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Partitioning of 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol activated pathways for stimulating human prostate cancer LNCaP cell proliferation

Eva H. Nunlist [a], Igor Dozmorov [b], Yuhong Tang [b], Rick Cowan [a], Michael Centola [b], Hsueh-Kung Lin [a,*]

[a] *Department of Urology, University of Oklahoma Health Sciences Center, 920 Stanton L. Young Blvd., WP3150, Oklahoma City, OK 73104, USA*
[b] *Arthritis and Immunology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA*

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Abstract

The growth and development of the prostate gland are regulated by androgens. Despite our understanding of molecular actions of 5α-dihydrotestosterone (5α-DHT) in the prostate through the *trans*-activation of the androgen receptor (AR), comprehensive analysis of androgen responsive genes (ARGs) has just been started. Moreover, expression changes induced by the androgen effects of 5α-androstane-3α,17β-diol (3α-diol), a metabolite of 5α-DHT through the action of 3α-hydroxysteroid dehydrogenases (3α-HSDs), remain undefined. We demonstrated that both 5α-DHT and 3α-diol stimulated similar levels of androgen sensitive human prostate cancer LNCaP cell proliferation. However, consistent with the fact that 3α-diol has low affinity toward the AR, 3α-diol did not elicit the same levels of AR *trans*-activation activity as that of 5α-DHT. Since LNCaP cells respond to androgen stimulation by transcriptionally activating the AR downstream genes, gene expression patterns between 0 and 48 h following 3α-diol and 5α-DHT stimulation were analyzed using cDNA-based membrane arrays to define the temporal regulation of ARGs. Array analysis identified 217 and 219 androgen-modulated genes in at least one time point following 3α-diol and 5α-DHTstimulation, respectively, including key regulators of cell proliferation. Only a subset of these genes (143) was regulated by both androgens. These data suggest that 3α-diol exerts androgenic effects independent of the action of 5α-DHT in steroid target tissues. Accordingly, 3α-diol might activate cell proliferation cascades independent of AR pathway in the prostate.

Keywords: 5α-Dihydrotesotsterone; 5α-Androstane-3α,17β-diol; Androgen receptor; Androgen receptor target genes; Array analysis

1. Introduction

Androgens mediate a wide spectrum of functions related to the development, growth, and maintenance of androgen responsive organs including prostate [1–9]. In mammals, the principal androgens are comprised of testosterone and its 5α-reduced metabolites such as 5α-dihydrotestosterone (5α-DHT) [1]. In order to exert their androgenic effects in the prostate, it has been well accepted that testosterone is first converted to 5α-DHT, which then binds to the androgen receptor (AR). The ligand-bound AR then translocates to the nucleus, interacts with androgen-response elements (AREs) of androgen-target genes and, ultimately, leads to the transcriptional activation or suppression of these genes [2].

5α-DHT can be further reduced to 5α-androstane-3α, 17β-diol (3α-diol) through the action of 3α-hydroxysteroid dehydrogenases (3α-HSDs). Unlike 5α-DHT, the mechanism behind 3α-diol-mediated androgenic effects remains unclear [10]. Generally considered as a weak androgen, 3α-diol has no defined hormonal functions. As of yet, no specific 3α-diol receptor has been identified [10]. Due to its low binding affinity to the AR with $K_d = 10^{-6}$ M [11], it has been widely accepted that 3α-diol must first be oxidized to 5α-DHT by 3α-HSDs before exerting its androgenic functions [12]; thus this reaction represents an alternative pathway for regulating steady-state levels of 5α-DHT in androgen target tissues. This hypothesis has been supported by the formation and accumulation of 5α-DHT in androgen target tissues including the prostate following 3α-diol

Abbreviations: AR, androgen receptor; ARE, androgen response elements; ARG, androgen responsive gene; 3α-Diol, 5α-androstane-3α,17β-diol; 3α-HSD, 3α-hydroxysteroid dehydrogenase; DHT, 5α-dihydrotestosterone (5α-androstan-17β-ol-3-one); Testosterone, 17β-hydroxy-androst-en-3-one

* Corresponding author. Tel.: +1 405 271 6900; fax: +1 405 271 3118.
E-mail address: hk-lin@ouhsc.edu (H.-K. Lin).

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administration in various animal models including dog [12] and rat [13,14]. It has further been shown that the AR antagonist, flutamide, prevents 3α-diol from virilizing the urogenital sinus of the tammar wallaby, suggesting that 3α-diol may be a precursor for 5α-DHT, which is responsible for virilizing in the wallaby [10].

Growing evidence suggests 3α-diol might function through pathways that are independent from the AR. It has been shown that 3α-diol forms a complex with sex hormone-binding globulin (SHBG) at the cell surface of certain androgen target tissues to stimulate rapid accumulation of intracellular cAMP and subsequent secondary activation of the AR [15]. This androgen has also been demonstrated to play a role in parturition of a murine model [16] as well as in prostate formation in marsupial mammals [17] through an unknown mechanism.

To determine the androgenic effects of 3α-diol, we compared 3α-diol and 5α-DHT stimulated cell proliferation in the LNCaP cells. We demonstrated that 3α-diol could be as potent as 5α-DHT in stimulating human prostate LNCaP cell proliferation as previously reported prostate hyperplasia in a dog model [15]. However, levels of 3α-diol-mediated AR *trans*-activation could not be correlated with 3α-diol-stimulated cell proliferation in the usual terms of androgens resorting to the AR pathway. To determine the potential 3α-diol-induced pathway(s) in LNCaP cells, gene expression profiles were analyzed over a course of 48 h following either 3α-diol or 5α-DHT treatment using cDNA-based membrane arrays. Although gene expression profiling in response to androgen stimulation has been reported recently [18], this is the first evaluation and comparison of temporal regulation of gene expression in LNCP cells following 3α-diol and 5α-DHT stimulation. From a total of 2370 genes analyzed, our results supported the hypothesis that 3α-diol may execute pathways other than classical AR *trans*-activation for prostate cell proliferation.

2. Materials and methods

2.1. Reagents and chemicals

LNCaP human prostate cancer cell line was obtained from American Type Culture Collection (ATCC # CRL-1740). 5α-DHT and 3α-diol were purchased from Sigma-Aldrich (St. Louis, MO). [α-^{32}P]dATP (3000 Ci/mmol, 10 μCi/μl) was acquired from Amersham Pharmacia (Piscataway, NJ). RPMI 1640 medium, OPTI-MEM, and penicillin–streptomycin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was acquired from Atlanta Biologicals (Norcross, GA); and charcoal-dextran treated (CD) FBS was obtained from HyClone (Logan, UT). p(GRE)-SEAP reporter gene construct, Atlas Human 1.2 Array, and Atlas Human Cancer 1.2 Array were obtained from Clontech Laboratories (Palo Alto, CA). pCMV-β-galactosidase was purchased from Stratagene (La Jolla, CA).

2.2. Cell culture

LNCaP cells were maintained at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were passaged every 3–5 days or whenever cells reached 70–80% confluence.

2.3. Cell proliferation assay

To determine LNCaP cell proliferation in response to androgen stimulation, 2×10^3 cells in 100 μl RPMI 1640 containing 10% FBS were seeded in each well of 96-well plates overnight for adherence. The medium was replaced with 100 μl of serum deficient medium consisting of phenol red-free OPTI-MEM, and 1% CD FBS. Following culture in serum deficient medium for 48 h, LNCaP cells were treated with 10^{-12} to 10^{-8} M 5α-DHT or 3α-diol in a total of 200 μl OPTI-MEM plus 1% CD FBS per well. Control wells received serum deficient medium without androgens. Cell number in these cultures was determined daily for a total of 6 days using an XTT cell proliferation kit (Roche; Indianapolis, IN). To perform the XTT assay, an aliquot of 100 μl culture medium was removed from each well, and 50 μl XTT colorimetric reagent was added to the well. Following incubation at 37 °C for 6 h, plates were read at dual wavelength 490 and 630 nm with μQuant microplate reader (Bio-Tek; Winooski, VT). Experiments were performed in triplicates and repeated at least three times. Results were present as fold increase in absorbance at days post-stimulation over that obtained at the day of androgen treatment.

2.4. Transient transfection and reporter gene assays

To perform transient transfection of reporter gene constructs, LNCaP cells grown in 100 mm dishes in RPMI 1640 with 10% FBS was replaced with serum deficient medium consisting of OPTI-MEM, 2% CD FBS, 200 μg/ml $CaCl_2$, and 100 units/ml penicillin–100 μg/ml streptomycin. After being cultured in the serum-deficient medium for 48 h, LNCaP cells were washed twice with PBS, trypsinized, washed again with OPTI-MEM containing 2% CD FBS, and resuspended with the serum deficient medium at the concentration of 1×10^7 cells/ml. Electroporation was performed with 400 μl of the cell suspension in a 4 mm gap electroporation cuvette (Bio-Rad; Hercules, CA) containing 4 μg pCMV-β-galactosidase and 10 μg p(GRE)-SEAP and pulsed once at 170 V for 70 ms with an electroporator (ECM 830, BTX; San Diego, CA). Followed by a 5 min incubation at room temperature, cells were seeded into 6-well plates at 0.5×10^6 cells per well in 2 ml OPTI-MEM supplemented with 2% CD FBS and 200 μg/μl $CaCl_2$. Cells were then

incubated at 37 °C for 6 h followed by 10^{-12} to 10^{-10} M 5α-DHT or 10^{-11} to 10^{-8} M 3α-diol stimulation. Supernatants were collected at 40 h post-treatment and stored at −80 °C for assessing secreted alkaline phosphatase (SEAP) activity. To collect cell lysates to measure β-galactosidase activity, cells in each well were incubated with 300 μl of 1× Reporter Lysis Buffer (Promega; Madison, WI) at room temperature for 15 min. Supernatants were cleared by centrifugation at 18,000 × *g* for 2 min, and stored at −80 °C until use.

SEAP activity was quantified using the Chemiluminescent SEAP Reporter Gene Assay Kit (Roche) following the manufacturer's protocol. Briefly, 50 μl culture supernatant was mixed with 150 μl dilution buffer and incubated at 65 °C for 30 min. An aliquot of 100 μl of the heat-inactivated sample was mixed with 100 μl inactivation buffer. After a 5 min incubation at room temperature, 100 μl of substrate reagent was added and the mixture was incubated at room temperature for at least 10 min with gently rocking. Samples were quantified using an FB12 single tube luminometer (Zylux Corporation, Oak Ridge, TN). β-Galactosidase activity was measured using the β-galactosidase assay system according to the manufacturer's procedures (Promega) as previously described [19]. SEAP activity was normalized against β-galactosidase level for each sample.

2.5. cDNA-based membrane array analysis

To identify androgen-activated transcription profiling in LNCaP cells, cDNA-based membrane arrays were used. Total RNA was extracted from LNCaP cells treated with 10^{-12} M 5α-DHT or 10^{-11} M 3α-diol at 0, 1, 3, 6, 12, 24, and 48 h after stimulation using Trizol according to manufacturer's instructions (Invitrogen). Equal amounts of total RNA were pooled from three repeats for each time point. Radio-labeled probes were reverse transcribed from 5 μg of the pooled total RNA from each time point in the presence of CDS primers (Clontech), 35 μCi [α-^{32}P]-dATP, 200 units Superscript II reverse transcriptase (Invitrogen), and dNTPs in a total of 10 μl. Reactions were performed at 42 °C for 1 h, and stopped by the addition of termination mix (Clontech). Radio-labeled first strand cDNAs were purified using NucleoSpin Extraction Spin Columns (Clontech) and eluted with 100 μl elution buffer (Clontech). Fractions containing [α-^{32}P]-dATP-labeled cDNAs were counted with LS 6000IC scintillation counter (Beckman Coulter; Fullerton, CA).

The array membranes were first prehybridized with 5 ml ExpressHyb™ solution (Clontech) in the presence of 0.5 mg denatured sheared salmon sperm DNA (Invitrogen) at 68 °C for 30 min. The Atlas Array membranes were hybridized with a mixture of denatured [α-^{32}P]-dATP-labeled probes (20×10^6 cpm) and 5 μg C_ot-1 DNA at 68 °C for 16–18 h. Membranes were then washed four times for 30 min each at 68 °C in 2× SSC plus 1% SDS, followed by one 30 min wash at 68 °C in 0.1× SSC plus 0.5% SSC and one final 5 min wash in 2× SSC at room temperature. Washed membranes were exposed to phosphor-imaging screens (Packard BioScience; Meriden, CT); and images were captured using a Cyclone storage phosphorimager system (Packard BioScience). Results from the phosphor-imaging screen were presented as digital light units (DLU) and interpreted using OptiQuant image analysis software (Packard BioScience). There were a total of 1185 genes present for each of the Atlas Human Cancer 1.2 and Human 1.2 cDNA Expression Arrays with 462 genes overlapping between the Human Cancer 1.2 and Human 1.2 arrays. For a complete list of genes present in these arrays, see http://www.clontech.com/atlas/genelists/index.html.

2.6. Array data analysis

2.6.1. Normalization and scaling

Signals from independent samples may vary on a global-basis and must be therefore adjusted to a common standard for comparison. Adjustment of expression levels in compared samples was performed as previously described [20]. Briefly, compared samples were first normalized using background signals to adjust overall expression levels. The parameters of the background were calculated from non-expressed genes whose signal values exhibited a normal distribution distinguishable from the rest of the values on an all-value histogram. The mean and standard deviation (S.D.) of the background was obtained by first excluding expressed genes from the distribution then using a nonlinear curve fitting algorithm on the remaining, predominantly background signals. In this algorithm, a given gene expression value exceeding the mean of the distribution of non-expressed genes (±2 S.D.) was excluded from the background set and a new mean and S.D. were determined for the remaining genes. This process was continued in an iterative manner until no new data could be discarded. Signal values of the expressed genes were then normalized to its background's mean and S.D. After the normalization step, the distribution of newly scaled intensities of background genes had a mean of zero and S.D. = 1. Genes which had values greater than mean + 3 S.D. of the background level are considered to be "expressed values" and used for further analysis. The normalized profiles of expressed genes in compared samples were then scaled to each other through a robust regression analysis [21]. This analysis is based on the fact that in a linear regression analysis between two compared samples the majority of genes are equally expressed and, therefore, randomly distributed around the regression line with a small portion of differentially expressed "outliers". The contribution of outliers to the regression analysis was down-weighted in an iterative manner. All expression profiles were then re-scaled to a common standard—the averaged profile of the control group. Our procedure for outlier exclusion was based on the selection of equally expressed genes as a homogenous family of genes with close to normally distributed

residuals (measured as deviations from the regression line).

2.6.2. Selection of "hyper-variable genes" (HV-genes)

Androgen-responsive genes were identified using an analysis of temporally-induced gene expression changes. This procedure utilized an internal standard to define both technologic and normal biologic variance and used this standard to identify statistically significant stimuli-induced changes in gene expression. The first step in this analysis was identification of a group of similarly expressed genes from control samples, denoted "the reference group" that was used for selection of differentially expressed genes in experimental samples and for the F-clustering procedure. The reference group was composed of a group of genes expressed above background in control samples with a low variability of expression (as determined by an *F*-test), and whose residuals approximated a normal distribution, based on the Kolmogorov–Smirnov criterion. The variability of expression of this reference group was due to both technical and normal biological variations. This reference group can be used to identify genes whose expression varies due to experimental conditions in a statistically significant manner using an *F*-test with a threshold at $P = 0.001$ that restricts the appearance of "just by chance" selections (false positives) to about one for 1000 comparisons. The differentially expressed genes varying in expression beyond the expected levels due to technological and normal biological variations were denoted "HV-genes".

2.6.3. F-means cluster analysis of HV-genes co-expression

Clustering procedure utilizing an *F*-test consisted of the following steps: Gene expression normalization, log-transformation and rescaling as described above, identification of, and limiting subsequent analyses, genes expressed above background (3 S.D. above background noise), in at least one time point, and identification and limiting of subsequent analysis to genes with expression levels that varied among time points (based on comparison with reference group variability by *F* criterion). Determination of a parameter, termed connectivity, for each HV-gene was then performed. Connectivity was defined as the number of genes whose expression behavior varied with respect to time in a manner similar to a given gene (based on an *F* test). A Monte Carlo simulation study was performed on randomized data having the same mean and S.D. as the experimental data to determine a statistically significant threshold for inclusion of genes in the same cluster. From this analysis threshold for the correlation coefficient was determined as 0.7.

Although genes could be associated with multiple clusters, inclusion was limited to the clusters of highest connectivity, such that the broadest biologic phenomenon that is those involving the largest number of genes, could be distinguished. A gene associated with two clusters might be a functional link between these clusters and was represented as a connecting point between clusters. Genes that had zero connectivity did not belong to any cluster. Matrices of correlation coefficients were calculated for these clusters and were represented in a graphical output termed a connectivity mosaic such that patterns of correlated and non-correlated behavior of genes can be identified by visual inspection.

3. Results

3.1. Both 3α-diol and 5α-DHT stimulated LNCaP cell proliferation

An elevated growth curve was observed when LNCaP cells treated with 10^{-12} to 10^{-8} M 5α-DHT on day 6 (Fig. 1A). Surprisingly, when LNCaP cells were stimulated with the same concentrations of 3α-diol, they also exhibited an exponential growth curve; and similar levels of elevated cell proliferation were observed when 10^{-12} to 10^{-9} M 5α-DHT and 3α-diol were used on day 6 (Fig. 1A). LNCaP cells had elevated cell proliferation on days 4 and 5 when treated with 10^{-11} M 5α-DHT as compared to those treated with the same concentration of 3α-diol (data not shown). There was no indication of a statistically significant difference in cell growth between 10^{-12} M 5α-DHT-treated and 10^{-11} M 3α-diol-treated LNCaP cells over the entire period (Fig. 1B); both groups of cells proliferated sharply from day 2 to day 3, and continued to grow in a nearly identical pattern. Thus, 10^{-11} M 3α-diol and 10^{-12} M 5α-DHT were chosen for the comparison of gene expression patterns regulated by these two androgens using array analysis.

3.2. 3α-Diol did not stimulate a similar level of AR trans-activation activity in LNCaP cells as compared to 5α-DHT

To determine if 3α-diol uses AR-dependent pathway, LNCaP cells were transiently transfected with a reporter gene construct, p(GRE)SEAP [a glucocorticoid response element (GRE)-directed SEAP expression vector], followed by androgen stimulation and reporter gene assays. It has been established that glucocorticoid receptors (GRs) share a high degree of homology with ARs in their DNA-binding domains [22]; and a GRE can be mediated through glucocorticoid-induced as well as androgen-induced gene expression [22]. Using the reporter gene assay system to assess the *trans*-activation activity of AR, transiently transfected LNCaP cells were stimulated with a series concentrations of either 5α-DHT or 3α-diol. An androgen-dependent SEAP activity was observed when LNCaP cells were tested with either 5α-DHT or 3α-diol (Fig. 2). Cells treated with 10^{-12} M 5α-DHT exhibited a 25 ± 7% higher SEAP activity than untreated controls after normalization. In contrast, when cells were treated with 10^{-11} M 3α-diol, an 8 ± 5% induction in SEAP activity over that of untreated cells was

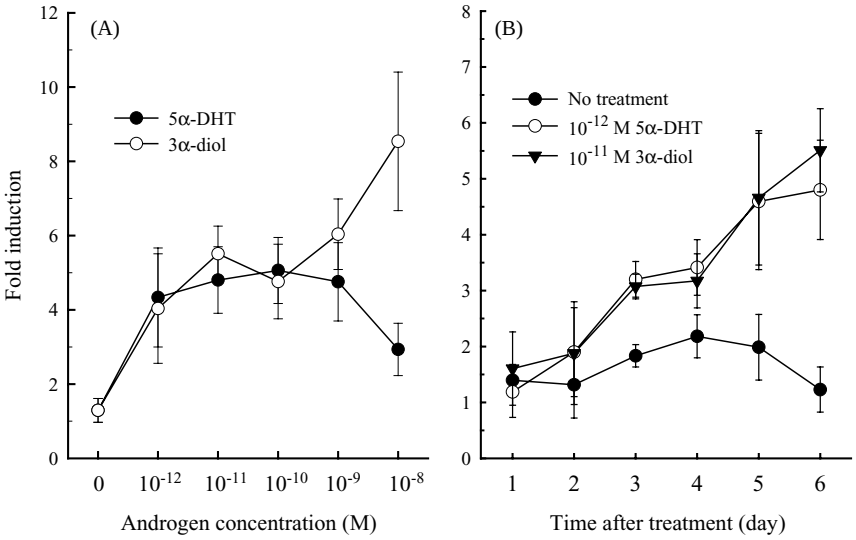

Fig. 1. Temporal- and dose-response effects of androgens on LNCaP cell proliferation. LNCaP cells were seeded in each well of 96-well plates at the concentration of 2×10^3 cells per well, and subjected to serum deprivation followed by 5α-DHT or 3α-diol stimulation. Cell proliferation was determined using XTT assay everyday for a period of 6 days. Data were calculated as absorbance at days following androgen stimulation divided by the absorbance at the day of androgen stimulation, and presented as fold induction in absorbance following androgen stimulation (A) LNCaP cells were treated with 10^{-12} to 10^{-8} M 5α-DHT or 3α-diol on day 6. Elevated proliferation was observed in both 5α-DHT and 3α-diol treated LNCaP cells in all concentrations tested ($P < 0.01$). (B) LNCaP cells were treated with either 10^{-12} M 5α-DHT or 10^{-11} M 3α-diol, where 5α-DHT and 3α-diol stimulated similar temporal changes in cell proliferation.

observed. The reporter gene activity was statistically significantly lower in cells treated with 10^{-11} M 3α-diol than that of cells treated with 10^{-12} M 5α-DHT. These results indicated that administration of 3α-diol to LNCaP cells slightly elevated the *trans*-activation activity of AR but not as potently as that was observed for 5α-DHT.

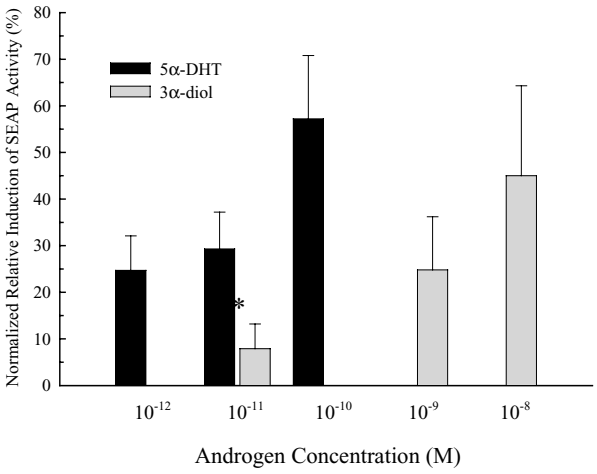

Fig. 2. AR *trans*-activation by 5α-DHT and 3α-diol measured by reporter gene assays. A GRE-regulated SEAP reporter gene construct was transiently transfected through electroporation into LNCaP cells which had been cultured in OPTI-MEM supplemented with 2% CD FBS and 200 μg/ml $CaCl_2$ for 24 h. 5α-DHT (10^{-12} to 10^{-10} M) or 3α-diol (10^{-11} to 10^{-8} M) was added at 6 h after transfection. Culture media and cell lysates were collected at 40 h after androgen treatment for SEAP and β-galactosidase activity assays, respectively. Data have been normalized to β-galactosidase activity co-transfected as pCMV-β-galactosidase. The mean ± standard error is given for 5–8 separate transfections.

3.3. Distinction of 3α-diol- and 5α-DHT-regulated gene expression in LNCaP cells

We were interested in determining 3α-diol- and 5α-DHT-regulated gene expression to understand the mechanisms of 3α-diol- and 5α-DHT-regulated LNCaP cells proliferation. A total of 1908 distinct genes were analyzed in LNCaP cells following either 3α-diol or 5α-DHT stimulation using cDNA-based membrane arrays. Using hypervariable analysis, 293 genes were found to be androgen-modulated. Of these, 143 genes (48.8%) were identified as HV-genes in both 3α-diol and 5α-DHT treated LNCaP cells. However, 150 genes (51.2%) were modulated differentially in response to the two treatments; 74 genes were modulated in response to 3α-diol only and 76 genes were modulated in response to 5α-DHT (Fig. 3). The genes

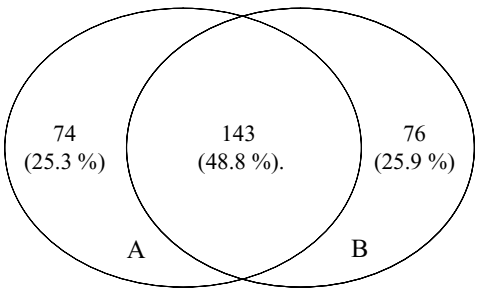

Fig. 3. Common and unique genes regulated by 3α-diol and 5α-DHT in LNCaP cells. Hypervariable genes were selected from 3α-diol (A) and 5α-DHT (B) treated LNCaP cells based on S.D. of their expression over a period of 48 h as described in Section 2.

Table 1
Genes that were modulated by (a) 3α-diol and (b) 5α-DHT treatments

Genbank number	Gene name	Cluster
(a) 3α-diol treatment only		
M3418d1	Protein kinase, cAMP-dependent, catalytic, β	−101
M21535	v-ets avian erythroblastosis virus E26 oncogene related	−99
M77830	Desmoplakin (DPI, DPII)	−48
L09210	Nitric oxide synthase 2A (inducible, hepatocytes)	−24
D30751	Bone morphogenetic protein 4	2
J03571	Arachidonate 5-lipoxygenase	2
J04177	Collagen, type XI, α 1	2
L12350	Thrombospondin 2	2
M19154	Transforming growth factor, β 2	2
M35296	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	2
M85289	Heparan sulfate proteoglycan 2 (perlecan)	2
L32976	Mitogen-activated protein kinase kinase kinase 11	3
U04847	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	3
X75621	Tuberous sclerosis 2	3
AF010309	Quinone oxidoreductase homolog	5
U02368	Paired box gene 3 (Waardenburg syndrome 1)	5
X52192	Feline sarcoma (Snyder-Theilen) viral (v-fes)/Fujinami avian sarcoma (PRCII) viral (v-fps) oncogene homolog	5
M21097	CD19 antigen	7
X69111	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	9
AF064019	DNA fragmentation factor, 40 kD, β polypeptide (caspase-activated DNase)	10
X74795	Minichromosome maintenance deficient (*S. cerevisiae*) 5 (cell division cycle 46)	10
AJ001189	Oligophrenin 1	13
U14417	ral guanine nucleotide dissociation stimulator	13
X98085	Tenascin R (restrictin, janusin)	17
Z23115	BCL2-like 1	19
X85030	Calpain 3, (p94)	21
Y00711	Lactate dehydrogenase B	21
D49547	Heat shock 40 kD protein 1	26
L07493	Replication protein A3 (14 kD)	35
L20688	Rho GDP dissociation inhibitor (GDI) β	37
Y12735	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	37
Y13115	Serine/threonine kinase 18	37
D21063	MCM2 DNA replication licensing factor; nuclear protein BM28; KIAA0030	39
M15796	Proliferating cell nuclear antigen	39
D10924	Chemokine (C-X-C motif), receptor 4 (fusin)	40
M12154	Antigen p97 (melanoma associated) identified by monoclonal antibodies 133.2 and 96.5	40
U62540	Anaplastic lymphoma kinase (Ki-1)	40
M57230	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	40
X03212	Keratin 7 (KRT7; KT7)	40
D85815	ras homolog gene family, member	43
U85245	Phosphatidylinositol-4-phosphate 5-kinase, type II, beta	44
U41816	Prefoldin 4	49
X74764	Discoidin domain receptor family, member 2	52
X03541	Neurotrophic tyrosine kinase receptor type 1 (NTRK1; TRK)	52
X60957	Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains	52
AJ010119	Ribosomal protein S6 kinase, 90 kD, polypeptide 4	53
M22995	RAP1A, member of RAS oncogene family	53
M74088	Adenomatosis polyposis coli	53
L05624	Mitogen-activated protein kinase kinase 1	62
D38305	Transducer of ERBB2, 1	63
X08004	RAP1B, member of RAS oncogene family	64
X07979	Integrin, beta 1 (fibronectin receptor, β polypeptide, antigen CD29 includes MDF2, MSK12)	78
X05562	Procollagen IV alpha 2 subunit (COL4A2)	79
U61262	Neogenin (chicken) homolog 1	80
M86400	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	81
M34356	cAMP responsive element binding protein 1	82
M26708	Prothymosin, α (gene sequence 28)	83
M25639	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	84
M16038	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	85
L31881	Nuclear factor I/X (CCAAT-binding transcription factor)	87
L31801	Solute carrier family 16 (monocarboxylic acid transporters), member 1	88

Table 1 (*Continued*)

Genbank number	Gene name	Cluster
L26584	cdc25, a res-specific nucleotide exchange factor	92
AF025409	Solute carrier family 30 (zinc transporter), member 4	94
Z29083	5T4 oncofetal trophoblast glycoprotein	95
Y09305	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 4	96
X67951	Peroxiredoxin 1	97
X62055	Protein tyrosine phosphatase, non-receptor type 6	98
X17644	G1 to S phase transition 1	99
U05040	Far upstream element (FUSE) binding protein 1	100
M99437	Neurogenic locus notch protein	101
M64595	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	102
L42450	Pyruvate dehydrogenase kinase, isoenzyme 1	103
D25274	Chromosome 4p clone PO2ST9 (human brain striatum cDNA)	104
D00763	Proteasome (prosome, macropain) subunit, α type, 4	105
(b) 5α-DHT treatment only		
AF006515	Chromodomain helicase DNA binding protein 3	−48
A00914	Angiotensin-converting enzyme (ACE)	1
L06801	Interleukin 13	1
D15057	Defender against cell death 1	3
M64673	Heat shock transcription factor 1	3
U12979	Activated RNA polymerase II transcription cofactor 4	3
U65928	COP9 (constitutive photomorphogenic, Arabidopsis, homolog) subunit 5	3
Z30094	General transcription factor IIH, polypeptide 2 (44 kD subunit)	3
U29656	Non-metastatic cells 3, protein expressed in	8
M21626	T cell receptor alpha variable 14/δ variable 4	9
D28468	Albumin D box-binding protein; D-binding protein (DBP); TAXREB302	10
D45132	Zinc-finger DNA-binding protein	10
D50405	Histone deacetylase 1	10
X16277	Ornithine decarboxylase 1	10
AF046873	Synapsin III	12
D13316	GA-binding protein transcription factor, β subunit 2 (47 kD)	12
D14520	Kruppel-like factor 5 (intestinal)	12
J02853	Casein kinase 2, α1 polypeptide	12
L76224	Glutamate receptor, ionotropic, *N*-methyl D-aspartate 2C	12
U15306	Nuclear transcription factor, X-box binding 1	12
U32376	Discs, large (Drosophila) homolog 2 (chapsyn-110)	12
L05515	cAMP response element-binding protein CRE-BPa	12
L14754	Immunoglobulin μ binding protein 2	12
M31523	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	12
M68520	Cyclin-dependent kinase 2	12
M97796	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	12
S75313	Machado–Joseph disease (spinocerebellar ataxia 3, olivopontocerebellar ataxia 3, autosomal dominant, ataxin 3)	12
X68274	Contactin 2 (axonal)	12
X91788	Chloride channel, nucleotide-sensitive, 1A	13
M77234	Ribosomal protein S3A	13
X95384	Translational inhibitor protein p14.5	13
X01057	Interleukin 2 receptor, α	14
AF077866	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	16
Y07683	Purinergic receptor P2X, ligand-gated ion channel, 3	16
U66838	Cyclin A1	16
X15949	Interferon regulatory factor 2	19
L01042	TATA element modulatory factor 1	20
M86492	Glia maturation factor, β	20
M92357	Tumor necrosis factor, α-induced protein 2	20
M86528	Neurotrophin 5 (neurotrophin 4/5)	20
M87503	Interferon-stimulated transcription factor 3, γ (48 kD)	20
AF016709	Purinergic receptor P2X, ligand-gated ion channel, 5	23
U07616	Amphiphysin (Stiff–Mann syndrome with breast cancer 128 kD autoantigen)	23
D31840	Dentatorubral-pallidoluysian atrophy (atrophin-1)	25
M81768	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 1 (antiporter, Na^+/H^+, amiloride sensitive)	26
M96824	Nucleobindin 1	30
X92669	Menage a trois 1 (CAK assembly factor)	30
D21878	Bone marrow stromal cell antigen 1	31

Table 1 (*Continued*)

Genbank number	Gene name	Cluster
M62843	ELAV (embryonic lethal, abnormal vision, Drosophila)-like 4 (Hu antigen D)	31
X58079	S100 calcium-binding protein A1	31
D17517	TYRO3 protein tyrosine kinase	32
X07767	Protein kinase, cAMP-dependent, catalytic, α	34
M25753	Cyclin B1	35
AF010127	Casper, a FADD- and caspase-related inducer of apoptosis (CASH-alpha + CASH-beta); FLAME-1; FLICE-like inhibitory protein	37
U77845	TRAF interacting protein	37
S87759	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	42
J04101	v-ets avian erythroblastosis virus E26 oncogene homolog 1	43
L06895	MAX dimerization protein	43
M76125	AXL receptor tyrosine kinase	43
U40370	Phosphodiesterase 1A, calmodulin-dependent	44
X54941	CDC28 protein kinase 1	49
Y15065	Potassium voltage-gated channel, KQT-like subfamily, member 2	50
M64930	Protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), β isoform	50
U18087	3′5′-cAMP phosphodiesterase HPDE4A6	50
AF001954	Inhibitor of growth 1 family, member 1	54
U43527	KiSS-1 metastasis-suppressor	54
U35143	Retinoblastoma-binding protein 7	55
M30773	Protein phosphatase 3 (formerly 2B), regulatory subunit B (19 kD), α isoform (calcineurin B, type I)	56
U47413	Cyclin G1	58
X75042	v-rel avian reticuloendotheliosis viral oncogene homolog	66
X12646	Protein phosphatase 2 (formerly 2A), catalytic subunit, α isoform	68
U10323	Interleukin enhancer binding factor 2, 45 kD	69
L05500	Adenylate cyclase 1 (brain)	70
K02268	Beta-neoendorphin-dynorphin; proenkephalin B; preprodynorphin	71
M21121	Small inducible cytokine A5 (RANTES)	74
L76200	Guanylate kinase 1	75

Cluster assignment is consistent for both 3α-diol and 5α-DHT treatments, same number indicating the same expression pattern.

that are modulated restricted to either 3α-diol or 5α-DHT were listed in Table 1 .

3.4. Identification of 3α-diol- and 5α-DHT-specific changes in gene expression

Although a significant number of genes were modulated in response to both 3α-diol and 5α-DHT stimulation, the regulation of 17 of these genes can be distinguished between treatments using cluster analysis. This method grouped genes that were modulated in a similar manner by a given stimulus into clusters. In this analysis, a quantitative measurement, denoted a correlation coefficient, of how related a given gene's expression profile was to the average profile of the cluster to which it belongs was calculated. Genes whose regulation are modulated differentially by various stimuli can be identified by their changes in correlation coefficients [21]; and the relative strength of the correlation coefficients represented in a color map [21]. Genes that respond to stimuli can be differentially identified by vision inspection on mosaic maps due to their variations in color observed. The 17 genes that were modulated by both 3α-diol and 5α-DHT stimulation but in a distinct manner could be discerned in a mosaic map of the data (Fig. 4A and B). The differences in regulation by these stimuli were confirmed as the correlation coefficients from these genes being somewhat different for 3α-diol- and 5α-DHT-treated cells (Fig. 4C). The most significant difference was observed for cyclin-dependent kinase inhibitor 2A [(melanoma, p16, inhibits CDK4); p16-INK4] whose expression switched from a strongly positive correlation with cluster 1 members in 3α-diol treated cells to a negative correlation in 5α-DHT treated cells.

Other genes whose expression varied in 3α-diol- and 5α-DHT-treated cells (correlations <0.7) included high-mobility group protein 17 (HMG17), zinc finger protein 9 (tb protein), protein tyrosine phosphate [(non-receptor type 11); HUMPTP], inhibitor of DNA binding 1 (Id-1H), histidine decarboxylase (HSHISDEC), wingless-type MMTV integration site family (WNT-8B), Ras-related associated with diabetes (Rad), Notch (Drosophila) homolog 1 [(translocation-associated; TAN-1], G-rich RNA sequence binding factor 1 (GRSF-1), membrane-type matrix metalloproteinase 1 (MT-MMP1), DNA (cytosine-5-)-methyltransferase 1 (HSDNMTASE), dopa decarboxylase (aromatic L-amino acid decarboxylase; ddc), glutathione peroxidase-like protein (HSGPLP), and proteasome [(prosome, macropain) subunit, α type, 1; HUMPSC2)] (Fig. 4).

In addition to the 293 HV-genes which were identified through statistical criteria in response to either 3α-diol and 5α-DHT stimulation, a list of 37 functionally important genes was specifically selected based on published database

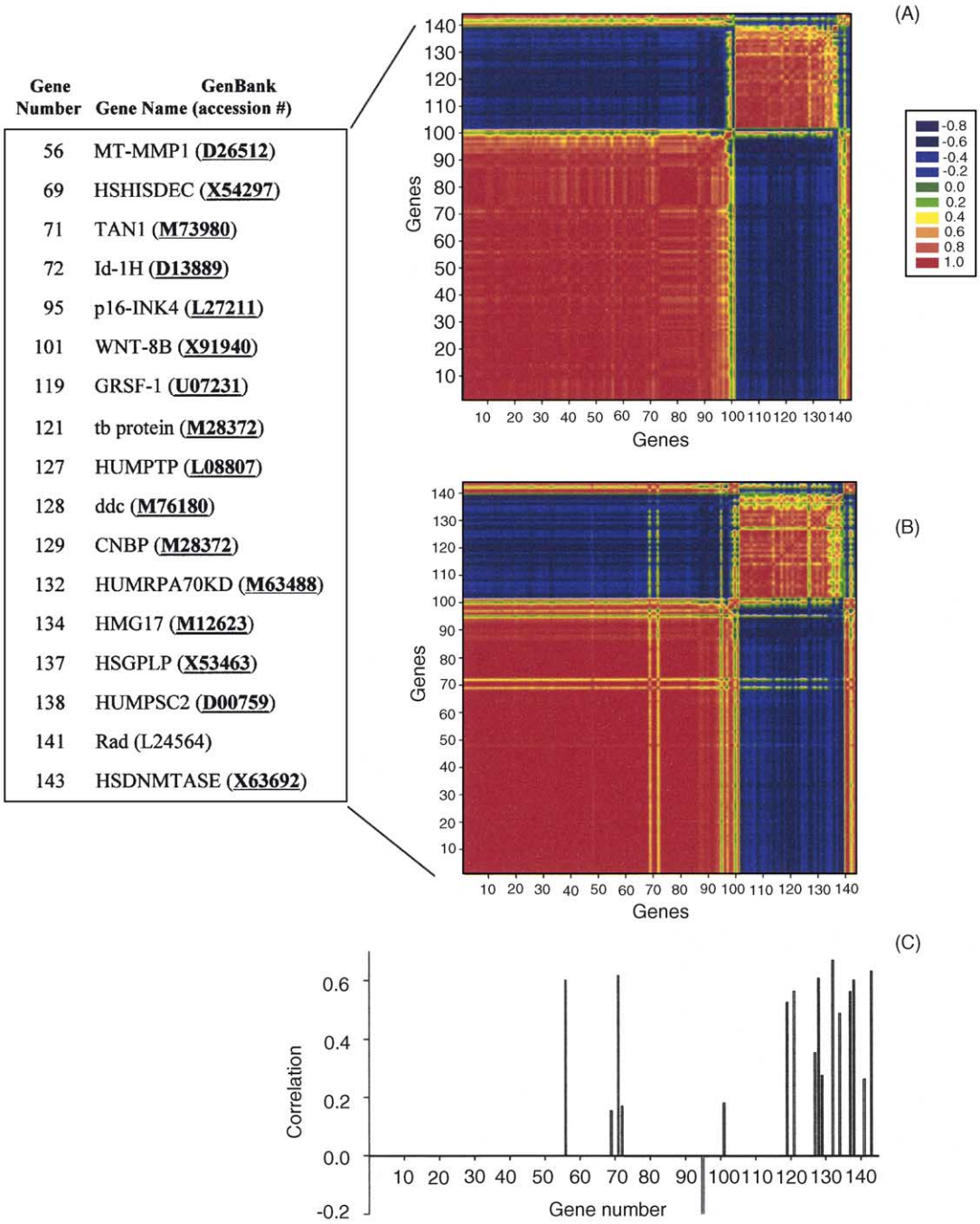

Fig. 4. Mosaic of correlation coefficients for the variable genes between 3α-diol and 5α-DHT. Correlation of expression pattern among different genes in LNCaP cells treated by either 3α-diol (A) or 5α-DHT (B) was calculated. Correlation of the most diverse genes (correlation <0.7) between these two treatments was presented (C). The numbers are the order of numbers as shown on the (A) and (B). Names of most diverse genes were listed.

for their roles involved in cell proliferation like the cyclin genes, and hormonal responses like the AR. Although for most of the 37 genes the expression variation between different time points might not be large enough to be selected as HV-genes, the correlation coefficients and mosaic maps indicated that their expression levels were modulated differently for at least one time point within the same treatment (Fig. 5A and B). Between these two androgens, low levels of correlations (<0.7) were found for v-raf-1 murine leukemia viral oncogene homolog 1 (c-raf-1), retinoblastoma-binding protein 4 (HSRBAP48), mitogen-activated protein kinase 3 (MAP kinase 3; ERK1), hepatocyte nuclear factor 4 (HNF4), heat shock protein 75 (TRAP1), glucocorticoid receptor DNA binding factor 1 (GRF-1), ATP synthase (ATP5A), cyclin D-binding Myb-like protein (hDMP1), tumor protein p53 (TP53), retinoblastoma 1 (RB1), cAMP-dependent

Gene Number	GenBank Gene Name (accession #)
1	Calmodulin (D45887)
2	c-raf-1 (X03484)
3	TCF5 (M64673)
4	AR (M20132)
5	HSPKA (X07767)
6	HSP27 (X54079)
7	PSM (M99487)
8	HUMRACPC (M63167)
9	Bbc6 (U66879)
10	PRKAR1A (M33336)
11	cyclin B (M25753)
12	HSRBAP48 (X74262)
13	cyclin G1 (U47413)
14	ERK1 (X60188)
15	HUMHSP40 (D49547)
16	cyclin H (U11791)
17	HNF4 (X76930)
18	TRAP1 (U12595)
19	GRF-1 (M73077)
20	EMS1 (M98343)
21	HSP70B (X51757)
22	β-catenin (X87838)
23	ATP5A (M37104)
24	ET2 (M65199)
25	SRC (NM_00541)
26	BSF-2 (X04602)
27	b-raf (M95712)
28	Fast (X86779)
29	ARAF1 (L24038)
30	VE-cadherin (X79981)
31	hDMP1 (AF084530)
32	MIC-1 (AF019770)
33	TP53 (M14694)
34	RB1 (M15400)
35	PRKACB (M34181)
36	PRKAR1B (M65066)
37	HSRB2P130 (X74594)

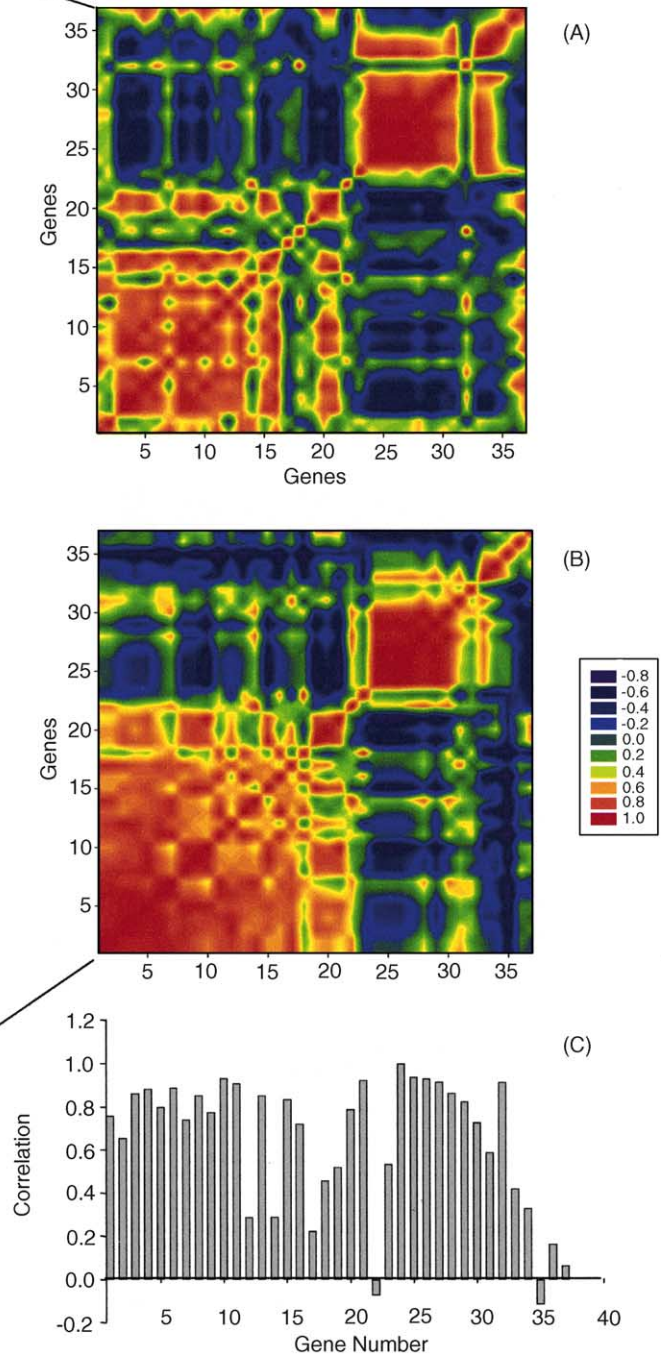

Fig. 5. Mosaic of correlation coefficients for the 37 functional important genes. Correlation of expression pattern among the 37 functional important genes obtained through literature searching was calculated for LNCaP cells following either 3α-diol (A) or 5α-DHT (B) treatment over a course of 48 h. Correlation of expression pattern of the same 37 genes was presented between two treatments (C).

protein kinase regulatory subunit type I-β (PRKAR1B), and retinoblastoma-like 2 (p130; HSRB2P130) (Fig. 5C). In addition, negative correlation was found in β-catenin and cAMP-dependent protein kinase catalytic subunit β (PRKACB) between 3α-diol- and 5α-DHT-treated cells.

4. Discussion

Androgens play important roles in mediating prostate growth, differentiation, and maintenance. It has been shown that LNCaP cells consistently respond to 5α-DHT

for proliferation [23] and AR *trans*-activation [24]. Our results re-confirmed that 5α-DHT induces prostate cell proliferation and activates AR *trans*-activation activity in the human prostate cancer cells at low concentration of 5α-DHT (10^{-12} M). Under the same experimental conditions, 10^{-11} M 3α-diol also stimulated similar levels of LNCaP cell proliferation to those were observed in 10^{-12} M 5α-DHT-stimulated cells. These results may correlate to the in vivo observation which demonstrated that 3α-diol, like 5α-DHT and testosterone, is capable of inducing prostatic hyperplasia in castrated dogs [17]. If 3α-diol needs to be converted to 5α-DHT before it can exert its androgenic effects [12], 3α-diol-induced LNCaP cells proliferation would occur through *trans*-activation of the AR in a manner similar fashion to that of 5α-DHT due to their similar temporal changes in cell proliferation. However, at these concentrations, 3α-diol did not activate similar levels of AR *trans*-activation activity like those observed in 5α-DHT-treated LNCaP cells. 3α-Diol has been classically regarded as an inert androgen metabolite reduced from 5α-DHT through 3α-HSDs [11], and acts via the AR only after being oxidized to 5α-DHT [12]. 3α-Diol's lower AR *trans*-activating potential observed in our results can be attributed to its efficiency to be oxidized to 5α-DHT in these cells. However, classical assumptions of the physiological actions of 3α-diol did not explain why 3α-diol was as effective as 5α-DHT in stimulating LNCaP cell proliferation.

Androgens regulate numerous processes in prostate epithelial cells that include cell division, cell quiescence, apoptosis, lipid metabolism, and the production of specialized secretory proteins to carry out physiological functions of the gland. The identification and quantitation of the complement of expressed genes in response to androgens provide a framework for distinguish biological properties of androgens and establish a tool for functional genomic studies. It has been reported using serial analysis of gene expression (SAGE) that a great majority of ARGs are involved in regulation of transcription, splicing, ribosomal biogenesis, mitogenesis, bioenergetics and redox processes [25]. Genes that are regulated by androgens are also responsible for these physiological functions as well as pathological development of the gland [26]. Comprehensive identification of ARGs in LNCaP cells using microarray analysis has been reported recently [18,27,28]. These reports demonstrate a temporal regulation of ARGs in LNCaP cells following the synthetic androgen R1881 as well as 5α-DHT stimulation [18,28]. Testosterone, 5α-DHT, and the R1881, induce a concentration dependent biphasic growth response in LNCaP cells that may be influenced by the relative activities of growth-promoting and growth-suppressing genes [29]. Different androgen ligands or ligand concentrations may also recruit distinct AR co-activators or co-suppressors that dictate the subset of gene regulation [30,31]. It is possible that different androgens and androgen concentrations activate or repress subsets of gene expression program [28]. Identification of ARGs from 3α-diol and 5α-DHT treated LNCaP cells in this report, therefore, may not be a subset of previous reported microarray results using R1881 and different concentrations of 5α-DHT. The low concentrations of 3α-diol and 5α-DHT have rarely been used previously but were used in here for identification of ARGs to minimize overlapping of ARGs through androgen conversions.

We used cDNA-based arrays to compare diversity of gene expression patterns between 3α-diol- and 5α-DHT-treated LNCaP cells to provide insight into 3α-diol's proliferative-inducing effects but weaker AR *trans*-activation capacity in these cells. Two recently developed bioinformatics procedures have been applied for this data analysis. First, normalization of gene expression patterns from different arrays was performed individually to their own "background" genes, based on the assumption of normal distribution of "unexpressed" genes. Previously, gene expression levels were always normalized to housekeeping genes like β-actin or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH); unfortunately, levels of these housekeeping gene transcript expression are not stable during the progress of cell proliferation [32]. This normalization to an internal standard eliminated the problematic aspect of using housekeeping genes as standards for normalization as the expression of these genes can vary. Moreover, this method used statistically robust thresholds for distinguishing differentially expressed genes and had false positive rates and false negative rates that are $\leq 1/N$. This is in start contrast to conventional analyses that use arbitrary thresholds and high *P*-values which generate differentially expressed gene lists with large numbers of false positive and false negative results. Second, to identify groups of genes whose expression levels varied in a temporally coordinated manner, we used a statistically robust clustering method that identified both positively and negatively correlated behaviors. The statistical criteria were established by first assessing gene expression behavior in response to stimulus. Then the relative behavior of genes was assessed using cluster analysis, which groups similarly regulated genes that are more likely to be functionally related.

As in previous studies, genes involved in the transcription machinery, cell cycle, and mitosis were modulated in 5α-DHT-stimulated cells. The up-regulation of positive cell cycle regulators, such as cyclin B [33], as well as its catalytic partners including CDK 4 [34], being previously demonstrated in androgen-treated LNCaP cells [33], was also observed in our system In addition, consistent with the phenomena of cell cycle progression following androgen stimulation, one of the most prominent cell cycle regulators, the regulatory cyclin H subunit in the cyclin H/cdk7/Mat1 complex occurring as a component of the transcription factor (TF) IIH transcription factor complex as well as in a free form [35], was up-regulated along with RNA polymerase II (L34587) and TFIID (U30504) in 5α-DHT-stimulated cell proliferation.

The potent androgen 5α-DHT also regulated growth factors, extracellular matrix (ECM), and apoptosis-related genes in LNCaP cells. It has been reported that steady state levels of transforming growth factor (TGF)-β mRNA was up-regulated in the rat ventral prostate after castration induced-androgen withdrawal [36]. In addition, elevated TGF-β1 expression has been correlated with 5α-DHT suppressed cell proliferation in human adrenocortical cell line, NCI-H295TGF [37]. We observed down-regulation of TGF-β1 mRNA in association with 5α-DHT stimulated LNCaP cell proliferation. The synthetic androgen R1881 up-regulates the expression of matrix metalloproteinase 2 (MMP2), which is a member of a family of proteolytic enzymes that are capable of degrading different substrates within the ECM [38]. The increase in steady state level of MMP2 transcripts might result from down-regulated expression of tissue inhibitor of MMP2 (TIMP) expression as observed in our array results. In addition, for genes involved in ECM remodeling and cell–cell or cell–matrix adhesion in LNCaP cells, aggrecan 1, a major proteoglycan important for the proper functioning and load-bearing capacity of articular cartilage [39], was repressed 5α-DHT treatment. Members of the caspase family (caspases 2, 3, 6, 7, 8, 9, 10 in human) of aspartate-specific cysteine proteases are best known for their involvement in apoptosis. Increased apoptosis has been associated with up-regulations of various caspases in prostate cells [40]; and down-regulated expression of several members of the caspase family is associated with acquired apoptotic resistance in cadmium-transformed human prostate epithelial cells [41]. Caspases 9 and 10 were down-regulated in response to 5α-DHT treated cells in our system. Our results were very close to what has been reported before in transcriptional regulation of ARGs and physiological functions of 5α-DHT.

In an effort to identify genes that respond to 3α-diol stimulation in LNCaP cells, we found that cyclin B, H, and G1 were also up-regulated by 3α-diol demonstrating that cells were going through similar cell cycle regulation. Down-regulation of TGF-β1 and caspases 9 and 10 programmed cell death genes were also similarly regulated by both 3α-diol and 5α-DHT. In addition, for genes involved in ECM remodeling, up-regulation of MMP2 and suppression of aggrecan 1 expression were also observed in 3α-diol-treated LNCaP cells. The similarity in 3α-diol- and 5α-DHT-regulated gene expression demonstrated similar physiological aspects of these androgens, although these gene regulation may or may not result from the same regulatory mechanism.

3α-Diol also regulated subsets of gene expression that was distinct from 5α-DHT-stimulated cells. It has been shown that androgen-dependent prostate cancer growth can occur through decreased retinoblastoma (Rb) protein expression via cyclinA/Cdk2 and cyclin B/Cdk1-mediated Rb phosphorylation and Rb degradation via the ubiquitin/proteasome pathway [33]. Several Rb associated proteins including Rb (M15400), RbAp48 encoding Rb binding protein (X74262), and Rb2/p130 protein (X74594) were differentially regulated by these two androgens suggesting that the differential usage of Rb by 3α-diol and 5α-DHT. β-Catenin signaling potentially contributes to multiple stimulatory inputs required for disease progression and has complex nature of its activity in prostate tissue. Although β-catenin stimulates T cell factor (TCF) signaling in most prostate cancer cell lines, functional suppression of β-catenin by overexpressed β-catenin mutant did not alter proliferative response to androgen in LNCaP cells [42]. In our array analysis, β-catenin was down-regulated by 5α-DHT at the first 24 h; however, it was slightly up-regulated by 3α-diol at early time points demonstrating the complex nature of the involvement of β-catenin in androgen stimulation. Furthermore, differentially regulation of cAMP-dependent protein kinase (PKA) subunits (M65066; M34181) was also observed between 3α-diol and 5α-DHT treated cells. 5α-DHT did not have much effect on steady state levels of PKA expression; whereas 3α-diol dramatically reduced levels of PKACB mRNA at 1 h and elevated levels of PKAR1B mRNA at 6 h. Increases in intracellular cAMP [43], activation of PKA [44], and PKA-mediated signaling [45] are required in acquisition of neuroendocrine phenotype by LNCaP cells. Regulation of PKA subunits by 3α-diol but not 5α-DHT suggested that 3α-diol might induce neuroendocrine differentiation of LNCaP cells.

In conclusion, we have shown 3α-diol may have unique functionality from 5α-DHT with regard to cell proliferation, AR *trans*-activation, and gene regulation. Our results suggest that 3α-diol-regulated genes may be involved in both AR-dependent and AR-independent pathways; and both pathways might be responsible for promoting prostate cell proliferation [15–17].

Acknowledgements

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