TBLR1 as an androgen receptor (AR) coactivator selectively activates AR target genes to inhibit prostate cancer growth

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Abstract

Androgen receptor (AR), a steroid hormone receptor, is critical for prostate cancer growth. However, activation of AR by androgens can also lead to growth suppression and differentiation. Transcriptional cofactors play an important role in this switch between proliferative and anti-proliferative AR target gene programs. Transducin β-like-related protein 1 (TBLR1), a core component of the nuclear receptor corepressor complex, shows both corepressor and coactivator activities on nuclear receptors, but little is known about its effects on AR and prostate cancer. We characterized TBLR1 as a coactivator of AR in prostate cancer cells and determined that the activation is dependent on both phosphorylation and 19S proteosome. We showed that TBLR1 physically interacts with AR and directly occupies the androgen-response elements of the affected AR target genes in an androgen-dependent manner. TBLR1 is primarily localized in the nucleus in benign prostate cells and nuclear expression is significantly reduced in prostate cancer cells in culture. Similarly, in human tumor samples, the expression of TBLR1 in the nucleus is significantly reduced in the malignant glands compared with the surrounding benign prostatic glands (P<0.005). Stable ectopic expression of nuclear TBLR1 leads to androgen-dependent growth suppression of prostate cancer cells in vitro and in vivo by selective activation of androgen-regulated genes associated with differentiation (e.g. KRT18) and growth suppression (e.g. NKX3-1), but not cell proliferation of the prostate cancer. Understanding the molecular switches involved in the transition from AR-dependent growth promotion to AR-dependent growth suppression will lead to more successful treatments for prostate cancer.

Key Words
- androgen
- androgen receptor
- gene expression
- gene transcription
- prostate

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Introduction

It has long been known that androgens are critical in the growth and progression of prostate cancer (Dehm & Tindall 2006). Androgens signal through the androgen receptor (AR), a member of the steroid receptor family of transcription factors that plays an important role in the regulation of genes controlling growth suppression/differentiation and growth of prostate cells. Androgens activate AR, facilitating its translocation to the nucleus, binding at androgen-response elements (ARE), and interacting with cofactors (coactivators or corepressors), leading to the activation or repression of AR target genes. Although androgen activation leads to increased cell proliferation in the prostate cancer cells, it can also lead to differentiation and growth suppression (Yuan et al. 1993, Whitacre et al. 2002). The specific mechanism responsible for this switch is not well understood, although transcriptional cofactors have been shown to be important in the activation and repression of AR target genes (Janne et al. 2000, Gelmann 2002, Heinlein & Chang 2002). The presence or absence of these cofactors may control the balance between proliferation and differentiation (Peng et al. 2008a, Ligr et al. 2010). Both ARA70α and p44/Mep50/WDR77 are AR coactivators that act as androgen-dependent tumor suppressors.

Transducin β-like-related protein 1 (TBLR1), a transcriptional cofactor, was initially identified as a core component of the nuclear receptor corepressor (NCoR) complex and also coimmunoprecipitates with silencing mediator of retinoic acid and thyroid receptor (SMRT) (Tomita et al. 2004). The NCoR complex also contains the closely related protein TBL1X, and GPS2, IR10, and histone deacetylase 3 (HDAC3) (Yoon et al. 2003). The NCoR complex is responsible for the repression of many different transcription factors, including AR and other nuclear hormone receptors (NHRs; Burke & Banaihammad 2000).

As an intrinsic component of the NCoR complex, TBLR1 has been shown to have essential functions as a corepressor. TBLR1 is necessary for targeting of the NCoR complex that binds to histones H2B and H4, preferentially to acetylated histones, allowing for repression via deacetylation by HDAC3 (Yoon et al. 2005). TBLR1 also has been shown to be necessary for the activation of liganded NHRs. Removal of TBLR1 by single-cell microinjection of TBLR1 antibodies leads to the repression of nuclear receptor transcription by β-gal reporter assay including thyroid receptor (TR), estrogen receptor, retinoic acid receptor (RAR), and peroxisome proliferator-activated receptor γ (PPARγ). This activation is mediated through NCoR and SMRT, as removal of these proteins by injecting antibody or siRNA rescues the repressive effect. TBLR1, an F-box-like-domain-containing protein, has been shown to recruit ubiquitin ligases to the NHR promoter and target NCoR for ubiquitination and dismissal, allowing for the binding of coactivators (Perissi et al. 2004). In addition, TBLR1 has been shown to be phosphorylated in 293T cells by PKCδ, and the ability to activate NHR transcription by TBLR1 is dependent on this phosphorylation (Perissi et al. 2008). Recent evidence has also shown that TBLR1 is sumoylated and that this sumoylation is required for the activation of Wnt target genes through β-catenin (Choi et al. 2011).

In this study, we investigated the role of TBLR1 in the modulation of AR-mediated transcription and prostate cancer growth. We show that TBLR1 is a coactivator of AR and acts as a tumor suppressor in prostate cancer, consistent with its decreased nuclear expression in human prostate cancer compared with benign glands in clinical samples by immunohistochemistry (IHC). TBLR1 selectively activates AR target genes important for growth suppression and differentiation but not proproliferative AR target genes.

Subjects and methods

Cell lines and culture

LNCaP, PC3, and 293T cells were procured from ATCC (Manassas, VA, USA) with authentication documented by short tandem repeat (STR) analysis. RC165, an immortalized benign prostate cell, was obtained from the laboratory of Dr J Rhim (Uniformed Services University of the Health Sciences) (Gu et al. 2005). LNCaP-AI, an androgen-independent derivative of LNCaP, was obtained from the laboratory of Dr A Ferrari (New York University Cancer Institute; Gao et al. 1999). PC3-AR was obtained from the laboratory of Dr Z Wang (MD Anderson Cancer Center; Peng et al. 2008b). RC165, RC170, LNCaP-AI, and PC3-AR were all previously used in published research by our laboratory (Peng et al. 2008b, Li et al. 2009, Ligr et al. 2012). Passages from 5 to 20 of each cell line were used for experiments. LNCaP, LNCaP-AI, PC3, RC165, RC170, and 293T cells were maintained in a regular medium (RPMI medium 1640 (High glucose DMEM for 293T) plus 10% charcoal-treated FBS), or androgen media (androgen-free medium with 10 nM R1881). The cell lines were maintained at 37 °C in the presence of 5% CO2.
Luciferase assay

Luciferase assays were carried out (Peng et al. 2008a) with Lipofectamine (Invitrogen) with four ARE (4× ARE) reporter, 10 ng pCDNA-AR, 100 ng reporter, 1 ng pRL-TK internal control plasmids, and varying amounts of pCDNA TBLR1 plasmid or mutants. The cells were treated with androgen media for 24 h before reading. For the appropriate experiments, the cells were also treated with 1 μM Rottlerin or 1 μM MG132 for 24 h before reading. Dual-Luciferase Reporter Assay System (E1910, Promega) was used according to the manufacturer’s instructions.

Coimmunoprecipitation

Immunoprecipitation was carried out using either anti-AR, anti-TBLR1, or M2FLAG agarose antibodies to precipitate complexes. The complexes were incubated overnight at 4 °C. Anti-AR/TBLR1 complexes were then incubated with protein G beads for 4 h at 4 °C. The beads/agarose were washed three times with lysis buffer and eluted with 2× Laemmli loading buffer at 100 °C for 5 min.

Whole-cell lysates, cell fractionation, and immunoblot analysis

Whole-cell extracts were prepared from LNCaP, RC165, and PC3 cells containing NLSTBLR1 (TBLR1 with a nuclear localization sequence (NLS) tag), pBabe, or native lysates in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.1% SDS). For cell fractionation, cells were lysed with cytoplasmic buffer (10 mM HEPES, pH 7.6, 50 mM NaCl, 0.5 M sucrose, 1 mM dithiothreitol, 5 mM MgCl₂, and 0.1% Triton X-100). After centrifugation, the supernatant could be retained as the cytoplasmic fraction and the nuclear pellet was washed three times with cytoplasmic buffer followed by lysis with RIPA buffer. The extracts were subjected to electrophoresis on SDS–PAGE and then transferred to nitrocellulose membranes for immunoblot analysis as described by Li et al. (2009) with antibodies for TBLR1 and ERK2 (Santa Cruz Antibodies (Dallas, TX, USA)), β-actin and anti-FLAG M2 (Sigma (St. Louis, MO, USA), H2B and GAPDH (Cell Signaling (Danvers, MA, USA)), KRT18, NKK3-1, p21, p27, HUS1, and JAG1 (Abcam (Cambridge, MA, USA)).

Immunofluorescence microscopy

The cells were fixed in 4% paraformaldehyde (PFA) for 20 min and permeabilized in 1:1 methanol:acetone for 20 min. The cells were incubated in 1:100 TBLR1 antibody in 10% sheep serum overnight, washed with PBS, and incubated in anti-mouse IgG-Cy2-conjugated antibodies (Molecular Probes (Grand Island, NY, USA) 1:500 in 10% sheep serum) for 1 h, washed with PBS, and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst for 2 min as described previously (Li et al. 2009).

Tissue microarray construction and IHC

The study protocol was approved by the New York University Institution Review Board. An IHC study with antibody against TBLR1 was used to characterize the expression pattern of TBLR1 in prostate cancer. Whole tissue sections from 30 cases of prostatic adenocarcinoma were studied. TBLR1 nuclear and cytoplasmic expression was compared between malignant glands and the surrounding benign prostatic glands on the same slide. The intensity of nuclear and cytoplasmic TBLR1 expression was scored semiquantitatively; 0 as negative, 1 as weak, 2 as moderate, and 3 as strong expression. The score represents the overall level of TBLR1 immunoreactivity including staining intensity and the percentage of positive cells. The nuclear staining and cytoplasmic staining were scored separately. The nuclear and cytoplasmic expression pattern of TBLR1 was studied in 86 prostatic adenocarcinoma samples on a tissue microarray (TMA). Each sample is represented by four 0.6 mm cores on the TMA. The samples on the TMA were stratified by various clinicopathological factors including age at diagnosis, preoperative PSA, grade, and stage.

Construction of retroviral pBabe vectors expressing NLSTBLR1 to construct stable cell lines

We created a TBLR1 fusion protein in which the strong NLS PKKKRKV was fused to the N terminus of TBLR1 to create NLSTBLR1. A retroviral-based mammalian expression vector, pBabe, was used to stably express NLSTBLR1 proteins in LNCaP cells as described previously (Peng et al. 2008a). Briefly, Phoenix A amphotropic packaging cells (American Type Culture Collection (ATCC)) were transfected with NLSTBLR1 and pBabe retroviral constructs to produce virus. The virus-containing supernatant was collected by centrifugation and filtered before retroviral infection. Stable cell lines were selected in 1 μg/ml puromycin.

Cell proliferation assays

For cell proliferation assay by WST1 (Roche), cells (1×10⁴) were plated into 24-well plates and measured at A450 every other day. For cell proliferation assays, cells (4×10⁴)
were plated into six-well plates and counted with a hemocytometer every other day, as described previously (Peng et al. 2008a).

Flow cytometry and apoptosis assay
The cells were grown in six-well plates and were dissociated with trypsin, resuspended in HBSS, and fixed in ice-cold 70% ethanol. The cells were incubated in propidium iodide/RNAse solution (1 mg propidium iodide, 10 mg EDTA, 250 µl Igepal, and 1 ml of 10 mg/ml RNAse dissolved in 50 ml PBS) at 37 ºC for 2 h. The cell-cycle analysis was performed on a FACSC flow cytometer (BD Biosciences (Bedford, MA, USA)) and analyzed using Weasel Software (The Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia). For measurement of apoptosis, Promega Apo-ONE homogeneous caspase 3/7 assay was used as per the manufacturer’s instructions. The cells were incubated in 10 nM R1881 media for 24 h before addition of reagent for 18 h incubation.

RNA isolation and qRT-PCR
Total RNA was extracted from the cell lines using RNAqueous-4PCR Kit (AM1914, Ambion). A RetroScript Kit (AM1710, Ambion) was used for cDNA synthesis with isolated RNA as template, according to the manufacturer’s instructions. RNA was isolated from LNCaP NLSTBLR1 and LNCaP pBabe cells after 48 h of hormone-free incubation, followed by 24 h of stimulation by 10 nM R1881. Primers for qPCR were kindly donated by Dr S K Logan as reported previously (Nwachukwu et al. 2009). PCR was carried out using the Bio-Rad CFX96 machine and Bio-Rad iQ SYBR Green supermix.

Gene knockdown by siRNA
siRNAs for AR, TBLR1, and control siRNA were purchased from Ambion and transfected with HiperFect Transfection Reagents (Qiagen) 48 h before immunoblot analysis of whole-cell extract, RNA extraction, or cell kinetic studies.

Anchorage-independent growth
LNCaP NLSTBLR1 and pBabe control were seeded into 0.35% agarose media, containing 10% charcoal-stripped FBS with 10 nM R1881 or ethanol vehicle at 4×10⁴ cells/60 mm plate. Fresh 0.35% agarose media was added every 3 days and colonies were allowed to grow for 3 weeks as described previously (Cai et al. 2008). The average number of colonies of the three duplicate plates is displayed. Pictures were taken of representative colonies for each condition.

Chromatin immunoprecipitation
The LNCaP cells were grown in androgen-free media for 72 h before 4 h of stimulation in 10 nM R1881 or ethanol vehicle. The cells were cross-linked for 10 min in 1% formaldehyde solution, followed by quenching with 0.125 M glycine. The cells were washed in PBS and lysed in 0.5% NP-40, 0.25% Triton X-100, 50 mM HEPES, pH 7.5, 10% glycerol, 140 mM NaCl, and 1 mM EDTA. Nuclei were resuspended in 50 mM Tris–HCl, pH 8, 10 mM EDTA, and 1% SDS and sonicated four times for 10 s each. Twenty five microgram of chromatin was used for each immunoprecipitation (IP) reaction: 2 µg of TBLR1, AR, or rabbit IgG antibody was used and incubated overnight at 4 ºC. The samples were incubated with Protein G beads for 4 h at 4 ºC and washed five times in wash buffer. Immunoprecipitated complexes containing DNA were eluted in 1% SDS–Tris buffer and reverse crosslinked at 65 ºC overnight. Quantitative PCR was carried out using the BioRad CFX96 machine and Biorad iQ SYBR Green supermix. Cycling conditions were 95 ºC for 10 minutes, followed by 40 cycles of 95 ºC for 15 seconds and 60 ºC for 1 minute. The primer sets used were NKX3-1 ARE I: F, GATGGGTGGGAGGAGATGA; R, TGTCCTTGA-CAAGCGGAG; NKX3-1 ARE II: F, GGTTCTGCTGTTACGTTTG; R, CTGCTGCTGCTGCTGCTG; HUS1 ARE: F, CTGCTGCTGCTGCTGCTGCTGCTG; R, CCCACGACCCA- GGGTGATG; and GAPDH 3'-UTR: F, ATGGTTGCCAC- TGGGGATCT; R, TGCCAAAGCCTAGGGGAAGA.

Nude mouse xenografts
Male nude mice (5 weeks old) were purchased from NCI (Frederick, MD, USA) and maintained in accordance with the Institutional Animal Care and Use Committee-approved protocol. Ten million cells were used for s.c. injection into the flank region of the mice with Matrigel ECM (reconstituted basement membrane) to support tumor growth. Each experimental group contained ten mice. Tumors were measured with calipers two times per week as described previously (Ligr et al. 2010). At the end of the experiment, tumors were removed and weighed.

Site-directed mutagenesis
Point mutations in pCDNA TBLR1 and pBabe NLSTBLR1 were made using the Stratagene Quickchange XL (200521)
mutagenesis kit as described previously (Cai et al. 2008). Mutation 1 is S123-A123 and mutation 2 is S199, T203, S204-A199, A203, A204 respectively.

**Statistical analysis**

Statistical analyses of the above results were performed by Student’s t-test. *P*<0.05, **P*<0.01, and ***P*<0.001. Differences are considered statistically significant if *P*<0.05.

**Results**

**TBLR1 functions as an AR coactivator in prostate cancer cells**

Although initially identified as a core component of the NCoR corepressor complex and necessary for repression of TR, TBLR1 has also been found to be important in the activation of several transcription factors, including but not limited to, RAR, PPARγ, and nuclear factor kappa B (NF-κB) (Perissi et al. 2004). We examined the role of TBLR1 in AR-mediated transcription using a dual luciferase assay, with a luciferase reporter containing 4× ARE and pRL Renilla internal control reporter in PC3 (AR negative) prostate cancer cells in the presence of synthetic androgen, R1881. In the absence of androgen or AR, increased TBLR1 expression had no effect on reporter activity. However, after transfection with AR, TBLR1 lead to up to 2.5-fold increase in AR activation in a dose-dependent manner (Fig. 1A).

We next determined if there was a physical interaction between AR and TBLR1. First, we overexpressed a FLAG-tagged AR plasmid or a GFP control plasmid in 293T cells. Using FLAG antibody to immunoprecipitate and immunoblotting for TBLR1, we identified an interaction between AR and TBLR1 (Fig. 1B1). Next, to confirm this interaction in prostate cancer cells, we used AR-positive LNCaP cells stably overexpressing NLSTBLR1. We used TBLR1 antibody for immunoprecipitation and AR for immunoblotting. The cells were grown in androgen-free media for 48 h before stimulation with 10 nM R1881 or ethanol vehicle for 6 h. We observed interaction between the two proteins in prostate cancer cells in the presence of R1881 (Fig. 1B2). In addition, we carried out the experiment in a reciprocal manner, using AR antibody to immunoprecipitate and TBLR1 antibody to probe to confirm the physical interaction (Fig. 1B3). Finally, to test this interaction in an endogenous system without any overexpression, we used the LNCaP-AI cell line. LNCaP-AI cells are an androgen-independent derivative of LNCaP cells that still express AR and are sensitive to androgens. By immunoprecipitation with AR antibody and immunoblotting for TBLR1, we were also able to observe the interaction between TBLR1 and AR in an androgen-dependent manner (Fig. 1B4).

**Posttranslational modifications important for TBLR1 action on AR-mediated transcription**

Several posttranslational modifications affect TBLR1 activity (Perissi et al. 2004, 2008). The ability of TBLR1 to activate RAR is dependent both on 19S proteosome and PKCδ phosphorylation. Phosphorylation sites of TBLR1 are marked on the diagram in Fig. 1C. Upon phosphorylation at S123 and/or S199/T203/T204, TBLR1 targets NCoR for ubiquitination and dismissal in the activation of RAR (Perissi et al. 2008). We tested if this was also true in the case of TBLR1 acting as a coactivator of AR in prostate cancer cell growth. In the presence of a specific 19S proteosome inhibitor, MG132, a specific PKC inhibitor, Rottlerin, or mutant TBLR1 constructs, S123A, S199A/T203A/T204A, or the double mutant TBLR1 activation of AR was diminished up to threefold (Fig. 1D, E and F). We found that mutation of site 1, site 2, or both had the same diminished transcriptional effect, suggesting that both sites are involved in transcriptional activation. It is important to note that the phospho-mutant TBLR1 plasmids did retain partial activation ability. We also performed a parallel western blot to check the overall NCoR levels and confirm overexpression of TBLR1 after transfection with WT TBLR1 and the mutant constructs (Fig. 1F). However, we found no variations in total NCoR levels among each condition.

Sumoylation is another posttranslational modification reported to affect TBLR1 activity (Choi et al. 2011). However, the mutation of the critical site for sumoylation K497 did not affect its AR-mediated transcriptional coactivator activities in our luciferase assay (Supplementary Fig. 1A, see section on supplementary data given at the end of this article).

**TBLR1 expression and subcellular localization in prostate cancer**

Altered cofactor expression and localization have been shown to correlate with prostate cancer (Peng et al. 2008a, Ligr et al. 2010). In this study, we have examined the expression levels and intracellular localization of TBLR1 in benign and malignant prostate cell lines and human tissue.
The level of expression in the benign prostate cell line, RC165 (Gu et al. 2005), and the malignant prostate cell line, LNCaP, was compared. TBLR1 protein expression in LNCaP was lower than in RC165 (Fig. 2A). By immunofluorescence microscopic methods, we have shown that TBLR1 was primarily localized in the nucleus of RC165 but distributed in both the cytoplasm and the nucleus in LNCaP cells (Fig. 2B).

We confirmed these results by western blot with cellular fractionation (Fig. 2C). To further confirm variable levels of nuclear TBLR1 expression in benign vs malignant prostate cancer cells, we also tested protein levels in two additional androgen-sensitive cell lines, benign RC170 cells and malignant LNCaP-AI cells, relative to LNCaP cells with cell fractionation followed by western blot (Supplementary Fig. 2A, see section on...
supplementary data given at the end of this article). Consistently, benign RC170 cells showed high nuclear TBLR1 and malignant LNCaP-AI showed low nuclear TBLR1 levels.

It has previously been shown that TBLR1 translocates to the nucleus upon serum starvation in NIH 3T3 cells (Zhang et al. 2006). These results, combined with the fact that we observed higher nuclear TBLR1 expression in benign RC165 vs malignant LNCaP cells, indicated that nuclear TBLR1 conveyed a growth arrest signal. To test if this phenomenon was true in the prostate, LNCaP-AI cells were serum starved for 16 h in 0.2% FBS. Serum-starved LNCaP-AI cells showed increased nuclear and reduced cytoplasmic staining compared with cells grown in normal 10% FBS growth media (Fig. 2D and E). Serum-starvation-induced growth arrest did not lead to changes in overall TBLR1 expression (Fig. 2F).

We next looked at TBLR1 expression in human prostate cancer tissue samples. TBLR1 nuclear and cytoplasmic expression was measured by IHC using whole tissue sections from 30 cases of prostatic adenocarcinoma. TBLR1 nuclear and cytoplasmic expression was compared between malignant glands and the surrounding benign prostatic glands on the same slide.

Comparing malignant glands with the surrounding benign prostatic glands on the same tissue section, the expression of TBLR1 in nucleus is significantly lower in the malignant glands (P < 0.005; Fig. 2G). Among the 30 cases of prostatic adenocarcinoma we studied, the levels of TBLR1 nuclear expression in malignant glands: decreased in 19 cases, was unchanged in ten cases, and increased in one case (Table 1). In addition, TBLR1 nuclear and cytoplasmic expression patterns were studied in 86 samples of prostatic adenocarcinoma on a TMA. When stratified with...
To study the functional effects of nuclear TBLR1 in prostate cancer, we aimed to overexpress nuclear TBLR1 in LNCaP cells and measure its effects. In order to achieve nuclear localization of exogenous TBLR1 expression, we created an NLSTBLR1 (containing a nuclear localization sequence PKKKRKV) fusion construct. GFP-NLSTBLR1 was confirmed to localize to the nucleus upon transfection into 293T cells (Supplementary Fig. 3A, see section on supplementary data given at the end of this article). In comparison, GFP-TBLR1 (WT) construct expression was exclusively observed in the cytoplasm (Supplementary Fig. 3B). Control cells were made using the pBabe vector alone. Overexpression of NLSTBLR1 is shown both at the RNA and protein level (Fig. 3A). We also confirmed that overexpression of nuclear TBLR1 had no effect on AR protein levels in these cells (Supplementary Fig. 3C). Nuclear TBLR1 dramatically reduced proliferation of LNCaP cells in 10 nM R1881 media and also showed reduced proliferation in hormone-free media (Fig. 3B), although the degree of inhibition is more substantial in androgen-containing media. The cell-cycle analysis by flow cytometry revealed that overexpression of nuclear TBLR1 in androgen-containing media led to a twofold decrease in S-phase cells, suggesting a G0/G1 arrest mechanism as the cause of reduced proliferation (Fig. 3C). Similar findings were observed in hormone-free media as well (data not shown). We found no evidence of increased apoptosis in LNCaP NLSTBLR1 compared with LNCaP pBabe in 10 nM R1881 media, using a caspase 3/7 activity assay (Supplementary Fig. 3D). We found similar proliferative effects of nuclear TBLR1 with both 1 and 10 nM R1881 (Supplementary Fig. 4A, B, C and D, see section on supplementary data given at the end of this article). In addition, we performed TBLR1 silencing with siRNA to test the effects on proliferation and cell cycle. Interestingly, loss of TBLR1 expression in LNCaP cell lines led to dramatic decrease in growth and G0/G1 cell-cycle arrest (Supplementary Fig. 5B and C). TBLR1 knockdown in LNCaP-AI cells also led to decreased growth and G0/G1 cell-cycle arrest (Supplementary Fig. 5D and E).

To test effect of nuclear TBLR1 on cellular transformation, we performed an anchorage-independent growth assay in soft agar, comparing LNCaP cells expressing NLSTBLR1 or control vector. After 14 days, significantly fewer colonies were observed in cells overexpressing NLSTBLR1. In hormone-free media, colony numbers of cells expressing NLSTBLR1 were reduced to 75%, and in androgen-containing media they were reduced to 67% (Fig. 3D). In addition, the average size of colonies was reduced as shown in representative pictures of colonies (Fig. 3E). Thus, increased nuclear TBLR1 not only led to reduced growth of LNCaP cells but also inhibited their anchorage-independent growth, an in vitro assay of transformation.

In addition, we created LNCaP cell lines expressing NLSTBLR1 phospho-mutants as described earlier; however, the growth inhibitory function of nuclear TBLR1 was not affected by either mutations (Supplementary Fig. 3E).

**Nuclear TBLR1 suppresses prostate tumor growth in vivo**

To determine if the inhibitory effect of nuclear TBLR1 on growth in culture could be replicated in vivo, we examined tumor growth in subcutaneous nude mouse xenografts with LNCaP pBabe and LNCaP NLSTBLR1 cells (Fig. 4A and B). In ten mice of each group, $1 \times 10^7$ cells were injected s.c. into the right flank and measured twice per week. Tumors produced by LNCaP pBabe cells exhibited steady growth over the 10-week time course of the experiment. However, tumors produced by LNCaP NLSTBLR1 cells exhibited minimal growth over the same time period (Fig. 4C). Two separate single clones of LNCaP NLSTBLR1 were used to confirm the inhibitory effect of

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**Table 1 Expression of nuclear TBLR1 in prostate cancer**

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<tr>
<th>TBLR1 nuclear staining (malignant vs adjacent benign gland)</th>
<th>Levels of TBLR1 nuclear expression</th>
<th>No. of cases</th>
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<tr>
<td>Decreased</td>
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Thirty cases of prostate cancer were stained for TBLR1 by immunohistochemistry in which both malignant and benign glands could be observed on the same slide. Benign and malignant cells were scored based on overall level of TBLR1 immunoreactivity including staining intensity and the percentage of positive cells in each sample. Data in the table indicate the number of the malignant glands vs benign glands samples that exhibited decreased, unchanged, or increased levels of TBLR1 nuclear expression in relation to clinicopathological factors, such as the levels of TBLR1 nuclear expression have a positive correlation with the Gleason scores ($R^2 = 0.9621$, $P < 0.01$; Supplementary Fig. 2C).

We also tested 14 additional cases with high-grade prostatic intraepithelial neoplasia (HGPIN), a precursor lesion of prostate cancer, adjacent to benign prostate glands on the same section. The data indicated decreased TBLR1 expression in ten of 14 cases (71%) of HGPIN (Supplementary Table 1 and Supplementary Fig. 2B). No statistically significant difference between the level of TBLR1 cytoplasmic expression in malignant prostatic glands and that in the benign prostatic glands was observed.

**Nuclear TBLR1 inhibits prostate cancer growth in vitro**

To study the functional effects of nuclear TBLR1 in prostate cancer, we aimed to overexpress nuclear TBLR1 in LNCaP cells and measure its effects. In order to achieve nuclear localization of exogenous TBLR1 expression, we created an NLSTBLR1 (containing a nuclear localization sequence PKKKRKV) fusion construct. GFP-NLSTBLR1 was confirmed to localize to the nucleus upon transfection into 293T cells (Supplementary Fig. 3A, see section on supplementary data given at the end of this article). In comparison, GFP-TBLR1 (WT) construct expression was exclusively observed in the cytoplasm (Supplementary Fig. 3B). Control cells were made using the pBabe vector alone. Overexpression of NLSTBLR1 is shown both at the RNA and protein level (Fig. 3A). We also confirmed that overexpression of nuclear TBLR1 had no effect on AR protein levels in these cells (Supplementary Fig. 3C). Nuclear TBLR1 dramatically reduced proliferation of LNCaP cells in 10 nM R1881 media and also showed reduced proliferation in hormone-free media (Fig. 3B), although the degree of inhibition is more substantial in androgen-containing media. The cell-cycle analysis by flow cytometry revealed that overexpression of nuclear TBLR1 in androgen-containing media led to a twofold decrease in S-phase cells, suggesting a G0/G1 arrest mechanism as the cause of reduced proliferation (Fig. 3C). Similar findings were observed in hormone-free media as well (data not shown). We found no evidence of increased apoptosis in LNCaP NLSTBLR1 compared with LNCaP pBabe in 10 nM R1881 media, using a caspase 3/7 activity assay (Supplementary Fig. 3D). We found similar proliferative effects of nuclear TBLR1 with both 1 and 10 nM R1881 (Supplementary Fig. 4A, B, C and D, see section on supplementary data given at the end of this article). In addition, we performed TBLR1 silencing with siRNA to test the effects on proliferation and cell cycle. Interestingly, loss of TBLR1 expression in LNCaP cell lines led to dramatic decrease in growth and G0/G1 cell-cycle arrest (Supplementary Fig. 5B and C). TBLR1 knockdown in LNCaP-AI cells also led to decreased growth and G0/G1 cell-cycle arrest (Supplementary Fig. 5D and E).

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nuclear TBLR1 on in vivo growth. After the 10-week time course, tumors were harvested and weighed. Weight of LNCaP pBabe tumors was significantly higher than that of LNCaP NLSTBLR1 tumors (Fig. 4D). IHC staining of xenograft tumors confirmed increased TBLR1 expression in the NLSTBLR1 tumors compared with pBabe control by IHC (Fig. 4E and F). There is a strong increase in the tumor suppressor NKX3-1, an AR target gene, reactivity in NLSTBLR1 tumors compared with pBabe control (Fig. 4E and F). There was no difference in cleaved Caspase 3 levels between NLSTBLR1 and control tumors (Supplementary Fig. 7A and B, see section on supplementary data given at the end of this article), consistent with our in vitro data (Supplementary Fig. 3D).

**AR-dependence of nuclear TBLR1 function on prostate cancer growth suppression**

To test if the effect of nuclear TBLR1 on prostate cancer cell growth is mediated through AR, we created cell lines expressing NLSTBLR1 in PC3 cells, an AR-negative prostate cancer cell line, and PC3-AR, a derivative of PC3 expressing WT AR. Increased nuclear TBLR1 slightly increased growth in AR-negative PC3 cells in 10 nM R1881 media, (Fig. 5A) but PC3-AR cells expressing NLSTBLR1 exhibited reduced growth in 10 nM R1881 media (Fig. 5B). In hormone-free media, PC3-AR NLSTBLR1 cells exhibited a slightly increased growth rate compared with PC3-AR control cells (Supplementary Fig. 8A and B).
Fig. 6A, see section on supplementary data given at the end of this article) similar to what we have observed in PC3-NLSTBLR1 cells in androgen media (Fig. 5A). Although AR-transfected PC3 cells lines primarily show a negative growth response to androgen (Yuan et al. 1993, Garcia-Arenas et al. 1995), more recently it has been observed that the level of AR expression can modulate the androgen response in these cells (Altuwaijri et al. 2007, Yu et al. 2009). The PC3-AR cell line used in our experiments showed a slight increase in growth rate upon stimulation with 10 nM R1881 compared with hormone-free media (Supplementary Fig. 6B).

In addition, we examined the effects of loss of AR using AR knockdown with siRNA in LNCaP pBabe control
cells (Fig. 5C) and LNCaP NLSTBLR1 cells (Fig. 5D), followed by cell proliferation assays. AR knockdown on LNCaP pBabe control cells reduced growth while AR knockdown in LNCaP NLSTBLR1 stimulated growth, partially reversing the negative growth effects of over-expressed nuclear TBLR1. Together, these data indicate that there is at least partial AR-dependence for nuclear TBLR1 suppression of prostate cancer growth.

Selective regulation of AR target genes by nuclear TBLR1

Because TBLR1 acts as a coactivator for AR in prostate cancer cells in a luciferase reporter assay and alterations in nuclear TBLR1 expression led to growth arrest, we sought to determine what specific AR-responsive genes were regulated by TBLR1. Quantitative RT-PCR was used to measure the effect of increased nuclear TBLR1 on the transcription of a set of androgen-regulated genes. AR target genes are involved in a broad spectrum of variable cellular processes including cell-cycle progression, tumor suppression, differentiation, and apoptosis. We selected a limited number of genes from different groups as examples to measure nuclear TBLR1 effects on AR-mediated transcriptional activation. This panel contained an assortment of exemplary androgen-regulated genes in various categories including CCNA2 (Cyclin A2) and CDC6 (promoting cell-cycle activation), HUS1 (involved in cell-cycle arrest), NKX3-1 (prostate-specific tumor suppressor), and KRT18 (luminal epithelial differentiation).

We compared gene expression between LNCaP NLSTBLR1 and LNCaP pBabe control cells after stimulation with androgen. The panel of genes we tested was divided into two groups. Genes that showed >1.7-fold activation by NLSTBLR1 in set 1 (Fig. 6A and B) and genes that showed little to no activation in set 2 (Supplementary Fig. 8A, see section on supplementary data given at the end of this article). Subset 1 included genes primarily associated with growth suppression/differentiation, including NKX3-1, PMEPA1, HUS1, and KRT18. These genes were confirmed to be androgen-regulated in our assay by comparing hormone-free with androgen-containing media in LNCaP control cells (Supplementary Fig. 8B). In addition, BMPR1B, a gene associated with reduced expression in prostate cancer (Kim et al. 2000) also showed activation. JAG1, the ligand for Notch1 receptor and suggested to have both oncogenic and tumor suppressor roles in varying tumor types, was highly upregulated by increased TBLR1. Subset 2, which showed
little to no activation from overexpression of NLSTBLR1, included genes primarily associated with cell proliferation, including CCNA2 and CDC6 (Supplementary Fig. 8A).

Changes in the expression of NKX3-1, KRT18, HUS1, and JAG1 after nuclear TBLR1 overexpression were also confirmed at the protein level by western blot. Overexpression of nuclear TBLR1 had no effect on p21, but there was a strong increase in p27 expression, suggesting a role of p27 in growth arrest initiated by nuclear TBLR1 (Fig. 6C). In addition, we tested the effect of increased nuclear TBLR1 on p27 levels in two additional androgen-sensitive cell lines, RC165 and LNCaP-AI. Overexpression of nuclear TBLR1 also led to increased p27 protein levels by western blot analysis in these cells (Supplementary Fig. 8C).

To further confirm these results of TBLR1 acting as a coactivator of AR, we also tested the effect of TBLR1 knockdown across this panel of genes. Loss of TBLR1 led to a reduction in expression across the panel of genes (Supplementary Fig. 9A, see section on supplementary data given at the end of this article). Loss of NKX3-1, KRT18, and HUS1 expression at the protein level was confirmed (Supplementary Fig. 9B). No change in p27 levels was observed upon TBLR1 knockdown.

We next used chromatin immunoprecipitation (ChIP) to test the direct regulation of the affected AR target genes by TBLR1. NKX3-1 has been confirmed to be a direct AR regulated gene. The expression of NKX3-1, KRT18, HUS1, and JAG1 was analyzed in LNCaP cells with and without overexpression of TBLR1 by western blot (Fig. 6C). The results showed a significant increase in the expression of these genes in the presence of TBLR1.

Figure 6
Nuclear TBLR1 upregulates growth suppressive AR target genes. (A and B) Comparison of expression of androgen-regulated genes between LNCaP pBabe and LNCaP NLSTBLR1 in 10 nM R1881 media by qRT-PCR. The figure is divided between genes for which expression was increased less than tenfold (A) and genes in which expression was increased more than tenfold (B) in LNCaP NLSTBLR1 cells compared with LNCaP pBabe cells in the presence of 10 nM R1881. (C) Comparison of protein expression of androgen-regulated genes between LNCaP pBabe and LNCaP NLSTBLR1 in hormone-free media or 10 nM R1881 media by western blot. *, P < 0.05, **, P < 0.01, ***, P < 0.001.
Endocrine-Related Cancer

Discussion

AR signaling is critical for prostate cancer growth and progression. Transcriptional cofactors, including both coactivators and corepressors, are important in the regulation of AR transcriptional activity. Tumor development and progression are affected by changes in expression, localization, and activation of various cofactors. In this study, we examined the role of the nuclear receptor cofactor, TBLR1, as a regulator of AR transcriptional activity and its expression and function in prostate cancer.

TBLR1 is ubiquitously expressed and has been shown to serve as a corepressor for a number of transcription factors of the NHR family, serving as a docking function for NCoR and SMRT. These interact with HDAC3 to form an active deacetylase and render chromatin less accessible. Little is known about the interactions of TBLR1 with AR and their ensuing function in prostate cancer. We showed that TBLR1 acts as a coactivator of AR in prostate cancer cells by both luciferase assay and AR target gene expression. TBLR1 interacts with AR in an androgen-dependent manner and directly cooccupies ARE with AR.

Overwhelming evidence in the literature indicates the importance of AR transcriptional activation in driving prostate cancer cell proliferation. Recently, a number of AR coactivators have been shown to have tumor suppressor effects, including ARA70α and nuclear p44/MEP50 (Peng et al. 2008a, Ligr et al. 2010). Understanding the balance between proliferation and growth suppression, and the role specific cofactors play in the regulation of this switch is critical in understanding prostate biology and prostate cancer treatment methods. The benign prostate cancer cell line, RC165, has high nuclear TBLR1 expression compared with the cancer cell line LNCaP. As observed in our cell line model, human tumor samples show significant reduction in TBLR1 in the nucleus of malignant glands in comparison with the neighboring benign glands. In addition, serum-starvation-
induced growth arrest leads to the translocation of TBLR1 from the cytoplasm to the nucleus in cancer cells. These data together strongly indicate a role of nuclear TBLR1 as a tumor suppressor in prostate cancer. Interestingly, although overall level of expression of TBLR1 is reduced in cancerous vs benign cells, we observed a correlation of increased nuclear TBLR1 with increased Gleason score. This could be indicative of a feedback mechanism in which higher grade cancers’ cells are producing more TBLR1 in an unsuccessful attempt to control cancer cell proliferation.

Indeed, overexpression of nuclear TBLR1 in AR-positive prostate cancer cells leads to growth suppression in vitro in cell proliferation and anchorage-independent assays, as well as tumor growth inhibition in nude mice xenografts in vivo. It appears that there is an androgen-independent component of tumor suppression by TBLR1 also, because the growth of LNCaP cells is reduced even in growth medium free of androgen (Fig. 3B). Importantly, there is a higher degree of androgen-dependent element of growth inhibition as nuclear TBLR1 promotes cell growth in PC3 cells and this process is reversed to growth inhibition in PC3-AR cells (Fig. 5A and B). To confirm an androgen-dependent mechanism of nuclear TBLR1-mediated growth inhibition, AR knockdown in LNCaP (Supplementary Fig. 9A) cells was also able to partially rescue growth suppressive effects (Fig. 5C and D).

AR can regulate selected sets of AR target genes (Agoulnik & Weigel 2009, Wang et al. 2011) under various conditions. A number of AR target genes have been shown to regulate cell growth including cell-cycle genes, apoptosis, tumor suppressors, and differentiation genes. In this study, we have shown that TBLR1 is an AR coactivator that acts as a tumor suppressor in prostate cancer cells by selectively activating growth suppression and differentiating androgen-regulated genes. Overexpressing nuclear TBLR1 in the prostate cancer model cell line LNCaP leads to increased expression of important prostate proliferation genes (i.e. KRT18) and tumor suppressor genes (i.e. NNX3-I and HUS1). Overexpression of nuclear TBLR1 in LNCaP cells also leads to increased expression of the cell-cycle inhibitor P27(CDKN1B), an androgen regulated gene which may be responsible for the G0/G1 cell-cycle arrest observed in our proliferation studies. Increased expression of NNX3-I and P27 has been previously reported to work synergistically to decrease prostate cancer cell proliferation (Wang et al. 2009). Our data indicate that TBLR1 interacts with AR in an androgen-dependent manner, and that TBLR1 directly occupies ARE of AR target genes NNX3-I and HUS1 in an androgen-dependent manner. Together, these data indicate that nuclear TBLR1 is a direct regulator of growth-suppressive AR target genes.

Interestingly, upon knockdown of TBLR1 in LNCaP or LNCaP-AI cells by siRNA, we also observed a reduction in proliferative ability, showing that either overexpression or knockdown of TBLR1 leads to G0/G1 growth arrest. Although these data at first seem contradictory, it appears that the growth arrest observed in each of these instances could be through different mechanisms. Overexpression of TBLR1 showed increased p27 levels whereas knockdown of TBLR1 did not affect p27 levels (Fig. 6C and Supplementary Fig. 9B). In addition, we showed that overexpression of nuclear TBLR1 leads to selective activation of growth suppression/differentiation AR target genes but not proproliferative genes (Fig. 6A); however, knockdown of TBLR1 leads to a more global downregulation of both sets of genes (Supplementary Fig. 9A). This shows that although increased nuclear TBLR1 does selectively activate a subset of AR target genes, a baseline level may be required for most AR target genes and cell proliferation. It is possible that the loss of expression of proliferation genes (i.e. CCNA2 and CDC6) upon TBLR1 knockdown overrides the effect of decreased tumor suppressor target genes (i.e. NNX3-I) on overall growth rate. In addition, TBLR1 functions in the context of a protein complex with HDAC and NCoR proteins, loss of expression of the TBLR1 component may disrupt the complex and interfere with transcription and cell growth.

Previous studies have shown that the mechanism of TBLR1 activation of nuclear receptors could be due to phosphorylation of TBLR1 by PKCα at two specific sites that target NCoR for ubiquitination and degradation by the 19S proteosome (Perissi et al. 2004). In prostatic tissues we showed by luciferase assay that activation of AR by TBLR1 also is dependent on both 19S proteosome activity and phosphorylation (Fig. 1C, D and E). Indirect evidence has suggested that TBLR1 serves as an F-box protein with E3 ligase activity on BCL3 (Keutgens et al. 2010). It has been suggested that as it contains an F-box like motif, it acts as an E3 ligase and promotes the degradation of NCoR, leading to increased chromatin accessibility. The F-box in TBLR1 is unusual, in that it lacks a tryptophan near the NH2-end of the motif that is required for Skp1 binding (Kipreos & Pagano 2000). Without this interaction E3 ligase activity does not develop. However, no interaction of TBLR1 with Skp1 could be demonstrated by our laboratory (Daniels & Lee, unpublished data 2013), indicating that the role of TBLR1 in the proteosome pathway may be indirect. When we tested LNCaP NLSTBLR1 phospho-mutants in

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proliferation assays, however, there was no difference in growth observed between NLSTBLR1 and all three phospho-mutants, revealing that phosphorylation at these sites alone is not critical for growth suppression by nuclear TBLR1 (Supplementary Fig. 3E), supported by our data indicating that phospho-mutants still retain some coactivator ability in our luciferase assay (Fig. 1F). Phosphorylation of TBLR1 may affect functions of TBLR1 other than growth inhibition. A recent report has also shown that TBLR1 is sumoylated at K497 and therefore required for activation of Wnt target genes through interaction with β-catenin (Choi et al. 2011). However, using a sumolation mutant K497R TBLR1 plasmid in our luciferase assay, we observed equal AR activation compared with WT TBLR1 (Supplementary Fig. 1A). Sumolation at K497 does not appear to be important for AR transcriptional activation in our limited luciferase system.

In conclusion, nuclear TBLR1 acts as a tumor suppressor in prostate cancer and AR coactivator directly and selectively activates androgen-regulated genes important for growth suppression/differentiation. Better understanding of AR regulation in the prostate and the molecular switches that occur in transition from growth suppression to proproliferation target genes will lead to more successful prostate cancer treatments.

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