Comparative Effects of DHEA and DHT on Gene Expression in Human LNCaP Prostate Cancer Cells

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Abstract. *Background: DHEA is widely used as a dietary supplement in older men. Because DHEA can be converted to androgens or estrogens, such use may promote prostate cancer. In this study, the effects of DHEA were compared with those of DHT using gene expression array profiles in human LNCaP prostate cancer cells. Materials and Methods: LNCaP cells were exposed to DHEA (300 nM), DHT (300 nM), or vehicle for 48 h, and mRNA expression was measured using Affymetrix HU-95 gene chips. Gene expression values were sorted in ascending order on the p-values corresponding to the extent of differential RNA expression between control and either hormone treatment. Results: S100 calcium binding protein, neurotensin, 24-dehydrocholesterol reductase, and anterior-gradient 2 homologue were the four most differentially expressed genes (p-values all $<3x10^{-5}$). Nested tests of differential expression revealed lesser effects of DHEA versus DHT treatment ($p<0.01$) for the S100 calcium binding protein and neurotensin genes. Microarray findings were confirmed by QRT-PCR. The top 83 genes exhibiting differential expression after DHEA or DHT were used for pathway analysis. DHT decreased expression of more genes involved in intercellular communication, signal transduction, nucleic acid binding and transport, and in structural components, such as myosin and golgin, than DHEA. Conclusion: These data revealed consistent, measurable changes in gene expression patterns following treatment of LNCaP prostate cancer cells with DHEA and DHT. Understanding the mechanisms of DHEA versus DHT actions in the prostate may help clarify the separate and interactive effects of androgenic and estrogenic actions in prostate cancer progression.*

Dehydroepiandrosterone (DHEA) is produced by the adrenal cortex and is the most abundant steroid in humans. DHEA exerts many of its actions *via* androgen receptor (AR)- and estrogen receptor (ER)-mediated effects following its enzymatic conversion to androgen or estrogen (1, 2), or *via* direct actions on the AR, ERα or ERβ (3-5), whereas other actions occur *via* steroid receptor-independent (6) or other mechanisms. Serum DHEA levels in aged men and women decrease by 75%-80% from peak levels (7). DHEA is widely used as a dietary supplement for its alleged anti-aging effects, although its reported beneficial effects on body composition and cardiometabolic, immune and neurobehavioral functions, and short- and long-term safety, remain unproved (8). Humans and other primates are unique among animal species in having adrenals that secrete large amounts of the inactive steroid precursors DHEA and its sulfate, DHEA-S (2, 9).

In older adults, the use of DHEA as a supplement is of potential concern in that its androgenic or estrogenic actions may stimulate proliferation of cancer cells within the prostate or breast. In that context, controversy exists as to whether DHEA enhances or reduces the risk of prostate and breast cancer (10, 11) DHEA has been reported to exert cancer protective effects in rodent models of prostate cancer (12-14). For example in one such chemoprevention study of prostate cancers induced by cyproterone acetate, MNU, and testosterone propionate, 1 or 2 g DHEA/kg diet was found to significantly decrease the incidence of dorsolateral prostate cancers by 45% and 60%, respectively,

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Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHT, dihydrotestosterone; FDR, false discovery rate; RT-PCR, reverse transcriptase polymerase chain reaction; LNCaP, lymph node derived cancer of prostate; DMEM, Dulbecco's Modified Essential Media; FBS, fetal bovine serum; ELISA, enzyme linked immunosorbent assay; SD, standard deviation; ANCOVA, analysis of covariance; CI, confidence interval.

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if given for the full 56 weeks following carcinogen exposure (13). In comparison, epidemiological data are confusing, in that the age-related decline in DHEA, testosterone and estrogens may protect against increasing occurrence of hormone-sensitive cancers (15); yet elevated serum levels of DHEA and DHEAS have been associated with decreased cancer rates (16). These apparently discrepant findings need to be resolved to understand effects on human prostate tissues. While epidemiological evidence does not support the role of DHEA in prostate cancer progression, the final mechanisms have not been delineated.

We previously examined the effects of DHEA, testosterone, DHT and estradiol on human LNCaP prostate cancer cells and human primary prostate stromal cells (17, 18). To confirm and extend these studies, we used microarray gene chip technology to compare the effects of DHEA *vs.* DHT on gene expression profiles in LNCaP cells. Such comparisons should yield new research targets for subsequent explorations into the possible role(s) of DHEA in human prostate cancer.

Materials and Methods

Cell culture. LNCaP-FGC cells (androgen-dependent, prostate adenocarcinoma cells derived from lymph node metastasis (CRL1435; American Type Culture Collection, Manassas, VA, USA) were grown in DMEM: F12 (1:1) medium, (Invitrogen, Gaithersburg, MD, USA) with penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (292 μg/mL) (Invitrogen) and 5% Fetal Bovine Serum (FBS, HyClone Laboratories, Inc., Logan, UT, USA) at 37˚C in 5% CO_2 and propagated at 1:5 dilutions.

Hormone treatments for gene and protein expression. LNCaP cells were seeded into three 100 mm tissue culture plates per treatment group at a density of $2x10^6$ cells/well with M199:F12 (1:1) media (Invitrogen), containing 2% dextran charcoal -treated FBS and antibiotics. After one day 300 nM DHEA, 300 nM DHT, or solvent (control) in media was added to groups of three plates each. Cells were treated for 48 h, and then harvested in asynchronous cultures to extract RNA. These doses and time of DHEA and DHT treatments previously elicited optimal cell proliferation responses for LNCaP cells (17). This experiment was conducted independently at two later dates and data from all three experiments were combined and analyzed, as described below.

RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The resulting RNA pellet was extracted for a second time with Trizol reagent to increase the RNA purity. Following this extraction, RNA in the aqueous phase was precipitated by slow addition of an equal volume of 70% ethanol at room temperature. The precipitate was loaded onto RNAeasy columns (Qiagen, Inc., Valencia, CA, USA). The subsequent washing and elution steps followed the manufacturer's instructions. Quality and concentration of the RNA were determined spectrophotometrically using absorption measurements at 260 and 280 nm wavelengths. Once purity and concentration were determined, the RNA samples were stored at –80˚C. To adjust the differences in cell proliferation resulting from hormonal exposure, equal amounts of total RNA were prepared for array analysis.

Sample preparation for gene expression analysis. Primer hybridization, cDNA synthesis and subsequent RNA synthesis were performed following the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA, USA) instructions for Eukaryotic RNA sample preparation. The poly(A) and mRNA present in the isolated sample were used to synthesize first single stranded and then double stranded DNA, using the Superscript II Double Stranded cDNA Synthesis kit (Invitrogen Corp., Carlsbad, CA, USA) and poly(T)-nucleotide primers that contained a sequence recognized by T4 DNA polymerase. After purifying the samples with a cDNA GeneChip Sample Cleanup Module (Qiagen), a portion of the resulting double stranded cDNA was used as a template to generate biotin tagged cRNA from an *in vitro* transcription kit (IVT), using the Bioarray High-Yield RNA Transcription Labeling Kit (T7) (Enzo Diagnostics, Inc., Farmingdale, NY, USA). Following cRNA purification, the amount of cRNA present was determined and 20 μg of cRNA was fragmented by incubation for 35 minutes at 94˚C. The fragmented cRNA, control RNAs and appropriate buffers from each group were loaded onto Affymetrix HG_U95Av2 microarray chips (Affymetrix) and incubated with rotation mixing at 45˚C overnight. After washing and staining the chips following the Affymetrix Fluidics Workstation protocols, they were scanned using an Affymetrix scanner resulting in image files in CEL format and the raw data were imported into the R statistical computing platform (19). For each gene, the probe set was summarized into a model based gene expression index (20), using the Bioconductor suite of add-on libraries for R (21). Within each experimental replicate and for each gene, the log base two of the expression ratios of treatment to control were calculated and used in subsequent analysis. The dchip data set for these data has been deposited at NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/projects/geo ; Series GSE3627).

RT-PCR analysis. RNA samples used in microarray studies were also prepared for cDNA synthesis and real time RT-PCR as described by Latil *et al.* (22) with some modifications. Briefly, RNA was reverse transcribed with Superscript III RNase H-reverse transcriptase from Invitrogen according to the manufacturer's indications, using 10 units of RNase inhibitor (Promega), 50 units of Superscript III, 150 ng of random hexamer (Amersham Biosciences) and 1.5 μg of total RNA. Real-time PCR was performed using SYBR Green PCR Core Reagents kit (Applied Biosystems, Foster City, CA, USA) and quantitated by ABI Prism 7900 Sequence Detection System (Applied Biosystems) according to the manufacturer's indications. Relative mRNA expression level was calculated using the ΔΔCt method, in which the calibrator is the *RPLPO* gene. *RPLPO* encodes human acidic ribosomal phosphoprotein P0 and was used as the endogenous RNA control to correct the amount of total RNA used in each PCR reaction. Primers for RPLPO and the top four gene sequences are summarized in Table II.

Statistical analysis of gene expression and pathway analysis. Genes were ranked in ascending order on the *p*-values corresponding to a variance stabilized Hotelling statistic (23) measuring the extent of differential expression in either treatment group (DHEA or DHT) *vs.* control. Very high statistical significance was established by applying the Benjamini and Hochberg (24) procedure at a False Discovery Rate (FDR) of 9%. This highly stringent criterion

identified the top four genes and resulted in per comparison p-values less than $3x10^{-5}$. Subsequently, for each of the four genes identified in this manner, nested tests for differential expression between the two treatment groups were done at a type I error of 1%. The Hotelling test, similar to the standard one way ANOVA test, allows for correlated groups with unequal within-group variances. Variance stabilization is a technique used in small sample micro-array studies that exploit the similarity in the within-gene error structure to form more stable estimates of the per gene variance parameters (25-27). Next, a longer list of genes for pathway analysis was obtained from the same list of sorted genes by applying the Benjamini and Hochberg (24) procedure at an FDR of less than 50%. This pathway analysis was done with Genespring 7 (Silicon Genetics, CA, USA), cross referencing this list of 83 genes with the simplified gene ontology gene lists. Pathways containing four or more genes from among the top 83 were considered important.

Results

Effects of DHT versus DHEA on human prostate LNCaP cancer cell gene expression. Extracted cellular RNA was highly pure with 260 nm/280 nm ratios very close to 2.0, as measured spectrophotometrically. LNCaP cells were treated with 300 nM of DHT or DHEA for 48 h, and exhibited major differences in gene expression from untreated cells, as measured by Affymetrix HU-U95Av2 gene chips experiments. In all three experiments at a FDR of 0.499 or less, 83 genes of the DHEA or DHT treated groups, including PSA, were significantly over- or under-expressed compared with control cells (Table I).

TOP four genes differentially expressed. The Benjamini and Hochberg (24) procedure at a FDR of 9% identified the top four genes in a sorted list of p-values corresponding to the above-mentioned statistic, measuring the extent of differential expression under one or both treatments relative to control. In all four cases the per comparison p-values were less than $3x10^{-5}$, indicating highly significant differential expression in one or both treatments relative to control. Fold changes relative to control and nested tests on between group differences for the four genes identified, are as follows. The most responsive gene, Calcium Binding Protein S100P, (Figure 1A) was more than 4-fold over-expressed after DHT *vs.* control treatment. In contrast DHEA treatment produced only a non-significant 1.7-fold increase. The nested test for differential expression between DHT and DHEA treatments was highly significant ($p=4.38x10^{-5}$). The second gene, neurotensin, (Figure 1B) exhibited a nearly 4-fold decrease in expression level following DHT treatment, whereas DHEA treatment reduced expression by only about 2-fold. A nested test of the difference between these two treatments was highly significant ($p=2.88x10^{-3}$). In comparison, 24-dihydroxy-cholesterol reductase (Figure 1C) expression was significantly and similarly increased by about 2-fold following DHT or DHEA *vs.* control treatment ($p=8.67x10^{-6}$). The final gene falling into this category was the Anterior-gradient 2 homologue gene, which was increased, approximately, 1.3- and 1.8-fold compared with control ($p=2.84x10^{-5}$) (Figure 1D) following treatment with DHEA or DHT, respectively. The difference between these two treatments was not significant.

RT-PCR analysis of gene expression compared to array expression patterns. Analysis of gene expression by real time RT-PCR revealed a very good correspondence with the results measured by microchip array analysis. RNA that was isolated from two independent experiments for each experimental group was used for the analysis. Using the primer sets shown in Table II, the mRNA was amplified and then the number of cycles required to amplify to a standard amount of cDNA was compared with control values. Table III illustrates the microarray results and RT-PCR results by measuring the relative gene expression of DHEA- to DHT-treated samples for the 4 top genes in two experiments. The data were similar between the two experiments, both qualitatively and quantitatively.

Pathway analysis of DHT vs. DHEA gene expression. The Benjamini and Hochberg (24) procedure at a FDR of 49.9% identified the top 83 genes in the list of genes sorted on p-values of the above-mentioned statistic, measuring the extent of differential expression under one or both treatments relative to control. Using GeneSpring 7 software, several major pathways were investigated to cross-reference this list of 83 genes with the simplified gene ontology lists, and for changes in gene expression following treatment with DHEA or DHT. In this 83 gene list, four genes were associated with intercellular communication (Figure 2A). DHEA and DHT led to similar decreases in gene expression of LPS-responsive vesicle trafficking, growth factor receptor bound protein 10, and human Na, K-ATPase β-1 subunit genes, whereas DHT doubled the expression of CDC45 compared with DHEA or control treatment. DHEA and DHT elicited similar effects on four genes associated with signal transduction (Figure 2B). Tumor necrosis factor alpha-induced protein 8 and human insulin induced protein 1 (INSIG1) were up-regulated by 2-fold and insulin induced gene 2 and growth factor receptor bound protein 10 were decreased by 50% compared with control. Of the 7 genes examined, involved in cellular processes (Figure 2C), transportin 1 and golgi SNAP receptor complex member 1 were expressed 20-50% above control, whereas collagen type XIV, nucleosome assemble protein 1, golgin-67, myosin heavy polypeptide 10, and human mitogen induced nuclear orphan receptor (MINOR) were expressed 20-40% below control levels. Two genes in the cancer pathway were aberrantly expressed following DHEA or DHT treatment (data not shown). Both treatments caused about an 80% increase in expression of the v-maf musculoaponeurotic fibrosarcoma oncogene homolog gene. Conversely, the gene

Table I. *Genes under or over expressed at FDR <0.499 compared to control.*

Affymetrix	Unicode	DHEA/control	DHT/control	*p*-value	FDR=0.499	Gene description
34319_at	AA131149	1.69	4.4	1.90E-007	3.95E-005	S100 calcium-binding protein P
36658_at	D13643	2.44	2.79	8.67E-006	7.91E-005	24-Dehydrocholestrol reductase
33998_at	U91618	0.519	0.275	1.08E-005	1.19E-004	neurotensin
38827_at	AF038451	1.31	1.84	2.84E-005	1.58E-004	anterior gradient 2 (Xenepus laevis) homolog
41242_at	AB011004	1.79	1.67	1.41E-004	1.98E-004	UDP-N-acteylglucosamine pyrophosphorylase 1
40040_at	AC002542	0.582	0.579	1.77E-004	2.37E-004	Human BAC clone RG114A06 from 7q31
40201_at	M76180	0.682	0.582	1.88E-004	2.77E-004	dopa decarboxylase (aromatic L-amino acid decarboxylase)
37841_at	M16541	0.676	0.636	2.34E-004	3.16E-004	butyrylcholinesterase
33370_r_at	U60205	1.84	1.95	2.83E-004	3.56E-004	sterol-C4-methyl oxidase-like
36432_at	AL079298	1.66	1.91	2.86E-004	3.95E-004	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)
35168_f_at	M92642	0.715	0.614	3.07E-004	4.35E-004	collagen, type XVI, alpha 1
34721_at	U42031	2.01	1.75	3.38E-004	4.74E-004	FK506-binding protein 5
41145_at	AB020721	0.7	0.612	3.51E-004	5.14E-004	KIAA0914 gene product
37984_s_at	M57763	1.33	1.52	3.65E-004	5.53E-004	ADP-ribosylation factor 6
41504_s_at	AF055376	1.88	1.78	4.80E-004	5.93E-004	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog
1556_at	U23946	0.85	0.693	5.42E-004	6.72E-004	RNA binding motif protein 5
38469_at	M35252	0.602	0.577	5.42E-004	6.32E-004	transmembrane 4 superfamily member 3
41491_s_at	AB028944	0.7	0.663	5.71E-004	7.11E-004	ATPase, Class VI, type 11A
1052_s_at	M83667	1.54	1.51	5.79E-004	7.51E-004	CCAAT/enhancer binding protein (C/EBP), delta
41544_at	AF059617	0.754	0.677	5.85E-004	7.91E-004	serum-inducible kinase
37692_at	AI557240	2.37	3.05	5.98E-004	8.30E-004	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)
38763_at	L29254	1.78	1.69	6.25E-004	8.70E-004	L-iditol-2 dehydrogenase
38409_at	M61199	0.696	0.627	6.50E-004	9.09E-004	M61199 sperm specific antigen 2
35371_at	M83822	0.787	0.676	6.85E-004	9.49E-004	cell division cycle 4-like
36526_at	AF000416	1.65	1.6	7.38E-004	9.88E-004	exostoses (multiple)-like 2
37615_at	D86962	0.616	0.559	7.52E-004	1.03E-003	growth factor receptor-bound protein 10
33836_at	AC002045	0.71	0.692	8.39E-004	1.07E-003	nuclear pore complex interacting protein
35643_at	X76732	0.688	0.576	8.96E-004	1.11E-003	nucleobindin 2
123_at	X75756	0.744	0.681	9.16E-004	1.19E-003	protein kinase C, mu
33821_at	AL034374	1.39	1.41	9.11E-004	1.15E-003	Human DNA sequence from clone 483K16 on chromosome 6p12.1-21.1
40690_at	X54942	1.97	2.47	9.55E-004	1.23E-003	CDC28 protein kinase 2
1602_at	L33881	1.5	1.3	9.66E-004	1.30E-003	protein kinase C, iota
40195_at	X14850	2.2	2.53	9.63E-004	1.27E-003	"H2A histone family, member X"
901_g_at	L41349	0.737	0.672	1.04E-003	1.34E-003	phospholipase C, beta 4
35710_s_at	U95006	1.51	1.61	1.20E-003	1.42E-003	Human D9 splice variant B mRNA, complete cds
40725_at	AF047438	1.54	1.39	1.20E-003	1.38E-003	golgi SNAP receptor complex member 1
35140_at	R59697	0.71	0.676	1.26E-003	1.46E-003	cyclin-dependent kinase 8
41733_at	AC003007	1.23	1.47	1.31E-003	1.50E-003	Human Chromosome 16 BAC clone CIT987SK-A-61E3
41468_at	M30894	2.38	2.49	1.32E-003	1.54E-003	T cell receptor gamma locus
36209_at	S78771	1.46	1.15	1.35E-003	1.58E-003	bromodomain-containing 2
38429_at	U29344	1.26	1.41	1.56E-003	1.62E-003	fatty acid synthase
34288_at	U67784	0.601	0.589	1.58E-003	1.70E-003	G protein-coupled receptor
37000_at	AL035304	1.6	1.64	1.57E-003	1.66E-003	DKFZP564B167 protein
39005_s_at	AB018257	0.644	0.542	1.60E-003	1.74E-003	zinc finger protein 294
32350_at	AB026118	1.4	1.44	1.64E-003	1.78E-003	mucosa associated lymphoid tissue lymphoma translocation gene 1
35833_at	AL080184	0.731	0.703	1.67E-003	1.82E-003	insulin induced protein 2
33243_at	AF099935	2.2	1.92	1.79E-003	1.86E-003	TNF-induced protein
571_at	M86667	0.869	0.727	1.83E-003	1.90E-003	nucleosome assembly protein 1-like 1
38218_at	M97347	1.87	1.86	1.89E-003	1.98E-003	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)"
38975_at	AF062534	0.749	0.717	1.89E-003	1.94E-003	genethonin 1
32190_at	AL050118	1.63	1.57	1.96E-003	2.02E-003	delta-6 fatty acid desaturase
32838_at	S67247	0.696	0.667	2.05E-003	2.06E-003	Homo sapiens cDNA: FLJ23324 fis, clone HEP12482
32336_at	X05236	1.34	1.45	2.09E-003	2.10E-003	aldolase A, fructose-bisphosphate

Affymetrix	Unicode	DHEA/control	DHT/control	*p*-value	FDR=0.499	Gene description
33442_at	AB002365	0.777	0.728	2.10E-003	2.21E-003	KIAA0367 protein
35823_at	M63573	1.35	1.3	2.10E-003	2.13E-003	peptidylprolyl isomerase B (cyclophilin B)
37669_s_at	U16799	0.74	0.648	2.11E-003	2.25E-003	ATPase, Na+/K+ transporting, beta 1 polypeptide
38716_at	AB018330	1.56	1.38	2.10E-003	2.17E-003	calcium/calmodulin-dependent protein kinase kinase 2, beta
1513_at	LBX1	1.81	1.6	2.22E-003	2.29E-003	Antigen, Prostate Specific, Alt. Splice Form 3
497_at	U32680	1.29	1.59	2.23E-003	2.33E-003	ceroid-lipofuscinosis, neuronal 3, juvenile (Batten, Spielmeyer-Vogt disease)
40038_at	W02490	0.686	0.638	2.28E-003	2.37E-003	family with sequence similarity 4, subfamily A, member 1
33894_at	AJ010046	0.706	0.717	2.32E-003	2.41E-003	guanine nucleotide regulatory protein (oncogene)
31773_at	U06715	1.32	1.52	2.45E-003	2.45E-003	cytochrome b-561
39324_at	AL050078	0.74	0.762	2.45E-003	2.49E-003	Homo sapiens cDNA FLJ10784 fis, clone NT2RP4000448
40510_at	AB007917	0.876	0.732	2.45E-003	2.53E-003	heparan sulfate 2-O-sulfotransferase
33369_at	AI535653	1.67	1.69	2.53E-003	2.57E-003	sterol-C4-methyl oxidase-like
1846_at	L78132	0.737	0.659	2.58E-003	2.69E-003	lectin, galactoside-binding, soluble, 8 (galectin 8)
40455_at	AB020637	1.53	1.49	2.55E-003	2.61E-003	KIAA0830 protein
40464_g_at	U70322	1.37	1.23	2.57E-003	2.65E-003	karyopherin (importin) beta 2
39706_at	AB014536	0.831	0.72	2.60E-003	2.73E-003	copine III
38792_at	AD001528	1.61	1.65	2.68E-003	2.77E-003	spermine synthase
33710_at	U72515	0.691	0.662	2.74E-003	2.81E-003	putative protein similar to nessy (Drosophila)
35007_at	AC004940	1.26	1.33	2.75E-003	2.89E-003	Homo sapiens PAC clone RP5-978E18 from 7p21
37458_at	AJ223728	1.18	2.05	2.77E-003	2.93E-003	CDC45 (cell division cycle 45, S.cerevisiae, homolog)-like
41767_r_at	AB020662	0.706	0.628	2.75E-003	2.85E-003	golgin-67
33436_at	Z46629	0.724	0.785	2.81E-003	2.96E-003	SRY (sex-determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)
34213_at	AB020676	1.66	1.45	2.83E-003	3.00E-003	KIAA0869 protein
36484_at	AI935146	0.67	0.606	2.87E-003	3.08E-003	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetyl-galactosaminyltransferase 3 (GalNAc-T3)
948_s_at	D63861	1.41	1.38	2.87E-003	3.04E-003	peptidylprolyl isomerase D (cyclophilin D)
35221_at	X91648	0.665	0.628	2.91E-003	3.12E-003	H.sapiens mRNA for pur alpha extended 3'untranslated region
40279_at	D50911	0.718	0.667	2.92E-003	3.16E-003	KIAA0121 gene product
40659_at	U12767	0.754	0.706	3.04E-003	3.20E-003	nuclear receptor subfamily 4, group A, member 3
35303_at	U96876	1.83	1.95	3.15E-003	3.24E-003	insulin induced gene 1
37426_at	U80736	0.698	0.648	3.25E-003	3.28E-003	trinucleotide repeat containing 9

for suppression of tumorigenicity 7 was 30-35% decreased in expression by both treatments. Twenty-two enzymes, representing a variety of reductases, transferases and kinases, were significantly up- or down-regulated compared to control (Table IV). The expression of 14 enzymes was significantly increased following DHEA or DHT exposure, while eight were significantly decreased. Of the 14 enzymes with increased expression DHT effects were greater than DHEA in 8 cases, while of the 8 down-regulated enzymes all were decreased more by DHT than by DHEA.

Discussion

The influence of DHEA on human prostate physiology is an active and important area of research. The purpose of this study was to compare the gene expression effects of exposure to DHEA with those of DHT on human prostate cancer cells. LNCaP cells represent one of only a few hormonally responsive *in vitro* models for understanding steroid hormone effects in the prostate (28). DHEA binds to the mutated androgen receptor characteristic of LNCaP cells, but with a lower binding affinity than DHT (29, 30). Using LNCaP cells, we found that DHEA and DHT treatment elicited substantial, though remarkable, similar gene expression changes compared to untreated cells, as measured by the Affymetrix microarrays. However, it is important to note that the magnitudes of these changes were generally less than those resulting from DHT exposure. The treatment dose of 300 nM steroid was chosen based on our previous experiments, in which the expression of IGF-IR and PSA mRNA were maximally separated in DHEA *versus* DHT treated LNCaP cells (17). In the current study we also found up-regulation of PSA and IGF-1 and down-regulation of AR, ERβ and IGFBP3 (data not shown) were qualitatively similar to those reported previously (17).

DHEA has multiple fates within the prostate. DHEA sulfate (DHEAS) and the sulfatase needed for conversion from DHEAS to DHEA are present in high levels in

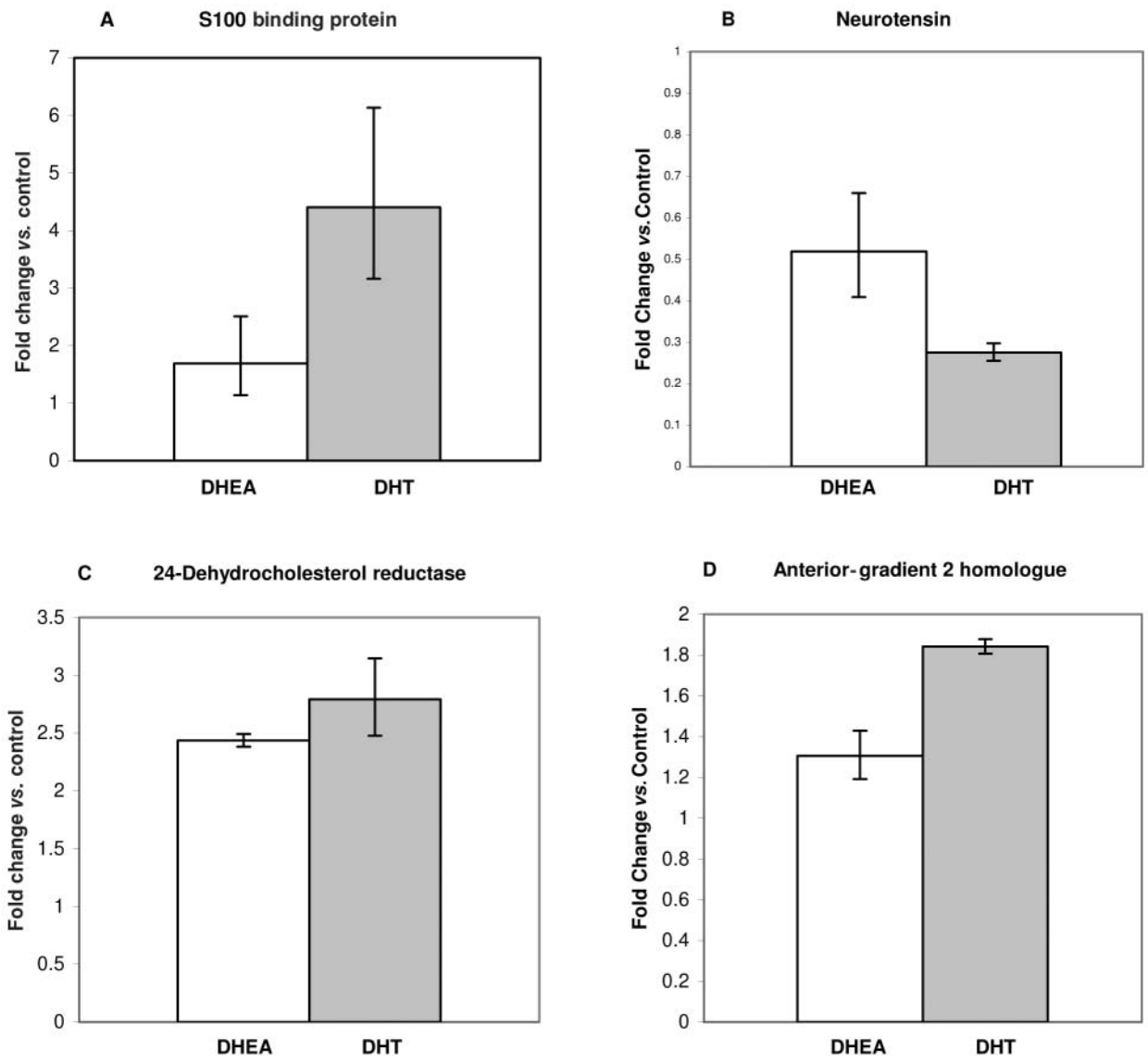

Figure 1. *Comparison of expression activities of the top four genes differentially expressed in LNCAP cells following treatment with* □ *DHEA or* ■ *DHT. (A) S100 binding protein; (B) Neurotensin; (C) 24-Dehydrocholesterol reductase; (D) Anterior-gradient 2 homologue. Error bars denote 95% confidence limits.*

the human prostate (31). Prostate cells contain 3β- and 17β-hydroxysteroid dehydrogenases and can metabolize DHEA to DHT (32), providing up to 17% of total prostatic DHT (33). In the experiments reported here, DHEA may have exerted its effects *via* the androgen receptor, either *via* direct binding, or indirectly after undergoing metabolism to DHT. Moreover, DHEA effects may also have been mediated *via* the estrogen receptor. LNCaP cells express ERβ (34) and ERβ is a target for DHEA metabolites including estradiol (30) and 7-hydroxy DHEA (35). DHT and DHEA can down-regulate ERb expression in LNCaP cells within 24 h (17). DHEA can be a direct ligand for ERβ and can inhibit DHT induced PSA production (5). Some of the differences in DHEA and DHT array expression patterns observed in the present study may have resulted from differences in ERβ-mediated effects.

The comparisons reported here used rigorous statistical methodologies to analyze the data and differences of three independent experiments. The novel statistical methods employed offer greater reliability in small sample microarray studies based upon the incorporation of stabilized per gene variance parameter estimates into the usual Hotelling test. Variance stabilization is a technique used in such microarray studies that exploits the similarity in the within gene error structure to form more stable

Table II. *Sequences of primers used for quantitative RT-PCR.*

Gene		Oligonucleotide sequence	Size
RPLPO (BC003655)	Forward	5'-GGCGACCTGGAAGTCCAACT-3'	149
	Reverse	5'-CCATCAGCACCACAGCCTTC-3'	
S100 Calcium Binding Protein (AA131149)	Forward	5'-TACCAGGCTTCCTGCAGAGT -3'	137
	Reverse	5'-GTGACAGGCAGACGTGATTG -3'	
Neurotensin (U91618)	Forward	5'-GCATGCTACTCCTGGCTTTC -3'	131
	Reverse	5'-CCAAGAGGGAACATGTGCTT -3'	
Anterior Gradient Homologue 2 (AF038451)	Forward	5'-TCTGCACTCCTGAACACACC-3'	151
	Reverse	5'-CCTCATCAACACGTCACCAC-3'	
24-Dehydrocholestrol (D13643)	Forward	5'-CAAAGGAAATGAGGCAGAGC-3'	148
	Reverse	5'-GTAGCAGTCGGCATACAGCA-3'	

estimates of the per gene variance, which lie between their raw estimates and the average of these raw values. This results in lower observed FDRs so that a list of genes that is sorted on values using this statistic can be considered more reliable. The use of this shrinkage variance estimator in conjunction with an ANOVA type test at the gene level has been proposed by other authors (25, 27).

These robust statistical tools were used to first compare gene expression in steroid-treated *versus* control groups. The list of 83 genes generated in which one or both hormone treatments were highly statistically different from control revealed a wide variety of interesting and some unknown genes. The top four genes (Table I) stood out as having expression values for DHT or DHEA treatments that were highly significantly different from those of control, and the expression of two of these genes were highly significantly different between DHEA and DHT treatments. These four genes may provide key insights into how DHEA and DHT differ in their actions on prostate cells. It is important to note that in all four cases the effect of DHEA was less in magnitude than that of DHT (either for up-regulation or down-regulation).

The expression of S100 calcium binding protein mRNA was increased more than 4-fold in cells treated with DHT compared with control cells. In contrast, DHEA led to a non-significant, less than 2-fold increase in expression of S100. The human S100P gene that locates on human chromosome 4p16 is expressed widely in many normal and tumorous human tissues (36), including placenta, lung, heart and skeletal muscle, and in the neural system. The human S100P binding protein exists as a series of proteins with similar properties that play important roles in calcium-mediated signal transduction, cell-cycle progression and differentiation. This gene codes for a calcium signaling molecule associated with the loss of senescence, and was one of the most highly overexpressed genes in the CWR22 human prostate xenograph model system (37). Moreover, the synthetic androgen R1881 regulates its expression in LNCaP cells (38), whereas decreases in the calcium binding protein S100P occur after androgen receptor blockade or androgen deprivation (39). Recently it was shown that S100P, which is up-regulated in metastatic androgen-refractory prostate cancer, could be induced by interleukin-6 (40). In oral cancer some of the S100 proteins appear to decrease with increasing malignancy (41). In a variety of human prostate tissues from normal to high grade PIN, immunohistochemical staining showed increased expression of *S100A4* to be associated with progression of prostate cancer in humans (42).

Table III. *RT-PCR results compared with MicroArray results.*

	DHEA/DHT			
	CBP	NEURO	DHC	AGH
Experiment 1				
RT-PCR results	0.25	2.34	0.67	0.46
Array results	0.42	2.65	1.01	0.74
Experiment 2				
RTR-PCR results	0.44	2.00	1.07	0.57
Array results	0.32	1.97	0.85	0.56

*AGH, anterior gradient homologue; CBP, S100 calcium binding protein; DHC, dihhydrocholesterol reductase; NEURO, neurotensin.

The expression of neuroendocrine markers correlates with tumor grade, loss of androgen sensitivity, and poor prognosis for prostate cancer. Neurotensin acts as a survival and migratory factor(s) for androgen-independent prostate cancers. It is present in and secreted from primary prostate cancer, resulting in high patient plasma neurotensin levels (43). Sehgal and others have shown that LNCaP cells produce and secrete neurotensin following androgen withdrawal (44). In this pattern, our androgen-depleted control cultures would have increased neurotensin levels before addition of DHT, which then produced a marked decline in neurotensin production compared to control. Again DHEA produced an effect that was of significantly lesser magnitude than that elicited by DHT.

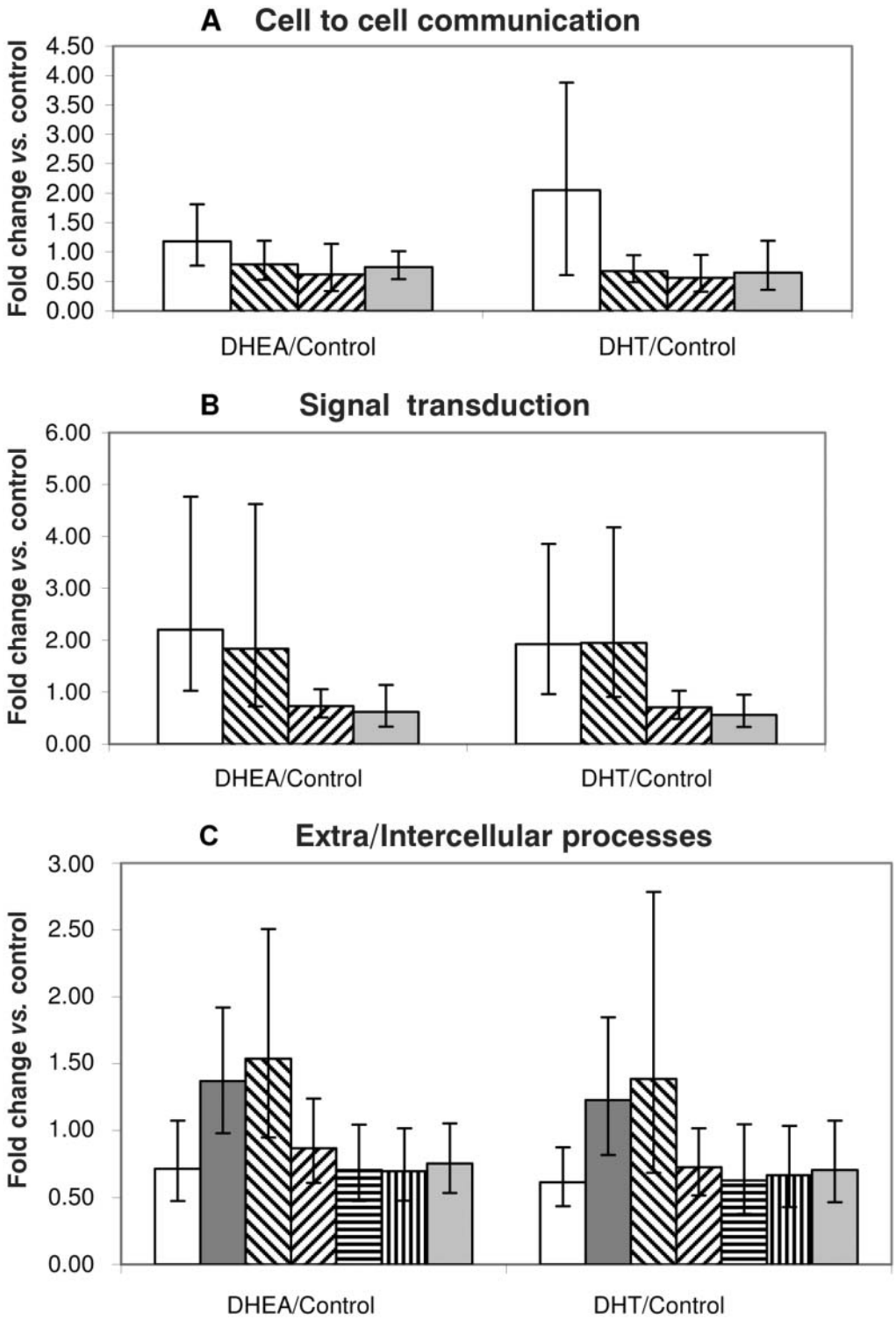

Figure 2. *Pathway analysis of highly differentially expressed genes in LNCaP cells following treatment with DHEA or DHT (FDR>0.5). (A) Biological Processes: CDC 45, cell division cycle 45-like, □; LPS responsive vesicle trafficking , ▧; growth factor receptor-bound protein 10, ▨; human Na, K-ATPase beta-1 subunit mRNA, ■. (B) Molecular Function – Signal Transduction Processes: Tumor necrosis factor, alpha-induced protein 8, □; human insulin-induced protein 1 (INSIG1) gene, ▧; human insulin-induced protein 2 (INSIG2) gene, ▨; growth factor receptor bound protein 10, ■. (C) Cellular Processes – Intracellular Processes/Extracellular structural proteins : Collagen type XVI –type 1, □; transportin 1, ■; golgi SNAP receptor complex member 1, ▧; nucleosome assembly protein 1, ▨; golgin-6 7, ▤; myosin, heavy polypeptide 10, non muscle, ▥; human mitogen induced nuclear orphan receptor (MINOR) mRNA, ■. Error bars denote 95% confidence limits.*

Table IV. *Comparative effects of DHEA vs. DHT on enzyme mRNA expression.*

DHEA/Control	DHT/Control	DHEA/DHT	Gene description
0.88	0.73	1.20	heparan sulfate 2-O-sulfotransferase 1
0.68	0.58	1.17	dopa decarboxylase (aromatic L-amino acid decarboxylase)
1.50	1.30	1.16	protein kinase C, iota
0.74	0.65	1.14	Diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)
1.56	1.38	1.14	calcium/calmodulin-dependent protein kinase kinase 2, beta
0.67	0.61	1.11	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
0.74	0.67	1.10	phospholipase C, beta 4
0.74	0.68	1.09	protein kinase C, mu
0.68	0.64	1.06	butyrylcholinesterase
0.70	0.66	1.06	ATPase, Class VI, type 11A
1.78	1.69	1.05	Human (clone D21-1) L-iditol-2 dehydrogenase gene, exon 9 and complete cds
1.35	1.30	1.04	peptidylprolyl isomerase B (cyclophilin B)
1.63	1.57	1.04	fatty acid desaturase 2
1.87	1.86	1.00	glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-acetylglucosaminyltransferase)
1.39	1.41	0.99	Human DNA sequence from clone RP3-483K16 on chromosome 6p12.1-21.1
1.61	1.65	0.98	spermine synthase
1.67	1.69	0.98	P9-C4.T3.P9.D4 conorm Homo sapiens cDNA 3', mRNA sequence
1.84	1.95	0.94	sterol-C4-methyl oxidase-like
1.34	1.45	0.93	unnamed protein product; aldolase A (AA 1-364); Human fibroblast mRNA for aldolase A
1.26	1.41	0.90	fatty acid synthase
2.44	2.79	0.87	24-dehydrocholesterol reductase
1.66	1.91	0.87	methylcrotonoyl-Coenzyme A carboxylase 2 (beta)

24-dehydrocholesterol reductase (also known as DHCR24 or *seladin*-1) is one of the five androgen inducible genes that are over expressed in prostate cancer (45). The other four are: PSA, KLK2, ABCC4 and GUCY1A3. It was increased in our study by 2-fold by both DHT and DHEA. Additionally, 24DCHR expression has been shown to be down-regulated in Alzheimer's disease and in adrenal cancers (46) and in granulosa cell tumors of the ovaries (47). The 24DHCR gene codes for an oxidoreductase which catalyzes the reduction of the Δ-24 double bond in the cholesterol biosynthesis pathway. 24DHCR also appears to be a key mediator of *ras*-induced senescence in human cells. In a recent study, wild type 24DHCR suppressed *ras*-induced transformation, whereas mutant 24DHCR permitted ras transformation (48). Deficiencies in this enzyme are thought to cause desmosterolosis characterized by multiple congenital abnormalities (49).

Anterior gradient homologue 2 (AGH2) gene expression has been shown to be inducible by androgen treatment in LNCaP cells, as well as being elevated in a majority of human prostate tumors compared with adjacent normal tissues (50). Differential protein analysis revealed that AGH2 is also significantly increased in colorectal cancer compared with normal tissues (51), as well as in the malignant human breast cancer cell line, MCF-7 (52). In our study, AGH2 gene expression was increased by 30-80% following treatment with DHEA or DHT respectively.

Pathway analysis of the effects of DHEA *vs.* DHT treatment revealed that four genes involved in cell communication were affected. DHT significantly increased CDC45 expression, whereas DHEA exerted little effect. Three other cell communication genes were significantly under expressed in the treatment groups. These data suggest that cell communication may decrease following DHEA or DHT administration. Of the signal transduction pathway genes evaluated, those for a tumor necrosis factor protein 8 and an insulin induced protein were increased similarly following DHEA and DHT treatments, whereas expression of another insulin induced gene and a growth factor receptor bound protein were decreased. The physiological relevance of these opposing expression patterns is as yet uncertain. Of the seven genes that were significantly over or under expressed in the Extra/Intercellular Processes pathway, most were under expressed. This may lead to structural differences in treated cells, but it remains to be determined how this would affect the overall phenotype of the cell line.

A wide variety of enzymes that were up- or down-regulated may lead to some insights into the actions of DHT and DHEA. In 16 of 22 significant enzyme mRNAs changes the effects of DHT were greater than DHEA.

In summary, microarray analysis revealed that DHEA and DHT exert qualitatively similar, yet quantitatively differing, expression levels of many genes in human LNCaP prostate cancer cells. For genes significantly over- or under-expressed, the effects of DHT were in most cases greater than that seen for DHEA exposure. These findings may have been due to differences in the affinity of DHEA *vs*. DHT for the androgen receptor, in the cellular metabolism of DHEA *vs*.

DHT, or in the potential interactions of DHEA *vs*. DHT or their metabolites with ERβ. Further inquiry into DHEA and DHT effects on prostate epithelial cells must consider the likely role of prostatic stromal cells, as the latter contribute to steroid hormone metabolism and production of paracrine factors important in the development, differentiation and maintenance of the normal prostate (53). Development of steroid-responsive *in vitro* models is warranted to distinguish the effects of DHEA compared with other androgens and/or estrogens on normal, premalignant, and malignant prostate epithelial and stromal cells of varying AR and ER status.

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References

1 Labrie F, Luu-The V, Labrie C and Simard J: DHEA and its transformation into androgens and estrogens in peripheral target tissues: intracrinology. Front Neuroendocrinol *22(3)*: 185-212, 2001.

2 Labrie F, Belanger A, Luu-The V, Labrie C, Simard J, Cusan L, Gomez JL and Candas B: DHEA and the intracrine formation of androgens and estrogens in peripheral target tissues: its role during aging. Steroids *63(5-6)*: 322-328, 1998.

3 Martin C, Ross M, Chapman KE, Andrew R, Bollina P, Seckl JR and Habib FK: CYP7B generates a selective estrogen receptor beta agonist in human prostate. J Clin Endocrinol Metab *89(6)*: 2928-2935, 2004.

4 Tan J, Sharief Y, Hamil KG, Gregory CW, Zang DY, Sar M, Gumerlock PH, deVere White RW, Pretlow TG, Harris SE, Wilson EM, Mohler JL and French FS: Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. Mol Endocrinol *11(4)*: 450-459, 1997.

5 Chen F, Knecht K, Birzin E, Fisher J, Wilkinson H, Mojena M, Moreno CT, Schmidt A, Harada S, Freedman LP and Reszka AA: Direct agonist/antagonist functions of dehydroepiandrosterone. Endocrinology *146(11)*: 4568-4576, 2005.

6 Williams MR, Dawood T, Ling S, Dai A, Lew R, Myles K, Funder JW, Sudhir K and Komesaroff PA: Dehydroepiandrosterone increases endothelial cell proliferation *in vitro* and improves endothelial function *in vivo* by mechanisms independent of androgen and estrogen receptors. J Clin Endocrinol Metab *89(9)*: 4708-4715, 2004.

7 Orentreich N, Brind JL, Vogelman JH, Andres R and Baldwin H: Long-term longitudinal measurements of plasma dehydroepiandrosterone sulfate in normal men. J Clin Endocrinol Metab *75(4)*: 1002-1004, 1992.

8 Alesci S, Manoli I and Blackman MR: Dehydroepiandrosterone (DHEA). *In*: Encyclopedia of Dietary Supplements (1st Ed.). Coates P, Blackman MR, Cragg G, Levine M, Moss J and White J (eds.). New York, New York: Marcel Dekker, Inc., pp. 167-176, 2005.

9 Martel C, Melner MH, Gagne D, Simard J and Labrie F: Widespread tissue distribution of steroid sulfatase, 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD), 17 beta-HSD 5 alpha-reductase and aromatase activities in the rhesus monkey. Mol Cell Endocrinol *104(1)*: 103-111, 1994.

10 Acacio BD, Stanczyk FZ, Mullin P, Saadat P, Jafarian N and Sokol RZ: Pharmacokinetics of dehydroepiandrosterone and its metabolites after long-term daily oral administration to healthy young men. Fertil Steril *81(3)*: 595-604, 2004.

11 Arnold JT and Blackman MR: Does DHEA exert direct effects on androgen and estrogen receptors, and does it promote or prevent prostate cancer? Endocrinology *146(11)*: 4565-4567, 2005.

12 McCormick DL and Rao KV: Chemoprevention of hormone-dependent prostate cancer in the Wistar-Unilever rat. Eur Urol *35(5-6)*: 464-467, 1999.

13 Rao KV, Johnson WD, Bosland MC, Lubet RA, Steele VE, Kelloff GJ and McCormick DL: Chemoprevention of rat prostate carcinogenesis by early and delayed administration of dehydroepiandrosterone. Cancer Res *59(13)*: 3084-3089, 1999.

14 Schwartz AG and Pashko LL: Mechanism of cancer preventive action of DHEA. Role of glucose-6-phosphate dehydrogenase. Ann N Y Acad Sci *774*: 180-186 1995.

15 Martin-Du Pan RC: Are the hormones of youth carcinogenic? Ann Endocrinol (Paris) *60(5)*: 392-397, 1999.

16 Comstock GW, Gordon GB and Hsing AW: The relationship of serum dehydroepiandrosterone and its sulfate to subsequent cancer of the prostate. Cancer Epidemiol Biomarkers Prev *2(3)*: 219-221, 1993.

17 Arnold JT, Le H, McFann KK and Blackman MR: Comparative effects of DHEA *vs.* testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. Am J Physiol Endrocrinol Metab *228*: E573-E584, 2005.

18 Le H, Arnold JT, McFann KK and Blackman MR: Dihydrotestosterone and testosterone, but not DHEA or estradiol, differentially modulate IGF-I, IGFBP-2 and IGFBP-3 gene and protein expression in primary cultures of human prostatic stromal cells. Am J Physiol Endocrinol Metab *290*: E952-960, 2006.

19 Research Development Core Team R: A language and environment for statistical computing. Research Foundation for Statistical Computing. Vienna, Austria 2004;ISBN 3-900051-00-3, URL http://www.R-project.org

20 Li C and Wong WH: Model-based analysis of oligonucleotide arrays: Expression index computation and outlier detection. Proc Natl Acad Science *98(1)*: 31-36, 2001.

21 Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y-C, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY-H and Zhang J-H: Bioconductor: open software development for computational biology and bioinformatics. Genome Biology 5: R80, 2004, URL http://genomebiology.com/2004/5/10/R80.

22 Latil A, Bieche I, Vidaud D *et al*: Evaluation of androgen, estrogen (ER alpha and ER beta) and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcriptase-polymerase chain reaction assays. Cancer Res *61*: 1919-1926, 2001.

23 Izmirlian G and Xu J-L: The shrinkage variance hotelling T^2 test for genomic profiling studies. Submitted, 2006.

24 Benjamini Y and Hochberg Y: Controlling the false discovery Rate - a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society B *57*: 289-300, 1995.

25 Cui X-G, Hwang J-TG, Qui J, Blades NJ and Churchill GA: Improved statistical tests for differential gene expression by shrinking variance components estimates. Biostatistics *6(1)*: 59-75, 2005.

26 Tusher VG, Tibshirani R and Chu G: Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sciences *98*: 5116-5121, 2001.

27 Wright GW and Simon RM: A random variance model for detection of differential gene expression in small microarray experiments. Bioinformatics *19*: 2448-2455, 2003.

28 Negri-Cesi P and Motta M: Androgen metabolism in the human prostatic cancer cell line LNCaP. J Steroid Biochem Mol Biol *51(1-2)*: 89-96, 1994.

29 Horoszewicz J, Leong S, Kawinski E, Karr J, Rosenthal H, Chu T, Mirand E and Murphy G: LNCaP model of human prostatic carcinoma. Cancer Res *43(4)*: 1809-1818, 1983.

30 Widstrom RL and Dillon JS: Is there a receptor for dehydroepiandrosterone or dehydroepiandrosterone sulfate? Semin Reprod Med *22(4)*: 289-298, 2004.

31 Klein H, Molwitz T and Bartsch W: Steroid sulfate sulfatase in human benign prostatic hyperplasia: characterization and quantification of the enzyme in epithelium and stroma. J Steroid Biochem *33(2)*: 195-200, 1989.

32 Labrie F: Intracrinology. Mol Cell Endocrinol *78(3)*: C113-118, 1991.

33 Geller J: Rationale for blockade of adrenal as well as testicular androgens in the treatment of advanced prostate cancer. Semin Oncol *12(1 Suppl 1)*: 28-35, 1985.

34 Lau KM, LaSpina M, Long J and Ho SM: Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation. Cancer Res *60(12)*: 3175-3182, 2000.

35 Martin C, Ross M, Chapman KE, Andrew R, Bollina P, Seckl JR and Habib FK: CYP7B generates a selective estrogen receptor beta agonist in human prostate. J Clin Endocrinol Metab *89(6)*: 2928-2935, 2004.

36 Jin G, Wang S, Hu X, Jing Z, Chen J, Ying K, Xie Y and Mao Y: Characterization of the tissue-specific expression of the s100P gene which encodes an EF-hand Ca^{2+}-binding protein. Mol Biol Rep *30(4)*: 243-248, 2003.

37 Mousses S, Bubendorf L, Wagner U, Hostetter G, Kononen J, Cornelison R, Goldberger N, Elkahloun AG, Willi N, Kiovisto P, Ferhle W, Raffeld M, Sauter G and Kallioniemi OP: Clinical validation of candidate genes associated with prostate cancer progression in the CWR22 model system using tissue microarrays. Cancer Res *62(5)*: 1256-1260, 2002.

38 Averboukh L, Liang P and Pardee AB: Regulation of S100P expression by androgen. Prostate *29*: 350-355, 1996.

39 Eder IE, Haag P, Basik M, Mousses S, Bektic J, Bartsch G and Klocker H: Gene expression changes following androgen receptor elimination in LNCaP prostate cancer cells. Mol Carcin *37*: 181-191, 2003.

40 Hammacher A, Thompson EW and Williams ED: Interleukin-6 is a potent inducer of S100P, which is up-regulated in androgen-refractory and metastatic prostate cancer. Int J Biochem Cell Biol *37*: 442-450, 2005.

41 Albuquerque RLC, Miguel M, Costa AL and Souza LB: Correlation of c-erbB-2 and S-100 expression with the malignancy grading and anatomical site in oral squamous cell carcinoma. Int J Exp Path *84*: 259-265, 2003.

42 Gupta S, Hussain T, MacLennan GT, Fu P, Patel J and Mukhtar H: Differential expression of S100A2 and S100A4 during progression of human prostate adenocarcinoma. J Clin Oncol *21*: 106-112, 2003.

43 Moody TW, Mayr CA, Gillespie TJ and Davis TP: Neurotensin is Metabolized by Endogenous Proteases in Prostate Cancer Cell Lines. Peptides *19*: 253-258, 1998.

44 Sehgal I, Powers S, Huntley B, Powis G, Pittelkow M and Maihle NJ: Neurotensin is an autocrine tropic factor stimulated by androgen withdrawal in human prostate cancer. Proc Natl Acad Sci *91*: 4673-4677, 1994.

45 Zhang H, Dong Y, Zhao H, Brooks JD, Hawthorn L, Nowak N, Marshall JR, Gao AC and Ip C: Microarray data mining for potential selenium targets in chemoprevention of prostate cancer. Cancer Genom Proteom *2*: 97-114, 2005.

46 Luciani P, Ferruzzi P, Arnaldi G, Crescioli C, Benvenuti S, Nesi G, Valeri A, Greeve I, Serio M, Mannelli M and Peri AL: Expression of the novel adrenocorticotropin-responsive gene selective Alzheimer's disease indicator-1 in the normal adrenal cortex and in adrenocortical adenomas and carcinomas. J Clin Endocrinol Metab *89*: 1332-1339, 2004.

47 Fuller PJ, Alexiadis M, Jobling T and McNeilage J: Seladin-1/DHCR24 expression in normal ovary, ovarian epithelial and granulosa tumours. Clin Endocrinol (Oxf) *63*: 111-115, 2005.

48 Wu C, Miloslavskaya I, Demontis S, Maestro R and Galaktionov K: Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. Nature *432*: 640-645, 2004.

49 Waterham HR, Koster J, Romeijn GJ, Hennekam RC, Vreken P, Anderson HC, FitzPatrick DR, Kelley RI and Wanders RJ: Mutations in the 3beta-hydroxysterol Delta24-reductase gene cause desmosterolosis, an autosomal recessive disorder of cholesterol biosynthesis. Am J Hum Genet *69*: 685-694, 2001.

50 Zhang JS, Gong A, Cheville JC, Smith DI and Young CY: AGR2, an androgen-inducible secretory protein overexpressed in prostate cancer. Genes Chromosomes Cancer *43*: 249-259, 2005.

51 Tomonaga T, Matsushita K, Yamaguchi S, Oh-Ishi M, Kodera Y, Maeda T, Shimada H, Ochiai T and Nomura F: Identification of altered protein expression and post-translational modifications in primary colorectal cancer by using agarose two-dimensional gel electrophoresis. Clin Cancer Res *10*: 2007-2014, 2004.

52 Liu D, Rudland PS, Sibson DR, Platt-Higgins A and Barraclough R: Human homologue of cement gland protein, a novel metastasis inducer associated with breast carcinomas. Cancer Res *65*: 3796-3805, 2005.

53 Cunha GR, Hayward SW and Wang YZ: Role of stroma in carcinogenesis of the prostate. Differentiation *70(9-10)*: 473-485, 2002.

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