Retinoic acid and androgen receptors combine to achieve tissue specific control of human prostatic transglutaminase expression: a novel regulatory network with broader significance

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ABSTRACT

In the human prostate, expression of prostate-specific genes is known to be directly regulated by the androgen-induced stimulation of the androgen receptor (AR). However, less is known about the expression control of the prostate-restricted TGM4 (hTGP) gene. In the present study we demonstrate that the regulation of the hTGP gene depends mainly on retinoic acid (RA). We provide evidence that the retinoic acid receptor gamma (RAR-G) plays a major role in the regulation of the hTGP gene and that presence of the AR, but not its transcriptional transactivation activity, is critical for hTGP transcription. RA and androgen responsive elements (RARE and ARE) were mapped to the hTGP promoter by chromatin immunoprecipitation (ChIP), which also indicated that the active ARE and RARE sites were adjacent, suggesting that the antagonistic effect of androgen and RA is related to the relative position of binding sites. Publicly available AR and RAR ChIP-seq data was used to find gene potentially regulated by AR and RAR. Four of these genes (CDCA7L, CDK6, BTG1 and SAMD3) were tested for RAR and AR binding and two of them (CDCA7L and CDK6) proved to be antagonistically regulated by androgens and RA confirming that this regulation is not particular of hTGP.

INTRODUCTION

Retinoic acid (RA) is a molecule with a wide variety of biological functions. In vertebrates, RA is known to control the differentiation process by altering the gene expression profile of cells (1). For example, RA participates in the transcriptional regulation of the Hox gene clusters (2,3), which are key to the development process in vertebrate and invertebrate organisms (4,5). RA is able to modify gene expression by activation of a family of non-steroid nuclear receptors known as retinoic acid receptors (RARs) and retinoid x receptors (RXRs), which function as heterodimeric units (6) and bind to the retinoic acid responsive elements (RAREs) present in the promoters or DNA regulatory elements of target genes, thus regulating their expression (7,8). Activated RARs are therefore responsible for promoting not only differentiation but also cell-cycle arrest and apoptosis (9–12), among other effects.

In prostate development and morphogenesis, androgens play a major role, for example in the stimulation of the mesenchyme to induce prostate formation and prostate secretory function (13,14). However, androgens are not the only molecule to regulate prostatic development. Retinoic acid controls both the proliferation and differentiation of prostate epithelium (15,16). To underline the importance of RA signalling in both prostate development and function, transgenic mice lacking RAR-G develop prostate squamous metaplasia (17) which also renders them sterile.

In different organs, tissue-specific gene expression is controlled by discrete sets of transcription factors and epigenetic mechanisms (18,19). In the prostate for example, most tissue specific expression is mediated by androgens, and modulated by the nuclear receptor for androgen (AR). Classical prostate specific genes such as KLK3 (PSA), FOLH1 (PSMA), NKX3.1 and TARP have all been shown to be directly regulated by androgen (20–24). Because of the role of androgen and the AR in prostate maintenance and development, it is usually assumed that classical androgen regulation is obligatory for prostate specific expression.

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Within the pool of prostate specific genes, TGM4 (hTGP) stands out as one of the most prostate specific genes reported to date (21). hTGP has been mapped to chromosome 3 in humans (21). Its function in rodents is related to fertilization and reduction of sperm antigenicity (25–27), while in humans hTGP expression has been linked to the invasive potential of prostate cancer cells (28). In vitro, hTGP transcription is controlled by androgen but only after extended treatment experiments in PC346C, but not in LNCaP prostate cancer cells, despite the presence of active AR in both cell lines. Promoter analysis also showed that the presence of an SP1-binding site in the proximal hTGP promoter region was needed for optimal expression, however no classical androgen control or functional androgen responsive elements (AREs) were found in the minimal promoter sequence (21,29).

Despite its critical role in prostate development, growth and differentiation, examples of the importance of retinoic acid in adult prostate gene expression are scarce; NKK3.1 is the only case of a prostate specific gene whose expression, while mainly regulated by androgen, can be affected by retinoic acid (30,31).

This work describes the first report of a prostate specific gene where androgen regulation plays a minor and negative role, and where RA is the main regulator of hTGP expression in prostate cancer cell lines. It provides evidence that other mechanisms apart from classical androgen–AR transcriptional control, regulate the expression of this highly prostate specific gene. It also implies that AR/RAR antagonistic effects are a widespread mechanism to regulate transcription within the prostate gland. These findings cast doubts on the primacy of classical androgen–AR regulation for prostate specific expression, while mainly regulated by androgen, can be affected by retinoic acid (30,31).

MATERIALS AND METHODS

Cell culture and treatments

LNCaP (ATCC), PNT1A and PNT2C2 cells (32) were grown in RPMI-1640 media (GIBCO) supplemented with 10% FCS (PAA Laboratories) and 2 mM L-Glutamine (Invitrogen). PC346C cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (GIBCO)/Ham’s F12 (Lonza) supplemented with 100 μg/ml streptomycin, 100 μ/ml penicillin G, 2% FCS, 0.01% (w/v) BSA, 10 ng/ml EGF, 1% (v/v) ITS-G, 0.1 mM R1881, 1.4 mM hydrocortisone, 1 mM triiodothyronine, 0.1 mM phosphoethanolamine, 50 ng/ml cholera toxin (Sigma), 0.1 μg/ml fibronectin and 20 μg/ml fetuin. All cells were certified free of mycoplasma and genotyped (using the ATCC-approved Powerplex 1.2 system (Promega) to ensure authenticity. Cells were routinely passaged in T25 flasks at 37°C with 5% CO₂. In advance of atRA (Sigma), R1881 (DuPont), Bicalutamide (Sigma) or TTNPB (Sigma) treatments, cells were grown for 24 h in charcoal stripped serum supplemented media.

Gene expression profile in human tissues

TissueScan Human Normal Tissue qPCR Arrays (OriGene Technologies, Rockville MD) were used to screen for hTGP, PSA and TMPRSS2 expression in 48 different tissues using Taqman gene expression assays Hs00162710_m1, Hs02576345_m1 and Hs01120965_m1 and following manufacturer’s instructions.

Gene expression

Total RNA was purified using the RNeasy mini Kit (Qiagen) following manufacturer’s protocol. cDNA was synthesized using Superscript II enzyme (Invitrogen) and random primers (Invitrogen) following manufacturer’s protocol. qPCR experiments were carried out using 50 ng cDNA as template, the Power SYBR Green PCR master mix and specific primer pairs. RT–PCR experiments were performed in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems) using Platinum taq DNA polymerase (Invitrogen), 10 ng of cDNA as template following manufacturers protocol (for primer sequences see Supplementary Table S3).

Western blot analysis

Whole cell lysate or nuclear proteins were isolated using Cytobuster reagent (Novagen) or the Nuclear and Cytoplasmatic Extraction Kit (Pierce) following manufacturer’s protocol. Antibodies used were anti-pan RAR (sc-773) anti-RAR-B (sc-552), anti-RAR-G (sc-550), anti-AR (sc-816) from Santa Cruz Biotechnologies, anti B-actin (A5316) from Sigma and anti-TBP (1TBP18) from Abcam.

Generation of hTGP promoter constructs and mutants

hTGP promoter sequence (4.5 kb) was amplified using the Expand High Fidelity PCR system (Roche) using specific primers (Supplementary Table S3) and the manufacturer’s protocol and cloned into the pEGFP-1 plasmid (Clontech) using the restriction enzymes XhoI and SacII. To clone the hTGp (4.5 kb) and the several deletion mutants into the pGL3 basic vector (Promega), the In Fusion cloning system was used (Clontech) following manufacturer’s protocol (see Supplementary Table S3 for primers sequence) and transformed into STBL3 (Invitrogen) chemically competent Escherichia coli. All sections of the promoter that involved PCR amplification were subject to DNA sequencing to confirm the fidelity of the amplification. For the generation of constructs with mutated RAREs and ARE, the QuickChange II XL kit (Stratagene) was used following manufacturer’s instructions.

Plasmid transfection and luciferase assay

Cells were seeded in 96-well plates and grown for 24 h in charcoal stripped media previous to transfection. To measure retinoic acid responsiveness, Cignal RARE reporter (luc) kit plasmids (CCS-016 L) from SABiosciences were transfected into LNCaP, PC346C, PNT1A and PNT2C2 cells using TransIT-LT1 for PC346C, PNT1A and PNT2C2 and TransIT-2020 for
LNCaP cells as transfection reagents, following manufacturer’s protocol. For the functional analysis of the hTGP promoter, plasmid mixtures containing the different versions of the hTGP promoter and the pRL-CMV Vector (Promega) (in a 1:1 copy number ratio) were co-transfected into LNCaP cells grown for 24 h in charcoal stripped media, using TransIT-2020 as a transfection reagent. Twelve hours after transfection cells were treated either with atRA or R1881 for further 24 h. Luciferase expression was measured using the Dual-Glo system (Promega) following manufacturer’s protocol using the Polarstar Optima micro-plate reader (BMG).

**siRNA knockdown**

LNCaP cells were grown in six-well plates coated with t-Polylysine and transfected with Silencer select (Applied Biosystems) siRNAs targeting RAR-B (siRNA ID: s11804), RAR-G (siRNA ID: s11807), AR (siRNA ID: s1538) or Negative control using DharmaFECT 2 (Dharmacon) as transfection reagent.

**Immunofluorescence**

PC346C or LNCaP cells were seeded in Poly-d-Lysine eight-well CultureSlides (BD) in charcoal stripped media for 48 h. Before fixation, cells were briefly washed with PBS, fixed by adding cold methanol (−20°C) for 5 min, then air-dried. Incubation in 10% goat serum (Sigma) for 1 h was used to block non-specific antibody binding. Cells were incubated with AR antibody (sc-816) or IgG rabbit isotype (Sigma) as negative control in 1% BSA/PBS for 1 h at room temperature. Secondary antibody goat anti-rabbit labelled with Alexa Fluor 488 (Invitrogen) was incubated for 30 min in 1% BSA/PBS at room temperature. Slides were mounted with VECTASHIELD with DAPI (Vector Laboratories).

**Chromatin immunoprecipitation**

Cells were grown in T175 flasks in charcoal stripped media for 24 h, then treated with either, atRA (500 nM), R1881 (10 nM) or vehicle (DMSO) for 10 h. After treatments cells were trypsinized and re-suspended in 5 ml media and treated with formaldehyde to a final concentration of 1% for 10 min at room temperature with gentle shaking. Glycine was added to stop fixation to a final concentration of 250 mM for 5 min at room temperature. Cells were washed with cold PBS and re-suspended in cold swelling buffer (5 mM Pipes pH 8, 85 mM KCl) supplemented with NP-40 (final concentration of 0.2%) and protease inhibitors (Roche). The cell suspension was incubated in ice with gentle shaking for 20 min. Suspension was centrifuged at 3000 rpm and resuspended in IP buffer TSE150 (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris–HCl pH 8, 500 mM NaCl), washing buffer (10 mM Tris–HCl pH 8, 0.25 M LiCl, 0.5% NP-40) and TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA). DNA was eluted by adding 100 μl of elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8) and a incubation step at 65°C for 15 min. Beads were centrifuged at 15 000 rpm for 1 min and supernatant transferred to a separate tube. Beads were rinsed with 150 μl TE (1% SDS, 10 mM EDTA) and vortexed, centrifuged at 15 000 rpm and supernatant pooled with previous one. Immunoprecipitated DNA was left at 65°C overnight, then treated with proteinase K (Invitrogen) and added glycogen (Roche). DNA was purified by phenol/chloroform extraction.

**Bioinformatic analyses**

*AR-binding sites and microarray data.* Time series microarray data autocorrelation from (33) (Supplementary Table S1) were used to select probes showing significant (autocorrelation ≥ 0.5) androgen-mediated expression alteration (GEO GSE18684). Probes were annotated to genes using the Bioconductor Illumia human BeadArray mappings (34) against the UCSC human reference genome hg18. Genes were assigned unique ENTREZ identifiers for comparison with other datasets.

Chromatin immunoprecipitation(ChIP)-seq data for both LNCaP and VCaP cell lines was downloaded from the same source. Peak positions for both datasets were mapped to unique ENTREZ identifiers based upon proximity to gene start sites. Data from both the LNCaP and VCaP cell lines were combined for downstream analysis.

**RARA ChIP-Seq data.** ChIP-seq data for RAR-alpha was obtained from (35), SRA study accession number SRA010193. Peak positions for both datasets were mapped to unique ENTREZ identifiers based upon proximity to gene start sites.

**RARA and RARG ChIP–chip data.** ChIP–chip data for both RAR-alpha and RAR-gamma was obtained from (36) (Supplementary Table S2), GEO database under GSE15244.
Peak positions for both datasets were mapped to unique ENTREZ identifiers based upon proximity to gene start sites.

**Combined dataset analysis**

Combined AR ChIP–chip data was created by finding the genes present in both the ChIP-seq and expression microarray datasets from (33). Combined RAR data was created using the intersection between the datasets determined from (35) and (36).

**Distance analysis**

Genes in both of the two gene sets were tested for the frequency and distance between AR-binding sites and putative RAR sites. All possible pairs of AR site and RAR site in each gene were checked for the distance between the sites. The number of distances less than 500 bases were recorded.

**RESULTS**

**hTGP is a highly prostate specific gene**

To assess with a quantitative method, such as qPCR, the extent to which hTGP gene expression is prostate-specific, its expression profile was compared to that of PSA and TMPRSS2 genes, which are regarded as prostate specific, in a qPCR array containing cDNA from 48 different normal human tissues. hTGP expression was ~200-fold higher in prostate in comparison to the next highly expressing tissue (testis). PSA also showed a highly prostate-specific profile. Expression levels were >400-fold enriched in the prostate, in comparison to the second highest expressing (adipose) tissue. In comparison, TMPRSS2 showed higher expression in the prostate but the fold enrichment was just 1.55-fold, while lungs and stomach also showed high expression (Figure 1). The number of tissues, apart from prostate, where expression could be detected showed that hTGP expression (21 tissues) was more restricted to the prostate than both PSA (32 tissues) and TMPRSS2 (39 tissues) (Figure 1).

![Figure 1](image-url)  
**Figure 1.** hTGP expression is highly prostate specific. hTGP, KLK3 and TMPRSS2 expression was analysed by qPCR using specific taqman probes and arrays containing cDNA from 48 different human tissues. Expression is shown relative to the second highest absolute value.
hTGP expression in the prostate cancer cell lines LNCaP and PC346C is regulated by atRA

Previous studies indicated that hTGP expression was controlled by androgen over long periods of androgen exposure (7–10 days) (21), but paradoxically no AREs could be found in a 2.1-kb region of the proximal promoter (29) which suggests an indirect androgen regulation of the hTGP gene. Bioinformatic re-analysis of a longer section of the hTGP promoter indicated the presence of a H3K4me2 region between −4898 and −3698. H3K4me2 is a histone mark associated with the presence of enhancers or transcription start sites (37,38). Since this was the only region within the hTGP promoter and gene with this feature, it was decided to find putative binding sites for transcription factors within this 5.5-kb upstream of the hTGP transcriptional start site. Bioinformatic analysis located not only AREs but also RAREs (Figure 2A). In the light of these results, it was decided to re-examine the effects of both hormones on hTGP expression in cell lines from normal and malignant prostate. First, to explore RA regulation, the prostate cell lines LNCaP, PC346C, PNT1A and PNT2C2 were treated with 500 nM all-trans retinoic acid (atRA), which is within the range of commonly used atRA concentrations (35,39,40), for 24 h to test whether this compound could regulate hTGP expression. LNCaP and PC346C are prostate cancer cell lines with luminal characteristics; they express PSA, AR and are responsive to androgen. PNT1A and PNT2C2 are benign prostate cell lines less differentiated than LNCaP and PC346C. PNT1A and PNT2C2 do not express AR and are not affected by the presence or absence of androgens (41). While LNCaP and PC346C cells treated with atRA showed a marked increase in hTGP expression, the normal, less-differentiated PNT1A and PNT2C2 cells showed a small decrease (Figure 2B). As the difference in hTGP regulation after atRA treatment could be the result of differential RAR expression in the different cell lines, total RAR protein and mRNA was measured in LNCaP, PC346C, PNT1A and PNT2C2 cells. PNT1A and PNT2C2 showed higher levels of total RAR protein in comparison to LNCaP and PC346C while mRNA for the different RARs did not show a clear tendency that could explain the lack of hTGP up-regulation after atRA treatment in PNT1A and PNT2C2 cells (Figure 2C and D). To functionally test the extent to which each cell line could sustain atRA-dependent gene activation, the cells were co-transfected with a reporter plasmid containing a tandem of RAREs controlling the expression of the firefly luciferase gene and a control construct where the CMV promoter controlled expression of the Renilla luciferase gene. Twenty-four hours after transfection cells were treated with increasing concentrations of atRA and luciferase activity was measured after a further 18 h. The results showed that LNCaP and PC346C cells could sustain atRA-dependent gene expression to a higher extent (up to 10.6- and 17.9-fold increase respectively) in comparison to PNT1A and PNT2C2 cells (up to 5.4- and 4.53-fold increase respectively) (Figure 2E). The greater extent to which LNCaP and PC346C sustain atRA-dependent transcription explains the lack of hTGP up-regulation observed in PNT1A and PNT2C2 cells.

Androgen has a minor and negative role in hTGP regulation

Next, to test whether androgen affects hTGP expression within a more physiological 24 h treatment period than in the previous studies, LNCaP and PC346C cells were treated with increasing concentrations of the synthetic androgen R1881. Surprisingly, hTGP expression decreased slightly after R1881 treatments in LNCaP, while in PC346C, hTGP expression decreased significantly down to 0.65-fold (P < 0.05) in cells treated with 0.1, 1 and 10 nM R1881 (Figure 3A). LNCaP and PC346C cells were treated with R1881 0.1 or 10 nM and/or atRA 500 nM for 24 h to assess whether the effects of R1881 and atRA were antagonistic. LNCaP and PC346C cells treated with atRA or R1881 showed an increase and decrease of hTGP expression respectively and co-treatment with 0.1 nM R1881 and 500 nM atRA decreased atRA-dependent hTGP expression in LNCaP but not in PC346C cells (Figure 3B, left panel). However co-treatment of 10 nM R1881 and 500 nM atRA resulted in complete abrogation of atRA-induced hTGP expression in LNCaP and a small decrease in PC346C cells (Figure 3B, right panel). To test whether AR knockdown could rescue R1881-dependent hTGP down-regulation, LNCaP cells were transfected with siRNA targeting the AR. AR was successfully knocked-down and mRNA levels remained low even 24 h after R1881 treatment (Figure 3C). Surprisingly, AR knockdown not only failed to rescue hTGP down-regulation after R1881 treatment but also had a significant (P < 0.05) negative effect on basal hTGP expression (Figure 3D).

AR presence but not its transactivational activity regulate hTGp expression

AR knockdown had a negative effect on atRA-dependent hTGP expression, when LNCaP cells were transfected with AR-specific siRNA, both in untreated conditions and 24 h after atRA treatment (500 nM) (Figure 4A). To evaluate if AR knockdown was down-regulating RAR levels and therefore acting to decrease hTGP expression, RAR-A, RAR-B and RAR-G mRNA levels in AR knockdown LNCaP cells were measured. AR knockdown up-regulated RAR-A and RAR-G levels but had no effect on RAR-B mRNA expression (Figure 4B). Next, LNCaP cells were treated with the AR inhibitor bicalutamide, which inhibits the receptor’s ability to recruit co-activators without affecting its DNA-binding ability, (42) to investigate if the AR knockdown effect was caused by the loss of AR transactivation. As expected, bicalutamide treatments had a negative effect on PSA transcription (a canonical AR responsive gene) even after R1881 treatment, confirming the block in AR activity (Figure 4C). Moreover, in LNCaP cells treated with atRA and bicalutamide, hTGP expression remained unchanged, suggesting that direct AR transcriptional activity did not affect hTGP expression. Although it has been reported that the AR is
Figure 2. hTGP expression is up-regulated by retinoic acid. (A) Schematic representation of a 4.5-kb hTGp promoter showing putative AREs and retinoic acid responsive elements (RARE). (B) hTGp relative expression in LNCaP, PC346C, PNT1A and PNT2C2 cells treated with vehicle or atRA (500 nM) for 24 h. Statistically significant T-test differences are denoted with the asterisk symbol (P < 0.05). (C) RAR protein expression in LNCaP, PC346C, PNT1A and PNT2C2 whole cell lysates, B-actin was used as a loading control. (D) mRNA expression of RAR-A, RAR-B and RAR-G in LNCaP, PC346C, PNT1A and PNT2C2 cells. (E) atRA responsiveness of each cell line was measured by co-transfecting a reporter plasmid where the firefly luciferase gene is under the control of a tandem of RAREs and a reporter plasmid where the Renilla luciferase expression is controlled by the constitutive CMV promoter. The negative control consisted in the same luciferase reporter gene construct as the test plasmid but lacking the RAREs elements. After transfection cells were treated with increasing concentrations of atRA for 18 h and luciferase activity was measured. All firefly luciferase values were normalized against Renilla luciferase expression.
Figure 3. Androgen has a minor and negative effect on hTGP mRNA expression while AR knockdown has a negative effect on its expression. (A) Relative hTGP mRNA expression, in LNCaP and PC346C cells treated with vehicle or increasing concentrations of the synthetic androgen R1881. (B) RT–PCR detection of hTGP, PSA and GAPDH in LNCaP and PC346C cells treated with atRA (500 nM), androgen (0.1 or 10 nM) or a combination for 24 h. (C) AR mRNA (left) and protein knockdown (right) in LNCaP cells. (D) hTGP mRNA expression in LNCaP cells after AR knockdown with or without R1881 treatment for 24 h. Statistically significant t-test differences are denoted with the asterisk symbol ($P < 0.05$). **Note the lower molecular weight band in the hTGP RT–PCR amplification is a known splice variant.
unable to bind atRA (43), we further confirmed this by immunostaining for the AR in LNCaP and PC346C cells treated with 10 nM R1881 or 500 nM atRA for 2 h. AR localization is both nuclear and cytoplasmic in LNCaP and PC346C cells in the absence of treatment (Figure 4D, left panels). Upon R1881 addition almost all of the detectable AR translocates into the nucleus (Figure 4D, middle panels). In atRA-treated cells the AR was present in both cytoplasm and nucleus in a similar pattern to that in untreated cells (Figure 4D, right panels). Statistically significant t-test differences are denoted with the asterisk symbol ($P < 0.05$).
right panels). Thus atRA did not affect the intracellular location of AR and did not activate this receptor.

RAR-G has a major role in atRA-dependent hTGP expression

To determine whether the hTGP gene could be controlled by the RARs, LNCaP and PC346C cells were treated for 24 h with 250 or 500 nM of the synthetic retinoid 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]-benzoic acid (TTNPB), which selectively binds to the RARs and induces transcription of its target genes (40,44). Both TTNPB concentrations enhanced hTGP expression, suggesting that the hTGP gene can be directly controlled by the RARs (Figure 5A). Among the RARs, RAR-B and RAR-G seem to play a major role in prostate biology. RAR-B and RAR-G are expressed in the rat prostate at an early stage; RAR-G deficient mice show prostate malformations and RAR-B is often down-regulated in prostate cancer (17,45,46) making these genes obvious candidates to control hTGP expression. Therefore, LNCaP cells were transfected with specific siRNA targeting either RAR-B or RAR-G mRNA, to determine the specific RAR(s) responsible for atRA induced hTGP expression. mRNA and protein knockdown were confirmed (Figure 5B). RAR-B knockdown had a negative impact on basal hTGP mRNA levels but had no effect after atRA induction (Figure 5C, left panel). RAR-G knockdown had a significant \( P < 0.05 \) negative effect on basal hTGP mRNA expression as well as in atRA treated cells (Figure 5C, right panel), implying that it is RAR-G, which plays the major role in atRA-dependent hTGP expression.

Localization of an upstream enhancer element and binding of the RAR-G and AR to the hTGP promoter

To analyse whether the elements that control atRA and AR-dependent hTGP regulation were present in the DNA sequence of the 4.5 kb hTGP promoter, the promoter was cloned, alongside several deletion mutants (Supplementary Figure S1), into a pGL3 basic luciferase reporter plasmid. Co-transfection of the different plasmids containing different lengths of the hTGP promoter and a construct where the CMV promoter controls the expression of the \( Renilla \) luciferase (as a transfection efficiency control) in LNCaP cells resulted in the detection of a positive regulatory region or enhancer in the upstream region of the promoter \((-4500 \text{ to } -3500 \text{ region})\) which is active in the absence of any treatment (Supplementary Figure S1). atRA (500 nM) and R1881 (10 nM) treatments up-regulated and down-regulated luciferase expression respectively only in cells transfected with the full 4.5-kb hTGP promoter, mimicking the behaviour of the endogenous gene (Supplementary Figure S1C and D). ChIP was carried out to map the binding sites of the RAR and AR, in untreated and atRA (500 nM) or R1881 (10 nM) treated LNCaP cells, using a set of primers that amplify both regions close to the predicted location of the RAREs and AREs and regions where there were no predicted binding sites (Figure 5B). RAR binding to the hTGP promoter presented the same pattern in vehicle-treated as well as atRA and R1881 treated cells (Figure 6B–D). The ChIP RAR-binding sites align with the predicted RAREs located at \(-3942\) and \(-1629\) (Figure 6B). AR binding to the hTGP promoter showed a similar pattern to RAR binding in vehicle-treated cells (Figure 6B), and decreased in atRA treated cells except for the region \(-3962\) where AR binding was maintained (Figure 6C). In R1881-treated cells, AR showed a higher binding in the \(-3962\) region, which is adjacent to a putative ARE element located at \(-4094\) (Figure 6D). To test whether the binding enrichment in each region was significant, values were normalized to the IgG control and tested using Student’s \( t \)-test for statistical significance (Supplementary Figure S2).

To further confirm these results, the putative responsive elements (RAREs and ARE), detected by promoter analysis and ChIP were next deleted, and the activity of the hTGP promoter measured following atRA or R1881 treatment. For this purpose the WT 4.5-kb hTGP promoter was subjected to site-directed mutagenesis, which deleted between 5 and 7 nt of the corresponding responsive element, in order to alter the binding of the receptors. The WT construct and the mutants (Figure 6E) were transfected into LNCaP cells and treated with 500 nM atRA or 10 nM R1881 for 24 h before measuring luciferase activity. As in previous experiments, the WT 4.5-kb hTGP construct activated luciferase expression following atRA treatment, and repressed luciferase expression after R1881 treatment. However, the disruption of the 5’ RARE (RARE1) resulted in down-regulation of luciferase expression following atRA treatment suggesting that the 3’ RARE (RARE2), being the unchanged RARE, had a negative effect on gene expression following atRA treatment. Disruption of RARE2, resulted in increased expression in basal conditions, and following atRA treatment, to more than twice the expression levels in the WT construct. Disruption of the ARE increased luciferase expression following atRA treatment. No changes in luciferase down-regulation were detected following R1881 treatment (Figure 6F), suggesting that either deletion of those particular nucleotides is not sufficient to alter the binding of the AR to that site or that the androgen mediated effects on the hTGP are exerted through other regulatory element that could not be detected. A model summarising the regulation of the hTGP gene is illustrated in Supplementary Figure S3.

Opposing effects of AR and RAR are not an hTGP exclusive mechanism

To investigate whether AR and RAR control the transcriptional regulation of other genes, a bioinformatic analysis was carried out to compare the occurrence of AREs and RAREs across the entire human genome (Supplementary Figure S4). AR-chip-Seq data from a recent manuscript describing AR-target genes in LNCaP and VCaP cells (33) was used to identify directly AR-responsive genes. To identify RAR-target genes, RAR-chip-Seq and RAR-chip-chip data was then used from two independent reports (35,36). AR-target genes
RAR-G plays a major role in atRA-dependent hTGP expression. (A) hTGP mRNA expression in LNCaP and PC346C cells treated with vehicle or the synthetic retinoid TTNPB for 24 h. (B) RAR-B (left panels) and RAR-G (right panels) mRNA and protein knockdown in LNCaP cells. (C) hTGP mRNA expression in RAR-B (left) and RAR-G (right) knockdown LNCaP cells treated with vehicle or atRA (500 nM) for 24 h. Statistically significant t-test differences are denoted with the asterisk symbol (P < 0.05).
and RAR-target genes were compared to obtain a list of candidate genes (Supplementary Table S1), which are likely to be controlled by both retinoic acid and androgen.

To validate this analysis, RAR and AR ChIP were performed on the predicted binding regions on selected genes. RAREs and AREs from CDCA7L, CDK6, BTG1 and SMAD3 were assessed for RAR and AR binding following atRA or R1881 treatment. Figure 7 demonstrates that all candidate genes have bound RAR and AR in their corresponding RAREs and AREs, following atRA or R1881 treatment. Since it was found that the role of atRA and R1881 had opposite effects on the regulation of hTGP, changes in the expression of CDCA7L, CDK6, BTG1 and SMAD3 were assayed, to evaluate whether any of these genes was regulated in a similarly opposing fashion. Retinoic acid and androgen treatment changed CDCA7L and CDK6 expression in opposite directions similar to the effect of these hormones on hTGP expression (Figure 7A and B). CDCA7L expression was down-regulated by R1881 and stimulated by atRA.
Figure 7. Analysis of other genes potentially regulated by androgen and retinoic acid. CDCA7L (A), CDK6 (B), BTG1 (C) and SMAD3 (D) were analysed for RAR and AR binding (upper panels) in selected RARE and ARE regions (middle panels). Gene expression was measured following R1881 or atRA treatment to analyse the effect of these hormones on the expression of selected genes (bottom panels). Statistically significant t-test differences in receptor binding between IgG control and AR or RAR antibodies and treatments are denoted with the asterisk symbol ($P < 0.05$). Statistically significant t-test differences in gene expression between vehicle and R1881 or atRA treatments are denoted with the asterisk symbol ($P < 0.05$).
Prostate specific genes such as PSA, PSMA and NKK3.1 are mainly regulated by androgen and the AR, which has led to the assumption that androgen regulation is necessary and sufficient for prostate specific gene expression (23,24,47). hTGP expression is highly prostate specific, even when compared to the expression profile of the archetypal prostate gene PSA (Figure 1). However, the previously reported role of androgen in hTGP regulation is reminiscent of an indirect regulation (21), since (i) no canonical AREs were found in the proximal promoter and (ii) the androgen stimulation was carried out over ten days in contrast to most direct AR stimulations, which require only 24h treatment. The presence of putative AREs and RAREs within the extended hTGP promoter we describe here, suggested a direct role for androgen and atRA in hTGP, regulation, as both compounds play a critical role in prostate development and differentiation (13,14,46,48).

Androgen was previously shown to have a positive effect on hTGP expression in PC346C cells, but not in LNCaP, and only over a period of 10 days’ treatment (21). Most AR-mediated transcriptional regulation results in expression changes detectable within 24h of steroid exposure. For hTGP, data describing a more rapid and physiological response to androgen was therefore lacking. Treatment of LNCaP and PC346C cells with R1881 for a 24 h period actually resulted in a small decrease in hTGP mRNA levels. Thus the effect of androgen was to antagonize that of atRA on hTGP expression (Figure 3B). Antagonistic effects of androgen and atRA have been previously described in both the prostate, where the activity of the AR was down-regulated, and expression of the PSA gene was negatively affected after atRA treatment (39,50,51) and in lacrimal gland cells (52). Interestingly, AR receptor knockdown showed that AR was positively regulating hTGP transcription in both luminally-differentiated prostate cancer cell lines LNCaP and PC346C, while the benign and basal cell lines PNT1A and PNT2C2 showed little response to atRA treatment (Figure 2B). Absence of response to atRA treatment was not due to lack of RAR expression (Figure 2C and D), but could be explained by the differential ability of each cell line to sustain atRA-dependent gene expression (Figure 2E). The observation that more luminal-like cell lines are principally capable of sustaining RAR-dependent gene activation could be related to the role of atRA and RAR in promoting and maintaining differentiation. This explanation is also in accordance with hTGP protein expression, which was found only in the luminal compartment of the prostate gland (49).
hypothesis is supported by the binding pattern of AR to the hTGP promoter (Figure 6D) where atRA treatment appears to maintain AR binding only in the region adjacent to the RARE where the RAR is bound. Such an interaction between the two nuclear receptors would explain why the low levels of AR affect not only basal hTGP transcription but also atRA-induced hTGP expression (Figure 7). A similar phenomenon has been recently described in breast cancer, where the estrogen receptor α (ER-A) cooperates with the RAR-A to promote transcription of a subset of ER responsive genes in the absence of retinoic acid. Here, the binding of the RAR-A was dependent on the presence of the ER and both proteins can be found in the same transcription complexes, although no direct interaction between the receptors was proven (35).

The primary role of RAR in hTGP transcriptional activation was confirmed by treatment of cells with the RAR-specific ligand TTNPB (Figure 5A), and when coupled with the knockdown of RAR-B and RAR-G (Figure 5B), the major role for RAR-G in RA-dependent hTGP expression was demonstrated.

Previously, basal activity of the hTGP promoter was mapped to the −113 to −61 bp relative to the transcriptional start site, and no functional ARE was found within the −13 to −2077 region (29). We here show by functional hTGP promoter analysis that a region in the distal promoter was responsible for an increase in transcriptional activity in the absence of treatment (Supplementary Figure S1B). Remarkably, both an ARE and RARE were located in the enhancer region and were responsive to atRA and R1881 treatments, while other ARE and RARE which mapped outside this 1 kb enhancer region (−4.5 to −3.5 kb) did not show a positive effect on gene expression (Supplementary Figure SIC and D). These results suggest that the 4.5-kb region of the hTGP promoter contains enough information to re-create the in vivo gene regulation in an in vitro model. Deletion of RAREs and ARE indicated the role of each site in the regulation of hTGP expression. Similar enhancer regions containing binding elements, such as AREs, have been described to control the prostate specific genes PSA and PSMA (22,24,53,54).

ChIP analysis confirmed that RAR binding was in close proximity to the predicted RAREs (Figure 6A). The RAR-binding pattern did not change when cells were treated with atRA or R1881, in accordance with the existing model, where RAR is bound to the RARE in the absence of ligand (7,8,55). Interestingly, the RARE proximal to the transcription start site was occupied by the RAR even after demonstration that the deletion of this site has a positive effect on promoter activity after atRA treatment (Figure 6C and F).

AR binding followed a similar pattern to that of RAR in untreated cells, but was up-regulated by androgen treatment in a region (−4015 to −3909) adjacent to an ARE. Thus AR binding to the distal hTGP promoter could antagonize RAR activity by sterically preventing the recruitment of co-activators or by actively recruiting co-repressors to the promoter.

Moreover, the binding of AR and RAR to a specific set of genes suggested the existence of a wide-spread regulatory mechanism. The validation of AR and RAR binding to key genes in cell cycle, proliferation and transcriptional control demonstrated that RAR and AR are capable of working together regulating a broad spectrum of genes. Interestingly, hTGP is not the only gene where retinoic acid and androgen have opposing effects. CDCA7L and CDK6 also show a similar pattern, being up-regulated by one hormone and down-regulated by the other. These findings help to explain previous evidence of antagonistic effects of these hormones. For example, the opposite effects of androgen and retinoic acid in cell death are well documented (56–59). In our analysis, genes important for apoptosis have been shown to be regulated by AR and RAR, consistent with a model in which AR and RAR regulate cell death-related genes in an opposite manner. A similar regulatory network has recently been described in breast cancer for the RAR-A and the ER, where binding sites for these receptors in a given gene exerted antagonizing effects on its transcription (35,36). The antagonistic co-regulation of AR and RAR activity, which is related to the presence of androgen and RA, could now play a role in prostate differentiation and cancer as well.

In human prostate, cancer tissues have been shown to contain less RA than normal tissues (60), while epidemiological studies revealed an inverse relationship between vitamin A, a precursor of RA, levels in serum and prostate cancer incidence (61–63). Since RA can inhibit growth and tumorigenic potential (59,64), it is likely that, as a natural mechanism, while androgen signalling promotes survival and growth in the prostate, RA signalling could be used to regulate growth and survival, and that a malfunction in the RA regulation could contribute to prostate cancer as previously suggested (65).

These data provide the first description of a prostate-specific gene where androgen plays a minor role in transcriptional regulation, raising the question of whether the classical transcriptional role of androgen–AR is sufficient as a mechanism to achieve prostate-specific expression. It also shows that RA might play an important role not only in prostate development but also in its function as a matured organ. Controlling the expression of prostatic genes and antagonizing the effects of androgen is important and perhaps necessary to control prostate gland homeostasis. By investigating how expression of the hTGP gene is restricted to the prostate, in comparison to PSA and other androgen regulated prostate specific genes, the role of RA in gene expression patterns of individual cell types and the binding pattern of RAR in human prostate will provide a valuable insight into the complex regulation of prostate development and differentiation as well as prostate cancer.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online: Supplementary Figures 1–4, Supplementary Tables 1–3.
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