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ORIGINAL ARTICLE

FKBP51 and Cyp40 are positive regulators of androgen-dependent prostate cancer cell growth and the targets of FK506 and cyclosporin A

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Prostate cancer (PCa) growth is dependent on androgens and on the androgen receptor (AR), which acts by modulating gene transcription. Tetratricopeptide repeat (TPR) proteins (FKBP52, FKBP51 and Cyp40) interact with AR in PCa cells, suggesting roles in AR-mediated gene transcription and cell growth. We report here that FKBP51 and Cyp40, but not FKBP52, are significantly elevated in PCa tissues and in androgen-dependent (AD) and androgen-independent (AI) cell lines. Overexpression of FKBP51 in AD LNCaP cells increased AR transcriptional activity in the presence and absence of androgen, whereas siRNA knockdown of FKBP51 dramatically decreased AD gene transcription and proliferation. Knockdown of Cyp40 also inhibited androgen-mediated transcription and growth in LNCaP cells. However, disruption of FKBP51 and Cyp40 in AI C4-2 cells caused only a small reduction in proliferation, indicating that Cyp40 and FKBP51 predominantly regulate AD cell proliferation. Under knockdown conditions, the inhibitory effects of TPR ligands, cyclosporine A (CsA) and FK506, on AR activity were not observed, indicating that Cyp40 and FKBP51 are the targets of CsA and FK506, respectively. Our findings show that FKBP51 and Cyp40 are positive regulators of AR that can be selectively targeted by CsA and FK506 to achieve inhibition of androgen-induced cell proliferation. These proteins and their cognate ligands thus provide new strategies in the treatment of PCa.
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Introduction

Prostate cancer (PCa) is the second leading cause of cancer death in American men (Landis *et al.*, 1999; Jemal *et al.*, 2006). Androgen signaling through androgen receptor (AR), a member of the nuclear receptor superfamily (Chang *et al.*, 1988; Tsai and O'Malley, 1994; Mangelsdorf *et al.*, 1995), is critical for the normal development of the prostate gland and for the aberrant onset of PCa (Balk, 2002). Androgen ablation is frequently used in the treatment of PCa to repress AR action (Scherr *et al.*, 2003), and this approach results in reduced expression of AR target genes and concomitant tumor regression (Amler *et al.*, 2000; Feldman and Feldman, 2001; Mousses *et al.*, 2001; Velasco *et al.*, 2004). Unfortunately, PCa often recurs after androgen ablation therapy—a state referred to as androgen independence (AI) or ablation resistance. Studies suggest that both AI and androgen-dependent (AD) tumors express AR (Chen *et al.*, 2004), indicating that AR-regulated gene expression might have a critical role in PCa growth and progression (Haag *et al.*, 2005; Han *et al.*, 2005; Liao *et al.*, 2005).

As with other steroid receptors, AR is a modulator protein that contains an N-terminal transactivation domain, a conserved DNA-binding domain and a C-terminal ligand-binding domain. The unliganded AR primarily exists in the cytoplasm in a complex with heat-shock protein 90 (Hsp90) and tetratricopeptide repeat (TPR) proteins (FK506-binding proteins, FKBP52 and FKBP51, and cyclosporin A-binding protein, Cyp40) (Heinlein and Chang, 2004; Febbo *et al.*, 2005; Periyasamy *et al.*, 2007; Veldscholte *et al.*, 1992 a, b). The ability of FKBP52/FKBP51 and Cyp40 to bind immunosuppressive drugs FK506 and cyclosporine A (CsA), respectively, has served to categorize these proteins as immunophilins. Binding of FK506 and CsA to FKBP51/FKBP52 and Cyp40, respectively, causes inhibition of the protein's peptidyl-prolyl cis-trans isomerase (PPIase) activity (Schreiber and Crabtree, 1992; Galat, 2003). A number of studies have shown contributions of FKBP52, FKBP51 and Cyp40 to protein folding, ligand binding and nuclear localization of glucocorticoid, estrogen and progesterone receptors (Reynolds *et al.*, 1999; Denny *et al.*, 2000; Cheung-Flynn *et al.*, 2003; Ratajczak *et al.*, 2003; Pratt *et al.*, 2004). Apart from the physical interaction of AR with Hsp90, FKBP52, FKBP51 and Cyp40, the molecular roles of these TPRs on AR action are poorly understood.

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Recently, new evidence for TPR control of AR function during reproductive organ development has come to light. Studies in our laboratory (Yong *et al*., 2007) and in others (Cheung-Flynn *et al*., 2005) have shown that male mice with targeted ablation of FKBP52 are infertile because of a developmental defect of the penis termed hypospadias—a condition prevalent in newborn boys and thought to arise from androgen insensitivity (Batch *et al*., 1992; Nakao *et al*., 1992). Complete prostate dysgenesis was also noted in FKBP52KO mice. We have shown overexpression of FKBP52, FKBP51 and Cyp40 in PCa cell lines and that TPR ligands, FK506 and CsA, inhibited androgen-induced cell growth and gene transcription in PCa cells through inhibition of hormone binding and nuclear localization of the AR (Periyasamy *et al*., 2007). Others have shown FKBP51 overexpression in PCa tissues compared with noncancerous controls (Zhu *et al*., 2001; Velasco *et al*., 2004; Tomlins *et al*., 2007). Moreover, reports exist showing that, similar to prostate-specific antigen (PSA) and KLK2 genes, FKBP51 is a highly sensitive AR-regulated gene (Amler *et al*., 2000; Vanaja *et al*., 2002; Magee *et al*., 2006), the upregulation of which serves to increase AR activity (Febbo *et al*., 2005). On the basis of these observations, we hypothesized that FKBP52, FKBP51 and Cyp40 may serve as potential activators of AR and that their upregulation might increase AR activity in PCa cells.

In this report, we show that expression of FKBP51 and Cyp40, but not of FKBP52, was elevated in PCa tissues and cell lines. Using knockdown and overexpression techniques, we also show that FKBP51 and Cyp40 are required for optimal AR activity in AD PCa cells, and that each TPR serves as the principal target for the inhibitory effects of FK506 and CsA on AR. Finally, we show that both FKBP51 and Cyp40 principally control AD, rather than AI, growth of prostate cells. These findings are the first of their kind and provide potential new targets in the treatment of PCa.

Results

Overexpression of Cyp40 and FKBP51 in human PCa specimens and cell lines

Previously, high levels of Cyp40, FKBP51 and FKBP52 were found in LNCaP, PC-3 and DU145 PCa cell lines (Periyasamy *et al*., 2007). To further validate the involvement of these TPRs in PCa, we measured the mRNA and protein expression of Cyp40, FKBP51 and FKBP52 in PCa specimens. Normal and PCa tissue samples were purchased from the Cooperative Human Tissue Network (CHTN). Analysis by real-time reverse transcription–PCR (RT–PCR) (Figure 1a) and western blotting (Figure 1b and Supplementary Figure S1B) revealed significant upregulation of Cyp40, FKBP51 and AR in most of the PCa samples. Interestingly, FKBP52 mRNA and protein levels were unaltered when comparing normal prostate tissues with PCa samples (data not shown). This suggests that FKBP51 and Cyp40, but not FKBP52, might have a critical role in PCa growth and progression. Although the FKBP51 results are consistent with earlier reports showing high FKBP51 expression in primary and recurrent PCa (Amler *et al*., 2000; Mousses *et al*., 2001; Velasco *et al*., 2004; Tomlins *et al*., 2007), these are the first observations showing overexpression of Cyp40 in cancerous prostate tissues.

We next measured protein expression levels of Cyp40, FKBP51 and AR in several PCa cell lines (Figure 2a). Normal prostate epithelial cells (PrECs) were used as controls and compared with AD (LNCaP, LAPC-4, VCaP and DuCaP) and AI (C4-2, C-81 and CWR22R) PCa cell lines. All PCa cell lines expressed the AR, although to different degrees, and in a manner that did not correlate with androgen growth sensitivity. As expected, the AR protein was not detected in normal PrECs (Periyasamy *et al*., 2007). With the exception of VCaP cells, high levels of Cyp40 and FKBP51 were found in all PCa cell lines compared with normal PrECs, providing further evidence that FKBP51 and Cyp40, rather than FKBP52 and other immunophilins, are the actual mediators of the inhibitory effects of FK506 and CsA on PCa cell growth. The data of Figure 2 also showed higher FKBP51 and Cyp40 levels in AI PCa cell lines compared with AD cells, suggesting that each TPR may be involved in PCa progression to androgen independence. In the study that follows, we test whether FKBP51 and Cyp40 are the targets of immunophilin drug action, and whether these proteins contribute to AD or AI growth of PCa cells, or both.

Inhibition of AR activity by FK506 and CsA is mediated by FKBP51 and Cyp40

In an earlier study, we showed inhibition of LNCaP cell proliferation by FK506 and CsA that correlated with reduced hormone-induced AR activity as measured by reporter gene assays (Periyasamy *et al*., 2007). To confirm that these results were not artifacts of reporter gene assays, expression of the androgen-regulated PSA (Figure 3a) and KLK2 (Figure 3b) genes was measured by real-time RT–PCR in LNCaP cells. The results show elevated PSA and KLK2 expression levels in response to R1881, which was effectively inhibited by the AR antagonist, casodex (bicalutamide). More importantly, both FK506 and CsA decreased the expression of PSA and KLK2 in response to R1881.

In the previous study, we also showed that FK506 inhibition of AR activity was not mediated by classical immunophilins such as FKBP12, but our results did not discriminate between FKBP51 and FKBP52. Here, this question was addressed by downregulating the expression of FKBP51 with short-interfering RNA (siRNA). Figure 4a shows effective knockdown of FKBP51 expression in LNCaP cells while leaving levels of FKBP52 unchanged. We then treated control-siRNA and FKBP51-siRNA LNCaP cells with FK506. FK506

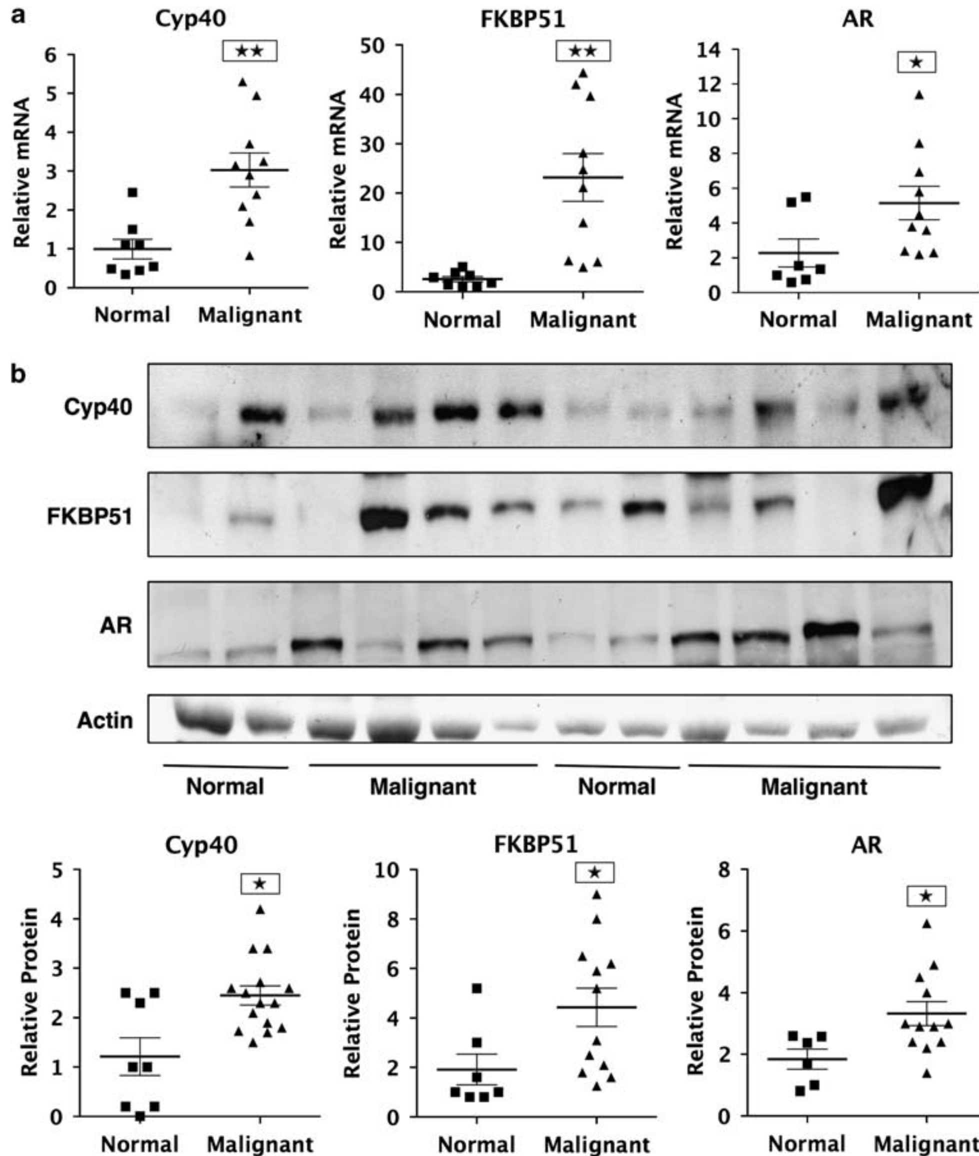

Figure 1 Overexpression of Cyp40 and FKBP51 in prostate cancer (PCa) specimens. (**a**) Total RNA was isolated from eight normal and 10 malignant human prostate tissues, followed by real-time–PCR using Cyp40-, FKBP51- and androgen receptor (AR)-specific primers and normalization to ribosomal 18S. RNA levels were expressed as relative to normal prostate tissue #1. $*P<0.05$ and $**P<0.01$. (**b**) Whole-cell extracts from normal and malignant prostate specimens were analyzed by western blotting for expression of AR, FKBP51, Cyp40 and actin. Quantitation was performed by densitometric scanning of the bands in eight normal and 15 malignant prostate specimens and normalization to actin. $*P<0.05$. Western blot results of remaining samples are seen in Supplementary Figure S1B.

had no effect on expression levels of FKBP51 and AR under basal conditions, but did inhibit the ability of R1881 to increase FKBP51 expression in control cells (Figure 4b). Interestingly, both FK506 and knockdown of FKBP51 had no effect on R1881 induction of the AR protein. With respect to AR activity at prostate-specific antigen luciferase (PSA-Luc), FK506 caused an approximate 50% reduction, as expected, in control cells (Figure 4c). However, FKBP51 knockdown alone also caused an ~50% reduction and this activity was not further reduced by treatment with FK506. These results show that FKBP51, but not FKBP52, is the target of FK506 action on AR.

To address the direct role of Cyp40 in AR function, siRNA was also used to downregulate Cyp40 in LNCaP (Figure 5). Cells transfected with Cyp40-siRNA showed a large reduction of the Cyp40 protein, with no change in the related cyclophilin Cyp18 or AR protein (data not shown). The latter result suggests that Cyp40, similar to FKBP51, is not essential for AR expression and stability. In control-siRNA cells, CsA treatment caused inhibition of AR activity, as expected. In response to Cyp40 downregulation, a large reduction of androgen-induced PSA promoter activity was seen, with no further inhibitory effect of CsA. These results identify Cyp40 as the target of CsA action on AR.

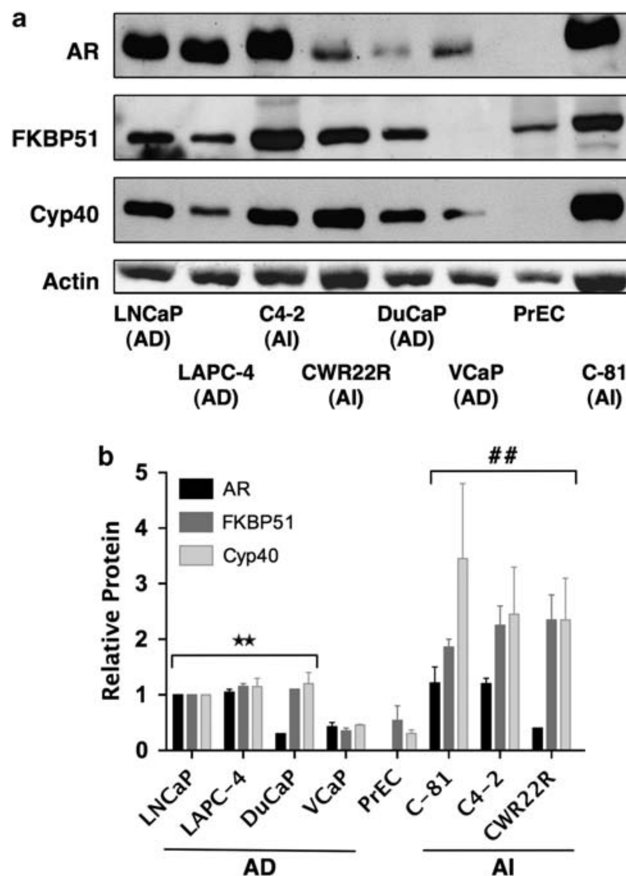

Figure 2 Overexpression of Cyp40, FKBP51 prostate cancer (PCa) cell lines. (**a**) Whole-cell extracts (WCEs) from normal prostate epithelial cells (PrEC), and from androgen-dependent (AD) (LNCaP, LAPC-4, VCaP and DuCaP) and -independent (AI) (C4-2, C-81 and CWR22R) human PCa cell lines were prepared and analyzed by western blot for expression of androgen receptor (AR), FKBP51, Cyp40 and actin as described in the Materials and methods section. (**b**) Densitometric scanning of the bands corresponding to protein expression of Cyp40, FKBP51 and the AR in PrEC, AD and AI human prostate cell lines. Cyp40, FKBP51 and AR protein levels were expressed as relative expression levels, taking the values obtained from LNCaP cells as 1. Results are representative of two independent experiments. AD vs PrEC, **P values ranged from <0.01 to <0.001. AI vs AD, ##P<0.01.

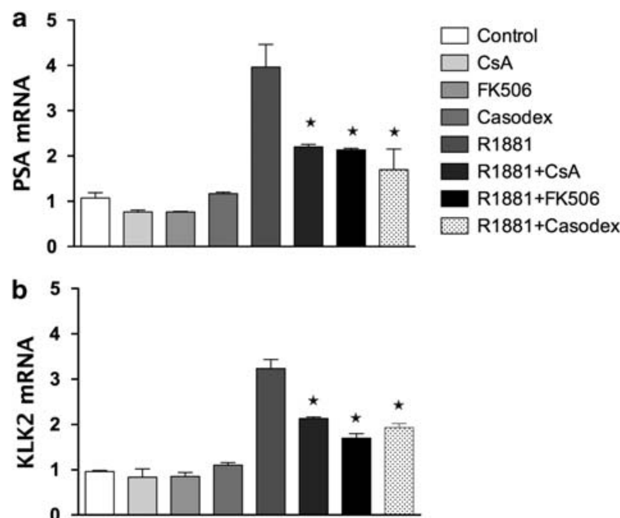

Figure 3 Inhibition of androgen receptor (AR) activity by FK506 and CsA is mediated by FKBP51 and Cyp40. LNCaP cells were treated with R1881 (1.0 nM) in the presence and absence of cyclosporine A (CsA) (5.0 μM) or FK506 (10.0 μM) or Casodex (10.0 μM) for 20 h. Total RNA was isolated and converted into cDNA. Real-time RT–PCR was performed using specific primers for prostate-specific antigen (PSA) (**a**) and KLK2 (**b**). PSA and KLK2 mRNA levels were normalized with the values obtained from 18S expression. PSA and KLK2 mRNA levels are expressed as relative expression values, taking the value obtained from cells treated with vehicle (control) as 1. Results are means ± s.e.m. of two independent experiments run in triplicate. *$P<0.05$ for ligand treated vs R1881.

FKBP51 and Cyp40 preferentially control AD proliferation of PCa cells

The results of Figure 2 showed overexpression of Cyp40 and FKBP51 in several AD PCa cell lines and even higher levels of each TPR in AI cells. The latter result suggested a possible role for the proteins in the AD to AI functional transition of PCa cells. To test this hypothesis, we compared AI C4-2 with AD LNCaP cells, as each expressed approximately equal amounts of AR, yet had differences with respect to FKBP51 (Figure 2). To confirm androgen sensitivity, C4-2 and LNCaP growth was measured in response to increasing concentrations of R1881. LNCaP cells showed a strong stimulatory effect by R1881 (200% at 1 nM), whereas C4-2 cells showed a small but reproducible growth response to hormone (data not shown). These findings are consistent with previously reported observations in LNCaP and C4-2 cells (Dehm and Tindall, 2006). However, C4-2 cells showed higher growth rates than LNCaP cells under hormone-free conditions (data not shown)—a key feature of AI PCa cells (Thalmann *et al.*, 2000; Igawa *et al.*, 2002). To assess the contribution of AR to the growth properties of C4-2 and LNCaP cells, AR activity at the PSA-Luc reporter gene was measured (Figure 6a). As expected, LNCaP cells showed a low basal activity by hormone-free AR and a strong response to ligand (~14 000%). In contrast, the defining feature of C4-2 cells was basal AR activity that was much higher (~2500%) than that of LNCaP cells (100%). Because of its high basal activity, C4-2 cells showed only a 10-fold induction in response to R1881, whereas LNCaP cells had a 130-fold induction of AR activity. These results show that, compared with LNCaP cells, the AR of C4-2 cells has a high constitutive activity and overall higher hormone-induced activity, but a lower degree of responsiveness to androgens.

To test the possibility that the high levels of FKBP51 in C4-2 cells contribute to their high basal AR activity, we overexpressed Flag-tagged FKBP51 in LNCaP cells. As expected, hormone treatment increased the expression of the AR and endogenous FKBP51 in both nontransfected (mock) and Flag-FKBP51-transfected LNCaP cells (Figures 6b and c). Overexpression of Flag-FKBP51 had no effect on AR protein levels, once again

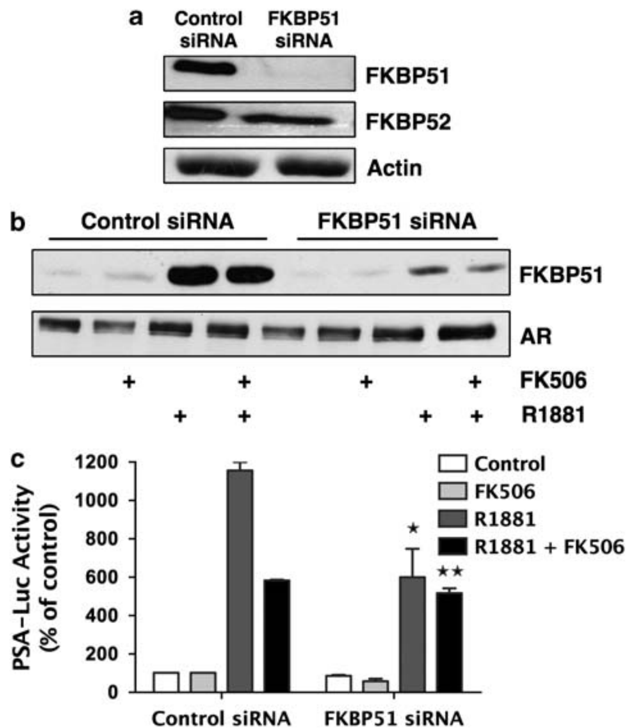

Figure 4 Downregulation of FKBP51 inhibits androgen receptor (AR) activity. LNCaP cells were transiently transfected with FKBP51 short-interfering RNA (siRNA) or nontarget control siRNA. After 96 h, whole-cell extracts (WCEs) were prepared, and expression of FKBP51, FKBP52 and actin (control) was measured by western blotting (**a**). LNCaP cells were transiently transfected with FKBP51 siRNA or nontarget control siRNA, and the prostate-specific antigen luciferase (PSA-Luc) promoter reporter construct along with a β-gal plasmid. After 48 h, cells were treated with or without R1881, FK506, FK506 and R1881 for 24 h. Lysates were prepared and analyzed for AR and FKBP51 expression by western blotting (**b**) and for AR reporter gene activity (**c**). Results are means ± s.e.m. of two independent experiments. *$P<0.05$ and **$P<0.01$ vs R1881 control siRNA.

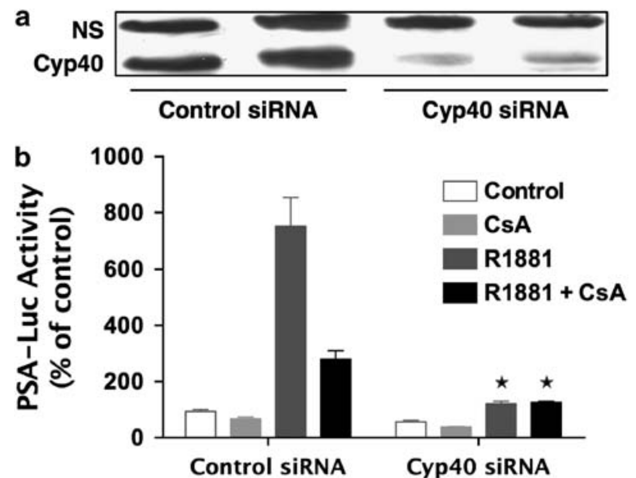

Figure 5 Downregulation of Cyp40 inhibits androgen receptor (AR) activity. LNCaP cells were transiently transfected with Cyp40 short-interfering RNA (siRNA) or nontarget control siRNA. After 96 h, whole-cell extracts (WCEs) were prepared, and expression of Cyp40 was measured by western blotting (**a**). LNCaP cells were transfected with Cyp40 siRNA or control siRNA, and with the prostate-specific antigen luciferase (PSA-Luc) reporter construct along with a β-gal plasmid. After 48 h, cells were treated with or without R1881, cyclosporine A (CsA), CsA and R1881 for 24 h. Lysates were prepared and analyzed for AR reporter gene activity (**b**). Results are means ± s.e.m. of two independent experiments. *$P<0.05$ vs R1881 control siRNA.

indicating that FKBP51 is not involved in the expression and stability of the AR. To our surprise, levels of Flag-FKBP51 were further enhanced by R1881 treatment, even though the Flag-FKBP51 plasmid is not under the control of the FKBP51 promoter. In mock-transfected LNCaP cells, the low basal reporter activity was increased by ~5–6-fold in response to hormone (Figure 6d). In FKBP51-transfected LNCaP cells, basal AR activity was ~2–3-fold higher than that of mock cells, and increased by only three folds in response to hormones. However, similar to C4-2 cells, the overall hormone-induced AR activity was higher than that of controls. These results substantiate the notion that FKBP51 functions as a positive regulator of the AR, serving to increase both the basal and overall activities of AR. The results also indicate that FKBP51 over-expression can cause the AR of AD LNCaP cells to mimic the AR found in AI C4-2 cells by increasing the constitutive and hormone-induced activities of the AR.

To confirm the significance of FKBP51 to AD and AI growth, levels of FKBP51 in LNCaP and C4-2 cells were reduced using siRNA (Figure 7). Western blot analysis showed a near-complete depletion of FKBP51 in both cell lines. As expected, FKBP51 expression was up-regulated by R1881 in control-siRNA-transfected cells in both cell lines, and this upregulation was significantly reduced by FKBP51-siRNA. In LNCaP cells, FKBP51 knockdown caused a greater than 50% reduction in androgen-induced cell proliferation compared with control-siRNA cells, while having no effect on hormone-free growth. The magnitude of this reduction is similar to the inhibitory effect that FKBP51 knockdown had on AR transcriptional activity in these cells (Figure 4). In contrast to LNCaP cells, FKBP51 knockdown in C4-2 cells had a smaller, but significant, reduction of proliferation (~15%) under basal and hormone-induced conditions, indicating that FKBP51 is critical for AD, but not for AI PCa cell growth.

Similar studies in LNCaP and C4-2 cells were conducted to determine the contribution of Cyp40 to AD and AI growth (Figure 8). In response to siRNA knockdown of Cyp40, LNCaP cells showed a dramatic decrease in androgen-induced proliferation. Indeed, loss of Cyp40 made LNCaP cells essentially unresponsive to hormone, while leaving basal growth unaltered. These results are in good agreement with the very strong inhibitory effect that Cyp40 knockdown had on hormone-induced AR transcriptional activity in LNCaP cells (Figure 5). The effect of Cyp40 loss on C4-2 cells, however, was much less dramatic, showing a decrease of ~20% in growth under all conditions. These results show that Cyp40, similar to FKBP51, is a positive regulator of AR activity, serving to preferentially

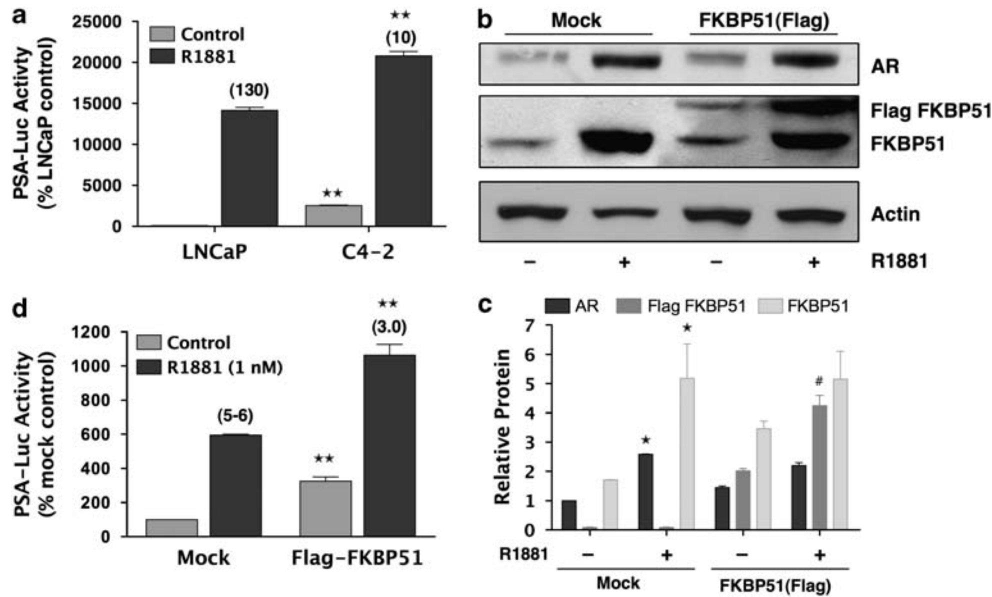

Figure 6 FKBP51 overexpression increases basal and hormone-induced androgen receptor (AR) activity. (**a**) LNCaP and C4-2 cells were transiently transfected with prostate-specific antigen luciferase (PSA-Luc) and β-gal constructs, followed by treatment with R1881 (1.0 nM) for 20 h and assays for luciferase activity normalized to β-gal. Parentheses show fold-increase relative to hormone-free cells. Results are means ± s.e.m. of three to four independent experiments. $^{**}P<0.01$, C4-2 vs LNCaP. (**b**, **c**, **d**) LNCaP cells were transfected with Flag-FKBP51 or control plasmid (mock), followed by treatment with or without R1881 for 20 h. (**b**) AR, Flag-FKBP51 and endogenous FKBP51 protein amounts were measured by western blotting with (**c**) quantization relative to actin and setting mock AR expression without R1881 as 1. $^{*}P<0.05$, mock AR and endogenous FKBP51 in the presence and absence of androgen. $^{\#}P<0.05$, Flag-FKBP51 in the presence and absence of androgen. (**d**) PSA-luc activity was normalized to actin and expressed as a percentage of mock control. Parentheses show fold-increase in response to hormones. Results are means ± s.e.m. of three independent experiments. $^{**}P<0.01$, Flag-FKBP51 vs Mock.

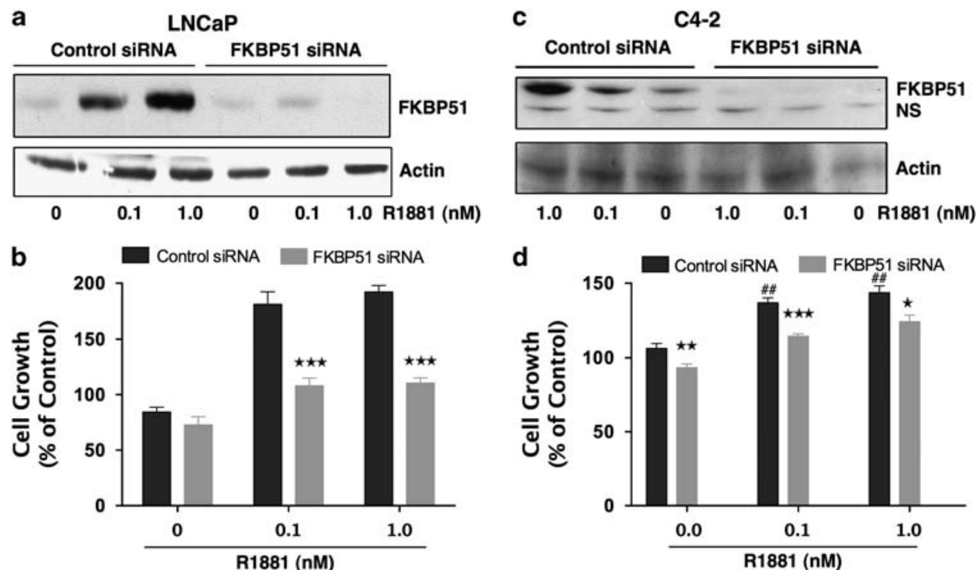

Figure 7 Downregulation of FKBP51 predominantly inhibits androgen-dependent (AD) growth in prostate cancer (PCa) cells. AD LNCaP and androgen-independent (AI) C4-2 cells were transiently transfected with FKBP51-short-interfering RNA (siRNA) or control-siRNA. At 6–8 h after transfection, the medium was changed. At 24 h after transfection, cells were plated (1500 cells per well) in 96-well (cell proliferation assay) and 6-well plates (western blotting). At 72 h after transfection, LNCaP (**a**) and C4-2 (**c**) cells were treated with R1881 (0.1 and 1.0 nM) or with vehicle (0) for 24 h. Whole-cell extracts were prepared, and expression of Cyp40 and actin (control) was measured by western blotting. LNCaP (**b**) and C4-2 (**d**) cells were treated with R1881 (0.1 and 1.0 nM) or vehicle (0) alone for 3 days. At 96 h after transfection, cell proliferation was determined by means of an MTT assay. Results are means ± s.e.m. of two independent experiments, each performed in quadruplicate. LNCAP: pairwise comparison of control siRNA vs FKBP51 siRNA at each condition, $^{***}P<0.001$. C4-2: pairwise comparison of control siRNA vs FKBP51 siRNA at each condition, $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$; comparison of control siRNA in the presence and absence of R1881, $^{\#\#}P<0.01$.

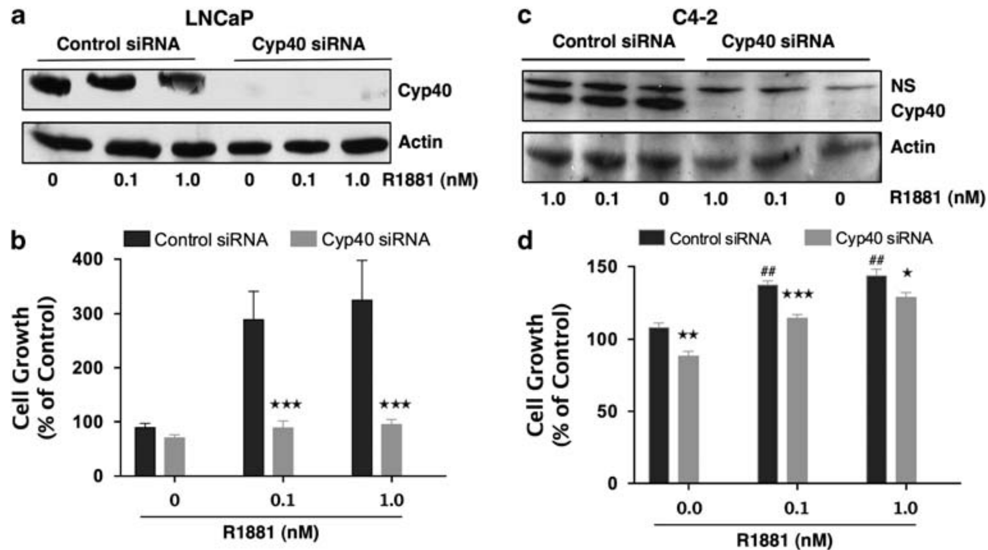

Figure 8 Downregulation of Cyp40 predominantly inhibits androgen-dependent (AD) growth in prostate cancer (PCa) cells. AD LNCaP and androgen-independent (AI) C4-2 cells were transiently transfected with Cyp40-short-interfering RNA (siRNA) or control-siRNA. At 6–8 h after transfection, the medium was changed. At 24 h after transfection, cells were plated (1500 cells per well) in 96-well (cell proliferation assay) and 6-well plates (western blotting). At 72 h after transfection, LNCaP (**a**) and C4-2 (**c**) cells were treated with R1881 (0.1 and 1.0 nM) or vehicle (0) for 24 h. Whole-cell extracts were prepared, and expression of Cyp40 and actin (control) was measured by western blotting. LNCaP (**b**) and C4-2 (**d**) cells were treated with R1881 (0.1 and 1.0 nM) or vehicle (0) alone for 3 days. At 96 h after transfection, cell proliferation was determined by means of an MTT assay. Results are means ± s.e.m. of two independent experiments, each performed in quadruplicate. LNCAP: pairwise comparison of control siRNA vs Cyp40 siRNA at each condition, $***P<0.001$. C4-2: pairwise comparison of control siRNA vs Cyp40 siRNA at each condition, $***P<0.001$, $**P<0.01$, $*P<0.05$; comparison of control siRNA in the presence and absence of R1881, $^{\#\#}P<0.01$.

promote androgen-induced PCa cell growth. The results also indicate that Cyp40 has a greater role than FKBP51 in the function of AR in LNCaP cells, consistent with our earlier observation that CsA is more effective than FK506 as an inhibitor of androgen-induced cell proliferation (Periyasamy *et al*., 2007).

Discussion

In a previous study, we showed the ability of immunophilin ligands, CsA and FK506, to inhibit the growth of PCa cell lines by attenuating AR hormone-binding and nuclear localization functions (Periyasamy *et al*., 2007). As Cyp40, FKBP51 and FKBP52 have been found in AR complexes (Veldscholte *et al*., 1992a; Febbo *et al*., 2005; Periyasamy *et al*., 2007), we speculated a direct targeting of these receptor-associated TPR proteins in the observed actions of FK506 and CsA. In this report, we extend these observations by measuring the expression of Cyp40, FKBP51 and FKBP52 in PCa tissue specimens and by altering their expression levels in PCa cells. We show a high-level expression of Cyp40 and FKBP51, but not FKBP52, in PCa tissues and in a variety of PCa cell lines, with expression being highest in AI cell lines. We also show that Cyp40 and FKBP51 are positive regulators of AR transcription that serve as the molecular targets of CsA and FK506, respectively. Finally, although both TPR proteins contributed to a smaller magnitude of AI growth, their principal roles seem to be control of AD PCa proliferation. Taken as a whole, the results suggest that selective and effective inhibition of androgen-induced growth is possible through CsA and FK506 drug action.

Recent results in FKBP52-deficient male mice showed aberrant penile development and prostate dysgenesis that correlated with reduced AR activity at endogenous and heterologous genes (Yong *et al*., 2007). It was therefore surprising that levels of FKBP52 were neutral in normal and PCa tissue samples (data not shown). Instead, we observed high levels of FKBP51 and Cyp40 in PCa tissues, suggesting that these proteins, rather than FKBP52, are prime contributors to the PCa phenotype. Interestingly, FKBP51-KO mice showed no defects of penile or prostate gland development (Yong *et al*., 2007), whereas similar negative results have been observed by our laboratories in Cyp40-KO male mice (Yong *et al*., unpublished observations). On the basis of these findings, we conclude that FKBP51 and Cyp40 must preferentially regulate AR activity in the mature prostate gland, whereas FKBP52 controls AR activity during prostate organogenesis.

Although it is clear that the PCa growth-suppressive effects of FK506 and CsA are mediated by the AR, the exact AR pathway affected remains unresolved. We have eliminated involvement by FK506 and CsA in apoptotic pathways, as no DNA fragmentation was observed in LNCaP cells treated with these drugs (Periyasamy *et al*., 2007). The classical immunosuppres-

sive pathways involving inhibition of calcineurin by Cyp18 or FKBP12 (Harding *et al*., 1989; Schreiber and Crabtree, 1992) have also been eliminated, as inhibition of calcineurin was found in PCa cell lines the growth of which was unaffected by CsA or FK506 (Periyasamy *et al*., 2007). Finally, no effect of CsA or FK506 on AR-mediated mammalian target of rapamycin (mTOR) activation was found (Periyasamy S and Sanchez E, unpublished observations), suggesting that the suppressive actions of CsA and FK506 on androgen-induced transcription and proliferation did not extend to all aspects of AR signaling. In this respect, TPR protein ligands represent an attractive alternative to androgen ablation in the treatment of PCa because of reduced potential for global hypoandrogenic side effects, such as impotence and reduced libido.

Evidence suggests that AR-mediated signaling has a key role in ablation-resistant PCa (Craft and Sawyers, 1998; Gregory *et al*., 2001; Grossmann *et al*., 2001), as re-expression of androgen-regulated genes is seen in recurrent PCa. Thus, factors other than androgens must activate the AR and contribute to PCa progression. Several laboratories have identified FKBP51 as a promising candidate for this function (Amler *et al*., 2000; Mousses *et al*., 2001; Velasco *et al*., 2004; Tomlins *et al*., 2007). FKBP51 is strongly repressed during hormone-ablation therapy. Conversely, FKBP51 can be twofold higher in recurrent compared with primary tumors, and a high-level expression of FKBP51 within sites of localized and/or metastatic PCa has been seen. Through our work here, a high-level expression of Cyp40 in PCa tissues and cells is now known. These results provided strong circumstantial evidence of a role for FKBP51 and Cyp40 in the pathogenesis and progression of PCa. As a direct test of this concept, we measured proliferation of PCa cells in the absence and presence of FKBP51 and Cyp40. The strongest role for each protein was seen for AD growth in LNCaP cells, although AI growth of C4-2 cells was affected to a much lesser degree. Taken together, the data indicate that FKBP51 and Cyp40 are predominantly involved in AD PCa cell proliferation. Although the etiology of FKBP51 and Cyp40 overexpression in PCa cells is not known, a possible candidate for FKBP51 overexpression is the AR itself. Androgens regulate the expression of the FKBP51 gene (*fkbp5*) through a direct interaction between the AR and distal enhancers located in the fifth and seventh introns of *fkbp5* (Magee *et al*., 2006; Makkonen *et al*., 2009 a, b). Thus, mutations to the AR that upregulate this function are likely to be important contributors to the AI phenotype.

An important question that arises from our study is with regard to the mechanisms by which FKBP51 and Cyp40 regulate AR transcription and proliferation in PCa cells. A mechanism likely to be shared by both proteins is alteration to AR conformation through their common PPIase function, which involves isomerization of the peptide backbone at select proline residues (Simental *et al*., 1991; Reid *et al*., 2002; McEwan, 2004; Lavery and McEwan, 2005). Interestingly, the N-terminal activation domain (AF-1) of AR has a high proline content and is required for both AD and AI activity (Dehm and Tindall, 2006), whereas the C-terminal AF-2 domain is critical only for ligand-dependent activity (Dehm and Tindall, 2006; Dehm *et al*., 2007). As such, the AF-1 domain of the AR is the most likely target for FKBP51 or Cyp40 actions. However, both the AF-1 and AF-2 domains of the AR contribute to a variety of AR functions, such as ligand binding, nuclear localization and recruitment of coactivators. Alterations to any one of these properties could account for our observed effects on AR transcription and cell proliferation. Indeed, we have already shown that CsA and FK506 caused a decrease in AR hormone-binding capacity and nuclear localization in LNCaP cells (Periyasamy *et al*., 2007). It should be noted, however, that the most recent data from studies of GR show that mutations that abrogate the PPIase function of FKBP52 do not block FKBP52 potentiation of GR (Riggs *et al*., 2007). Similarly, the PPIase activity of FKBP51 is not important for its inhibitory effect on GR. Yet, it is clear that the PPIase domain (rather than catalytic activity) is critical to FKBP52 actions on GR and PR (Riggs *et al*., 2003; Tranguch *et al*., 2005; Wochnik *et al*., 2005). A similar relationship may exist for FKBP51 and Cyp40 control of the AR in PCa cells. However, we cannot exclude the possibility that PPIase catalytic activity may still be required for the AR, as the mechanism of TPR control of nuclear receptors need not be universal. Indeed, FKBP51 actions on the AR are distinctly different from that on GR and PR, in which FKBP51 is a negative modulator (Reynolds *et al*., 1999; Denny *et al*., 2000; Hubler *et al*., 2003). Our results (Periyasamy *et al*., 2007 and this study) and others (Febbo *et al*., 2005) all point to positive regulation of the AR by FKBP51. Therefore, it is likely that FKBP51 exerts receptor-specific actions that could also vary across tissues or cell types. Further experiments are clearly needed to resolve these differences and define the possible mechanisms by which FKBP51 and Cyp40 augment AR transcription and cell proliferation in PCa cells.

In addition to the above-mentioned direct mechanism on the AR, FKBP51 and Cyp40 proteins might indirectly activate AR transcription and cell proliferation through other signaling pathways. For example, FKBP51 has been shown to activate STAT5 (Giraudier *et al*., 2002; Komura *et al*., 2003) and NF-κB signaling pathways (Romano *et al*., 2004; Avellino *et al*., 2005; Jiang *et al*., 2008), overactivity of which correlates with several non-PCa malignancies. Because cross-talk between STAT5 and AR (Ahonen *et al*., 2003; Li *et al*., 2007), as well as NF-κB and AR signaling (Chen and Sawyers, 2002; Cinar *et al*., 2004; Lee *et al*., 2005), in PCa cells has been shown, careful consideration of these and other nondirect pathways will be incorporated into future mechanistic studies.

In conclusion, our findings are the first evidence that CsA and FK506 negatively modulate AD activity and PCa proliferation by directly targeting Cyp40 and FKBP51, respectively. Cyp40 and FKBP51 can now be considered positive regulators of androgen-induced

AR-mediated cell growth and transcription, providing new and potentially important targets for the drug treatment of PCa.

Materials and methods

Cell culture
The human PCa cell lines, LNCaP, C4-2, C-81, CWR22R, DuCaP and VCaP, were routinely cultured and maintained in RPMI 1640 medium containing 5–10% FBS and 0.05% vol/vol penicillin/streptomycin (pen/strep). The human PCa LAPC-4 cells were routinely cultured and maintained in Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS and 0.05% vol/vol pen/strep. The normal human prostate epithelial cells (hPrECs) were cultured and maintained in media recommended by the manufacturer (Cambrex Corp., San Diego, CA, USA).

Prostate tissues
Normal and PCa tissues were purchased from the CHTN as frozen samples. The CHTN represents a group of hospitals that obtain tissues from either surgeries or autopsies and make these tissues available for research. The information obtained from the tissues indicates a diagnosis of the stage of cancer, which may include a Gleason score and type of therapy used (radiation and/or chemotherapy).

Real-time RT–PCR
Total RNA from normal and PCa tissues and from control and treated LNCaP cells was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription (2 μg total RNA) was performed by the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. Real-time RT–PCR (RT–PCR) was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7700 sequence detection system (Applied Biosystems). Gene-specific forward and reverse primer sequences were selected using the TaqMan probe and primer design function of the Primer Express version 1.5 software (Applied Biosystems). The primers used are listed in Supplementary Table 1. The fold-change in expression levels (using ribosomal 18S as control) was determined by a comparative C_t method using the formula $2^{-\Delta\Delta C_t}$, where C_t is the threshold cycle of amplification.

SDS–PAGE and western blot
Whole-cell extracts (WCEs) of prostate cells and tissues were prepared as previously described (Periyasamy *et al*., 2007). Equal amounts of WCEs were resolved by denaturing SDS–PAGE using either 7% or 10% polyacrylamide gels, followed by transfer to Immobilon polyvinylidene difluoride membranes and immunoblotting, as previously described (Banerjee *et al*., 2008). The human anti-AR antibody (SC815, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to probe for AR, whereas various antibodies were used to probe for FKBP51 (SC11514), actin (SC8432) and Cyp40 (PA3–022; Affinity BioReagents, Golden, CO, USA). After being probed, blots were incubated with appropriate HRP-conjugated counter antibodies. Proteins were detected using luminol (A8511; Sigma, Saint Louis, MO, USA), coumaric acid (C9008; Sigma) and hydrogen peroxide (H1009; Sigma) through enhanced chemiluminescence.

Transient transfection and luciferase reporter assays
LNCaP and C4-2 cells were plated either at a density of 5×10^5 in 6-well plates or 1×10^6 in 6 cm culture dishes and incubated with RPMI 1640 medium containing 5% dextran-coated charcoal-treated (DCC) serum. At 95% confluence, the cells were cotransfected with a PSA-Luc construct and a β-gal plasmid using Lipofectamine 2000 reagent (Invitrogen Corp.) according to the manufacturer's protocol, and incubated for 6 h at 37 °C in a serum-free RPMI 1640 medium. For TPR protein overexpression and knockdown studies, PCa cells were cotransfected with Flag-tagged FKBP51 (2.0 μg) or FKBP51-siRNA or Cyp40-siRNA or control siRNA (100 nM; Dharmacon RNA Technologies, Lafayette, CO, USA), along with and without a PSA-Luc construct and a β-gal plasmid as described above. At 24–48 h after transfection, cells were washed and refed with RPMI 1640 medium containing 5% DCC serum, and then treated with CsA (5.0 μM) or FK506 (10.0 μM) for 3 h, followed by R1881 (1 nM) for an additional 20 h as previously described (Periyasamy *et al*., 2007). Cell extracts were prepared with reporter lysis buffer (Promega Corp., Madison, WI, USA), and luciferase activity was measured using a luminometer and expressed as a percentage of control after normalization to β-gal activity.

Proliferation assays
LNCaP and C4-2 cells (3×10^3 cells per well) were plated in 96-well plates in RPMI 1640 medium containing 5% DCC serum, and the growth rate was determined as a function of time. To determine the effect of androgen-stimulated cell growth, LNCaP and C4-2 cells grown in RPMI 1640 medium containing 2% DCC serum were treated with increasing concentrations of R1881 (0–100 nM) with media and ligand changes on days 2, 4 and 6. At the end of day 7, cell proliferation was determined by a calorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazoline bromide) as previously described (Periyasamy *et al*., 2007). In experiments assessing the effects of TPR protein knockdown on cell growth, PCa cells were transfected with FKBP51-siRNA or Cyp40-siRNA or control siRNA as described. At 24 h after transfection, cells were plated (1500 cells per well) in 96-well plates and treated with R1881 (0.1 and 1.0 nM) or vehicle (0) alone for 3 days. At 96 h after transfection, cell proliferation was determined by means of an MTT assay and protein expression was determined by western blot as described.

Statistical analysis
Student's unpaired *t*-test was used throughout this study. The threshold for significance was set at $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)