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PI3K-AKT-mTOR pathway is dominant over androgen receptor signaling in prostate cancer cells

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Abstract. *Background:* Androgen receptor (AR) and the phosphatidylinositol-3 kinase (PI3K) signaling are two of the most important pathways implicated in prostate cancer. Previous work has shown that there is crosstalk between these two pathways; however, there are conflicting findings and the molecular mechanisms are not clear. Here we studied the AR–PI3K pathway crosstalk in prostate cancer cells *in vitro* as well as *in vivo*.

Methods: Quantitative PCR, Western analysis, reporter assays, and proliferation analyses *in vitro* and *in vivo* were used to evaluate the effect of PI3K pathway inhibition on AR signaling and cell growth.

Results: Transcriptional activity of AR was increased when the PI3K pathway was inhibited at different levels. In the androgen responsive prostate cancer cell line LNCaP, androgen and the mTOR inhibitor rapamycin synergistically activated androgen target genes. Despite increased androgen signaling, rapamycin treatment reduced LNCaP cell growth; the AR antagonist bicalutamide potentiated this effect. Furthermore, the rapamycin derivative CCI-779 reduced the growth of CWR22 prostate cancer xenografts while increasing AR target gene expression.

Conclusion: These findings suggest that inhibition of the PI3K pathway activates AR signaling. Despite the increase in AR signaling which has proliferative effects, the result of PI3K pathway inhibition is antiproliferative. These findings suggest that the PI3K pathway is dominant over AR signaling in prostate cancer cells which should be considered in developing novel therapeutic strategies for prostate cancer.

Keywords: Androgen receptor, PI3K pathway, mTOR

Abbreviations

AF	Activation function;
AR	Androgen receptor;
ARE	Androgen response element;
CT	Charcoal treated;
DBD	DNA-binding domain;
i.p.	Intraperitonially;
LBD	Ligand binding domain;
LTR	Long terminal repeat;
MMTV	Mouse mammary tumor virus;
mTOR	Mammalian target of rapamycin;
NTD	N-terminal domain;
PCa	Prostate;
PI3K	Phosphatidylinositol-3 kinase;
PIP_2	Phosphatidylinositol 4,5-bisphosphate;
PIP_3	Phosphatidylinositol 3,4,5-trisphoshate;
PKB	Protein kinase B;
PSA	Prostate specific antigen;
PTEN	Phosphatase and tensin homolog deleted; on chromosome ten;
RTK	Receptor tyrosine kinase;
RTV	Relative tumor volumes;
STAMP	Six transmembrane protein of prostate;
TBP	TATA-binding protein.

1. Introduction

Prostate cancer (PCa) remains the most diagnosed non-cutaneous cancer and is the second leading cause of cancer-related deaths in men in most western in-

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dustrialized countries [1]. The initial stages of prostate cancer are regulated by androgens and the androgen receptor (AR); thus, decreasing the levels of circulating androgens and inhibition of AR have been the main focus for treatment of hormone-dependent prostate cancer [2,3]. Unfortunately, in most patients, androgen-independent PCa develops for which there is no effective therapy available at present. AR signaling is also important in the transition from androgen-dependent to androgen-independent PCa [4].

AR is a ligand-dependent transcription factor and a member of the nuclear receptor superfamily (for a review, see [5]). Upon ligand binding, AR translocates to the nucleus and binds androgen response elements (AREs) through a highly conserved DNA-binding domain (DBD). AR then mediates transcriptional activation through two transactivating domains, activation function-1 (AF-1) and AF-2, localized in the N-terminal domain (NTD) or the ligand binding domain (LBD) within the C-terminus, respectively (reviewed in [5]).

In addition to AR signaling, other signaling pathways and their crosstalk with AR signaling may also have a critical role in prostate cancer progression and development (for a review, see [6]). One of these is the PI3K/AKT/mTOR pathway that has been implicated in the development of many cancers, including prostate cancer (for reviews, see [7,8]). Upon activation of receptor tyrosine kinases (RTKs) on the cell surface, RTKs interact with p85 Src homology 2 (SH2) domains, and recruits PI3K to the cell membrane. When activated, PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-trisphoshate (PIP_3) which acts as a secondary messenger that promotes growth, proliferation and survival (for a review, see [9]).

The tumor suppressor phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a plasma membrane associated lipid phosphatase that acts as an antagonist of the PI3K pathway (for a review, see [10]). It dephosphorylates PIP_3, and thereby prevents the activation of PI3K downstream targets. PTEN is one of the most frequently lost or mutated tumor suppressors in human cancer.

An important downstream target of PI3K is AKT, also known as protein kinase B (PKB) (for a review, see [9]). Once generated, PIP_3 recruits AKT and PDK1 to cellular membranes where AKT is phosphorylated and activated by PDK1 and by the mammalian target of rapamycin (mTOR) kinase in complex with rapamycin-insensitive companion of mTOR (Rictor). Active AKT in turn phosphorylates a myriad of downstream substrates, such as FOXO, BAD, TSC2 and GSK3, to increase cell proliferation, growth and survival.

AKT activation also leads to the activation of the mTOR-rapamycin-sensitive companion of mTOR (Raptor) complex, which phosphorylates downstream targets, such as 4E-BP1 and the S6 kinases (for a review, see [11]). mTOR-Raptor is efficiently inhibited by the antifungal antibiotic rapamycin and its derivatives. Cell lines with mutated PTEN are particularly sensitive to rapamycin or the derivative CCI-779 [12]. CCI-779 and two other rapamycin derivatives, RAD001 and AP 23573, have shown promise as potential therapeutic agents in treatment of different cancers *in vivo* [13,14]. For example, *Pten* $\pm$ null mice treated with CCI-779 have reduced number of intestinal lesions, and AKT transgenic mice treated with RAD001 completely recovered from a PIN phenotype [13,14]. Based on these data, mTOR inhibitors are being evaluated in clinical trials as anticancer agents (reviewed in [15]).

Previous work has resulted in alternative views on the crosstalk between different components of the PI3K/AKT/mTOR pathway and AR signaling. For example, work in cell culture models demonstrated that selective inhibition of PI3K with LY294002 enhances AR transactivation [16–19]. In contrast, other studies have reported that inhibition of PI3K activity by LY294002 suppressed AR-mediated gene expression in androgen-dependent prostate cancer [20–22]. In another study, prolonged inhibition of PI3K increased while shorter treatment decreased the AR target Prostate Specific Antigen (PSA) mRNA levels [16]. Furthermore, in LNCaP cells PI3K was shown to either activate or inhibit AR signaling depending on the passage number of the cells [19]. Other studies demonstrated a direct synergy between AKT and AR signaling in transforming naive prostate epithelium into androgen insensitive, but AR-dependent, carcinoma [23].

Given the importance of both the AR and PI3K signaling in prostate carcinogenesis and the conflicting findings regarding their interactions, we evaluated this crosstalk *in vitro* and *in vivo*. We show that inhibition of the different components of the PI3K pathway increases expression of AR target genes in androgen-responsive/dependent cells, which is largely due to an increase in AR transactivation potential *in vitro* and *in vivo*. However, despite an increase in AR signaling, which has proliferative effects, the end result of PI3K pathway inhibition is reduced prostate cancer cell growth which is potentiated when AR signaling is independently blocked.

2. Materials and methods

2.1. Cell culture and reagents

LNCaP and HeLa cells were obtained from the American Type Culture Collection (ATCC). LNCaP cells were cultured in RPMI 1640 medium (Lonza) which was supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine (Lonza), 100 units (U)/ml penicillin and 100 µg/ml streptomycin (Lonza). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza), supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. For inhibition of the PI3K pathway at different levels, LNCaP cells were grown in RPMI and 10% FCS and treated with 10 µM LY294002, 10 µM AKT inhibitor or 100 nM rapamycin for the indicated time points. For androgen-induction experiments, cells were grown for 48 h in RPMI 1640 containing 2% charcoal treated (CT)-FCS (to remove steroids), followed by an additional 24 hrs in RPMI 1640 containing 0.5% CT-FCS prior to treatment with 10^{-8} M R1881 (synthetic androgen) and/or 100 nM rapamycin for 48–72 h. LY294002 and AKT-inhibitor were purchased from Calbiochem (San Diego, CA, USA), R1881 was obtained from NEN and rapamycin was purchased from Sigma. Bicalutamide was obtained from Astra Zeneca. CCI-779 was kindly provided by Wyeth (Pearl River, NY, USA) and sustained release testosterone pellet was obtained from Innovative Research of America (Sarasota, FL, USA).

2.2. Plasmids

The reporter plasmids -285-PB-LUC containing a deletion derivative of the rat probasin gene promoter driving expression of the luciferase (LUC) gene and MMTV-LUC, in which the mouse mammary tumor virus (MMTV) LTR drives expression of luciferase, were previously described [24,25]. The expression vectors pSG5-AR, N-terminal deletion mutants, C-terminal deletion mutants and C-terminal point mutants of AR have been described previously [26,27].

2.3. Transient transfection and luciferase assays

Transfections were carried out with FuGENE6 Transfection Reagent (Roche) according to manufacturer's recommendations. The luciferase experiments were performed in 6-well plates. For LNCaP cells, 2.0×10^5 cells were plated out per well in RPMI with 10% FCS one day prior to transfection. The cells were transfected with 150 ng reporter plasmid (-285-PB-LUC) and 850 ng of pUC18 to a total of 1 µg per well. Transient transfection assays with wtAR or its mutants were performed in HeLa cells. 7.5×10^4 cells were plated out per well in DMEM with 10% FCS one day prior to transfection and were co-transfected with 2 or 10 ng of pSG5-AR (or equimolar ratio of empty expression vector pSG5), 150 ng -285-PB-LUC or MMTV reporter plasmid, respectively and pUC18 to a total of 1 µg. The medium was removed 6 h after transfection, cells were washed with PBS and cultured in RPMI or DMEM, respectively, containing 0.5% CT-FBS. The cells were treated with R1881 (10^{-8} M) and/or rapamycin (100 nM) as indicated. After 48 h, cells were washed with PBS, harvested and lysed in 25 mM Tris-HCl, pH 7.8, 2 mM DTT, 10% glycerol and 1% Triton X-100 and assayed for expression of luciferase activity using the Luciferase Assay system (Promega). The luciferase activity of each sample was then normalized against the total level of protein. At least three independent experiments in triplicate were carried out for all constructs. To investigate the expression of AR in HeLa cells under the conditions described above, 1.5×10^5 cells were plated out in 4 ml DMEM with 10% FCS in 6 cm plates one day prior to transfection and were transfected with 2 µg pSG5-AR. The medium was removed 6 h after transfection, cells were washed with PBS and cultured in DMEM containing 0.5% CT-FBS and treated with R1881 (10^{-8} M) and/or rapamycin (100 nM) as indicated. The experiment was performed in triplicate twice. The differences between the groups were evaluated using a one-tailed, paired Student's *t*-test, with $P < 0.05$ being considered as significant.

2.4. Production of anti-hSTAMP2 antibody

A C-terminal mSTAMP2 (six transmembrane protein 2) peptide (CNSKYT-QSALNGKSDI) was used as an antigen to raise the anti-hSTAMP2 antiserum (Medprobe). The antiserum was affinity purified before use.

2.5. Quantitative RT-PCR

Upon harvest, total RNA was extracted from cells with Trizol reagent (Invitrogen) according to manufacturer's recommendations. RNA was used for first-

strand cDNA synthesis with the SuperScript II system (Invitrogen). AR, STAMP2, PSA, KLK4, NKX3.1 and TATA-binding protein (TBP) mRNA expression levels were determined using the LightCycler 2.0 and LightCycler 480 instruments (Roche) with the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche) and LightCycler 480 SYBR Green I Master (Roche). A standard curve made from serial dilutions of cDNA was made to calculate the relative amount of STAMP2, PSA, KLK4 and NKX3.1 cDNA for each sample. These values were then normalized to the relative amount of the internal standard TBP in the same samples. The STAMP2 primers were: forward 5′-ATG ACA GCA AAG CCA AGC AA-3′, reverse 5′-GCA AAG CAT CCA GTG GTC AA-3′. The PSA primers were: forward 5′-CCC TGA GCA CCC CTA TCA AC-3′, reverse 5′-TGA GTG TCT GGT GCG TTG TG-3′. The KLK4 primers were: forward 5′-ATG GAA AAC GAA TTG TTC TGC TC-3′, reverse 5′-ATC TGG CTC CCT GGC TCT T-3′. The NKX3.1 primers were: forward 5′-GGC CTG GGA GTC TCT TGA CTC CAC TAC-3′, reverse 5′-ATG TGG AGC CCA AAC CAC AGA AAA TG-3′. The TBP primers were: forward 5′-GAA TAT AAT CCC AAG CGG TTT G-3′, reverse 5′-ACT TCA CAT CAC AGC TCC CC-3′. The experiments were performed three times in triplicate. The differences between the groups were evaluated using a one-tailed, paired Student's *t*-test, with $P < 0.05$ being considered as significant.

2.6. Protein extraction and Western blot analysis

Whole cell extracts were prepared by resuspending cell pellets in 100 µl lysis buffer (20 mM HEPES (pH 7.7), 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothretol (DTT)) and a mixture of protease inhibitors and incubated at 4°C ON. The lysate was then centrifuged at 13,000 × *g* for 15 min at 4°C for removal of cell debris. 100 µg of total protein was resolved on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad). 40–150 µg of total protein extracted from xenograft tumors were run on a 8–12% polyacrylamide gel and used in Western analysis as previously described [28]. The membrane was blocked in 5–10% skimmed milk powder in TBST, followed by incubation with the primary antibodies against AR (1:500) (N-20, Santa Cruz), STAMP2 (1:1000), PSA (Santa Cruz) (1:300), KLK4 (1:600) [28], NKX3.1 (1:3000) [29] and GAPDH (1:500) (Santa Cruz) or tubulin 1:1000 (Santa Cruz). Horseradish peroxidase (HRP) conjugated secondary antibodies anti-rabbit IgG (whole molecule) produced in goat (Sigma-Aldrich), anti-goat IgG produced in donkey, ECL anti-mouse IgG produced in sheep (Amersham) and ECL Western Blotting Detection Reagents (Amersham) were used for detection of proteins. Quantification of protein levels was performed on scanned blots using the ImageQuant TL software (Amersham Biotech). The differences between the groups were evaluated using a one-tailed Student's *t*-test, with $P < 0.05$ being considered as significant.

2.7. Cell growth analysis

2.7.1. Effect of rapamycin on androgen-induced proliferation of LNCaP cells

Exponentially growing LNCaP cells were trypsinized and plated out at a density of 1.4×10^5 cells per well in 6-well culture plates. Cells were grown to 50% confluence, washed with PBS and starved in 2% CT-FCS. After 72 h, the medium was changed to 5% CT-FCS and cells were treated with 10^{-8} M R1881 and/or 100 nM rapamycin as indicated. Cell growth was measured by counting the number of cells in duplicate in a haemocytometer every second day for eight days. The experiment was performed three times in triplicate. The differences between the groups were evaluated using one-tailed, paired Student's *t*-test, with $P < 0.05$ being considered as significant.

2.7.2. Effect of rapamycin and bicalutamide on androgen-induced proliferation of LNCaP cells

LNCaP cells were plated out in 6-well culture plates as described above and cultured in 2% CT-FCS. After 72 h, the medium was changed to 0.5% CT-FCS and the cells were further cultured for 24 h prior to treatment with vehicle, 10^{-10} M R1881, 100 nM rapamycin, and/or 100 µM bicalutamide as indicated. Cell proliferation was measured by counting the number of cells in duplicate in a haemocytometer at the indicated time points. The experiment was performed twice in triplicate with consistent results. The statistical differences between the groups were evaluated using a one-tailed, paired Student's *t*-test, with $P < 0.05$ being considered as significant.

2.8. Xenograft studies

Xenograft experiments were carried out at Department of Tumor Biology, Institute for Cancer Research,

Norwegian Radium Hospital Oslo, Norway and all procedures and experiments were approved by The National Animal Research Authority, and conducted according to the European Convention for the Protection of Vertebrates used for Scientific Purposes. The animals were maintained under specific pathogen-free conditions, with filtered and humidified air (55% relative humidity). Androgen-dependent CWR22 prostate xenografts were implanted in castrated Balb/c nu/nu mice, bred at the nude rodent facility at the Norwegian Radium Hospital, weighing 20–25 g and were four to six weeks old [30]. The mice were implanted with sustained release testosterone pellet (2.5 mg for concentration of 4 ng/ml) prior to implantation of xenografts. Anesthesia was obtained with intraperitonally (i.p.) injection of 0.1 mg/kg fentanyl, 5 mg/kg fluanisone and 2.5 mg/kg midazolam. Fragments, about $2\times2\times2$ mm^3, of CWR22 tumor tissue were transplanted subcutaneously and bilaterally into both rear flanks of the mice. The animals were randomized for treatment according to tumor size when the average tumor diameters were about 6 mm (after approximately three weeks). Animals bearing tumors with diameters <4 mm or >8 mm were excluded. CCI-779 was dissolved in ethanol and diluted with 5% Tween-80 and 5% PEG 400 in sterile water. The drug or vehicle were administered i.p. at a concentration of 20 mg/kg for a period of five days, then two drug free days, and this cycle was repeated for two additional weeks. Tumor diameters were observed twice a week for the whole treatment period and the tumor volume was calculated by the formula $0.5 \times \text{length} \times \text{width}^2$. Relative tumor volumes (RTV) were defined as 1 for each individual tumor at the start of the treatment, day 0. Tumors were then collected and flash frozen by immersion in liquid nitrogen. Total protein was extracted from each tumor using the NucleoSpin kit (Macherey-Nagel) according to the manufacturer's instructions.

3. Results

3.1. The PI3K-AKT-mTOR pathway inhibits AR signaling

To investigate possible crosstalk between the PI3K-AKT-mTOR pathway and the regulation of the AR target gene STAMP2, an androgen target gene that is overexpressed in prostate cancer [31], we used inhibitors that are specific for distinct components of the PI3K pathway in the androgen-responsive prostate cancer cell line LNCaP, which expresses functional AR but not PTEN [32]. As shown in Fig. 1A, the specific PI3K inhibitor LY294002 increased expression of STAMP2 mRNA in a time-dependent manner reaching a three-fold increase at 3 h. Since AKT is the major downstream target of activated PI3K, we tested whether AKT is involved in activation of STAMP2 expression. A specific AKT inhibitor increased the accumulation of STAMP2 mRNA levels in a time-dependent manner reaching a 4.5 fold increase after 1 h of treatment (Fig. 1B). At later time points, the STAMP2 mRNA levels decreased and declined back to basal levels by 4 h of treatment. These results indicate that PI3K pathway inhibits AR signaling and AKT is involved in this process.

A major downstream target of AKT is mTOR (for a review, see [9]). To investigate whether mTOR is involved in the PI3K-AKT-mediated regulation of STAMP2 expression, the mTOR-specific inhibitor rapamycin was used. Treatment of LNCaP cells with rapamycin significantly induced STAMP2 mRNA levels in a time-dependent manner, reaching a 20-fold increase after 72 h (Fig. 1C). Under the same conditions, cyclin D1, which is a known target for mTOR [33], was inhibited (Fig. 1D). Similar to STAMP2, the androgen target genes PSA, KLK4 and NKX3.1 were significantly increased in response to rapamycin (Fig. 1E–G). These data indicate that similar to PI3K and AKT, mTOR inhibition activates AR signaling in prostate cancer cells.

3.2. mTOR inhibition synergizes with androgen to increase expression of AR target genes

We next investigated whether there were combinatorial effects of mTOR inhibition and androgen-treatment on expression of androgen target genes. LNCaP cells were cultured in steroid depleted medium and were either left untreated or treated with synthetic androgen R1881 in the absence or presence of rapamycin. As shown in Fig. 2A, whereas R1881 alone resulted in a robust activation of STAMP2 mRNA expression, rapamycin alone did not have any effect compared to non-treated cells. The combination of R1881 and rapamycin resulted in an approximately three-fold increase in STAMP2 mRNA expression compared to R1881 alone. Qualitatively similar results were obtained for PSA and KLK4 (Fig. 2B and C). A similar trend was also observed for NKX3.1, but this did not reach statistical significance (data not shown).

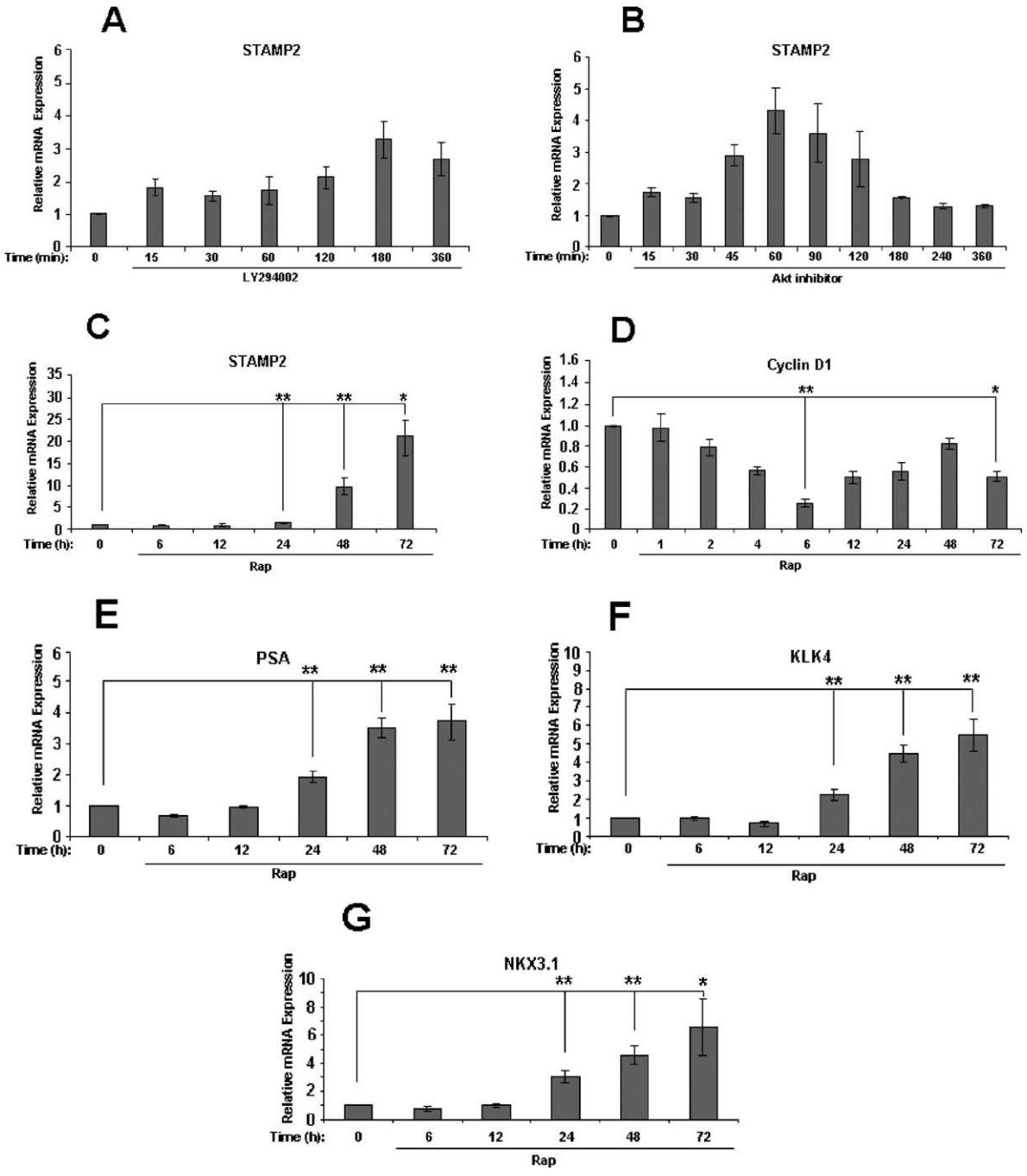

Fig. 1. The PI3K-AKT-mTOR pathway negatively regulates AR target gene expression. Quantitative RT-PCR (qPCR) was performed on cDNA made from RNA of LNCaP cells grown in 10% FCS and treated with the respective drugs as indicated; TBP was used as a housekeeping gene. (A) Cells were treated with PI3K-inhibitor LY294002 (10 μM) for different time points and STAMP2 mRNA levels were determined. (B) Cells were treated with AKT-inhibitor (10 μM) for different time points and the differences in STAMP2 mRNA levels were investigated. (C–G) Cells were treated with the mTOR-inhibitor rapamycin (100 nM) for different time points. Differences in the STAMP2 (C), cyclin D1 (D), PSA (E), KLK4 (F), and NKX3.1 (G) mRNA levels were determined. The experiments were performed three times in triplicates, error bars represent ± SEM. The expression levels are relative to non-treated cells (set to 1). The differences between the groups were evaluated using one-tailed, paired Student's *t*-test compared to non-treated samples, with $P < 0.05$ being considered as significant. Significance is indicated by asterisks; one asterisk indicates $P < 0.05$, two asterisks indicate $P < 0.01$.

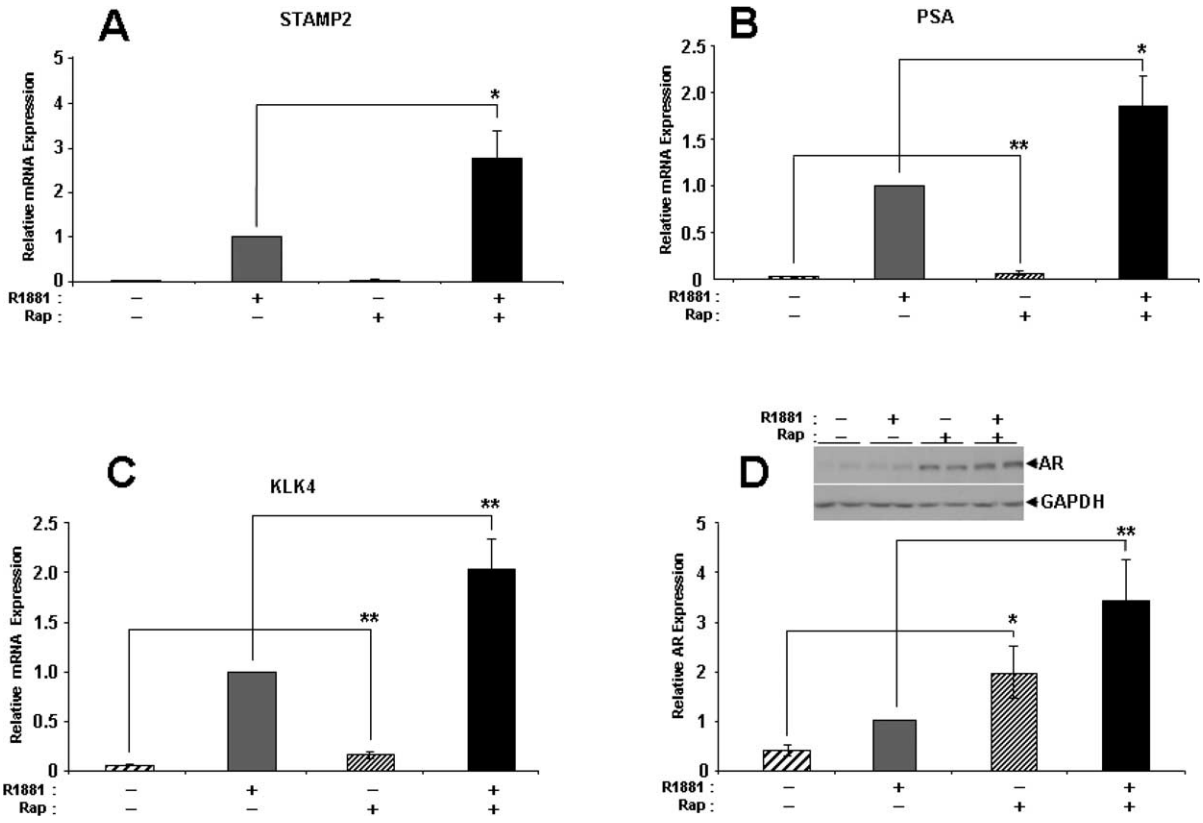

Fig. 2. mTOR inhibition synergizes with androgen to increase expression of AR target genes. (A–C) qPCR was performed on cDNAs made from RNA isolated from LNCaP cells treated with rapamycin (100 nM) in the presence or absence of R1881 (10^{-8} M) for 48 h. Note that the cells were grown in low serum conditions to maximize androgen effects (see Section 2). TBP was used as housekeeping gene, the expression levels are relative to R1881 treated cells (set to 1). Differences in the expression of mRNA levels of STAMP2 (A), PSA (B), and KLK4 (C) were determined. The experiments were performed three times in triplicate, with error bars shown as $\pm$ SEM. (D) Western analysis was performed on whole-cell extracts made from cells treated as described above to investigate any changes in AR levels. GAPDH was used as a loading control. A representative Western blot, as well as quantification from two independent experiments done in triplicate, is shown. The expression levels are relative to R1881 treated cells (set to 1). (E) Western analysis were performed on whole-cell extracts made from cells treated with rapamycin (100 nM) in the presence or absence of R1881 (10^{-8} M) for 72 h as indicated. Antibodies against STAMP2, PSA, KLK4 and GAPDH were used sequentially on the same blot. The expression levels were normalized to GAPDH and the values presented are relative to R1881 treated samples (set to 1) (F–H). The differences between the groups were evaluated using one-tailed, paired Student's *t*-test compared to non-treated samples, with $P < 0.05$ being considered as significant. Significance is indicated by asterisks, $P < 0.05$ and double asterisk, $P < 0.01$.

It has been reported that treatment of LNCaP cells with rapamycin increases the expression of AR. This could explain, at least in part, the increased expression of AR-target genes [34]. We thus investigated the effect of androgen and rapamycin on endogenous AR levels in LNCaP cells under our experimental conditions. LNCaP cells were treated with androgen and/or rapamycin for 48 h as above, and whole cell protein extracts were prepared and subjected to Western analysis. As depicted in Fig. 2D, AR protein levels increased approximately two-fold after androgen-treatment consistent with previous reports [35]. Rapamycin alone increased AR expression a further two-fold compared to androgen alone whereas androgen plus rapamycin increased AR expression 3.5 fold compared to androgen alone. These results indicate that rapamycin alone and in combination with R1881 affects the expression of AR in LNCaP cells. The increase of STAMP2, KLK4 and PSA mRNA observed by androgen and rapamycin may thus be, at least in part, a result of increased expression of AR. However, whereas rapamycin alone induced AR expression approximately four-fold compared with non-treated cells, this increase in AR expression did not result in significant changes on the mRNA level of STAMP2, while a modest increase for PSA and KLK4 mRNA expression was observed

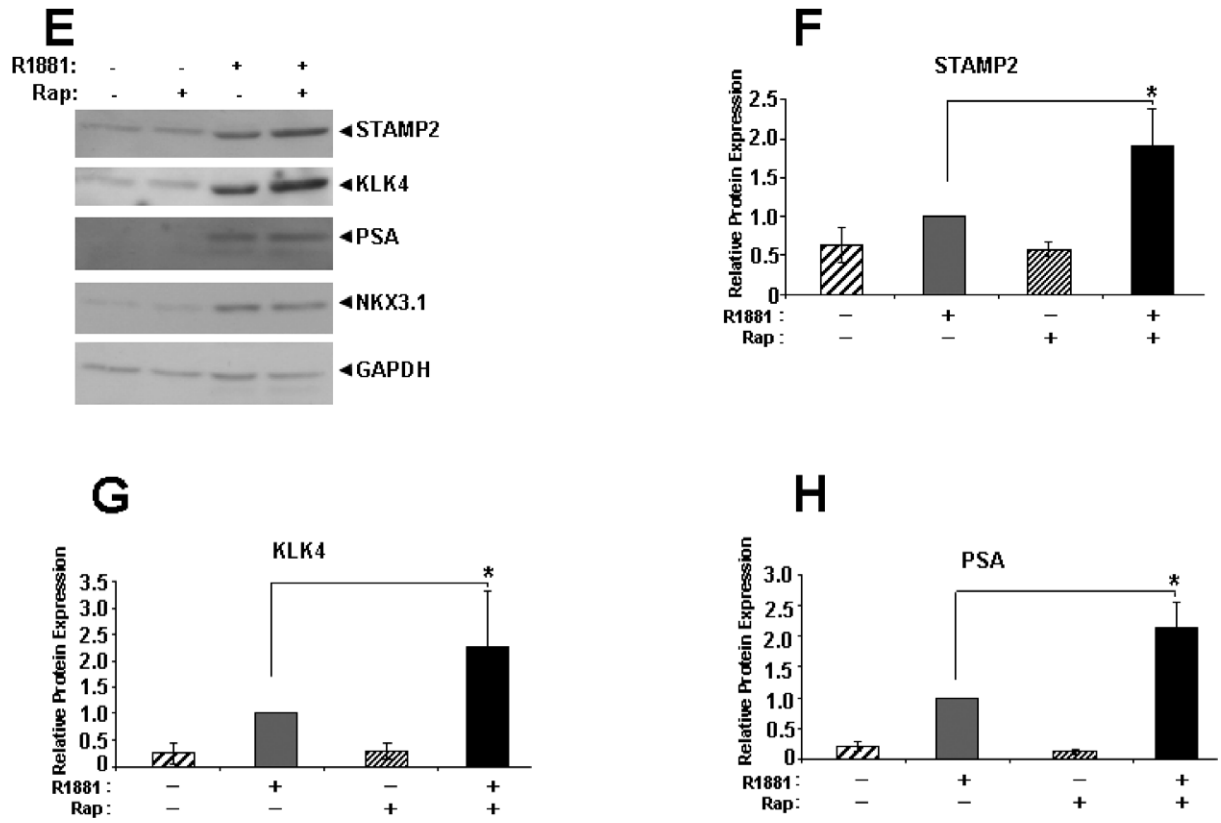

Fig. 2. (Continued.)

(Fig. 2A–C). In addition, increased expression of androgen target gene products was observed at the protein level in the presence of androgen and rapamycin compared with androgen alone (Fig. 2E–H). Taken together, these data suggest that inhibition of mTOR by rapamycin potentiates androgen induction of AR target genes, and this may in part be due to induction of AR expression itself.

3.3. Inhibition of mTOR increases AR transcriptional activity

Since there was no direct correlation between rapamycin treatment and AR expression that coincided with AR target gene expression, we evaluated whether mTOR inhibition may affect AR transcriptional activity. To that end, we used the androgen responsive reporter construct -285-PB-LUC in transient transfection assays. LNCaP cells were transfected with -285-PB-LUC and were either left untreated or treated with R1881 and/or rapamycin. As expected, the -285-PB-LUC expression significantly increased (∼400 fold) in cells treated with R1881 compared to untreated cells or cells treated with rapamycin alone (Fig. 3A). However, in the presence of R1881 and rapamycin, -285-PB-LUC expression increased a further four-fold compared to R1881 alone indicating that androgen and rapamycin act synergistically.

The same experiment was performed in HeLa cells which expressed very low endogenous AR (data not shown). HeLa cells were cotransfected with -285-PB-LUC and either with an empty expression vector (pSG5) or an expression vector encoding AR (pSG5-AR). After transfection, cells were treated as above. In response to R1881, -285-PB-LUC expression increased 40-fold compared to untreated cells and there was a further two-fold increase in this activity in the presence of rapamycin, consistent with the data obtained in LNCaP cells (Fig. 3B). To assess whether these findings are specific to the -285-PB-LUC reporter, a similar experiment was carried out using another AR dependent reporter construct; MMTV-LUC. HeLa cells were transfected with MMTV-LUC and

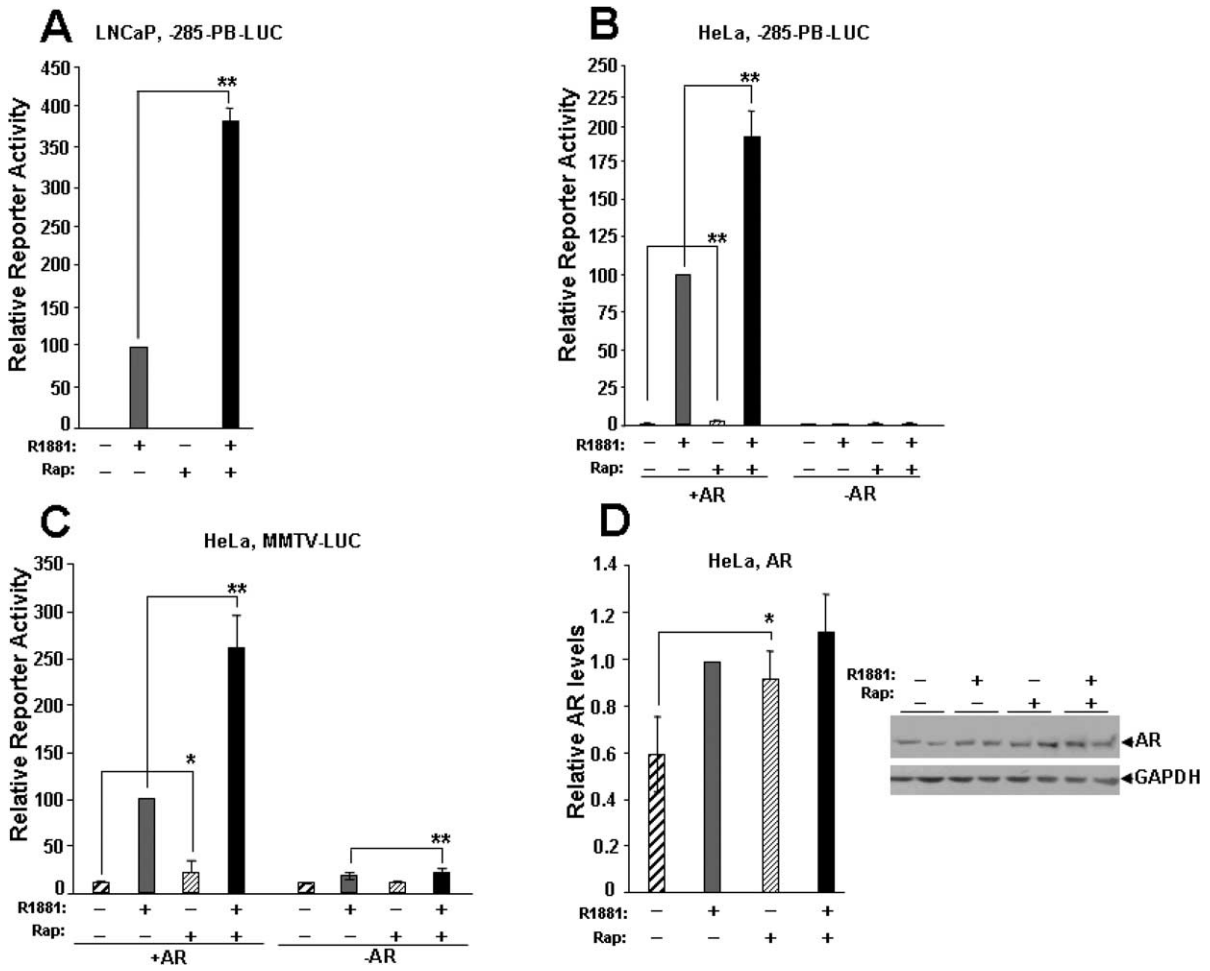

Fig. 3. mTOR inhibition increases the transactivation potential of AR. (A) LNCaP cells were cultured and transfected with -285-PB-LUC as described in Section 2. After transfection, cells were either left untreated, treated with rapamycin (100 nM), the synthetic androgen R1881 (10^{-8} M), or both, for 48 h as indicated. AR transcriptional activity was normalized to total amount of protein. Activity in the presence of R1881 was set to 100. (B) -285-PB-LUC was cotransfected into HeLa cells, either with an empty expression vector (pSG5) or an expression vector encoding AR (pSG5-AR). After transfection, cells were either left untreated, treated with rapamycin (100 nM), the synthetic androgen R1881 (10^{-8} M), or both, for 48 h as indicated. Cells were then harvested, luciferase activity was determined and normalized to total amount of protein. Activity in the presence of R1881 was set to 100. (C) Transfection into HeLa cells was performed as in B, but MMTV-LUC was used as the reporter. (D) Western analysis was performed with AR antibody on whole-cell extracts from HeLa cells transfected with 2 μg pSG5-AR and treated as in (B) and (C). Fold differences of AR levels were normalized to GAPDH levels, the values presented are relative to the AR level in cells treated with R1881 (set to 1). A representative Western blot, as well as quantification from two independent experiments done in triplicate, is shown. The differences between the groups were evaluated using one-tailed, paired Student's *t*-test compared to non-treated samples, with $P < 0.05$ being considered as significant. Significance is indicated by asterisks, $P < 0.05$ and double asterisk, $P < 0.01$.

the experiment was performed as for -285-PB-LUC. Similar to that observed for -285-PB-LUC, rapamycin acted synergistically with R1881 in the induction of MMTV-LUC compared to R1881 or rapamycin alone (Fig. 3C). Rapamycin itself modestly increased basal levels of MMTV-LUC expression but to a much lower extent than in the presence of R1881. The modest increase in the basal level of MMTV-LUC expression in response to R1881 and rapamycin, in the absence of AR, is probably due to a low level of endogenous AR in Hela cells. These data indicate that androgen and rapamycin act synergistically to increase AR transcriptional activity.

High affinity androgen binding has been shown to stabilize exogenously expressed AR [36]. To investigate the effect of R1881 and rapamycin on ex-

ogenously expressed AR, Hela cells were transfected with pSG5-AR and cultured in the presence of R1881 and/or rapamycin. As shown in Fig. 3D, AR protein levels modestly increased in response to androgen (1.7 fold) but there was no further increase in the presence of rapamycin. Taken together with the data presented above, these results indicate that rapamycin activates androgen induction of AR target genes primarily by increasing AR transcriptional activity.

3.4. *Delineation of AR domains required for induction of AR transcriptional activity by rapamycin*

To determine the domain(s) of AR that is required for the rapamycin effect on AR activation, we tested different AR deletion and point mutant constructs which differentially affect AR function (Fig. 4A). These included two N-terminal deletion mutants, one of which is inactive, a ligand binding domain deletion mutant which is a constitutive activator that does not respond to hormone, as well as two point mutants of the AF-2 core which have significantly impaired transactivation function [26,27,29].

The mutant AR constructs were tested in transient transfection assays in HeLa cells to assess their transactivation properties in response to R1881 and/or rapamycin. -285-PB-LUC was cotransfected into HeLa cells with either the empty expression vector pSG5 or expression vectors for wild type AR or its mutants. Cells were either left untreated or treated with R1881 and/or rapamycin as indicated. After 48 h, cells were

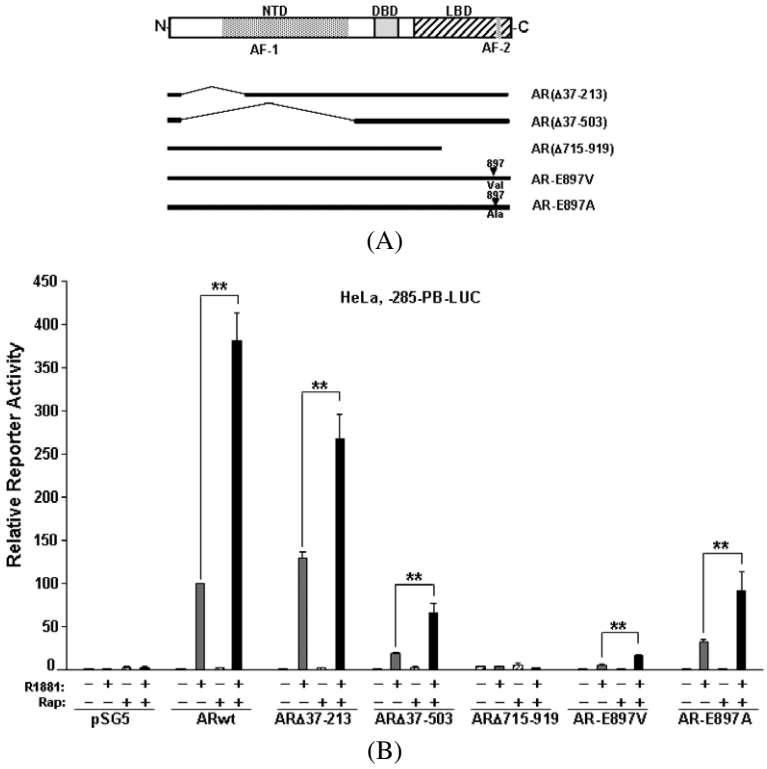

Fig. 4. Mapping the domains in AR that are necessary for the synergistic effect of androgen and rapamycin on AR transcriptional activity. (A) Schematic illustration of AR and AR mutant constructs. The N-terminal domain (NTD), DNA binding domain (DBD) and ligand binding domain (LBD) are indicated. The transactivation function domains, AF-1 and AF-2, are located within the NTD and LBD, respectively, as depicted (regions with dotted black). (B) Mapping the domains of AR that respond to androgen and rapamycin. HeLa cells were co-transfected with -285-PB-LUC and pSG5 with or without wt or mutant AR as indicated. Starved cells were either treated with R1881 (10^{-8} M) and/or rapamycin (100 nM) as indicated, harvested after 48 h and luciferase activities were determined and normalized to the total amount of protein. The values presented are relative to the activity of wt AR in the presence of R1881 (set to 100). The results shown represent three independent experiments in triplicate with $\pm$ SEM indicated as error bars. The differences between the groups were evaluated using a one-tailed, paired Student's t-test, significance is indicated by asterisks, $P < 0.05$ and double asterisk, $P < 0.01$. There were also significant differences in luciferase activity for samples treated with rapamycin compared to non-treated samples for all constructs, except for AR(Δ715-919); however the activities for these were very close to the basal activity levels observed in the presence of pSG5 and were thus not indicated.

harvested and luciferase activities were determined. As shown in Fig. 4B, rapamycin efficiently increased androgen-induced activity of wild type AR approximately four-fold, consistent with data presented above. Despite different levels of androgen-induced activity, consistent with published data [26,27,29], all mutant ARs, with the exception of the AR(Δ715-919), responded to rapamycin in a similar manner as the wild type AR; however, there were some quantitative differences in the fold-increase in activity in response to rapamycin and R1881 compared with R1881 alone. The inability of AR(Δ715-919) to respond to rapamycin indicates that only the liganded AR can respond to mTOR inhibition. These data suggest that activation of AR by rapamycin requires an intact ligand binding domain and that AF-1 is not sufficient for this response.

3.5. Rapamycin inhibits LNCaP cell growth whereas androgen counteracts this effect

Whereas rapamycin is a well known cell growth inhibitor and has earlier been reported to reduce LNCaP cell growth, androgens are known to increase LNCaP proliferation [37–39]. It was therefore of interest to assess the effect of rapamycin in combination with androgen on growth characteristics of LNCaP cells. To investigate this, LNCaP cells were cultured in the absence or presence of R1881 and/or rapamycin and cell growth was assessed (Fig. 5A). Whereas cells treated with vehicle alone did not appreciably grow during the course of the experiment, there was a significant increase in cell growth in response to R1881 at all time points. Rapamycin alone inhibited cell growth compared with vehicle control. Furthermore, rapamycin also reduced the R1881-induced cell growth. These data suggest that although inhibition of mTOR by rapamycin increases the activity of androgen signaling, which has been implicated in cell growth of prostate cancer cells, additional and independent inhibitory effects of rapamycin on cell proliferation override these effects and result in reduction of LNCaP cell growth.

3.6. AR antagonist bicalutamide potentiates growth inhibitory effects of rapamycin in LNCaP cells

We next assessed the possible effect of AR inhibition on rapamycin-induced repression of LNCaP cell growth. The non-steroid AR antagonist bicalutamide is a commonly used drug in prostate cancer therapy (reviewed in [2]) which inhibits LNCaP cell growth [40, 41]. To evaluate the combined effect of rapamycin and bicalutamide, LNCaP cells were cultured with R1881, rapamycin and/or bicalutamide and cell-proliferation was assessed after three and six days. Similar to the experiment described above (see Fig. 5A), cells treated with vehicle alone did not show any significant change in growth during the course of the experiment (Fig. 5B). There was a significant increase in cell growth in response to R1881 after six days compared to all treatments. Rapamycin inhibited androgen-induced LNCaP cell-growth significantly after three and six days compared with androgen only. Treatment with bicalutamide resulted in significantly reduced cell-growth by compared to rapamycin alone for both time points. R1881-induced cell growth was most strongly inhibited when the cells were treated with a rapamycin + bicalutamide reaching approximately 80% inhibition by six days. The AR inhibitory effect of bicalutamide was confirmed on AR target genes (data not shown). These data indicate that rapamycin and bicalutamide have additive effects in inhibition of androgen-induced cell growth of LNCaP cells.

3.7. Inhibition of mTOR reduces tumor growth and increases expression of androgen target genes in vivo

To investigate the interplay of androgen and mTOR signaling on expression of AR target genes and assess their effects on cell growth *in vivo*, we used the CWR22 xenograft model derived from a primary human prostate carcinoma [30]. CWR22 cells are androgen-dependent and regress rapidly upon androgen ablation, thus providing a good model for human prostate cancer in the androgen-dependent stages. For these experiments, we used the synthetic rapamycin derivative CCl-779 which has improved aqueous solubility and pharmacokinetic properties and is in clinical trials for various types of cancers [42–44]. Expression analysis of AR target genes in LNCaP cells and reporter assays confirmed that CCI-779 acts in a similar manner to rapamycin on transcriptional activity of AR (data not shown); due to its improved pharmacokinetic properties CCI-779 was used in the xenograft experiments described below.

Androgen dependent CWR22 human prostate cancer xenografts [30] were grown subcutaneously in nude mice in the presence of androgen and were either treated with vehicle or with CCl-779; tumor growth was monitored throughout the procedure. As shown in Fig. 6A, the xenografts in mice which were treated with CCI-779 were significantly retarded in tumor

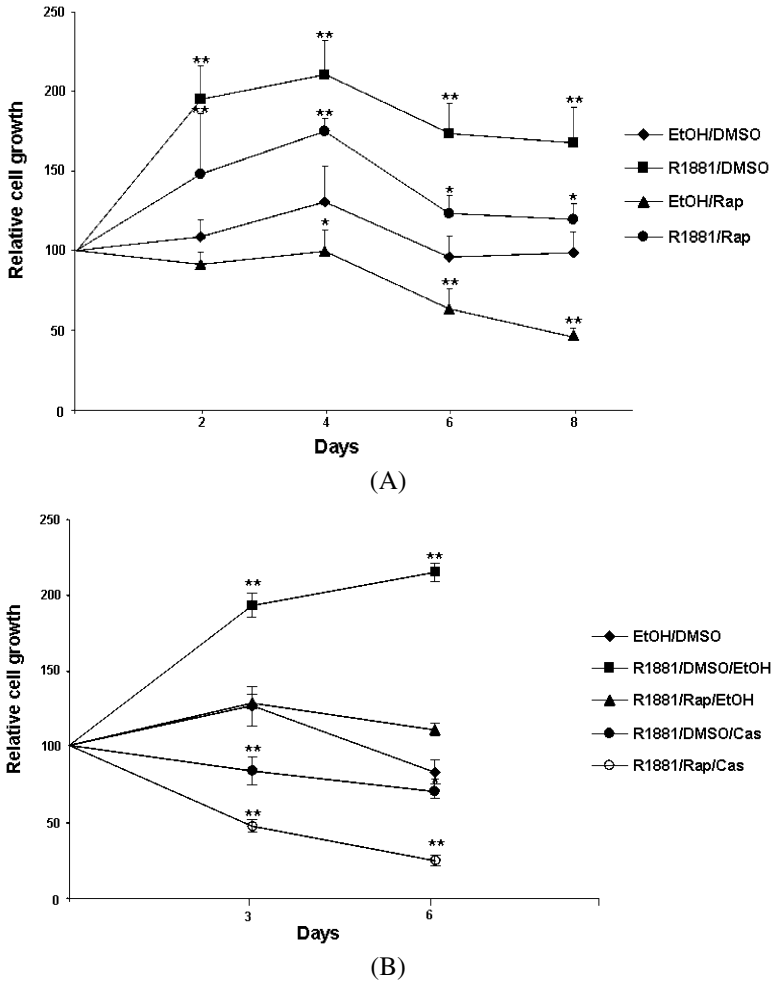

Fig. 5. Effect of mTOR inhibition and antiandrogen treatment on growth of LNCaP cells. (A) mTOR inhibition overrides androgen-induced growth of LNCaP cells. LNCaP cells were grown in 6-well plates as described in Materials and Methods. Cells were treated with either ethanol or DMSO (diamonds), R1881 (10^{-8} M) and DMSO (squares), ethanol and rapamycin (100 nM) (triangles), or R1881 (10^{-8} M) and rapamycin (100 nM) (circle) for eight days. Cells were then counted in a haemocytometer in duplicate. The average number of cells for each experiment was set to 100 at day 0. Data represent the relative mean cell numbers from three independent experiments done in triplicate with error bars shown as $\pm$ SEM. The significance between vehicle and each respective treatment was calculated by one-tailed, paired Student's t-test; asterisk: $P < 0.05$; double asterisk: $P < 0.01$. (B) Potentiation of rapamycin-induced inhibition of LNCaP cell growth by bicalutamide. LNCaP cells were grown in 6-well plates as described in Section 2. Cells were treated with either ethanol or DMSO (diamonds), R1881 (10^{-10} M), DMSO and ethanol (squares), R1881, rapamycin (100 nM) and ethanol (triangles), R1881, DMSO and casodex (100 μM) (circle), or R1881, rapamycin, and casodex (open circle) for three and six days. Viable cells were then counted in a haemocytometer in duplicate. The average number of live cells for each experiment was set to 100 at day 0. Data represent the relative mean cell numbers from one representative experiment done in triplicate, with error bars shown as $\pm$ SEM. The experiment was repeated twice with similar results. The significance between R1881 and rapamycin-treated samples and each respective treatment was calculated by one-tailed, paired Student's t-test; asterisk: $P < 0.05$; double asterisk: $P < 0.01$.

growth compared to vehicle-treated mice (Fig. 6A). To assess any changes in AR target gene expression after three weeks of treatment, tumors were harvested, protein extracts were prepared, and used in Western blot analysis. Compared to xenografts from mice that received vehicle only, CWR22 tumors from mice treated with CCI-779 displayed a significant increase in the protein levels of all AR target genes tested: STAMP2, KLK4, NKX3.1 and PSA (Fig. 6B–E). In contrast, there were no significant changes in AR expression under these conditions (Fig. 6F). In light of the data described above, these data indicate that CCI-779 synergizes with androgen to increase AR target gene expression by potentiating the transcriptional activity of AR

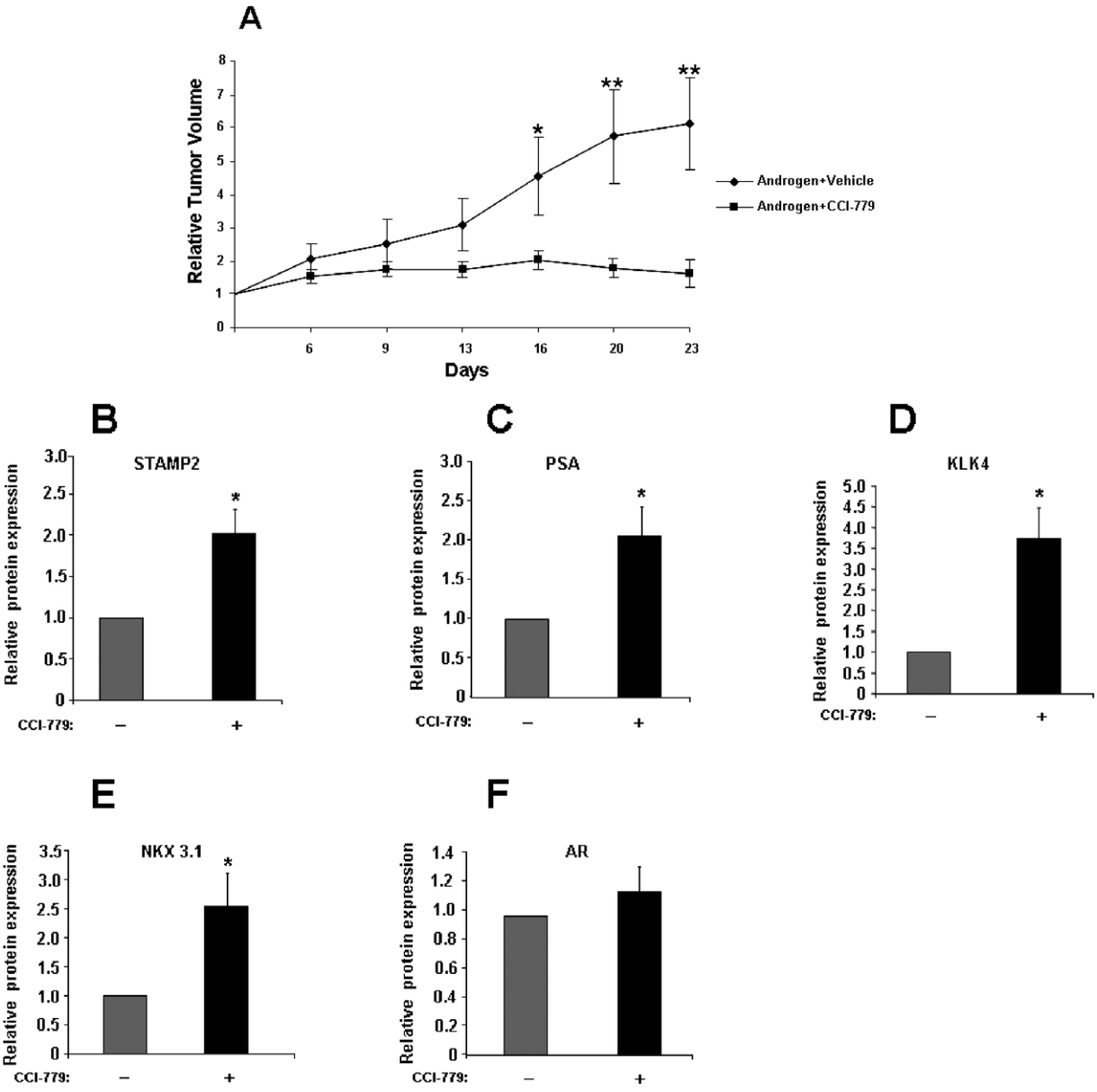

Fig. 6. mTOR inhibition represses the growth of CWR22 xenografts. (A) Relative tumor volume of CWR22 xenografts treated with androgen and CCI-779. CWR22 xenografts were grown in nude mice as described in Section 2 and were treated with testosterone (Tes) plus vehicle (triangles) or testosterone plus CCI-779 (squares). Mice were treated for five days per week for three consecutive weeks. Relative tumor volumes (RTV) were set as 1 for each individual tumor at the start of the treatment, day 0, with $\pm$ SEM indicated as error bars. The significance between the two groups was calculated by one-tailed, unpaired Student's t-test; single asterisk: $P < 0.05$; double asterisk: $P < 0.01$, $n = 5$–8 per sample. (B–E) Expression analysis of AR target genes in CWR22 xenografts treated with testosterone (Tes) and CCI-779. Total protein was extracted and the expression of STAMP2 (B), KLK4 (C), NKX3.1 (D), PSA (E) and AR (F) was investigated by Western blotting, normalized to GAPDH or tubulin, and quantified. The expression values are relative to cells that were treated only with testosterone (set to 1), with $\pm$ SEM indicated as error bars. The significance between the two groups was calculated by one-tailed, unpaired Student's t-test; asterisk: $P < 0.05$; $n = 4$–7 individual samples.

in the CWR22 xenografts which results in a decrease in tumor growth.

4. Discussion

There has been conflicting data in the literature on the crosstalk between PI3K and AR signaling pathways. Data that are presented herein suggest that inhibition of PI3K signaling activates AR transactivation potential.

We showed that mTOR inhibition by rapamycin increased AR target gene expression at both mRNA and protein levels in LNCaP cells *in vitro* and CWR22 xenografts *in vivo*. There was an increase in AR expression levels in LNCaP cells upon mTOR inhibition, which may at least in part, account for the observed effects. However, in HeLa cells, in which the effect of rapamycin on AR transactivation potential was studied, there was no significant effect on AR levels indicating cell-type specific differences. This suggests that HeLa cells might lack some other molecular factors essential for the rapamycin-induced effect on AR expression. Similarly, in the CWR22 xenograft mTOR inhibition did not affect AR levels. Taken together, these data suggest that rapamycin sensitizes prostate cancer cells for androgen treatment primarily by affecting AR transcriptional activity and not AR expression levels.

Previous studies have arrived at different views on the molecular mechanism of PI3K/AKT/mTOR–AR signaling interactions. Whereas some studies found increases in AR levels upon inhibition of the PI3K pathway [18,45], others have reported no changes on AR expression [34]. Interestingly, whereas rapamycin increased AR expression in LNCaP cells, knockdown of mTOR by siRNA did not affect AR levels [34]. This suggests that rapamycin may affect the expression of AR not through the PI3K/Akt/mTOR pathway, but by an as yet unknown mechanism. These data also converge upon changes in AR transcriptional activity as the primary mechanism through which PI3K inhibition mediates activation of AR signaling.

Mutational studies indicated that both AF-1 and AF-2 domains and an intact ligand binding domain of AR are required for rapamycin induction of AR transcriptional activity. How PI3K pathway inhibition affects AR activity is currently not known. Direct phosphorylation of the FOXO subfamily of Forkhead transcription factors by AKT inactivates FOXOs and thus inhibits the expression of FOXO regulated genes [46]. It was found that FOXO3a can activate transcriptional activation of AR and expression of AR target genes [18]. This provides one mechanism in which inhibition of the PI3K/AKT pathway induces AR transcriptional activity. In addition, it has been suggested that AKT directly interacts with AR and thereby suppresses AR activity [16,17,47]. *In vitro* studies have implicated AKT and Mdm2 as important factors in ubiquitination of AR by a proteasome-dependent mechanism, providing another possible mechanism as to how the PI3K/AKT pathway may negatively regulate AR action.

In addition to these possibilities, there are additional mechanisms through which PI3K/AKT/mTOR may regulate AR transcriptional activity. It is possible that mTOR inhibition can affect posttranslational modulation of AR, such as phosphorylation and acetylation, both of which have been implicated in regulating AR activity [5]. Furthermore, mTOR inhibition can affect the activity or the levels of AR cofactors that are required for AR-mediated transcription. Another possibility is that, since both the AF-1 and AF-2 are required for the rapamycin effects, it is tempting to speculate that mTOR inhibition may influence the intramolecular interactions between the N- and C-termini of AR that are required for AR transcriptional activity [48,49] and which has been demonstrated to occur at the AR targets in living cells [50]. Further work is required to test these possibilities.

Whereas there have been different data with opposing conclusions regarding the PI3K pathway – AR signaling interactions, PI3K/AKT/mTOR inhibition uniformly resulted in growth inhibition in prostate cancer cells *in vitro* and *in vivo* [38,39,51]. The data we present here in LNCaP cells suggest that although inhibition of mTOR by rapamycin increases the activity of androgen signaling, the growth inhibitory effects of rapamycin could not be overcome by increased AR activity. Consistently, androgen-dependent CWR22 tumors treated with androgen plus CCI-779 showed significantly reduced growth, concomitant with increased expression of AR target genes compared to those treated with androgen alone. Taken together, these results indicate that rapamycin, under the conditions of these experiments, might affect cell growth that are more dominant than androgen-induced cell proliferation, resulting in a net inhibitory effect on prostate cancer cell growth.

In addition, we showed that LNCaP cells treated with a combination of rapamycin and bicalutamide resulted in further reduced cell-growth compared to these agents used alone. The exact molecular mechanisms of bicalutamide action are not known. In addition to pre-

venting androgen binding to the AR as an antagonist, it has been suggested that bicalutamide induces release of cytochrome C by Bax-dependent and -independent mechanisms resulting in apoptosis of LNCaP cells [40]. Furthermore, previous work from our laboratory suggests that AR antagonists, including bicalutamide, may function by affecting AR–chromatin interaction dynamics [50]. Further work is required to determine at what level bicalutamide and the PI3K/Akt/mTOR pathway interact to bring about the added inhibitory effects on prostate cancer cells.

Reduced proliferation of LNCaP cells in response to rapamycin and bicalutamide treatment has also been recently observed in another study [52]. Coinciding with our results, rapamycin increased AR transactivation potential, although to a lesser extent compared with our data, and inhibited LNCaP cell growth. In this study, rapamycin treatment inhibited both mTORC1 and mTORC2, and inhibition of both complexes induced apoptosis, whereas rapamycin-induced AR transcriptional activity resulted from the inhibition of mTORC1, but not mTORC2. These data indicated that there are two parallel cell-survival pathways in prostate cancer cells: a strong Akt-independent, but rapamycin-sensitive, pathway downstream of mTORC1, and an AR-dependent pathway downstream of mTORC2 and Akt, which is stimulated by mTORC1 inhibition. In contrast, our data indicate that inhibition of the PI3K/Akt pathway, therefore affecting both mTORC1 and mTORC2, increases AR transactivation potential. At present, we do not know the basis for these differences. One possibility is that to obtain optimal androgen response, we culture LNCaP cells in low serum conditions for all experiments involving androgens, whereas Wang et al. [52] used 5% serum. Whether this or some other reason is at the basis of these different observations requires further investigation.

The major problem in prostate cancer therapy today is that although tumor cell growth is inhibited by androgen ablation therapy, some cells survive and invariably progress to an androgen-independent phenotype, for which there is no effective therapy. Recent studies have shown that AR signaling, even upon androgen ablation therapy, has a central role in all stages of prostate cancer, in part due to targeting of AR and other aspects of the proliferative machinery by a variety of signaling pathways (reviewed in [6]). Other studies have shown that depletion of mitochondrial DNA (mtDNA) induces prostate cancer progression with increased cell migration by upregulation of PI3K/Akt2 and some of its downstream molecules [53,54]. Thus, in addition to inhibition of AR with antiandrogens, inhibition of signaling pathways shown to be important for prostate cancer progression have been the main focus in the development of novel prostate cancer therapies. PI3K/AKT/mTOR constitute a major pathway in this regard [7]. Thus simultaneous inhibition of AR and PI3K/AKT/mTOR has a stronger antiproliferative effect which may also be useful in prostate cancer therapy. Further experiments in *in vivo* models, such as xenografts as well as transgenic animals, should be performed to check this hypothesis.

In light of the complex crosstalk between PI3K/AKT/mTOR and AR pathways and the fact that PI3K pathway inhibition activates AR which has a role in all stages of prostate carcinogenesis, pharmacologic agents that are targeting the PI3K/AKT/mTOR pathway for use in prostate cancer therapy should be considered with care as there could be unanticipated effects. The cumulative data indicate that unique survival and compensatory mechanisms are activated in prostate cancer cells when subjected to pharmacologic agents which underscore the importance of combinatorial targeted therapies. Further understanding of the molecular details of the crosstalk between the two pathways may result in the identification of novel targets that may increase the efficacy of such therapies.

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