

ORIGINAL ARTICLE

PSGR promotes prostatic intraepithelial neoplasia and prostate cancer xenograft growth through NF- κ BM Rodriguez¹, W Luo¹, J Weng¹, L Zeng¹, Z Yi², S Siwko¹ and M Liu^{1,2}

Prostate-specific G-protein-coupled receptor (PSGR), a member of the olfactory subfamily of G-protein-coupled receptors, is specifically expressed in human prostate tissue and overexpressed in prostate cancer (PCa). This expression pattern suggests a possible role in PCa initiation and progression. We developed a PSGR transgenic mouse model driven by a probasin promoter and investigated the role of PSGR in prostate malignancy. Overexpression of PSGR induced a chronic inflammatory response that ultimately gave rise to premalignant mouse prostate intraepithelial neoplasia lesions in later stages of life. PSGR-overexpressing LnCaP cells in prostate xenografts formed larger tumors compared with normal LnCaP cancer cells, suggesting a role of PSGR in the promotion of tumor development. Furthermore, we identified nuclear factor- κ B (NF- κ B) or RELA as a key downstream target activated by PSGR signaling. We also show that this regulation was mediated in part by the phosphatidylinositol-3-kinase/Akt (PI3K/AKT) pathway, highlighting a collaborative role between PI3K/AKT and NF- κ B during tumor inflammation downstream of PSGR in the initial phases of prostate disease.

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INTRODUCTION

Prostate cancer (PCa) is a heterogeneous disease, with multiple biologic processes and genetic alterations as likely underlying causes.¹ Several lines of evidence link chronic inflammation both pathologically and epidemiologically² to PCa etiology. Administration of the heterocyclic amine PhIP (2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine) induces a chronic inflammatory response and results in increased hyperplasia and prostate intraepithelial neoplasia (PIN).³ Further pathologic evidence includes the prostatic lesion known as proliferative inflammatory atrophy, a precursor to mouse PIN (mPIN), characterized by atrophic epithelium and proliferation concomitant with an inflammatory response. Thought to be the prostatic response to repeated injury, proliferative inflammatory atrophy lesions are commonly seen in association with PIN and carcinoma.^{4,5} Epidemiologically, diets rich in fats and meat, but low in fruits and vegetables, have been linked to PCa incidence,⁶ possibly mediated through inflammation owing to increased prostate eicosanoid and prostaglandin (such as COX2) production in response to the high fatty acid intake.²

Chronic inflammation may lead to cancer through diverse mechanisms, including DNA damage resulting from increased reactive oxygen and nitrogen species, increased cytokine-mediated proliferation and downregulation of antitumor immune surveillance.⁷⁻¹⁰ The constant response to injury could lead to somatic alterations that ultimately give rise to cancer.^{9,11} Inflammation converges on several regulatory transcription factors, including signal transducer and activator of transcription 3 (STAT3) and nuclear factor- κ B (NF- κ B).¹²⁻¹⁴ NF- κ B is canonically activated through phosphorylation of its regulatory I κ Ba kinase complex (IKK). Activated IKK then phosphorylates the NF- κ B inhibitor I κ Ba, facilitating I κ Ba release from NF- κ B and subsequent

degradation. This release from inhibition enables NF- κ B nuclear translocation and gene transcription initiation.¹⁴ G-protein-coupled receptor (GPCR) signaling can interact with NF- κ B either to promote or inhibit an inflammatory response.^{15,16} For example, protein kinase C zeta (PKC ζ) can phosphorylate NF- κ B directly at Ser311^{17,18} enabling its interaction with CREB binding protein. The scaffold protein CARMA3 links GPCRs and NF- κ B by forming a complex with BCL10 and MALT1 (the CBM complex) capable of activating IKK as a result of G-protein alpha-q subunit activation.¹⁹⁻²¹ The Ras homolog family member A has also been linked to NF- κ B activation, but the underlying mechanisms remain unknown.¹⁶ Finally, oncogenes such as AKT/PKB also activate NF- κ B through the stimulation of intrinsic inflammatory pathways,²² in this case, through interaction with IKK via mammalian target of rapamycin in a phosphatase and tensin homolog-deleted environment.^{23,24}

PSGR, a member of the GPCR family, has a high structural homology to the olfactory GPCR subfamily.²⁵ Sequence analysis shows an open reading frame of 320 amino acids and localization to chromosome 11p15, a GPCR cluster region.^{26,27} Two separate promoters drive PSGR expression.²⁸ PSGR expression is limited to prostate tissue with increased transcript levels in prostate tumor samples.^{25,26,29,30} This overexpression was found in an average of 62% of prostate tissue/tumor samples, with the most significant difference being the benign tissue to PCa transition.^{27,31,32} This suggests that PSGR may have a role in the initiation of a specific subset of PCas and can be used as a potential biomarker in early PCa.

Here, we sought to elucidate the role of PSGR in PCa. Using a transgenic mouse model, we found that PSGR is expressed in the apical membrane of luminal epithelial cells. PSGR overexpression leads to an inflammatory reaction and increased reactive stroma

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that continues to varying degrees as mice age. Eventually PSGR transgenic mice develop mPIN but not PCa. In an orthotopic mouse model, PSGR overexpression in LnCaP cells promotes the formation of larger tumors. PCR screening shows increased NF- κ B target gene expression, suggesting NF- κ B regulation by PSGR. Furthermore, we demonstrated that NF- κ B activation is mediated through the phosphatidylinositol-3-kinase//Akt (PI3K/AKT) pathway. Our findings are the first to reveal a functional role for PSGR in the regulation of chronic inflammation and in the initiation of prostate pathogenesis using animal models.

RESULTS

Generation of PSGR transgenic mice

We have previously reported elevation of PSGR expression in human prostate tumors and PIN.³¹ To further extend these studies, we used Oncomine (Compendia Bioscience, Ann Arbor, MI, USA) to analyze microarray databases for PSGR expression in PCa. These databases show that PSGR expression is increased in PCa tissues when compared with normal prostate (Figure 1a), in accordance with our previous findings. Next, we sought to determine whether PSGR overexpression played a causal role in PCa initiation or progression. We generated a mouse model that specifically overexpressed human PSGR in the mouse prostate epithelium by inserting the PSGR coding region under the control of an ARR₂PB promoter into the mouse genome (Figure 1b). We confirmed insertion of the transgene in two different mouse

lines by PCR of genomic DNA and expression in the prostate by reverse transcription-PCR (Figures 1c and d). As shown in Figure 1e, overexpression of PSGR was highest in the ventral and anterior prostate with low levels in dorsolateral prostate. PSGR expression was localized to the apical membrane of luminal epithelial cells. Wild-type littermates showed no increase in PSGR.

PSGR overexpression induces inflammation and low-grade PIN in transgenic mice

We analyzed the PSGR transgenic mice at five different time-points (3, 6, 9, 12 and 15 months of age) and observed distinct morphologic changes at each age. Young (3 months) transgenic mice showed acute prostatitis with an increase in infiltrating inflammatory cells as well as epithelial cell shedding into the lumen (Figure 2a). This inflammatory reaction eventually subsided as the mice aged. At 9 months, however, we first observed low-grade mPIN lesions, with characteristic atypical cells (changed to a columnar shape, were taller, with more abundant cytoplasm, growing in foci of one or two layers, with hyperchromatic and occasionally pleomorphic nuclei) as described by Shappell *et al.*³³ and Park *et al.*³⁴ in the ventral prostate. These persisted and increased in number at later ages (12–15 months) (Figure 2b). The PIN lesions, however, did not appear to progress to high-grade PIN or localized adenocarcinoma. At the last two time-points, there was a persistent inflammatory reaction present in the stroma with visible thickening around the glands (Figure 2a). Proliferation

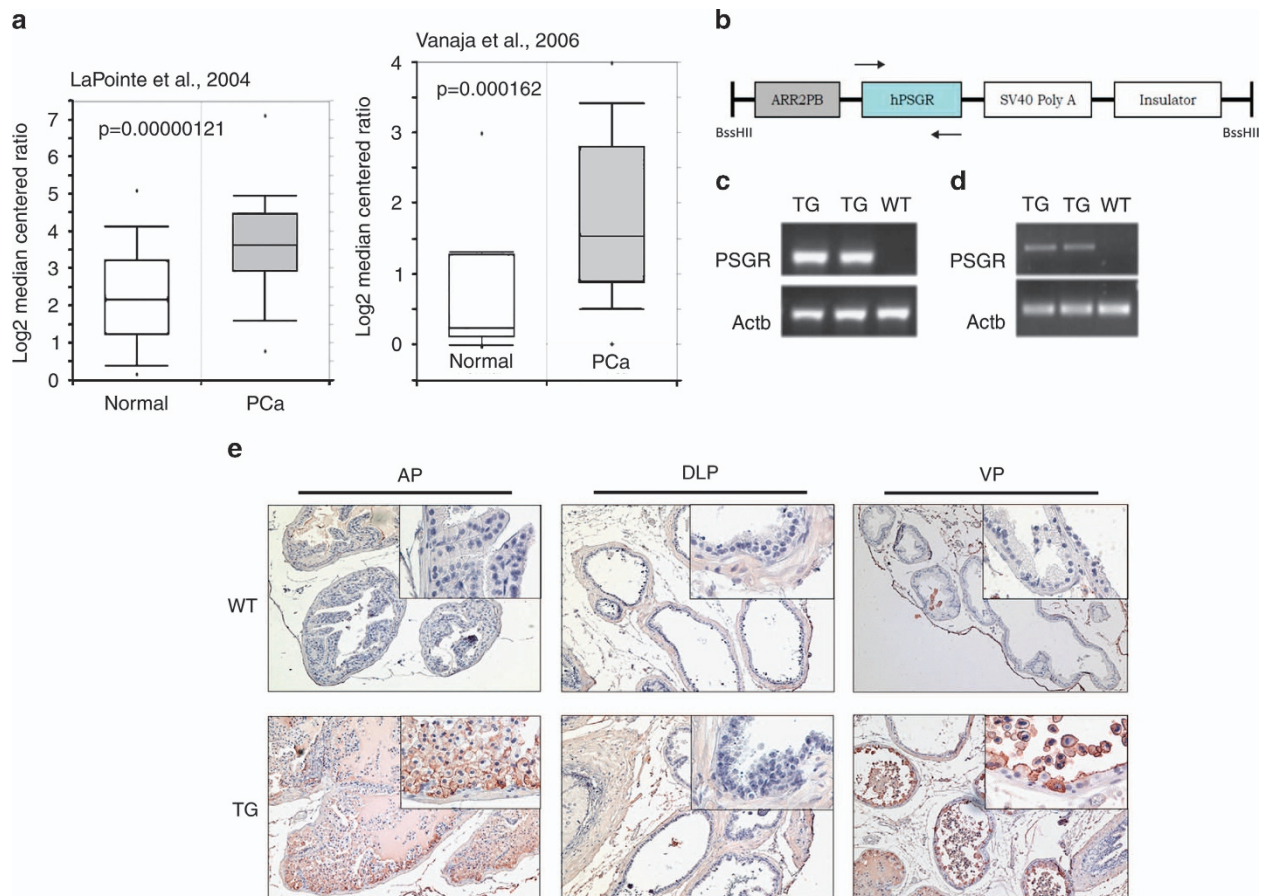


Figure 1. Generation of the PSGR transgenic mouse line. **(a)** Oncomine microarray data shows that PSGR is overexpressed in prostate cancer samples compared with normal prostate tissue. **(b)** Diagram of human PSGR insertion into mouse genome under ARR₂PB promoter control. **(c)** Genotyping PCR of mouse tail DNA to screen for PSGR transgenics. **(d)** Reverse transcription-PCR of mouse prostates screening for PSGR overexpression. **(e)** Immunohistochemical analysis of PSGR in all lobes at 12 months of age shows expression localized to apical membranes of epithelial cells in both ventral and anterior prostate.

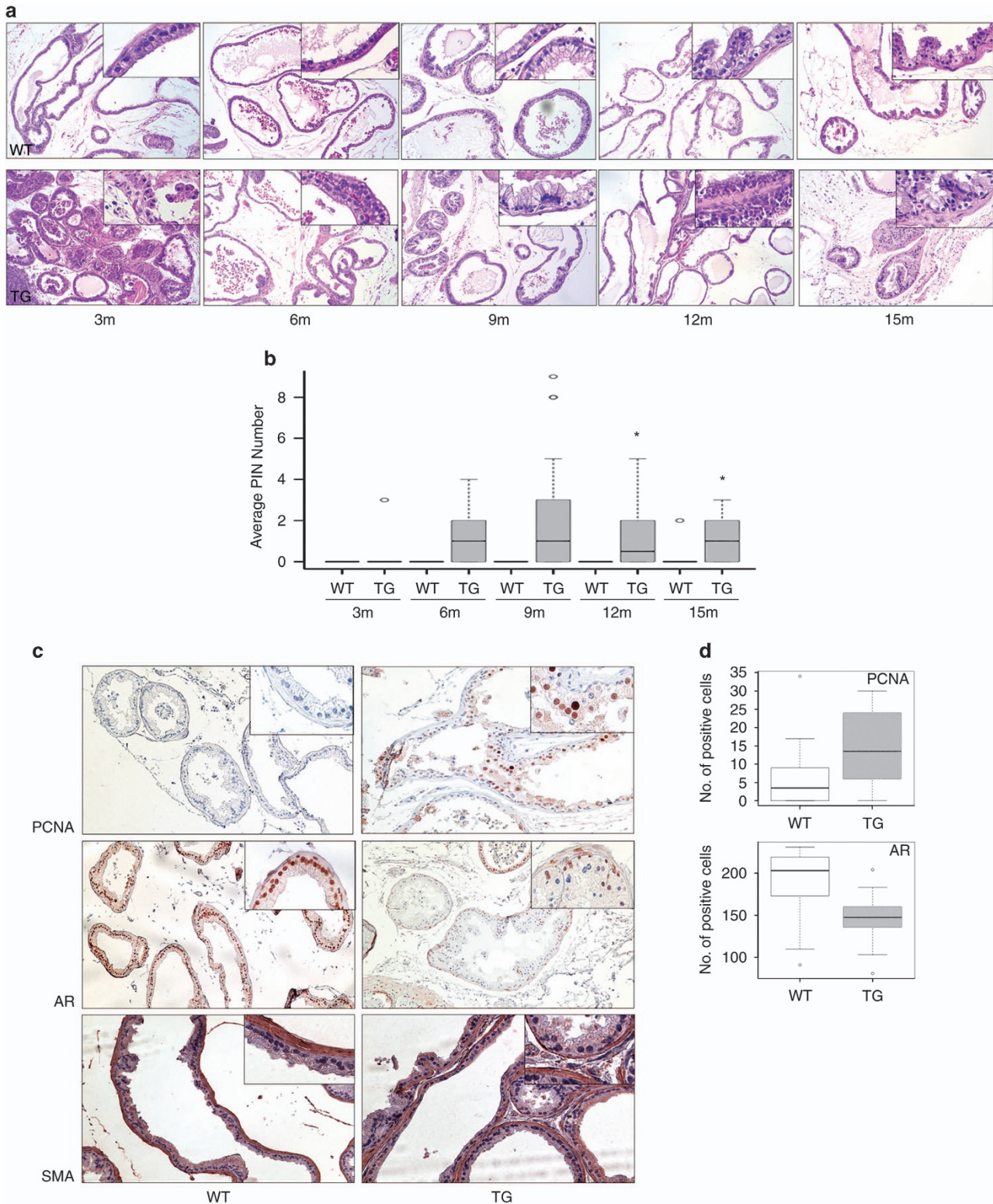


Figure 2. Development of mPIN and increased inflammatory stromal reaction in transgenic mice. **(a)** Hematoxylin and eosin sections of prostate glands from wild-type and transgenic littermates at different time intervals. Pictures show ventral lobe, magnification $\times 100$ (outer box) and $\times 400$ (inner box). Low-power view shows an inflammatory reaction and shedding of cells into the lumen consistent with chronic prostatitis, beginning at 3 months of age. This gradually progresses to LGPIN (mPIN I and mPIN II), seen at 9–12 months of age, with increased density and cellularity of the stroma. **(b)** Box plot shows median \pm upper and lower quartiles of mPIN lesions from 10 fields per mouse, 5 mice per genotype ($*P < 0.05$). **(c)** Characterization of PSGR transgenics, including proliferation (proliferating cell nuclear antigen (PCNA)), AR and smooth muscle actin (SMA) at 12 months of age. **(d)** Box plots show median \pm upper and lower quartiles of 10 fields per mouse, 6 mice per genotype. PCNA, $P = 0.09$; AR, $P = 0.01$.

markers showed increased proliferation in 12-month-old transgenic mice (Figures 2c and d). Also, there was a decrease in epithelial androgen receptor (AR) (Figures 2c and d) but no significant differences in smooth muscle actin expression in these mice (Figure 2c).

PSGR overexpression increases NF- κ B activity

Given the evidence of chronic inflammation in PSGR transgenic mice, we hypothesized that PSGR may regulate inflammatory pathways that will ultimately enhance mPIN initiation and potentially contribute to prostate cancer development in conjunction with additional oncogenic insults. First, we analyzed our mouse tissues for inflammatory gene expression using real-time PCR. As shown in Figure 3a, there was a significant increase in the expression of inflammatory genes under the control of NF- κ B, such as inflammatory cytokines (interleukin-1 β (IL-1 β) and tumor necrosis factor- α), proinflammatory interleukins (IL-23 and IL-12) and immunosuppressant interleukins (IL-10 and IL-4). Consistent with this, there was a significant macrophage infiltration in the gland lumens of PSGR transgenic mice as well as an increase in the number of pNF- κ B-positive cells (Figures 3c and d). Finally, we observed NF- κ B activation in older (15-month-old) transgenic

mice as indicated by elevated phosphorylation of NF- κ B and its upstream regulator the IKK complex (Figure 3b).

PSGR activates NF- κ B transcription factor

To further evaluate the relationship between PSGR and NF- κ B in prostate cancer cells, we examined several human cell lines (LnCaP, PC3, DU145, VCaP, LAPC4, MDA Pca2a and MDA Pca2b) for PSGR expression as well as relative phosphatase and tensin homolog levels. Only LnCaP and MDA Pca2a cells expressed PSGR (Figure 4a). AR mRNA levels were also examined for the different cell lines studied (Supplementary Figure S1A). We generated cells stably overexpressing PSGR by transducing LnCaP cells with a lentiviral vector for PSGR expression (LnCaP-PSGR cells). As shown in Figure 4b, there was a significant increase in PSGR expression in cells transfected with the PSGR virus. AR mRNA levels showed no significant changes upon PSGR overexpression (Supplementary Figure S1B). When PSGR was overexpressed in immortalized, non-tumorigenic PNT1a cells, which do not normally express PSGR (Supplementary Figure S3A),³⁵ we noted an increase in proliferation but no change in cell migration (Supplementary Figures S3C and D). To further confirm regulation of NF- κ B activity by PSGR, we performed a luciferase assay to test for NF- κ B promoter activation

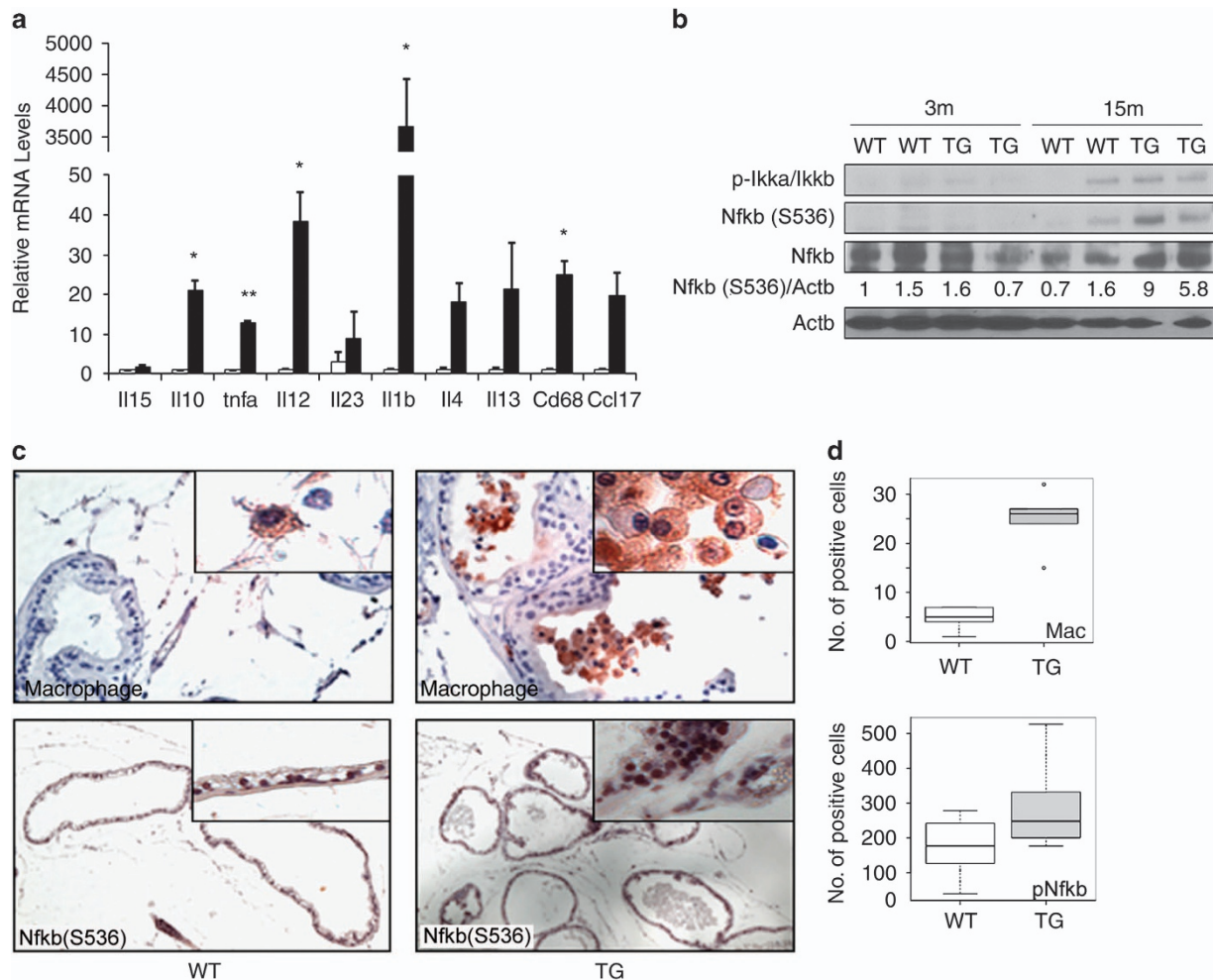


Figure 3. PSGR overexpression activates inflammatory signaling pathways with NF- κ B phosphorylation. **(a)** Real-time PCR analysis of ventral prostates shows an increase in NF- κ B-dependent cytokines and ILs. Data show mean \pm s.e.m. of three mice per genotype for three different experiments. * $P=0.01$ and ** $P=0.001$. **(b)** Western blot analysis shows increased phosphorylation of IKK1/IKK2 accompanied by the activation of NF- κ B in transgenic mice. **(c)** Immunohistochemistry shows increased infiltration of macrophages (Mac) (P -value=0.001) as well as an increased number of cells with active NF- κ B in their nuclei (S536) (P -value=0.04). **(d)** Box plots show median \pm upper and lower quartiles of 10 fields per mouse, 5 mice per genotype.

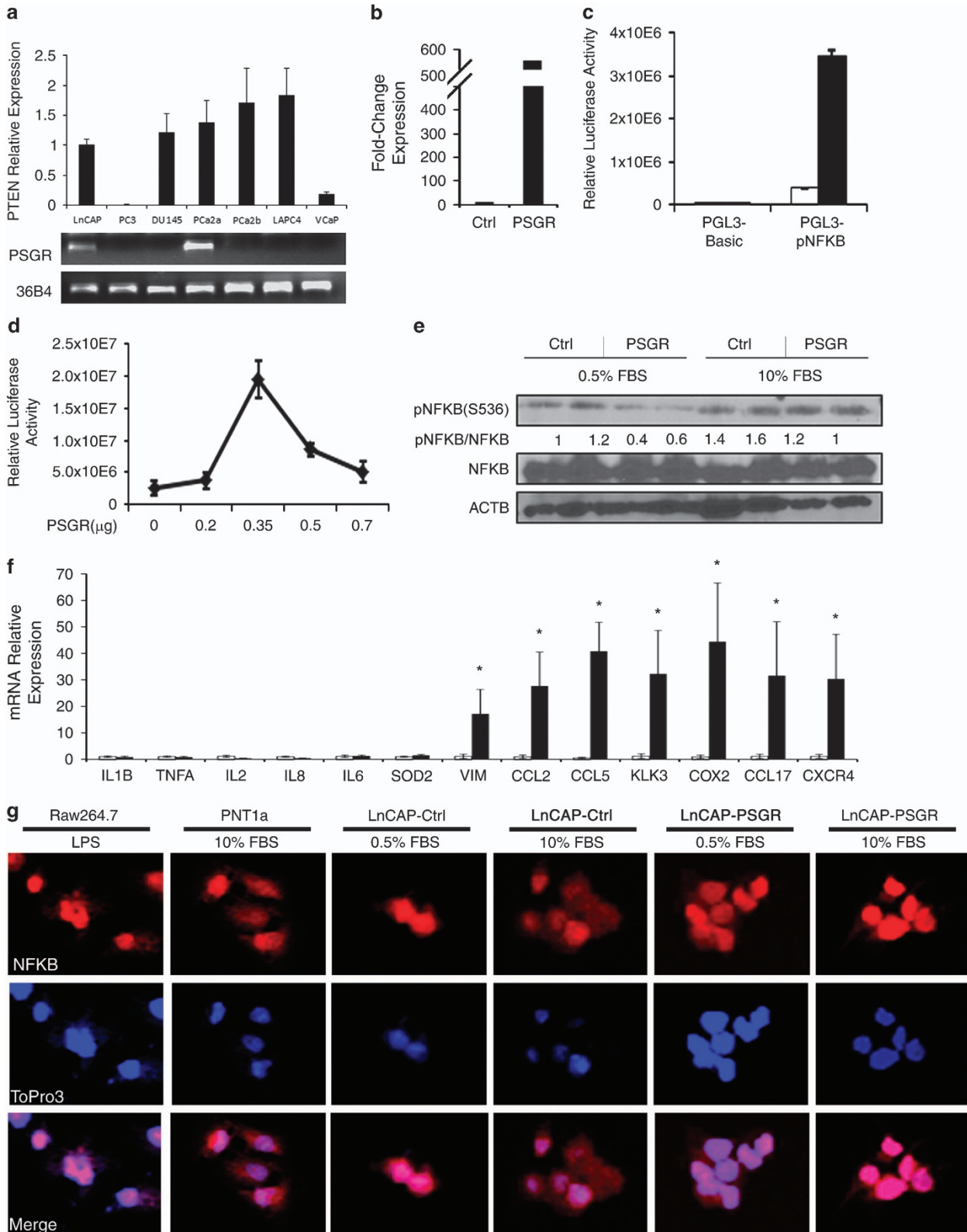


Figure 4. PSGR overexpression activates NF-κB. **(a)** PSGR and phosphatase and tensin homolog (PTEN) expression in prostate cancer cell lines by RT and quantitative PCR (qPCR). Bar graph shows qPCR of PTEN expression levels as mean ± s.e.m. of three mice per genotype. **(b)** Relative PSGR gene expression of LnCAP-overexpressing cells. Data are shown as fold change relative to control. **(c)** Luciferase assay in HEK293 cells shows significant activation of NF-κB with PSGR overexpression. White bar indicates HEK293-Ctrl cells; black bars indicate HEK293-PSGR cells. **(d)** NF-κB activity is dose-dependent on increasing concentration of PSGR plasmid DNA transfected into HEK293 cells. **(e)** Western blot shows NF-κB activation upon 10% FBS stimulation in LnCAP cells. **(f)** Activation of NF-κB in LnCAP-PSGR-overexpressing cells induces expression of several NF-κB target genes including cytokines and cytokine receptors (**P*-value < 0.0002). **(g)** Serum stimulation in PSGR-overexpressing cells induces nuclear localization of NF-κB.

upon serum stimulation. Human embryonic kidney 293 (HEK293) cells lack endogenous PSGR expression (data not shown), and exhibit minimal NF- κ B promoter activity. Upon exogenous PSGR expression, we observed a significant activation of NF- κ B in HEK293 cells (Figure 4c). NF- κ B activity increased with increasing PSGR expression up to a saturation point for this activity (Figure 4d). Also, NF- κ B phosphorylation (S536) was increased upon 10% fetal bovine serum (FBS) stimulation in LnCaP – PSGR cells (Figure 4e), confirming NF- κ B pathway activation. Next, we examined the RNA levels of different inflammatory pathway genes. We confirmed upregulation of cytokines, including CCL2, CCL5 and COX2, but saw no production of ILs, including IL-1b, IL-2 and IL-6 (Figure 4f). Finally, in response to serum stimulation, we observed nuclear translocation of NF- κ B in PSGR-expressing LnCaP cells (Figure 4g), further supporting NF- κ B activation downstream of PSGR.

PSGR enhances oncogenesis in prostate tissue

To test PSGR function in tumor progression, we injected stable LnCaP-PSGR cells directly into the prostates of nu/nu mice and analyzed orthotopic prostate xenografts 1 month after surgery ($n = 7$ mice per group). Although we did not observe a statistically significant increase in orthotopic tumor incidence in LnCaP-PSGR cells (control: 3/7 (42% incidence); PSGR: 5/7 (72% incidence)), supporting our transgenic mouse data suggesting that PSGR alone is not an oncogene, there was a fivefold increase in tumor prostate mass upon PSGR overexpression (control 1.05 g (95% confidence interval: 0.296–1.817) and PSGR 5.34 g (95% confidence interval: 1.633–9.052) ($P = 0.023$)) (Figures 5a–c). This supports a role for PSGR in promoting prostate tumor progression, and argues against the PSGR elevation observed in human prostate cancers being merely a bystander effect. Histologic analysis of these tissues showed altered morphologies, including small tumorigenic foci, in all injected prostates of both groups, but only LnCaP-PSGR-injected mice showed predominant adenocarcinoma in all prostatic lumens (Figure 5d). LnCaP-PSGR-injected prostate showed increased proliferation in both the stromal and epithelial compartments (Figure 5e). These mice also had increased inflammatory cell infiltration (Figure 5f) compared with controls, in accord with the data from PSGR transgenic mice (Figure 3c), as well as increased AR expression in the stroma (Figure 5g).

PSGR activates NF κ B through the AKT pathway

LnCaP-PSGR cells activated both AKT and NF- κ B upon 10% FBS stimulation (Figure 6a). Transgenic mice also showed activation of AKT (Figure 6b). To test whether AKT mediates PSGR regulation of NF- κ B, we stimulated LnCaP cells (LnCaP-Ctrl and LnCaP-PSGR) with 10% FBS for 20 min in the presence or absence of specific pathway inhibitors including MK-2206 (AKT), H-89 (protein kinase A (PKA)), GF109203X (PKC), C3 (Ras homolog family member A) and SB203580 (p38), and tested for NF- κ B activation. We found downregulation of NF- κ B activity with AKT inhibitor (MK-2206) treatment ($P < 0.05$) (Figure 6c). Other GPCR canonical pathways, including the PKA-cAMP-CREB pathway and the PLC-PKC-Ca²⁺ pathway, were also analyzed for NF- κ B activity. The former showed increased NF- κ B phosphorylation upon PKA inhibition, while the latter showed a mild decrease in NF- κ B phosphorylation in the presence of PSGR (Figure 6d). We tested the functional relationship of PSGR, AKT and NF- κ B using two approaches. First, through quantitative PCR, we measured NF- κ B-dependent gene expression upon pathway inhibition and observed a decrease in NF- κ B target gene (CCL2, CCL5, CXCR4 and Vimentin) expression with AKT inhibition, only in PSGR-overexpressing cells (Figure 6e). Second, we stimulated our cells with 10% FBS with or without AKT inhibitor and observed NF- κ B intracellular localization. Addition of the inhibitor abrogated NF- κ B nuclear translocation in LnCaP-PSGR cells (Figure 6f), suggesting that PSGR induces an

inflammatory reaction through PI3K/AKT-mediated NF- κ B activation in the prostate.

DISCUSSION

The expression pattern observed for PSGR, which is highly specific to human prostate and significantly increased in PCa,³¹ raised the question whether PSGR has an important role in the initiation of this disease. We hypothesized that indeed, owing to its changes in expression, PSGR possesses oncogenic properties that aid in the development of prostate cancer. PSGR overexpression induced a prostatitis reaction relatively early in life (3 m), with inflammatory cell infiltration persisting beyond this time point. At 9 months of age, transgenic mice developed mPIN and an increased reactive stroma. However, even beyond 12 months of age, the prostatic lesions in these mice did not progress to adenocarcinoma, suggesting that PSGR can alter the prostate microenvironment toward an inflammatory phenotype and induce initial prostatic lesions, and also that further genetic mutations may be needed to produce prostate cancer. Chronic inflammation is one of several biologic processes thought to have a key role in prostate cancer initiation.¹ It can increase proliferative signals of both epithelial and stromal cells, and also create an oxidative stress-prone environment that could easily facilitate the appearance of new genomic alterations, including mutations that drive further disease progression.³⁶ Using prostate xenografts, we demonstrated a strong inflammatory and tumor-promoting effect of PSGR overexpression in human cancer cells. PSGR-overexpressing cells produced a larger tumor burden and had increased stromal proliferation. Inflammatory cell infiltration was also much higher in PSGR-overexpressing tumors when compared with controls, similar to what was seen in PSGR transgenic mouse models. Expression of AR was increased in stromal cells of PSGR-overexpressing tumors compared with controls, suggesting a possible relationship between PSGR and AR. Analysis of several prostate cancer cell lines showed different levels of mRNA expression for AR, but overexpression of PSGR had no direct effect on AR mRNA levels, suggesting an indirect or posttranslational relationship between these proteins. While the nature of the interaction between the AR with PSGR in prostate cancer remains a crucial unresolved question, taken together, these data suggest that PSGR can function to promote tumor formation and that it may use modulation of inflammatory pathways in conjunction with AR to cause this effect.

The transcription factor NF- κ B is central to inflammation. NF- κ B signaling has been linked to prostate cancer cell survival and disease progression to hormone independence.³⁷ Its activity is reported to be higher in androgen-independent cell lines as well as in metastatic disease, and it correlates with poor prognosis,^{37,38} but little has been studied on the role of NF- κ B in initial phases of prostate disease. We were able to confirm the regulatory relationship between PSGR and NF- κ B in the prostate by showing PSGR-dependent NF- κ B activity and phosphorylation upon serum stimulation. We observed increased expression of NF- κ B-dependent cytokines such as CCL2, CCL5 and the cytokine receptor CXCR4 in epithelial LnCaP-PSGR-overexpressing cells, but saw no significant changes in IL levels. However, we did find significantly increased levels of ILs when we analyzed the prostate tumor samples from mice. This suggests that the lack of ILs in the epithelial cells could be because it is not the epithelium but the infiltrating inflammatory cells that ultimately produce these mediators of inflammation. We also showed nuclear localization of NF- κ B upon 10% FBS stimulation in the presence of PSGR, and that the presence of PSGR, with or without 10% FBS, induced p65 phosphorylation (S536). Protein levels do not directly correlate with pAKT (S473), but we found increased pAKT levels in transgenic mice at 12 months of age, suggesting it may have a potential role in PSGR-induced mPIN. When AKT is blocked with

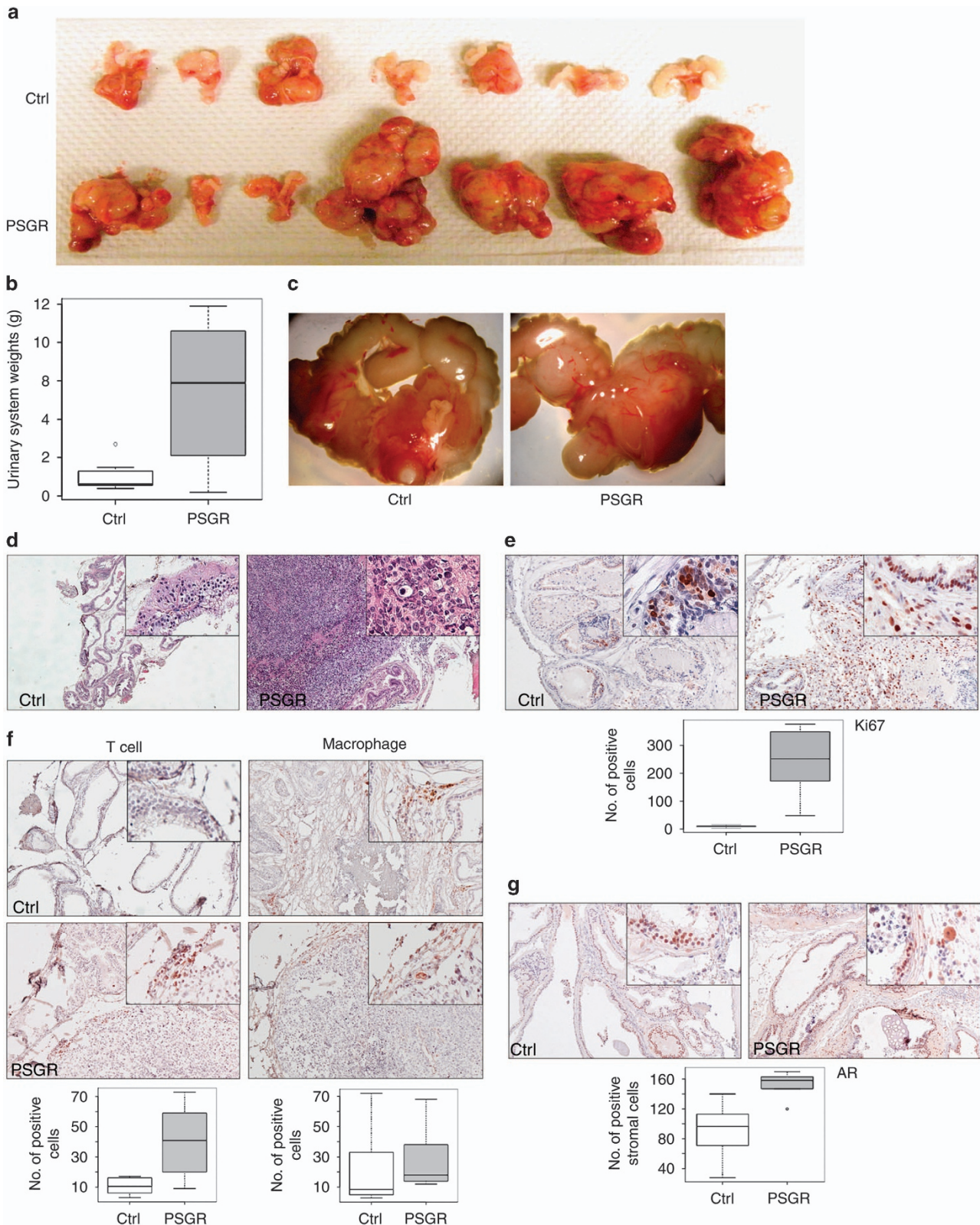
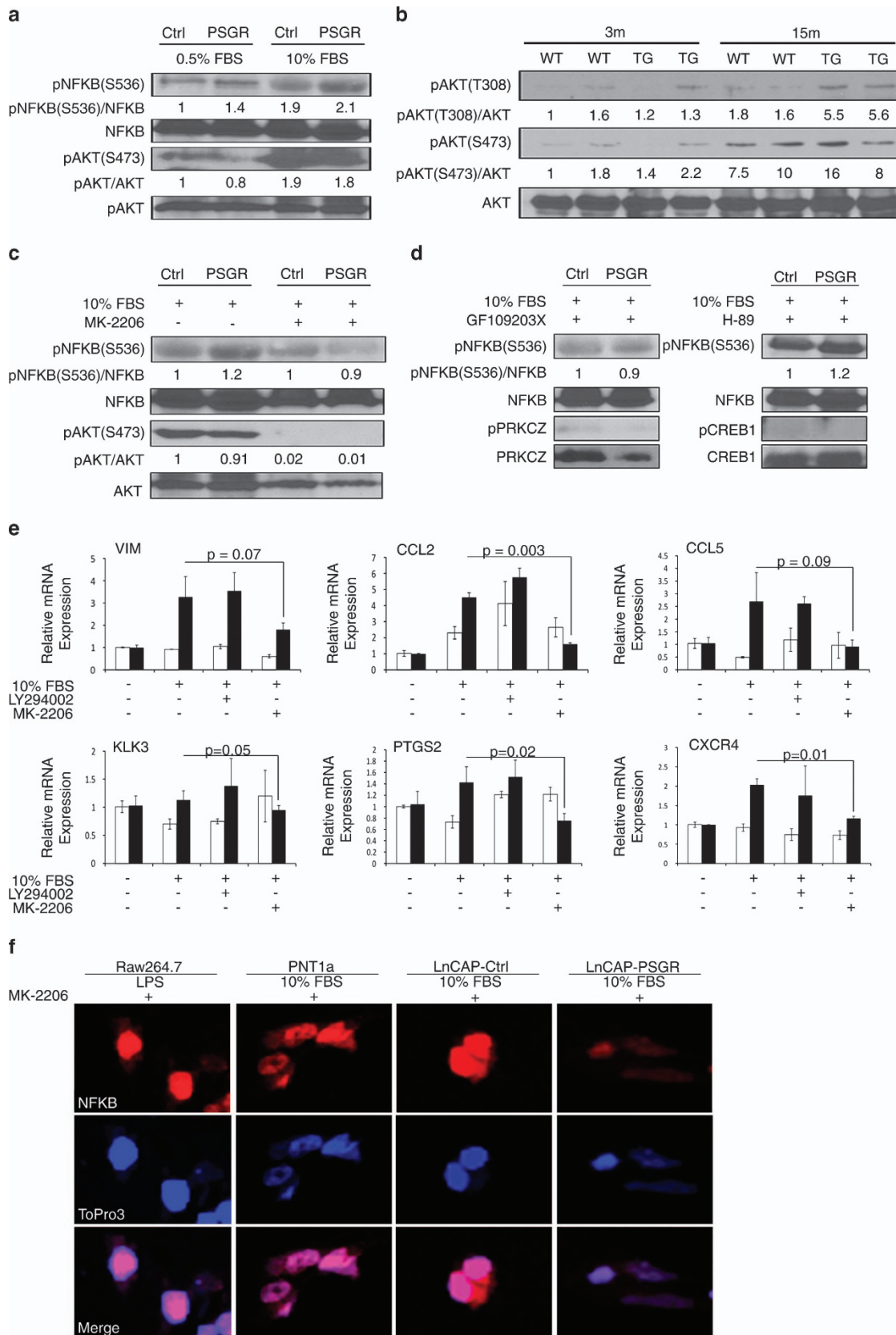


Figure 5. PSGR enhances oncogenesis in prostate tissue. **(a)** Prostate orthotopic cell implantation shows PSGR-overexpressing cells produce larger tumors than controls. **(b)** Urinary system weights are in accordance with tumor progression ($*P=0.023$). Data of $n=7$ per group. **(c)** Representative images of tumor sizes in xenografts injected with LnCAP-Ctrl vs LnCAP-PSGR cells. **(d)** Morphologic differences between xenograft tissues show tumor formation in the presence of PSGR ($n=7$ per group). Data show representative median box plots \pm upper and lower quartiles (P -value = 0.001). **(e)** PSGR xenografts show increased proliferation compared with Ctrl prostates ($n=7$ per group). Data show representative median box plots \pm upper and lower quartiles (P -value < 0.0001). **(f)** Immunohistochemistry of xenograft tissue for T cells (CD3) and macrophages (Mac) shows elevated T-cell infiltration in PSGR-overexpressing xenografts ($n=7$, $P < 0.05$). **(g)** Immunohistochemistry of xenograft tissue for AR shows increased stromal AR expression in PSGR-overexpressing xenografts ($n=7$, $P < 0.05$).

an AKT-selective inhibitor, MK-2206, p65 (S536) further decreased in the presence of PSGR. Inhibition of AKT also functionally blocked NF- κ B-dependent cytokine production and nuclear

localization in our PSGR-overexpressing cell lines as well. We analyzed other canonical GPCR pathways for NF κ B activation and found that inhibition of the PKA-cAMP pathway increased NF- κ B



phosphorylation, in accordance with published results.¹⁶ High concentrations of GF109203X (6 μ M), sufficient to inhibit all PKC isoforms, showed a modest decrease in NF- κ B phosphorylation. A possible link between PKC and AKT cannot be ruled out with our data. Nonetheless, PKC inhibition does not affect AKT phosphorylation levels nor does AKT inhibition affect PKC phosphorylation (Supplementary Figure S2B), suggesting that this could represent an alternate pathway directly between PKC and NF- κ B.¹⁸ However, we cannot rule out nonspecific effects of GF109203X on AKT at this concentration or the possibility of biased PSGR ligands in the serum capable of activating NF- κ B through PKC. Further experiments are needed to elucidate the exact role of PKC downstream of PSGR.

AKT can activate NF- κ B in the presence of androgens to induce normal prostate epithelial cell proliferation.³⁹ Overexpression of PSGR can thus cause prolonged NF- κ B activation through AKT, leading to chronic inflammation and increased cell proliferation, linking two important survival pathways to initial disease. Importantly, PSGR overexpression modulates these pathways to change the prostate microenvironment before disease onset. PSGR overexpression was also clinically correlated to higher non-organ-confined (pT3) disease and higher PSA levels,³² suggesting that it may have a further role in prostate cancer progression, with possible links to the AR. Our work for the first time uses mouse models to demonstrate NF- κ B linkage to premalignant prostate lesions as a direct result of GPCR activation. We found no activation of NF- κ B in the presence of PSGR by stimulating the receptor with β -ionone (data not shown), its currently described agonist.⁴⁰ This is in agreement with the hypothesis that GPCRs can bind to multiple ligands, which have different potentials for activating downstream signaling pathways.¹⁵ It would be of significant interest to identify the ligand(s) that can activate NF- κ B through PSGR, which would represent an attractive new therapeutic target both for initial and potentially more advanced stages of prostate cancer.

MATERIALS AND METHODS

Generation of PSGR transgenic mice

All experiments involving animals were conducted according to a protocol approved by the Texas A&M Health Science Center institutional animal care and use committee. The human PSGR cDNA was cloned into the pBSK-SSI plasmid under the control of a rat ARR2PB promoter with a poly A site and a chicken insulator sequence. The transgenic fragment was excised by digestion with *Bss*HI enzyme and an 8.5 kb fragment was used for pronuclear microinjection into FVB/N mice. Genomic DNA was purified from the tails of founder mice (F1) at day 14 after birth and screened with human PSGR-specific primers: PSGR-ClonF (5'-ATGGTGGATCCCAATGGCAA-3') and PSGR-ClonR (5'-CTAGGGCTCTGAAGCGTGTG-3'). To test for PSGR expression, total RNA was isolated from the prostate, reverse transcribed and amplified with PSGR-specific primers. Screening was performed through genomic DNA extraction from tail tissue digested with proteinase K (20 mg/ml) overnight and then heat-inactivated. PSGR genotyping primers include: PSGR-F (5'-CCGATATCGCCACCACCATGAGTTCCTGC-3'); PSGR-R (5'-CCGATATCGGGTCACTTGCCCTCCAC-3'). Animals were inbred for three generations (F3) and only littermates were used for experimental comparisons.

Reverse transcription-PCR and real-time PCR

RNA was extracted using TRIzol (Ambion-LifeTechnologies, Grand Island, NY, USA). A total of 3 μ g RNA was used for cDNA, together with a PSGR-specific primer sequence (5'-TGAGTCTCAGCTTGACTGAGC-3') and oligo(dT) primers (Promega, Madison, WI, USA). PSGR expression was later detected using real-time PCR primers: rt-OR51E2-F1 (5'-TTCCTGCAACTTCACACATGCCAC-3') and rt-OR51E2-R1 (5'-TGGATGTGGATAAGGCCAGGTCAA-3'). Other real-time PCR primers used are included in the Supplementary Data. Inhibitors used included: H-89 (1 μ M) (Enzo Life Sciences, Farmingdale, NY, USA), GF109203X (6 μ M) (Tocris Biosciences, Bristol, UK), MK-2206 (1 μ M) (Selleck, Houston, TX, USA), C3 (0.5 mg/ml), Y27632 (Selleck) and SB239063 (1 μ M) (Calbiochem, Millipore, Billerica, MA, USA). All inhibitors were used as per the manufacturer's recommended concentrations.

Histology

Prostate tissue was dissected together with the genitourinary system and separated by lobes under the microscope. Tissues were fixed in zinc formalin (Richard-Allan Scientific, Kalamazoo, MI, USA) for 7 h and transferred to 70% ethanol for 48 h. Next, samples were dehydrated through graded alcohols, paraffin embedded and cut into 5 μ m sections. Morphologic analysis was performed after hematoxylin/eosin staining. For immunohistochemistry, slides were baked at 65 $^{\circ}$ C for 3 h and rehydrated in graded alcohols (100%, 95%, 85%, 70% and water), and then boiled for 30 min in preheated 10 mM sodium citrate. Vectastain ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) was used to stain samples, according to the manufacturer's instructions. Antibodies used included: PSGR (1:1000; ab13383; Abcam, Cambridge, MA, USA), AR (1:200; N-20; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), CK5 (1:1000; ab53121; Abcam), CK8 (no dilution required; rdi-pro61038; Fitzgerald, Acton, MA, USA), α -smooth muscle actin (1:1000; A2547; Sigma, St Louis, MO, USA), NF- κ B-p65 (S276, 1:100; no. 4764; Cell Signaling Technologies (CST), Danvers, MA, USA), Ki67 (1:500; NB110-89719; Novus Biochemicals, Littleton, CO, USA), macrophage (1:200; ab22506; Abcam). Photomicrographs were taken using a Nikon Eclipse 80i microscope (Nikon, Tochigi, Japan) and acquired using NIS-Elements BR 3.2. For immunocytochemistry, cells were seeded on sterilized Histobond Microscope Slides (VWR, Radnor, PA, USA) and fixed with zinc formalin for 15 min. Cells were then permeabilized with phosphate-buffered saline containing 0.25% Triton X-100 for 10 min and blocked in 1% bovine serum albumin for 1 h. Cells were left in primary antibody overnight at 4 $^{\circ}$ C. The next day, cells were washed and incubated in secondary antibody, washed, stained in To-Pro3 (Invitrogen, Carlsbad, CA, USA) for 10 min and mounted. Antibodies used include NF- κ B-p65 (1:100; no. 4764; CST). Immunocytochemical photomicrographs were taken using a Zeiss Axiovert 200 M microscope with Zeiss EC Plan Neofluar lenses equipped with a Zeiss LSM 510 camera and using the manufacturer's LSM 510 image acquisition software program (Zeiss, Jena, Germany).

Immunoblotting

Protein was extracted from tissue and cell samples using RIPA buffer supplemented with proteinase inhibitors (1:1000) aprotinin, leupeptin, pepstatin A, PMSF, EDTA, EGTA, Na fluoride and Na orthovanadate (Sigma). Samples were then diluted in 5 \times sodium dodecyl sulfate buffer and boiled for 5 min. Fifty micrograms of protein was used per sample. Antibodies used include β -actin (1:1000; Santa Cruz Biotechnologies), I κ B α (1:1000; no. 4814; CST), phospho-I κ B (S176/180) (1:1000; no. 2697; CST), phospho-p65 (S536) (1:1000; no. 3033; CST), p65 (1:1000; no. 4764; CST), phospho-PKC ζ (1:1000; no. 9378; CST), PKC ζ (1:1000; no. 9368; CST), phospho-CREB (1:1000; no. 9198; CST), CREB (1:1000; no. sc-186; Santa Cruz Biotechnologies), phospho-AKT (S473) (1:1000; no. 4060; CST), phospho-AKT (T308) (1:1000; no. 9275; CST), pan-AKT (1:1000; no. 4691; CST). Western blots were quantified using ImageJ software (<http://imagej.nih.gov/ij/>), according to the software's instructions. Total protein levels were used as normalization controls.

Figure 6. PSGR regulates NF- κ B activity through the PI3K/AKT pathway. (a) NF- κ B and AKT are phosphorylated upon 10% FBS stimulation of LnCaP (Ctrl) and LnCaP-PSGR (PSGR) cells. (b) Prostates from transgenic mice show increased AKT phosphorylation at 15 months of age. (c) Treatment of cells with AKT inhibitor MK-2206 decreases NF- κ B phosphorylation in the presence of PSGR. (d) Treatment of cells with PKC inhibitor (GF109203X) slightly decreased NF- κ B phosphorylation, whereas PKA inhibitor (H-89) treatment increased NF- κ B phosphorylation. (e) AKT inhibition (MK-2206) blocks transcription of NF- κ B-dependent target genes, including cytokines CCL2 and CCL5, as well as the EMT marker Vimentin (VIM). PI3K inhibition (LY294002) does not show the same effect. Data show mean \pm s.e.m. of three repeats per experiment for three different experiments. White bars denote LnCaP-Ctrl cells and black bars show LnCaP-PSGR cells. (f) Nuclear localization of NF- κ B is blocked upon AKT inhibition.

Lentivirus cell lines

The 293FT, HEK293 and LnCaP cell lines were purchased from ATCC (Manassas, VA, USA) and passaged for fewer than 6 months. PNT1A cells were a gift from Dr Fen Wang (Texas A&M Health Science Center, Houston, TX, USA). All stable cells were kept in RPMI-1640 growth media (Hyclone, Logan, UT, USA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA). For overexpression, hPSGR was cloned into the pLVX lentivirus plasmid (Clontech Laboratories, Mountain View, CA, USA) and transfected together with packaging plasmids pMD2.G (Addgene, Cambridge, MA, USA) and psPAX2 (Addgene) using calcium phosphate (Clontech CalPhos Transfection Kit, Clontech Laboratories) into 293FT cells (Invitrogen). Cloning primers included: PSGR-ClonF (5'-TGCGAATTCATGAGTCC TGCAACTTAC-3') and PSGR-R (5'-TATTCTAGATCACTTGCTCCACAG CCTG-3'). Infection efficiency was measured under fluorescence microscopy for the presence of green fluorescent protein-positive cells. Cell media were then harvested, supplemented with Polybrene (Millipore) and used to infect LnCaP cells directly.

Luciferase assays

PSGR was cloned into pcDNA3.1 expression plasmid (Invitrogen) using specific primers (pcDNAPSGR-F: 5'-TTTTCTAGAGTCGACAGTGTGACCTCACC CTCTCCAGTC-3'; pcDNA-PSGR-R: 5'-TGGAAGCTTAAGGGTCACTTGCTCCACAG-3'); the RTPS1 plasmid (for membrane localization) was a gift from Dr H Matsunami (Duke University, Durham, NC, USA), and NF- κ B-Luc (SA Biosciences, Qiagen, Valencia, CA, USA) and pGL3-Basic (Promega) were purchased. HEK293 cells were cultured in Dulbecco's modified Eagle's medium (Hyclone) with 10% FBS. They were seeded (5000 cells per well) in 24-well plates and transfected with 0.8 μ g DNA (pcDNA/PSGR, RTPS1 and luciferase plasmid) with Lipofectamine 2000 for 7 h and left incubating for 48 h. Afterwards, cells were serum starved for 3 h in Dulbecco's modified Eagle's medium with 0.5% FBS and subsequently stimulated with 10% FBS as described for specific experiments.

Xenograft assays

PSGR-overexpressing LnCaP cells were prepared at 100 000 cells per 100 μ l in a mixture of RPMI-1640 (Hyclone) with 10% FBS (Atlanta Biologicals) (50%) and growth factor-enriched Matrigel (50%) (BD Biosciences, San Jose, CA, USA). Nu/nu mice (Charles River Labs, Wilmington, MA, USA) were anesthetized, and an incision was made in the abdominal skin and musculature to expose the genitourinary tract. Cells (100 μ l per mouse) were injected directly into a single site of the ventral prostate. Mice were observed for prostate cancer progression for a month after surgery. Mice were then killed and tissues processed for histologic examination.

Statistical analysis

Data analysis is shown as mean with standard error. Statistical differences between groups were compared using two-tailed Student's *t*-test. *P*-values and confidence intervals were obtained using the R statistical software package (R Foundation, Vienna, Austria).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MR: experimental design, data collection, data analysis and manuscript preparation; WL: data collection; JW: experimental design and data collection; ZY: data collection; SS: data analysis and manuscript preparation; ML: experimental design, data analysis, funding and final approval of manuscript.

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