector of PKC and RasGRP. We have evaluated the ability of HGF and other growth factors (EGF, V-EGF and TNF-α) to modulate phosphorylation of ERK or AKT in both PC-3 and DU 145 cells and the involvement of RasGRP3 in this activity. In the case of the PC-3 cells, the basal level of AKT phosphorylation was already high, reflecting the PTEN mutation in this cell line (31). Although AKT phosphorylation was not significantly enhanced by the addition of HGF or EGF in the PC-3 cells (Supplementary Figure 5A), AKT phosphorylation was nonetheless decreased by the suppression of the endogenous RasGRP3 expression by shRNA (Figure 4A) and by siRNA (Figure 4C). Additionally, HGF and EGF treatment increased phosphorylation of ERK1/2 in the PC-3 cells (Supplementary Figure 5A). We confirmed that down regulation of RasGRP3 reduced the HGF-induced ERK phosphorylation (Figure 4C). In the DU 145 cells, HGF treatment significantly increased phosphorylation of both AKT and ERK1/2 (Supplementary Figure 5B). Conversely, down regulation of RasGRP3 with either the shRNAs (Figure 4B) or siRNA pool (Figure 4D) reduced the HGF induced AKT phosphorylation in these cells and variably reduced the basal level of AKT phosphorylation. ERK1/2 activation and HGF induced ERK1/2 activation were also inhibited (Figure 4D). Once again, introducing the exogenous RasGRP3 mutant resistant to shRNA854 restored the level of phosphorylated AKT in both cell lines (Figure 4A and B). Consistent with the central role of AKT pathway in the survival of PC-3 and DU 145 cells, decreasing the level of p-AKT using an AKT inhibitor resulted in significant inhibition of cell proliferation in both cell lines (Supplementary Figure 5C and 5D).

Endogenous RasGRP3 expression contributes to resistance to carboplatin in the PC-3 and DU 145 cells

The effect of RasGRP3 on the sensitivities of both the PC-3 and DU 145 cells to the chemotherapeutic drugs carboplatin, etoposide and doxorubicin was evaluated. In the PC-3 cell line, induction of either the shRNA236 or shRNA854 cells with tetracycline sensitized the cells to carboplatin, shifting the dose response curve to the left, representing a 3–4 fold decrease in the IC_{50} (Figure 5A). Introducing the mutated RasGRP3 resistant to shRNA854 blocked the increase in sensitivity of the cells. Likewise, no change in sensitivity upon tetracycline addition was observed for the cells containing the control shRNA. Unlike sensitivity to carboplatin, sensitivity to etoposide and doxorubicin was unaffected by the suppression of endogenous RasGRP3 (data not shown). For the DU 145 cells, the uninduced sh236 and sh854 cell lines already showed increased sensitivity to carboplatin relative to the control or sh854m cells, consistent with the reduced RasGRP3 levels caused by leakage of the promoter (Figure 5B). There was no additional increase of sensitivity to carboplatin upon the further reduction of RasGRP3 induced by tetracycline treatment (data not shown). As was the case for the PC-3 cells, suppression of RasGRP3 did not affect the sensitivity of the DU 145 cells to etoposide or doxorubicin (data not shown).

Transformation of LNCaP cells overexpressing RasGRP3

To further explore the potential effects of RasGRP3 overexpression in prostate cells, we established a LNCaP cell subline expressing V5 tagged human RasGRP3 (RasGRP3-LNCaP) as described before (32). RasGRP3 expression was confirmed by western blotting (Figure 6A) and was accompanied by the activation of Ras, reflected in the elevated level of Ras-GTP, and an elevated level of phosphorylated ERK1/2 (Figure 6A). Like the PC-3 and DU 145 cells, the RasGRP3-LNCaP cells lost or showed greatly diminished expression of prostate specific antigen (PSA) and of the androgen receptor (Figure 6A). Like the PC-3 and DU 145 cells, they had highly elevated expression of vimentin (Figure 6A) and loss of E-cadherin (Supplementary Figure 6), both markers of the epithelial–mesenchymal transition (EMT). Like the DU 145 cells and unlike the PC-3 cells, the RasGRP-LNCaP cells did not differ appreciably from the LNCaP cells in their expression of Slug and Snail, transcription factors contributing to the epithelial-mesenchymal transition (Supplementary Figure 6). Relative to the LNCaP cells, the RasGRP3 overexpressing LNCaP cells also showed a flattened, cuboidal shape with pseudopodia (Supplementary Figure 7A). These morphological properties resemble those of PC-3 and DU 145 cells (Supplementary Figure 7A).

In the presence of normal medium the RasGRP3-LNCaP showed a markedly enhanced rate of growth compared to the LNCaP cells (Figure 6B). Likewise, the RasGRP3-LNCaP cells grew in the presence of charcoal-stripped serum at a rate similar to that under usual culture conditions (Figure 6B). In contrast, the parental LNCaP cells were sensitive to culture in the presence of charcoal-stripped serum, which is used to deplete the serum of steroids.

PMA has been reported to induce apoptosis in the LNCaP cells but not in the PC-3 cells and DU 145 cells (33,34). Like the PC-3 and DU 145 cells, the RasGRP3-LNCaP cells were resistant to PMA induced apoptosis (Supplementary Figure 7B). The more aggressive behavior of the RasGRP3-LNCaP cells in culture was reflected in their behavior in a tumor xenograft system. The RasGRP3-LNCaP cells were able to form tumors in both male and female immunodeficient mice within five weeks, when 5×10^6 cells were injected subcutaneously, whereas little growth was evident for the control LNCaP cells at this time (Figure 6C and Table 2).

Reduction of RasGRP3 expression by siRNA treatment partially reverts the phenotype of the RasGRP3-LNCaP cells

To confirm that RasGRP3 expression caused phenotypic transformation, we knocked down the exogenous RasGRP3 expression with siRNA. We found that changes in RasGRP3 expression were linked to changes in Ras-GTP levels. This was the case both with the individual RasGRP3-siRNAs (Supplementary Figure 1B) as well as with the RasGRP3-siRNA pool (Figure 6D).

Reduction in the level of RasGRP3 expression in the RasGRP3-LNCaP cells reduced the phosphorylated ERK1/2 (Figure 6D and Supplementary Figure 1B). Similarly, the morphology of the RasGRP3-LNCaP cells upon treatment with RasGRP3 siRNA assumed a spindle shape with a smaller nucleus, resembling the parental LNCaP cells (Supplementary Figure 7C). In addition, the vimentin expression was reduced upon the RasGRP3-siRNA treatment (Figure 6D). Finally, cell proliferation was reduced in response to treatment with the pooled RasGRP3-siRNA and the cells became more sensitive to culture in the presence of charcoal stripped medium (Figure 6D).

Our results argue that the overexpression of RasGRP3 was directly responsible for enhancing the activity of Ras –ERK pathway and shifting the morphology and growth properties of the RasGRP3-LNCaP cells to more resemble the PC-3 and DU 145 cells.

Discussion

Activation of Ras signaling has long been recognized to be important for carcinogenesis and progression of prostate cancer cells with respect to growth, androgen dependency, and tumorigenicity. Nonetheless, activating gene mutations of ras alleles are infrequent in the development or progression of carcinoma of the prostate (35,36). Weber and Gioeli have thus suggested that wild-type Ras at its physiological expression level can be chronically activated by upstream autocrine and paracrine growth factors (37). We show that RasGRP3, a Ras activator, is an important signaling element upstream of Ras, at least in two metastatic human androgen-independent prostate cancer cells.

The involvement of RasGRP family members in cancer development and progression is proving to be ever more extensive (9,19,38,39). Our findings of RasGRP3 expression in the prostate tumor cell lines PC-3 and DU 145 further broaden the scope of tissue types where RasGRP family members are of importance. Our demonstration of an elevated level of RasGRP3 mRNA in several of a small collection of prostate tumor samples and in 26% of a larger panel of tumor cDNAs supports the relevance of these findings in the two prostate cell lines, as do the cDNA microarray gene expression profiling studies of Yu *et al*. (21) and Tomlins *et al.* (22). Their data showed that RasGRP3 was overexpressed in metastatic prostate cancer compared to normal prostate.

Our finding that endogenous RasGRP3 expression makes an important contribution to the proliferation and tumorigenicity of the PC-3 and DU 145 cells not only demonstrates its role in prostate but implies that this level of expression of RasGRP3 can be physiologically important. The PC-3 and DU 145 cell lines are members of the NCI 60 tumor cell line panel. Comparable or higher levels of RasGRP3 expression are reported for a number of cell lines from multiple tumor types in the NCI 60 tumor cell line panel (http://dtp.nci.nih.gov/mtweb/browse.jsp). Although the functional potential of RasGRP3 will necessarily depend on the cellular context, our results suggest that RasGRP3 is of relevance in an appreciably broader range of tissue types than had previously been anticipated. This is particularly significant in the context of chemotherapy resistance, as our

data show that reduction of RasGRP3 in both PC-3 and DU 145 cells enhanced sensitivity to carboplatin by 3–4 folds.

Although RasGRP3, when present in meaningful levels, should contribute to the signaling of those many receptors in cells which activate phospholipase C, the focus of studies so far has been on its role in B cell receptor and T cell receptor signaling. In the case of the prostate, serum levels of hepatocyte growth factor (HGF) have been reported to be elevated in patients with advanced-stage prostate cancer (40); c-Met, a HGF receptor, has been detected in androgen-independent PC-3 and DU 145 human prostate cells but not in the androgen-dependent LNCaP cells (41). HGF has been shown to induce cellular proliferation and promote the invasiveness of PC-3 and DU 145 prostate cancer cells (42–45); and HGF is coupled to phospholipase C activation (46). Here, we demonstrated that RasGRP3 contributed to signaling downstream of HGF, as reflected in the DU 145 cells by its role in the level of HGF induced phosphorylation of AKT and ERK1/2. In the PC-3 cells, HGF did not enhance the level of AKT phosphorylation, perhaps reflecting the already high basal level of AKT phosphorylation caused by the loss of function PTEN mutation in these cells. Nonetheless, down regulation of RasGRP3 still decreased the level of phosphorylated AKT, suggesting that RasGRP3 might be involved in alternative drivers of AKT phosphorylation in the PC-3 cell lines. Additionally, HGF treatment induced ERK1/2 activation in both PC-3 and DU 145 cells. Down regulation of RasGRP3 partially blocked this activation. AKT and ERK1/2, downstream targets of Ras, play a critical role in activation of both cell proliferation and anti-apoptotic signaling (47,48) and enhance tumor progression by promoting cell invasiveness and angiogenesis (49,50). The effects of RasGRP3 on AKT and ERK1/2 phosphorylation in both the DU 145 and PC-3 cells are consistent with our demonstration that RasGRP3 is contributing to Ras activation in these cells and likewise support the effects we observe on cell proliferation, chemoresistance and tumorigenesis.

The identification of RasGRP3 as an additional important signaling element contributing to the cancer phenotype in the PC-3 and DU 145 prostate cancer cells, together with the evidence for its elevated expression in a subpopulation of prostate tumors, reveals an additional potential target for pathway directed chemotherapy in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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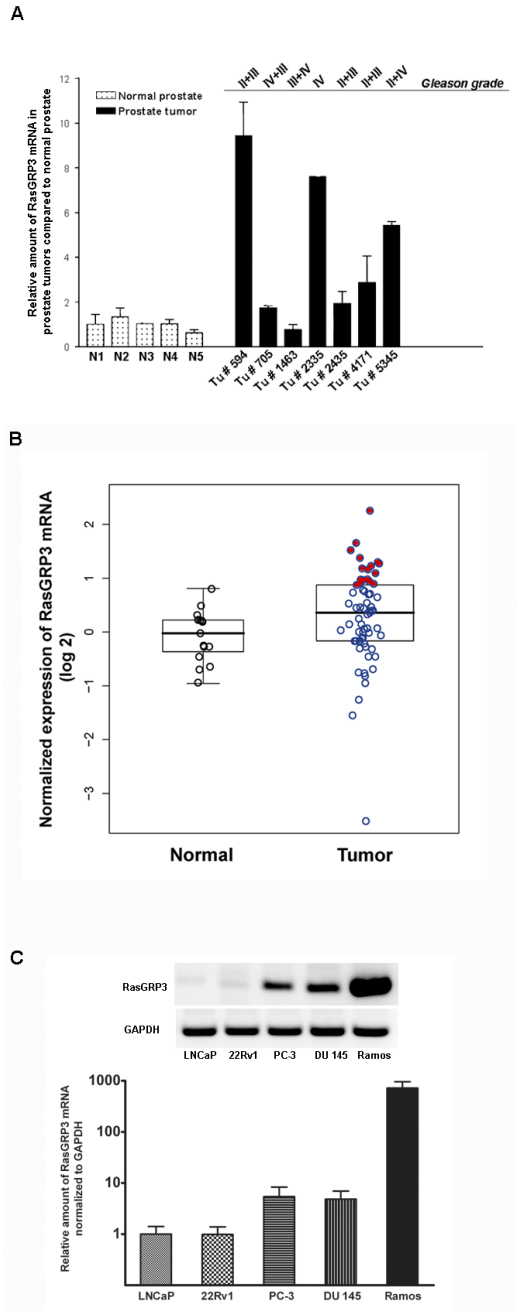

Figure 1.

RasGRP3 expression in prostate tumors and prostate cancer cell lines. (A) Q-PCR analysis of RasGRP3 gene expression in human normal prostate (N) and prostate tumor (Tu). Values represent mean ± SEM (mean ± SD for #2335). (B) RasGRP3 expression in normal prostate and prostate tumor cDNAs. The log2 of normalized (to actin) expression values relative to the mean expression of the normal are presented. The line in the middle of the box represents the median with the lower and upper edges showing the 1st and 3rd quartile, respectively. The whiskers in the normal group to the 1*IQR (interquartile range) indicate the cancer outlying expression as defined by the outlier robust t-statistics (23). The red circles indicate outliers for RasGRP3 over-expression. (C) RT-PCR and Q-PCR analysis of RasGRP3 gene expression in prostate cancer cells. Results are representative of 3

independent experiments for RT-PCR and two independent experiments for Q-PCR. Values represent average ± SD.

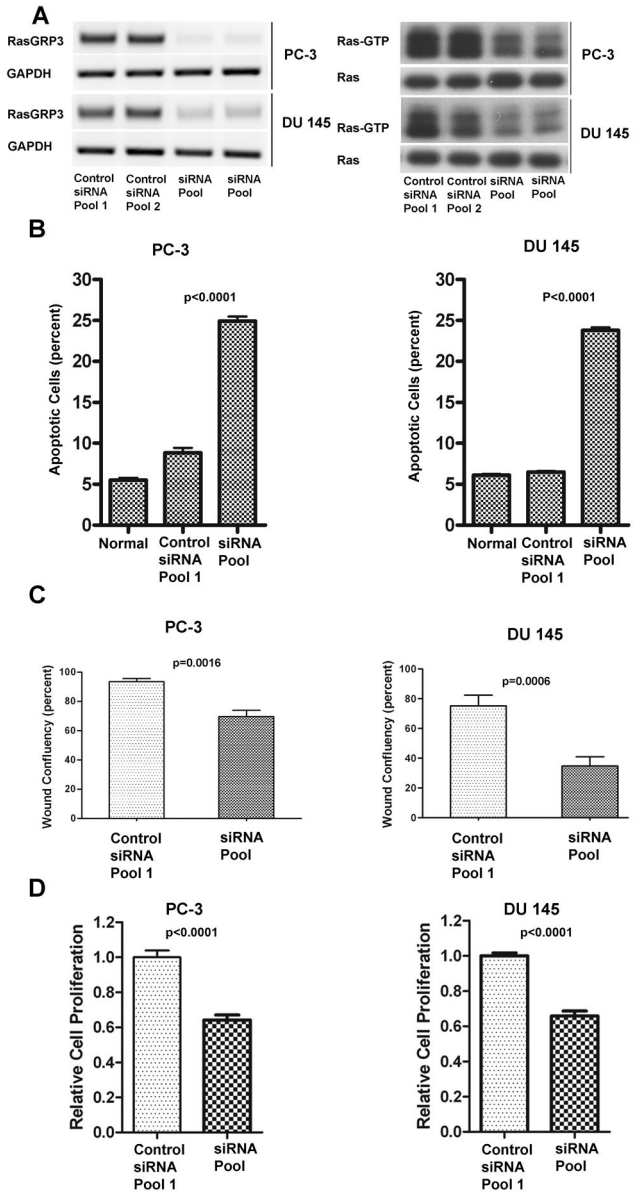

Figure 2.

Blocking endogenous RasGRP3 expression caused reduction of Ras activation, cell apoptosis, cell migration, and inhibition of cell proliferation in PC-3 and DU 145 cells. The cells were treated with RasGRP3-siRNA pool or Control siRNA pools 1 and 2 for 96 hours unless stated otherwise. (A) Endogenous RasGRP3 expression was determined by RT-PCR. Ras-GTP levels were detected by pull-down assay and immunoblotting. (B) The siRNA treated cells were stained with YO-PRO-1 and 7-AAD and analyzed by flow cytometry. (C) Scratch wound assay. (D) The proliferation of siRNA-treated cells was determined using the CyQuant NF cell proliferation assay, with values normalized to the levels of non-treated cells. All results are representative of three experiments. All values represent the mean ± SEM for three independent experiments.

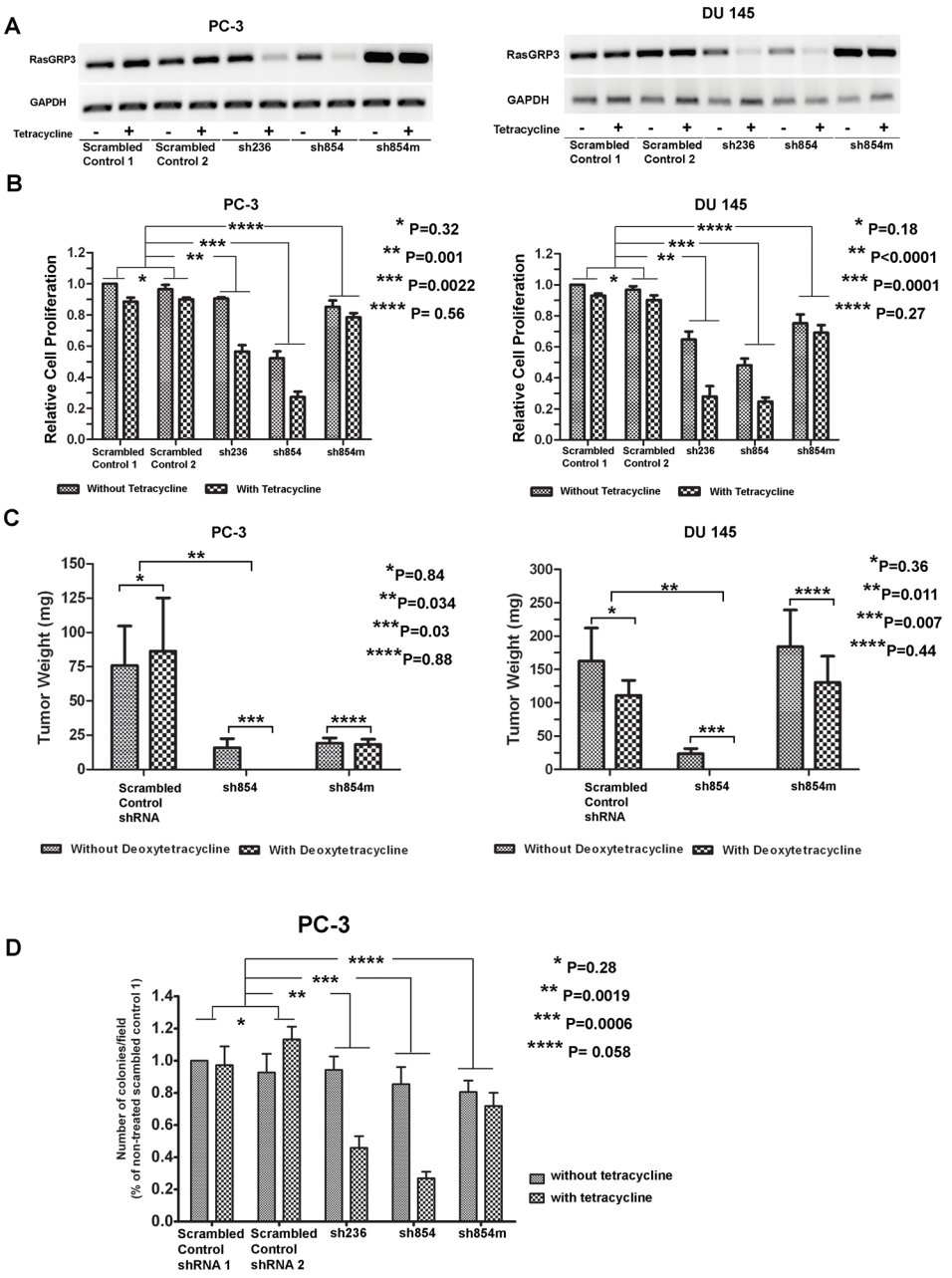

Figure 3.

Inhibition of endogenous RasGRP3 expression retarded cell proliferation, inhibited xenograft tumor growth of both PC-3 and DU 145 cells and impeded colony formation of PC-3 cells in soft-agar. (A)The PC-3 and DU 145 Tet-on stable cell lines were treated with/without tetracycline for 120 hours. Endogenous RasGRP3 expression was determined by RT-PCR. Results are representative of three experiments. (B) The proliferation of PC-3 and DU 145 derived cells treated as above was determined using the CyQuant NF cell proliferation assay. The values were normalized to the non-treated scrambled shRNA 1 control. Values represent the mean ± SEM of four independent experiments. Addition of tetracycline caused a statistically significant ($p<0.002$) decrease in proliferation relative to the control in both the sh236 and sh854 derived line. (C) The NOD.SCID/NCr male mice

were injected subcutaneously with 1×10^7 cells of the indicated PC-3 and DU 145 derived lines. On the eighth day, half the animals in each group were shifted to food containing deoxytetracycline. The animals were sacrificed 5 weeks (PC-3) or 8 weeks (DU 145) after injection. (D) Colony formation of the PC-3 derived cells in soft agar. Values represent the mean +/− SEM of the four independent experiments.

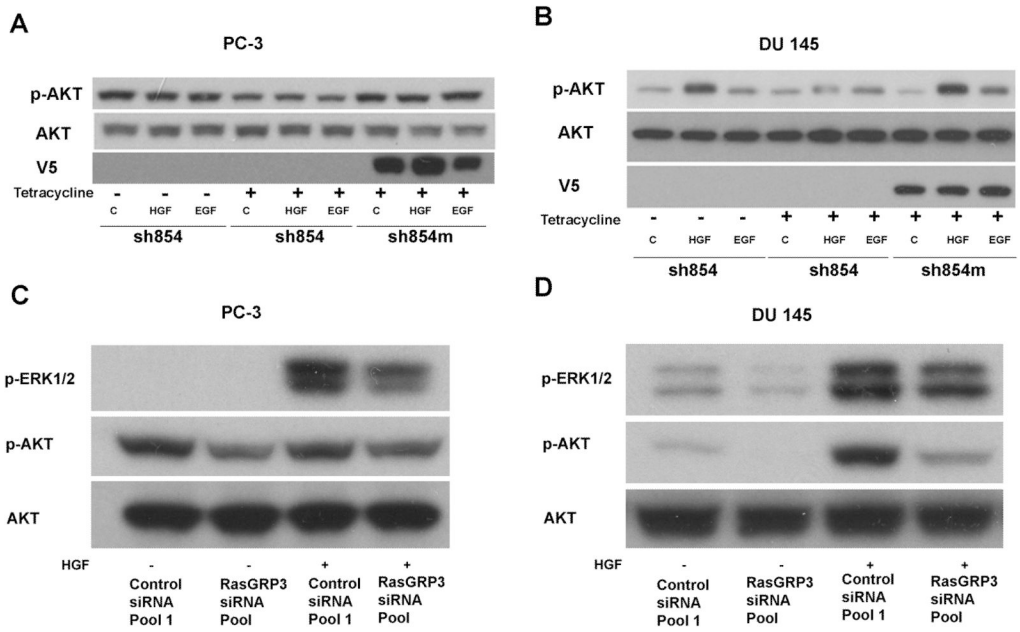

Figure 4.

RasGRP3 is involved in both HGF dependent and independent AKT activation. (A, B) The PC-3 and DU 145 tet-on stable cell lines were treated with/without tetracycline for 5 days. The cells were induced with/without HGF (20 ng/mL) or EGF (100 pg/ml) for 20 minutes. The AKT and phosphorylated AKT were detected by immunoblotting. (C, D) PC-3 cells and DU 145 cells were transiently transfected with RasGRP3 siRNA pool and the Control siRNA pool 1 and 48 hours later were stimulated with/without HGF (20 ng/mL) for 20 minutes. The AKT, phosphorylated-AKT, and phospho-ERK1/2 were detected by immunoblotting. All results are representative of 3 independent experiments.

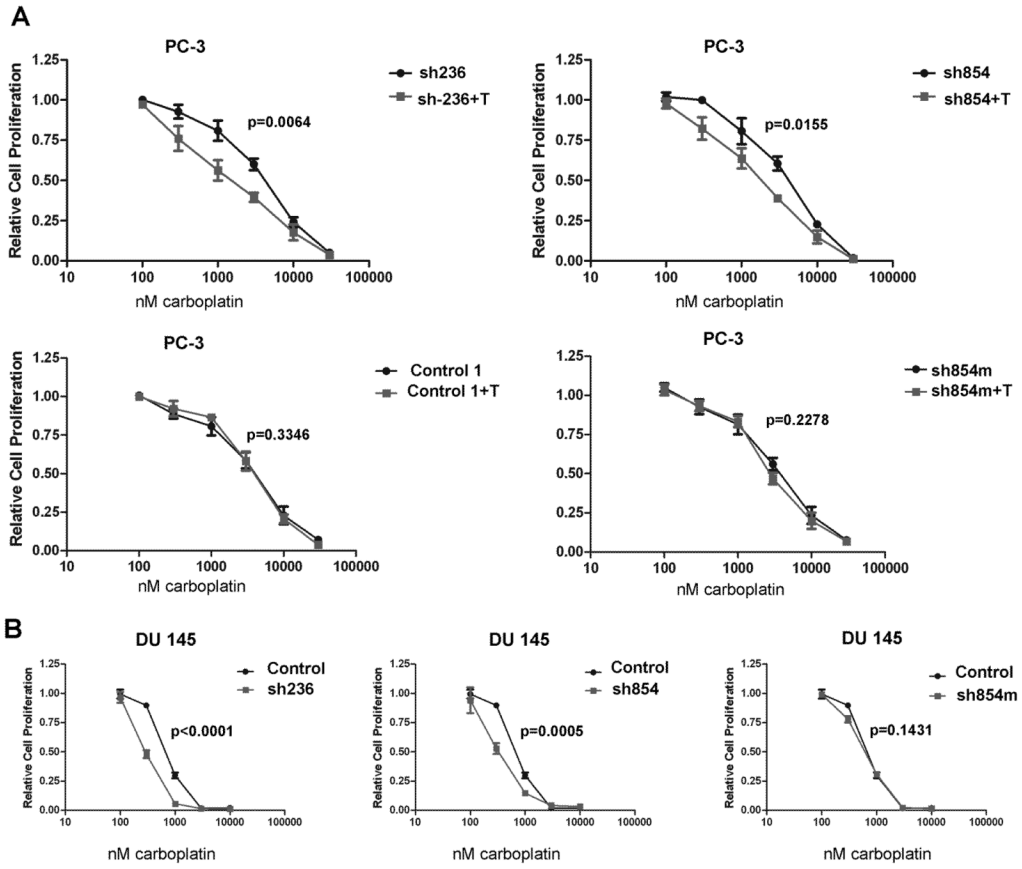

Figure 5.

Down regulation of RasGRP3 increased the sensitivity to carboplatin of both the PC-3 and DU 145 cell lines. The indicated PC-3 (A) and DU 145 (B) derived cell lines were treated with/without tetracycline (T) for 24 hours, followed by carboplatin treatment (PC-3 cells: 100–30,000 nM; DU 145 cells: 100–10,000 nM). After 72 hours cell proliferation was determined using the CyQuant NF cell proliferation assay. The results were normalized to no treatment with carboplatin. All results are representative of 3 independent experiments.

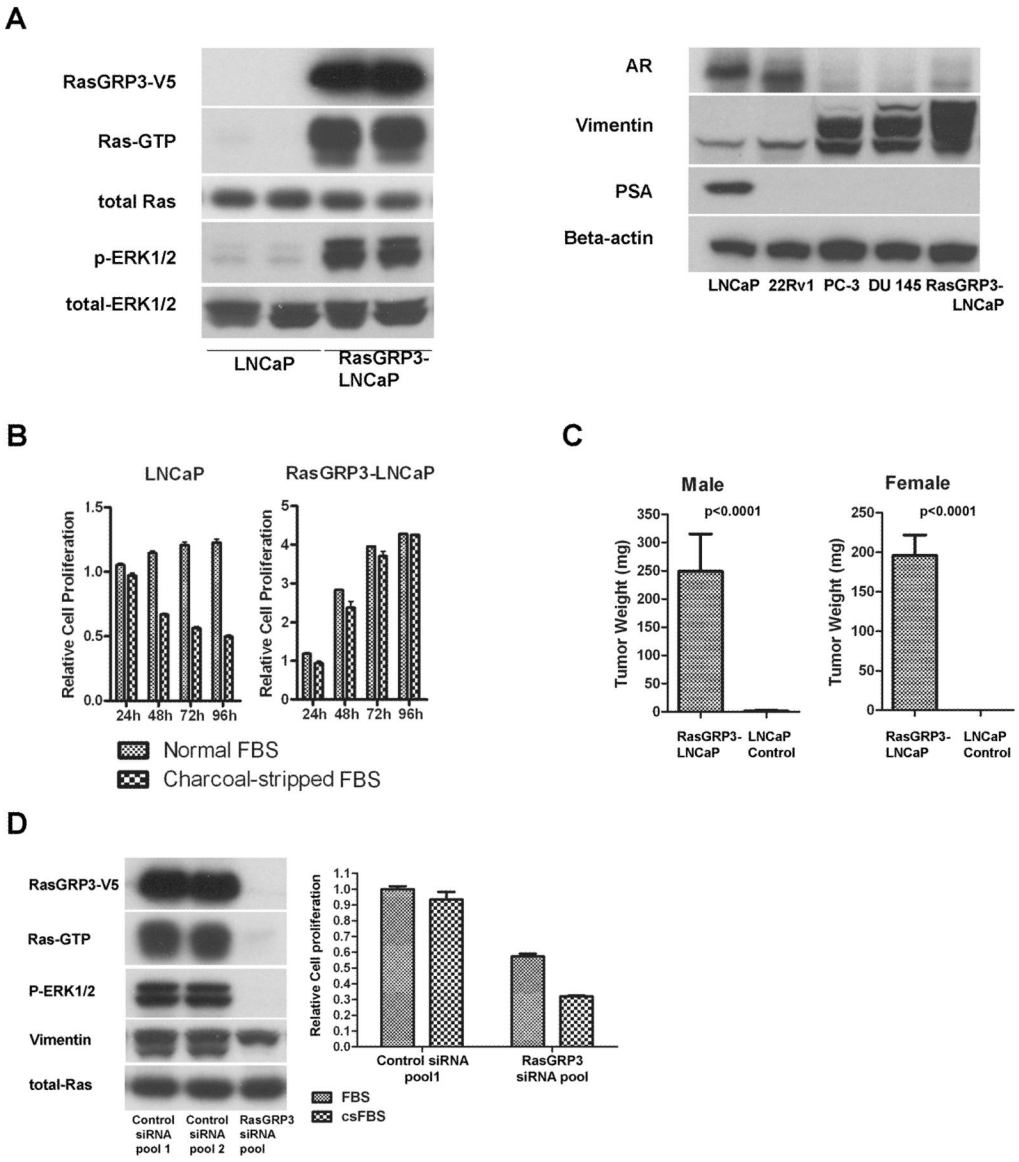

Figure 6.

Phenotypic alterations and effects of inhibiting RasGRP3 expression in the RasGRP3-LNCaP cells. (A) Over-expression of V5 tagged RasGRP3 and its effects on the Ras pathway. V5 tagged RasGRP3, phosphorylated ERK1/2, ERK1/2, androgen receptor (AR), vimentin, and prostate specific antigen (PSA) levels were determined by immunoblotting. Ras-GTP levels were determined by pull-down assay followed by immunoblotting. Levels of total Ras were used as control. Results are representative of 3–4 independent experiments. (B) LNCaP and RasGRP3-LNCaP cells were cultured in 10% FBS containing medium to approximately 30% confluency. The medium was then replaced with either normal medium or steroid-depleted medium containing 10% charcoal-stripped FBS. Cell proliferation was determined daily using the CyQuant NF cell proliferation assay, with values normalized to the levels on day 0. Results are representative of 3 independent experiments. (C) NOD.SCID/NCr male and female mice were injected subcutaneously with 5×10^6 cells RasGRP3-LNCaP or LNCaP cells. 5 weeks later the mice were sacrificed and the weight of the tumors was determined. (D) RasGRP3-LNCaP cells were transiently transfected with

RasGRP3-siRNA pool or Control siRNA pools. 96 hours later V5 tagged RasGRP3, phosphorylated ERK1/2, vimentin and Ras were detected by immunoblotting. GTP bound Ras was analyzed by pull-down assay. RasGRP3-LNCaP cells were transiently transfected with RasGRP3 siRNA pool and control siRNA pool 1. The cell culture medium was changed to normal medium or steroid-depleted medium and cell proliferation was determined 96 hours after transfection using the CyQuant NF cell proliferation assay. The values are normalized to that of the “non-treated + FBS” group. All results are representative of 3 independent experiments.

Table 1

Inhibition of endogenous RasGRP3 expression inhibited xenograft tumor growth of both PC-3 and DU 145 cells

Cell Line		Total tumor/Total injection		Tumor weight (mg)	
		Deoxytetracycline		Deoxytetracycline	
		−	+	−	+
PC-3	Control	10/12	9/12	76±29	86±39
	sh854	5/12	0/14	16±7	0
	sh854m	9/12	8/12	19±4	18±4
DU145	Control	10/12	10/12	162±50	111±22
	sh854	8/12	0/12	24±8	0
	sh854m	9/12	8/12	184±55	130±40

Table 2

Overexpression of RasGRP3 enhances tumorigenicity of LNCaP cells

Cell line	Total tumor/Total injection		Tumor weight (mg)	
	Male	Female	Male	Female
LNCaP Control	1/7	0/7	1.5±1.5	0
RasGRP3-LNCaP	7/7	7/7	249±66	196±26