

Increased Expression of Androgen Receptor Sensitizes Prostate Cancer Cells to Low Levels of Androgens

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Abstract

Androgen receptor (AR) is known to be overexpressed in castration-resistant prostate cancer. To interrogate the functional significance of the AR level, we established two LNCaP cell sublines expressing in a stable fashion two to four times (LNCaP-ARmo) and four to six times (LNCaP-ARhi) higher level of AR than the parental cell line expressing the empty vector (LNCaP-pcDNA3.1). LNCaP-ARhi cell line grew faster than the control line in low concentrations, especially in 1 nmol/L 5 α -dihydrotestosterone (DHT). Microarray-based transcript profiling and subsequent unsupervised hierarchical clustering showed that LNCaP-ARhi cells clustered together with VCaP cells, containing endogenous AR gene amplification and overexpression, indicating the central role of AR in the overall regulation of gene expression in prostate cancer cells. Two hundred forty genes showed >2-fold changes on DHT treatment in LNCaP-ARhi at 4 h time point, whereas only 164 and 52 showed changes in LNCaP-ARmo and LNCaP-pcDNA3.1, respectively. Many androgen-regulated genes were upregulated in LNCaP-ARhi at 10-fold lower concentration of DHT than in control cells. DHT (1 nmol/L) increased expression of several cell cycle-associated genes in LNCaP-ARhi cells. ChIP-on-chip assay revealed the presence of chromatin binding sites for AR within \pm 200 kb of most of these genes. The growth of LNCaP-ARhi cells was also highly sensitive to cyclin-dependent kinase inhibitor, roscovitine, at 1 nmol/L DHT. In conclusion, our results show that overexpression of AR sensitizes castration-resistant prostate cancer cells to the low levels of androgens. The activity of AR signaling pathway is regulated by the levels of both ligand and the receptor. [Cancer Res 2009;69(20):8141–9]

Introduction

Prostate cancer is the most common male malignancy in many western countries (1, 2). The growth and the differentiation of normal prostate epithelial cells as well as development of prostate cancer are dependent on androgens (3). Androgen ablation, the gold standard treatment for advanced prostate cancer, initially inhibits tumor growth but ultimately fails and leads to emergence of castration-resistant prostate cancer (CRPC), which has also been

called as androgen-independent prostate cancer. However, a recent clinical trial on abiraterone indicated directly that CRPC is still androgen-dependent (4). It has been shown that CRPC cells upregulate the expression of many of enzymes involved in steroidogenesis, suggesting that cancer cells themselves produce androgens during androgen withdrawal (5–7). In addition, the experimental models of CRPC have shown that many of the androgen-regulated genes are upregulated in CRPC (8–11).

Androgen action is mediated by the androgen receptor (AR; ref. 12). It has been shown that AR is overexpressed in vast majority of CRPC (13, 14). In addition, ~30% of CRPC carry AR gene amplification (15). Somatic mutations of AR in prostate cancer have also been extensively studied. The mutations seem to be rare in untreated tumors but are found in 10% to 30% of tumors treated with antiandrogens, such as flutamide and bicalutamide (16, 17). Receptor mutations may broaden the ligand specificity converting even the antagonist effect of antiandrogens to agonist one (10, 18). It has also been suggested that crosstalk between AR signaling and other pathways, such as mitogen-activated protein kinase, epidermal growth factor receptor, and Akt pathways, takes place, especially in androgen-depleted environment (12). In addition, alterations in the expression of AR coregulators have been suggested, but not proven, to be involved in the progression of prostate cancer (19). Functional evidence that AR is involved in the emergence of CRPC was presented by Chen and colleagues (20) who showed that ectopic expression of a high AR content was sufficient to transform androgen-dependent prostate cancer cells to androgen-independent ones. Also, Kokontis and colleagues (21) have shown previously that adaptation of LNCaP cell line to low levels of androgens is associated with increased expression of endogenous mutant AR. Together with the findings that AR overexpression is common in CRPC, the experimental data suggest that the overexpression of this receptor is a key mechanism for the progression of prostate cancer.

To mimic the conditions of high AR expression in CRPC, we have established a cell line model by a stable transfection of AR into an androgen-responsive prostate cancer cell line, LNCaP. Two sublines with moderate (LNCaP-ARmo) and high (LNCaP-ARhi) levels of AR overexpression were produced. The model cell lines were subsequently used to examine the influence of AR levels on cell growth and expression of downstream genes of the AR signaling pathway. The aim was to investigate whether AR overexpression hypersensitizes cells to the low levels of androgens, as we have suggested previously (15), as well as to identify the candidate downstream genes that are involved in the emergence of CRPC.

Materials and Methods

Cell culture protocols and transfections. LNCaP cells (American Type Culture Collection) were cultured under the recommended conditions.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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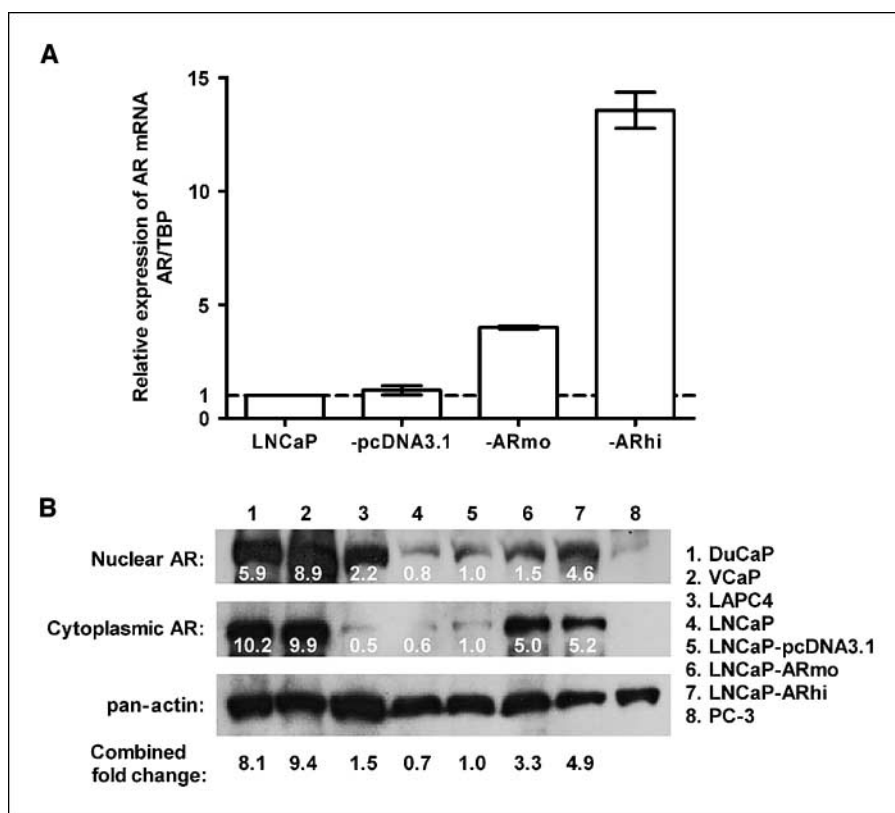


Figure 1. A, relative expression levels of AR mRNA (*AR/TBP*) as measured by Q-RT-PCR. The AR mRNA level is ~13 times higher in LNCaP-ARhi and 4 times higher in LNCaP-ARmo cells than in controls (parental LNCaP and LNCaP-pcDNA3.1). Mean \pm SE of three replicates. B, Western analysis of the AR protein levels. LNCaP-ARhi cells had 4- to 6-fold and LNCaP-ARmo cells had 2- to 4-fold higher AR protein levels than control cells (LNCaP and LNCaP-pcDNA3.1). Nuclear and cytoplasmic protein levels were quantified by ImageJ software program and normalized against pan-actin (loading control). The combined fold change is also shown. PC-3 cells were used as a negative control.

Either pcDNA3.1(+) empty expression vector (Invitrogen) or pcDNA3.1(+) inserted with the *AR* coding region [accession #_M23263; digested with *Sa*I and *Nhe*I from pSG5 expression vector and subcloned pTarget vector (Invitrogen) and finally into *Not*I/*Bam*HI site in pcDNA3.1] were stable transfected into LNCaP with Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's instructions. Transfected clones were selected with 400 μ g/mL geneticin (G418; Invitrogen), and several clones were expanded. AR mRNA level was determined by using Northern blot analysis and quantitative real-time reverse transcription-PCR (Q-RT-PCR). Subsequently, dozen clones, showing the highest overexpression of AR mRNA, were analyzed further for their AR protein levels using Western blotting. Finally, two clones, expressing moderately (LNCaP-ARmo) and highly (LNCaP-ARhi) increased levels of AR protein, were selected for further analyses. Cells transfected with an empty vector (LNCaP-pcDNA3.1) were used as a control. The transfected cells were cultured in medium containing geneticin (200 μ g/mL). DuCaP and VCaP cells were kindly provided by Dr. Jack Schalken (Radboud University Nijmegen Medical Center) and grown under the recommended conditions. LAPC4 cell line was kindly provided by Dr. Charles Sawyers (Sloan-Kettering Institute) and cultured under the recommended conditions.

Before each experiment with hormone exposure, cells were grown in charcoal-stripped serum (CSS; Hyclone) in medium without phenol red for 4 days. Subsequently, the medium was replaced for the experiment with that containing various concentrations of 5 α -dihydrotestosterone (DHT; Steroids) or roscovitine (Calbiochem, EMD Chemicals).

Cell proliferation assays. After 3 days of incubation in charcoal-stripped serum medium, the cells were trypsinized, counted, and placed in 12-well dishes in charcoal-stripped serum medium with desired concentration of DHT. The amount of cells at each time point was analyzed using Alamar Blue reagent (AbD Serotec) and luminometric detection using a fluorometer (Wallac 1420 Victor; Perkin-Elmer). Alternatively, the cells were trypsinized and the number of cells was calculated with Beckman Coulter Z2-series particle counter according to the manufacturer's instructions. Each experiment was done in quadruplicate for DHT-induced growth

analysis and in triplicate for roscovitine exposures. For the relative growth curves, the luminometric values or the number of the cells in each well in each follow-up day were divided by the mean values or number at day 1.

Q-RT-PCR. Subconfluent cells were collected from dishes and their total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out from total RNA using AMV reverse transcriptase (Finnzymes) according to the manufacturer's instructions. The primers for Q-RT-PCR were designed with Primer3 program.³ Primer sequences are listed in Supplementary Table S1. SYBR Green II-Fast Start kit (Roche Diagnostics) and Light Cycler apparatus (Roche Diagnostics) were used for Q-RT-PCR essentially as described previously (13). *TBP* (TATA box binding protein) mRNA was used as a reference. The specificity of the reactions was confirmed, in addition to the melting curve analysis, with 1.5% agarose gel electrophoresis.

Western blot. The soluble cytoplasmic and nuclear proteins were extracted from subconfluent cells using the modified method of Dignam and colleagues (22). Both cytoplasmic and nuclear proteins (12.5 μ g each) were separated in 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane (Immobilon-P; Millipore). After blocking, membranes were incubated with the primary antibody (mouse anti-AR 441 and mouse anti-pan-actin, clone ACTN05; NeoMarkers), washed, and incubated with the secondary antibody (anti-mouse IgG-horseradish peroxidase conjugate; DAKO). After washing, the protein bands were visualized on autoradiography film (Kodak) using chemiluminescence detection (Western Blotting Luminol reagent; Santa Cruz Biotechnology). Intensity differences were quantified by ImageJ image analysis software program.⁴ Equal loading was confirmed by staining with antibody against pan-actin.

Microarray analysis. Microarray hybridizations were done in the Finnish DNA Microarray Centre at Turku Centre for Biotechnology. First,

³ <http://frodo.wi.mit.edu/primer3/input.htm>

⁴ <http://rsb.info.nih.gov/ij/index.html>

300 ng total RNA of each sample was transcribed *in vitro*, biotinylated and amplified with Illumina RNA TotalPrep Amplification kit (Ambion), and hybridized to Illumina Sentrix HumanRef-8_V2 Expression BeadChip according to the manufacturer's instructions. The probes of Illumina HumanRef-8 v2 chip are based on the content from the National Center for Biotechnology Information RefSeq database 1, release 17 containing >22,000 well-annotated transcripts. The data were analyzed with GeneSpring Analysis Platform version GX 7.3.1 (Agilent Technologies). First, lowest signal value was set to be 3-fold higher compared with negative control (water) signals. All individual values below that were set to this lowest signal value.

For unsupervised hierarchical clustering, samples were normalized per chip by the 50th percentile and per gene by the median. Average linkage method was used for clustering, and the similarities were estimated with Pearson's correlation. For the analyses of DHT dose responses, data were normalized with repeated median polishing per chip and gene. Subsequently, signal values in each treatment were divided by the signal value of the 0 nmol/L DHT treatment of the same cell line at the same time point. To identify differently expressed genes in the faster-growing LNCaP-ARhi cells at 1 nmol/L DHT, the data were normalized with intensity dependent Lowess normalization. Twenty percent of the data were used to calculate the Lowess fit at each point. This curve was used to adjust the control value (LNCaP-pcDNA3.1 or LNCaP-ARmo were used as control samples for LNCaP-ARhi) for each measurement. For ontology classifications, all gene ontology lists containing at least 10 genes with $P < 0.001$ (hypergeometric P value without multiple testing correction) in either LNCaP-pcDNA3.1, or

LNCaP-ARmo, or LNCaP-ARhi were filtered and organized with GeneSpring Ontology browser.

The array data were submitted using MIAMEExpress to the ArrayExpress database (accession number E-MEXP-2286).

ChIP-on-chip assays. ChIP-on-chip assays were done with anti-AR antibody (BJ14-AR3; ref. 23) in nontransfected LNCaP-1F5 cells that were cultured in the absence of hormone for 4 days and then exposed to 100 nmol/L DHT or vehicle for 2 h. The immunoprecipitation enriched and input chromatin samples were amplified by ligation-mediated PCR followed by fragmentation and labeling of DNA. Three micrograms of samples from input and immunoprecipitated samples were hybridized to Affymetrix whole-genome tiling arrays (GeneChip Human Tiling 2.0R Array Set; Affymetrix). The regions enriched for AR-binding sites were identified by MAT algorithm (24) and mapped to the most recent human genome sequence (Hg18), and sites that were enriched above input were scored at a P value of 10^{-4} . These AR-binding sites were then subsequently mapped to the differentially expressed genes in the AR overexpression model.

Results

Establishment of AR-overexpressing LNCaP. Two AR-overexpressing clones were selected for experiments. These were LNCaP-ARmo, with ~4-fold higher AR mRNA and ~2- to 4-fold higher AR protein level, and LNCaP-ARhi, with 13-fold higher AR

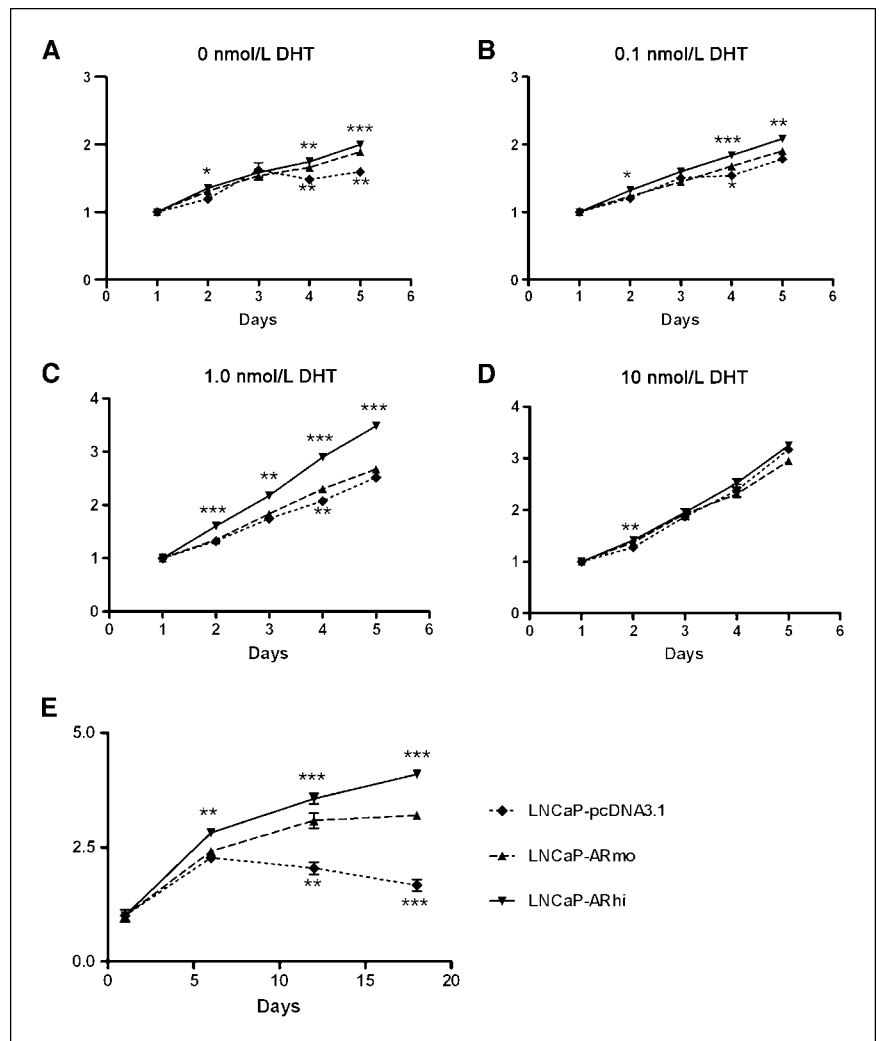


Figure 2. Relative growth of the LNCaP-AR model cells in the presence of (A) 0 nmol/L DHT, (B) 0.1 nmol/L DHT, (C) 1 nmol/L DHT, and (D) 10 nmol/L DHT. The greatest advantage in proliferation rate for AR-overexpressing cells was seen in 1.0 nmol/L DHT. E, growth of cells in charcoal-stripped serum medium without androgens during 3 wk. The highest growth rate was seen in LNCaP-ARhi cells. The growth of LNCaP-ARmo was between LNCaP-ARhi and control cells. Mean \pm SE of four replicates. Y axis, relative growth against day 1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, below the curves for LNCaP-ARmo versus LNCaP-pcDNA3.1 and above the curves for LNCaP-ARhi versus LNCaP-pcDNA3.1.

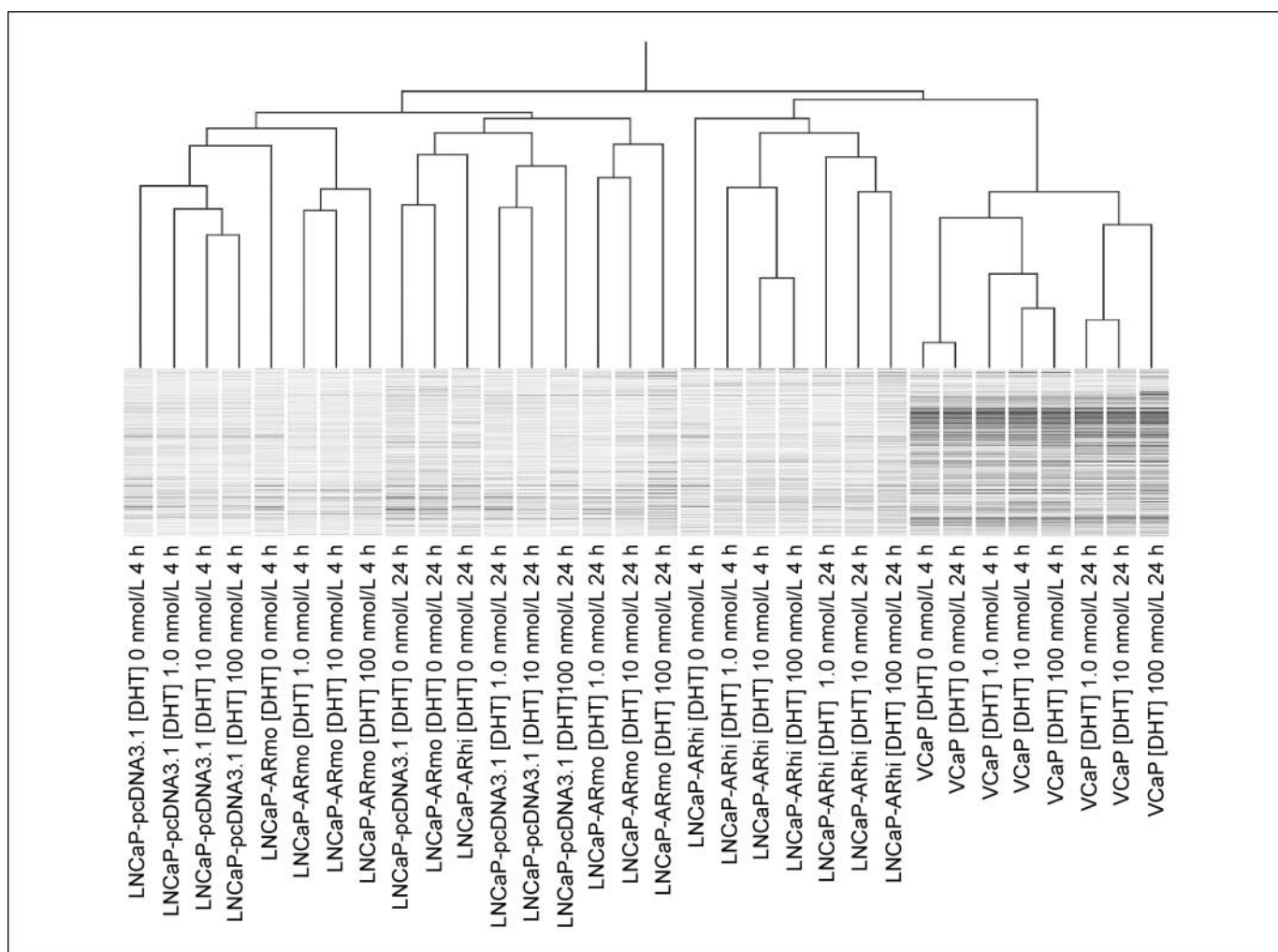


Figure 3. Unsupervised hierarchical clustering of mRNA expression in DHT-treated LNCaP-AR and VCaP cells. LNCaP-ARhi cells cluster together with VCaP cells (except 0 mol/L DHT treated), and LNCaP-pcDNA3.1 cells together with LNCaP-ARmo cells.

mRNA and 4- to 6-fold higher AR protein level, than those in the control LNCaP-pcDNA3.1 cells (Fig. 1). Immunofluorescence staining showed that the AR protein is located in nucleus when cells are grown in the presence of androgens (Supplementary Fig. S1). In addition, overexpression of AR seemed to enhance the nuclear transport after DHT exposure. The most intense nuclear staining was seen in the LNCaP-ARhi cells after 1 h exposure to 10 nmol/L DHT.

Growth curve analyses. The proliferation rates of these cells were analyzed in the presence of various DHT concentrations. The growth of LNCaP-ARhi cells was stimulated with lower concentrations of DHT than control LNCaP-pcDNA3.1 or LNCaP-ARmo cells (Fig. 2). Especially, in the presence of 1 nmol/L DHT, LNCaP-ARhi cells grew clearly faster than LNCaP-ARmo or LNCaP-pcDNA3.1 cells ($P < 0.01$). The cells overexpressing AR were also capable of growing better in charcoal-stripped serum medium without DHT (Fig. 2E). However, the growth finally plateaued in all cell lines.

Expression profiling. For genome-wide expression profiling, LNCaP-ARhi, LNCaP-ARmo, and LNCaP-pcDNA3.1 cells were grown in the presence of various DHT concentrations (0, 1, 10, and 100 nmol/L), and total RNA was extracted at 4 and 24 h

points. In addition, VCaP cells that contain endogenous *AR* gene amplification and strong overexpression of AR (ref. 24; Fig. 1) were treated and analyzed in a similar fashion. Unsupervised hierarchical clustering of androgen-regulated transcripts revealed that VCaP and LNCaP-ARhi cells clustered together, whereas androgen-dependent transcripts of LNCaP-ARmo cells clustered together with those of LNCaP-pcDNA3.1 cells (Fig. 3).

More genes were upregulated than downregulated in the LNCaP-AR cells by DHT. Venn diagrams for upregulated and downregulated genes are shown in Fig. 4. In LNCaP-pcDNA3.1 cells, expression of 52 and 118 genes was changed >2-fold at any DHT concentration compared with vehicle at 4 and 24 h, respectively. In LNCaP-ARmo and LNCaP-ARhi, the number of genes with altered expression was higher; in ARmo 164 and 379 at 4 and 24 h time points, respectively, and in ARhi 240 and 475 at 4 and 24 h time points, respectively ($P < 0.0001$, χ^2 test). In VCaP cells, expression of 430 genes was changed >2-fold at the 4 h time point and 428 genes at the 24 h time point. The upregulated and downregulated genes by DHT exposure in LNCaP-pcDNA3.1, LNCaP-ARmo, LNCaP-ARhi, and VCaP are listed in Supplementary Tables S2 to S5.

Next, we analyzed gene ontology categories for all genes upregulated and downregulated at 24 h of DHT treatments. In

LNCaP-pcDNA3.1 cells, DHT upregulated transcripts belonged to the following five gene ontology categories (at least 10 upregulated genes with $P < 0.001$): intracellular signaling cascade, cell cycle, cell division, protein metabolism, and DNA metabolism (Supplementary Table S6). In LNCaP-ARhi and LNCaP-ARmo cells, the same five gene ontology categories were also upregulated as those in LNCaP-pcDNA3.1 cells, but the number of upregulated genes was significantly higher in each category. In addition, six other main ontologies were enriched in LNCaP-ARmo and/or LNCaP-ARhi cells. These categories were lipid metabolism, secretory pathway, cell organization and biogenesis, chromosome segregation, response to endogenous stimulus, and cell proliferation (Supplementary Table S6). Ontologies that showed highly significantly ($P < 0.0001$, χ^2 test) more upregulated genes in LNCaP-ARhi cells compared with LNCaP-pcDNA3.1 or LNCaP-ARmo cells were genes associated with mitotic cell cycle, regulation of progression through cell cycle, organelle organization and biogenesis, cellular protein metabolism, and DNA metabolism (Supplementary Table S6). Of upregulated ontologies, intracellular signaling cascade, cell cycle, and lipid metabolism were also upregulated significantly ($P < 0.01$) already at 4 h time point. Only one ontology category, apoptosis, was significantly upregulated at 4 h time point but not at 24 h time point. The downregulated genes were not enriched in any of the categories with more than two genes in any of the cell lines.

When upregulated ontologies were examined separately at various DHT concentrations (Fig. 5), particularly DNA metabolism, cell cycle, cell organization and biogenesis, cell division, and intracel-

lular signaling cascade were found to be highly significantly upregulated in LNCaP-ARhi cells already in 1 nmol/L DHT concentration (Fig. 5A). At 10 and 100 nmol/L DHT concentrations, the same ontologies were upregulated but with higher number of genes. At 10 and 100 nmol/L DHT concentrations, cell cycle, DNA metabolism, lipid metabolism, cell organization and biogenesis, cell division, and chromosome segregation were highly significantly upregulated also in LNCaP-pcDNA3.1 or LNCaP-ARmo cells. The number of upregulated genes in these categories were, however, always highest in LNCaP-ARhi cells. The most significantly upregulated ontologies in LNCaP-ARmo cells were lipid metabolism and secretory pathway.

Expression of known AR target genes. Expression of well-known AR target genes, such as *PSA*, *TMPRSS2*, *NKX3-1*, and *TMEPAI*, was found to be increased by 4- to 10-fold in LNCaP-ARhi and LNCaP-ARmo compared with LNCaP-pcDNA3.1 at 4 and 24 h time points (Fig. 6). Likewise, expression of well-known downregulated AR target genes, such as *PAP* and *PSMA*, was attenuated more in LNCaP-ARhi and LNCaP-ARmo cells than in control cells. On average, for an equal level of upregulation of genes, a 10-fold higher DHT concentration was required for LNCaP-pcDNA3.1 cells than for LNCaP-ARhi or LNCaP-ARmo cells. Q-RT-PCR of *PSA* and six selected genes, unknown previously to be regulated by androgens, confirmed the microarray data (Supplementary Fig. S2).

LNCaP and VCaP cells contain genetic rearrangements affecting Ets transcription factors, *ETV1* and *ERG*, respectively (25, 26). In VCaP cells, *ERG* expression was upregulated by androgens

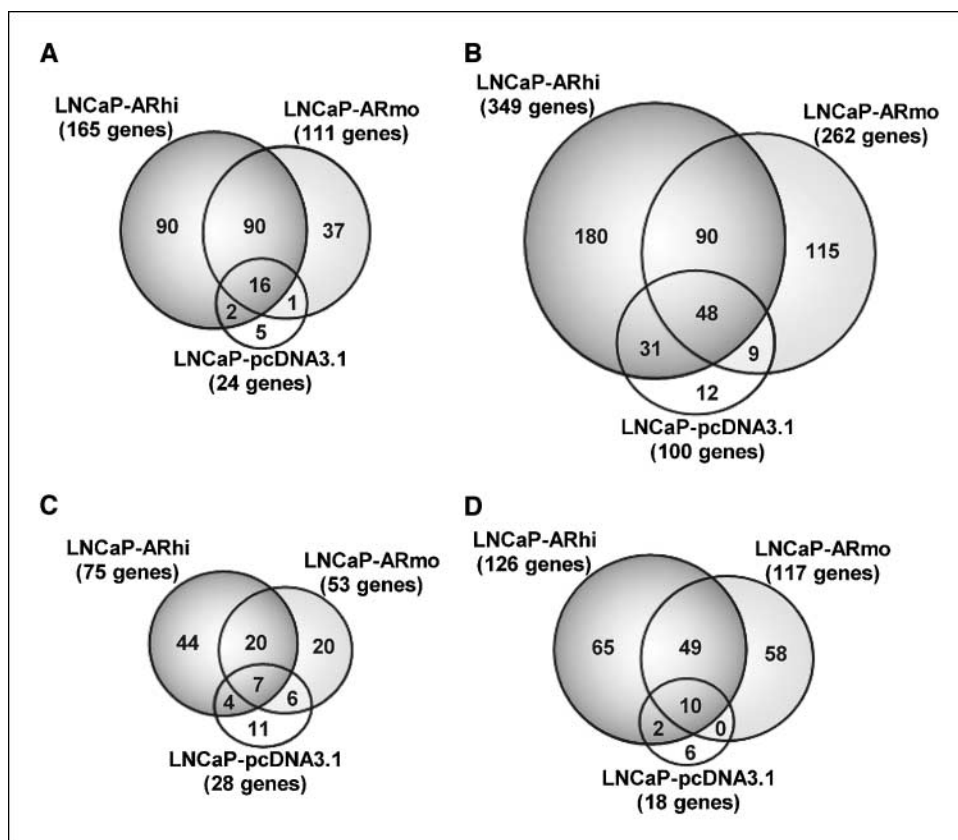


Figure 4. Venn diagrams of DHT responding genes in LNCaP-AR cell panel for upregulated genes at (A) 4 h and (B) 2 h. Ninety and 180 genes were found to be upregulated (>2-fold) only in LNCaP-ARhi at 4 and 24 h time points, respectively. Venn diagrams of genes downregulated by DHT in LNCaP-AR panel at (C) 4 h and (D) 24 h. Forty-four and 65 genes were found to be downregulated (>2-fold) only in LNCaP-ARhi at 4 and 24 h time points, respectively.

(Supplementary Fig. S3). Unfortunately, Illumina RefSeq8-v2 probes for *ETV1* failed to detect any expression. However, Q-RT-PCR analysis showed >2-fold increase of *ETV1* mRNA (Supplementary Fig. S3). Of Ets family members, only *ELK4* mRNA level was increased with androgens in LNCaP-ARhi cells at 24 h time point (Supplementary Fig. S3). The *ELK4* gene is known to be an AR target in human prostate cancer cells with association to cell growth *in vitro* (27).

Kokontis and colleagues (21) have shown previously that LNCaP cells overexpressing endogenous AR show higher expression of *MYC* in low levels of androgens, and the expression is decreased in lower concentrations of the androgens than in the parental cell line. In similar fashion, here the highest *MYC* expression was found in VCaP and LNCaP-ARhi cells in the low DHT levels, and the

expression was decreased in higher concentrations of DHT (Supplementary Fig. S4).

Identification of candidate AR downstream genes. Because the LNCaP-ARhi cells gained a growth benefit *in vitro* with 1 nmol/L DHT concentration compared with LNCaP-ARmo cells or empty vector-transfected LNCaP-pcDNA3.1 cells, we were particularly interested in the genes that are upregulated or downregulated in LNCaP-ARhi cells at 1 nmol/L DHT. Expression of 173 genes were found to be altered >2-fold (127 upregulated and 46 downregulated) in LNCaP-ARhi cells and compared with LNCaP-pcDNA3.1 or LNCaP-ARmo cell at 24 h after DHT exposure (Supplementary Table S7). Of these genes, we examined whether any of the upregulated genes were expressed in VCaP cells to the same or higher level than in LNCaP-ARhi cells. Ninety-nine such genes

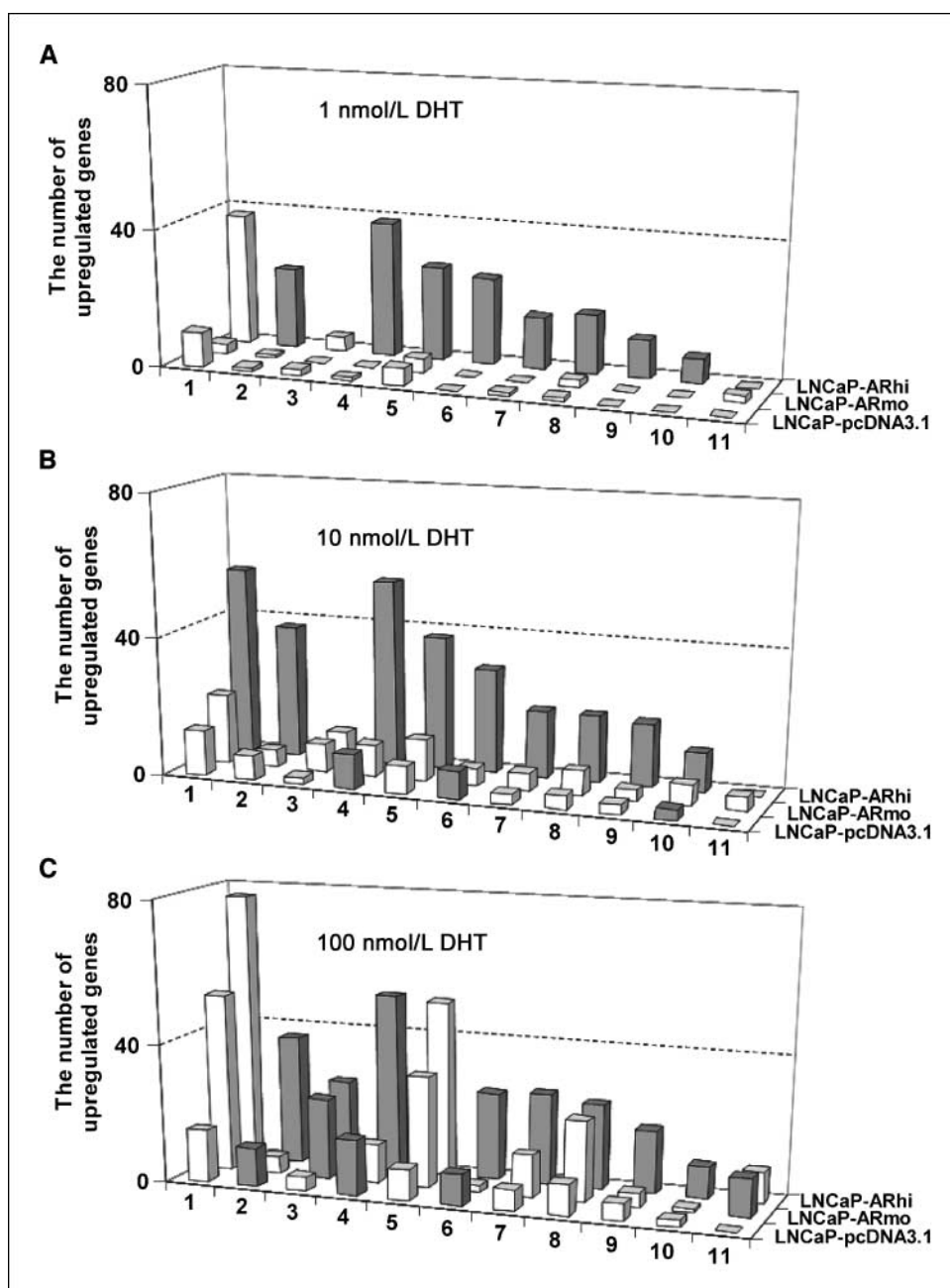


Figure 5. Androgen upregulated (>2-fold) gene ontology classes at different DHT concentrations. The histograms show the number of upregulated genes in LNCaP-pcDNA3.1, LNCaP-ARmo, and LNCaP-ARhi at 1 nmol/L (A), 10 nmol/L (B), and 100 nmol/L (C) DHT concentrations at the 24 h time point. Gray columns, highly significantly ($P < 0.001$, a hypergeometric P value without multiple testing correction) upregulated ontologies. Different ontology classes are numbered as follows: 1, GO:19538 protein metabolism; 2, GO:6259 DNA metabolism; 3, GO:6629 lipid metabolism; 4, GO:7049 cell cycle; 5, GO:16043 cell organization and biogenesis; 6, GO:51301 cell division; 7, GO:8283 cell proliferation; 8, GO:7242 intracellular signaling cascade; 9, GO:9719 response to endogenous stimulus; 10, GO:7059 chromosome segregation; 11, GO:45045 secretory pathway.

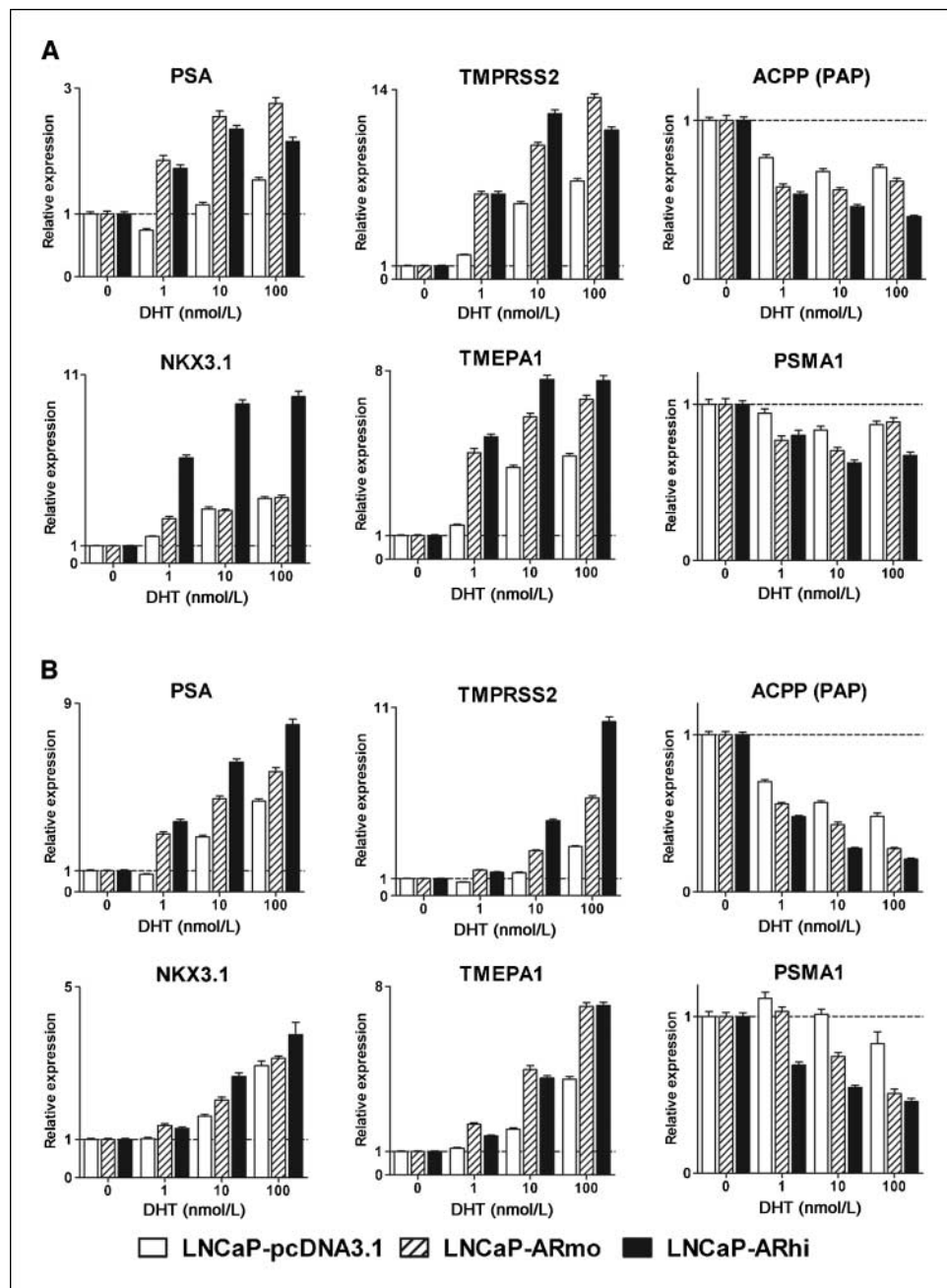


Figure 6. Expression of well-known androgen-regulated target genes (*PSA*, *TMPRSS2*, *TMEPA1*, *NKX3-1*, *ACPP*, and *PSMA1*) in LNCaP-AR cell panel in different concentrations of DHT at (A) 4 h and (B) 24 h according to microarray analysis. Mean \pm SE.

were found (Supplementary Table S8), and 66 of those were also >2-fold upregulated with 1.0 nmol/L DHT compared with vehicle-treated LNCaP-ARhi cells. The expression of 56 of these 66 genes were also >1.5-fold upregulated by DHT already at 4 h time point in LNCaP-ARhi. These genes were determined using Oncomine Research Edition.⁵ Almost all of them (51 of 56, 91%) showed significantly upregulated expressions in prostate cancer, at least, in one of the data sets, and 49 of 56 (88%) genes were upregulated, at least, in two independent sets in metastasized prostate cancer compared with primary prostate cancers (Supplementary Table S9). ChIP-on-chip analysis of genome-wide AR binding in LNCaP-1F5 cells after a 2 h DHT exposure indicated that a majority of these 56

genes (34 of 56, 61%) possessed AR-binding site within a 200-kb window from transcription start sites of the genes (Supplementary Table S9). Two of 34 had an AR-binding site in the proximal promoter, 15 of 34 had an AR-binding site within -100 kb upstream of transcription start site, and 16 of 34 had AR-binding sites downstream of transcription start site. Eight of 34 had AR-binding site both upstream and downstream of transcription start site. The androgen response elements within the AR-binding sites were analyzed using MotifMatch (28). Using a high cutoff score of 9 and with a strict 3-bp spacing for typical class I canonical androgen response element (AGAACAnnnTGTTCT), we could find a canonical androgen response element in following genes: *NDC80*, *NCAPG*, *FANCI*, *CI2orf48*, *PRIMI*, *IQGAP3*, *KIF20A*, *CDC2*, *HMGB2*, *SPAG5*, *TKI*, *PRCI*, *MCM4*, and *PCNA*.

⁵ <http://www.oncomine.org>

Effect of roscovitine on growth of LNCaP-AR cells. Because the expression of both *CDC2* (alias CDK1) and CDK2 were increased in 1 nmol/L DHT, especially in LNCaP-ARhi, and the genes have AR-binding site according to ChIP-on-chip data, the effect of CDK1/2 inhibition was tested. The growth of LNCaP sublines was assayed in different concentrations of CDK1/2 inhibitor roscovitine and DHT. The growth of LNCaP-pcDNA3.1, LNCaP-ARmo, and LNCaP-ARhi cells was significantly ($P < 0.001$, unpaired two-tailed t test) inhibited with 15 $\mu\text{mol/L}$ roscovitine in 10 nmol/L DHT (Supplementary Fig. S5). The LNCaP-ARhi cells showed the growth inhibition also by 7.5 $\mu\text{mol/L}$ roscovitine ($P = 0.0002$). In addition, the growth of LNCaP-ARhi ($P = 0.0253$), but not LNCaP-pcDNA3.1 or LNCaP-ARmo, was suppressed by roscovitine in 1 nmol/L DHT.

Discussion

AR is a key protein in both development and progression of prostate cancer. It is expressed in almost all prostate carcinomas from the beginning of the disease to the castration-resistant stage (8, 13, 14). The standard treatment of advanced prostate cancer is hormone ablation. Although androgen withdrawal attenuates AR-mediated signaling and initially prevents tumor growth, several changes occur in AR action during the treatment that lead to reactivation of AR signaling and eventually to emergence of the lethal form of the disease, the CRPC (12). One of the key mechanisms in the emergence of CRPC is amplification of the *AR* gene leading to overexpression of AR protein (13, 15). This suggests that CRPC cells are not androgen-independent but may be hypersensitive to low androgen level. To study the functional consequences of AR overexpression and the role of the AR level in more detail, we generated two LNCaP cell lines that overexpressed AR to different levels.

AR overexpression seemed to increase the ability of prostate cancer cells to grow and proliferate in the absence of or at a low concentration of DHT. The level of AR affected the growth in different androgen concentrations. The LNCaP-ARhi cells with the highest level of AR expression had the fastest growth rate, whereas the growth of LNCaP-ARmo cells was between that of the LNCaP-ARhi and the control cells (LNCaP-pcDNA3.1). Both LNCaP-ARhi and LNCaP-ARmo were also able to grow longer in the medium without androgens. Instead, control cell proliferation ceased after the first week. The data indicate that increased expression of AR sensitized the growth of the cells to low hormone concentrations. Even a modest increase in AR expression level can help tumor cells to proliferate at a low androgen concentration as has also been suggested previously by Chen and colleagues (20). Kokontis and colleagues (21) have shown previously that androgens have biphasic effect on the growth of LNCaP cells. Androgens stimulate the growth, but in higher concentrations the induction of proliferation is diminished. In addition, they showed that, in the LNCaP cells overexpressing endogenous AR on adaptation to growth in low levels of androgens, the repression of proliferation takes place in lower androgen levels than in parental cell lines. Here, we found similar AR level-dependent biphasic effect of androgens (Supplementary Fig. S6). Thus, the optimal level of growth induction is dependent on the level of both ligand and the receptor.

Several microarray studies have addressed androgen regulation of gene expression in LNCaP cells (20, 29, 30). However, the data in these studies are only partially concordant. Reasons for the discrepant findings could be different time points used, different ligands, different ligand concentrations, and heterogeneity of the LNCaP cells themselves in different laboratories. For example, in

our data, several genes, such as *TNFRSF10B*, *APRIN*, *TNFAIP3*, and *SGK*, which were upregulated strongly at the 4 h time point, showed very little, if any, upregulation anymore at 24 h. An important aspect is also that AR in the parental LNCaP cells is mutated allowing other steroids and even antiandrogens, such as flutamide, function as agonist (18). To alleviate these problems, we transfected wild-type AR cDNA into LNCaP cells and used the natural ligand at two different time points with four different concentrations. In our experimental model, we cannot fully separate the effect of wild-type and mutated AR. However, at least in LNCaP-ARmo and LNCaP-ARhi cells, the majority of AR is wild-type. Thus, the differences between these two sublines can be assumed to be due to the different levels of the expression of wild-type AR.

In our models, the number of androgen-responsive genes was clearly associated with AR expression level. LNCaP-ARhi cells possessed more androgen-responsive genes than LNCaP-ARmo cells that, in turn, had more those genes than LNCaP-pcDNA3.1 cells. We analyzed also VCaP cells, which contain high-level amplification of the *AR* gene leading to strong (up to 12-fold) overexpression of AR protein (ref. 24; Fig. 1). Its growth is androgen-sensitive (31). Interestingly, the number of the androgen-responsive genes in VCaP cells was even higher than in LNCaP-ARhi. In unsupervised hierarchical clustering, VCaP and LNCaP-ARhi cells clustered together despite that they are different cell lines with different genetic backgrounds. This indicates a very strong influence of AR to the genome-wide expression of prostate cancer cells. Of the well-known androgen-regulated genes, such as *PSA* and *TMPRSS2*, induction of gene expression took place at a 10-fold lower concentrations of DHT in LNCaP-ARhi and LNCaP-ARmo cells than in LNCaP-pcDNA3.1 cells. Thus, it seems that the level of AR sensitizes the cells to androgens not only in terms of growth but also by increasing number of genes responding to DHT.

Because the growth advantage in LNCaP-ARhi cells was especially prominent at 1 nmol/L DHT, we were interested in genes whose expression was altered at that concentration. Because VCaP cells are also androgen-sensitive and contain *AR* gene amplification, we postulated that androgen target genes that are important for progression of CRPC should be detected in both LNCaP-ARhi and VCaP cells. All in all, 56 genes were induced in 1 nmol/L DHT in LNCaP-ARhi (but not in LNCaP-ARmo or LNCaP-pcDNA3.1) already at 4 h and were also highly expressed in VCaP. According to Oncomine data resource, 51 of 56 (91%) of these genes have been shown to exhibit significant upregulation in primary prostate cancer and/or metastatic prostate cancer samples (Supplementary Table S9). Unfortunately, the Oncomine data from studies comparing directly hormone-naive and castration-resistant cancers were not available for all of those genes. The list of genes consists of cell cycle genes, for example, *CDK1*, *CDK2*, *cyclin B*, *cyclin E*, and *aurora kinase A* and *B*. All of them are known to have an effect on cell proliferation or chromosome condensation and be upregulated in prostate cancer. ChIP-on-chip assay revealed that the majority (61%) of these genes have AR-binding sites within a 200-kb window from transcription start sites. Our experiments with roscovitine, a CDK1/2 inhibitor, showed that LNCaP-ARhi cells were more sensitive to the inhibition than the LNCaP-pcDNA3.1 and LNCaP-ARmo, indicating the importance of these androgen-regulated genes for the growth benefit of the AR-overexpressing cells. Because it has been shown previously that CDK1 phosphorylates and stabilizes AR (32), there may be a positive feedback mechanism between expression of AR and CDK1.

Previous studies have shown that increased AR expression is associated with the growth of castration-resistant cancers and with

transformation of androgen-dependent prostate cancer cells to androgen-independent ones (10, 20, 21). In addition, association of AR expression level with cell invasion has also been suggested (11). These studies have been performed with different cell lines or xenograft models. In our LNCaP-AR model cells, high AR level was significantly associated with increased proliferation at a low androgen concentration and with an increased number of genes being associated to cell cycle and DNA replication. The finding that only LNCaP-ARhi cells showed the growth advance, and high number of cell proliferation associated genes that were androgen-responsive, is in good agreement with observations that clinical CRPC often have >10-fold overexpression of *AR* (13, 14).

The results also indicated that AR can have an effect on different cellular processes depending on the receptor level. In LNCaP-ARmo cells, the number of responding genes associated with cell cycle was not increased; instead, genes in other ontology categories, such as lipid, sterol, and cholesterol biosynthesis, were highly significantly enriched. For example, the androgen-regulated lipid metabolism genes reviewed by Chen and colleagues (33) were all androgen-regulated, at least, in one of the cell line used in our data. Of those lipid metabolism genes, especially *DHCR24*, *FASN*, *HMGCSI*, *LDLR*, *PPAP2A*, and *SCAP* were strongly androgen-upregulated in LNCaP-ARmo. Also, the ontology category of secretory pathway, including endoplasmic reticulum to Golgi transport genes, showed higher number of responsive genes in LNCaP-ARmo compared with LNCaP-ARhi. These observations suggest that the amount of AR may also have other effects rather than simple sensitization of cells to lower levels of androgens.

In conclusion, increased expression of AR seems to sensitize prostate cancer cells in multiple ways and give them several biological benefits during hormone ablation. High AR protein level helped the cells to sustain and increase their proliferation in environment with no androgen or a low androgen concentration. Microarray analyses of AR-regulated genes gave further evidence for the biological benefits of AR overexpression. They showed enhanced expression of several cell cycle-associated genes at 1 nmol/L DHT, especially in LNCaP-ARhi cells. In addition, expression of genes associated with biosynthesis of lipids and other cellular structures was elevated in LNCaP-ARhi cells. AR expression level seemed also to predict the activity of AR; the more AR expression, the more androgen responsive genes. Further studies are warranted to investigate whether the genes upregulated at low androgen concentrations could function as drug targets for CRPC treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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