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Androgen-induced expression of endoplasmic reticulum (ER) stress response genes in prostate cancer cells

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Evaluations of androgen regulated gene (ARG) repertoire provide new insights into the androgen receptor (AR) mediated signaling at the transcriptional level. Definition of ARGs having critical functions in the biology of normal and malignant prostate should aid in identifying new bio-markers and therapeutic targets for prostate cancer (CaP). Using Affymetrix HuGene FL oligonucleotide arrays, temporal expression profiles of ARGs in widely used hormone responsive LNCaP cells, were analysed by hierarchical clustering methods and functional classification. ARGs in response to different androgen concentrations showed temporal co-regulation of genes involved in specific biochemical pathways. This study focuses on our new observations of the coordinated androgen induction of genes (NDRG1, PDIR, HERPUD1, ORP150) involved in the endoplasmic reticulum (ER) stress response pathway. Expression analysis of the two selected ER stress responsive genes, NDRG1 and HERPUD1 in primary CaPs revealed a significantly reduced tumor associated expression. Intriguing linkage of the androgen signaling to ER stress responsive genes, a protective response to protein unfolding or protein damage resulting from cellular stress signals, suggests that androgens may induce such stress signals in CaP cells. Decreased CaP associated expression of two ER stress responsive genes also suggests that possible abrogation of this pathway in prostate tumorigenesis.
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Keywords: prostate cancer; androgen regulated genes; gene chip; endoplasmic reticulum stress response

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Introduction

Since Huggins' seminal discovery showing that growth of the prostate gland depends on male sex hormones, androgen deprivation therapy has been the gold standard for the treatment of advanced prostate cancer (CaP) (Huggins and Hodges, 1941). However, the vast majority of the patients eventually relapse after a period of initial response to hormone therapy. Molecular mechanisms involved in the cell proliferation and differentiation of the prostate by androgen, as well as androgen ablation mediated prostate cell death and emergence of androgen independent cancers are under active investigations (Coffey, 1992; Hakimi *et al*., 1996; Augustus *et al*., 1999; Tindall and Scardino, 1999; Grossmann *et al*., 2001).

Biologic effects of androgen on target cells, e.g., prostatic epithelial are in part mediated by transcriptional regulation of ARGs by the androgen receptor (AR) (Coffey, 1992; Hakimi *et al*., 1996; Augustus *et al*., 1999; Tindall and Scardino, 1999; Grossmann *et al*., 2001). Gain of AR functions is believed to play some role in prostate tumorigenesis. Alterations of the AR gene by mutations in the hormone-binding domain have been reported in a subset advanced stage of CaP (Tilley *et al*., 1996; Grossmann *et al*., 2001). Amplifications of the AR gene in hormone refractory CaP represent yet another scenario where gain of AR functions may be associated with tumor progression (Visakorpi *et al*., 1995). Germ-line alterations of CAG repeat length in AR may influence the risk of CaP (Hakimi *et al*., 1996; Hardy *et al*., 1996; Stanford *et al*., 1997). Growth factors commonly involved in cell proliferation and tumorigenesis, e.g., IGF I, EGF and KGF have been shown to activate the transcription transactivation functions of the AR (Culig *et al*., 1994). Recent studies analysing expression of ARGs in hormone sensitive and refractory CWR22 nude mice xenograft model have also shown sustained expression of some ARGs in AR positive recurrent tumors following castration suggesting constitutive activation of AR signaling in these tumors (Nagabhushan *et al*.,

Table 1 Temporal expression profiles of androgen regulated genes in LNCaP

Accession	*gene*	*Function*	*0.1 nM treatment* 1 h	6 h	12 h	24 h	*10 nM treatment* 1 h	6 h	12 h	24 h
Up-regulated genes (>threefold)										
S39329	KLK2	Protease (prostate specific)	1.0	3.5	6.9	19	1.0	21	62	**111**
HT2351	KLK3	Protease (prostate specific)	−1.1	3.1	6.8	18	1.1	11	33	**83**
X07730	KLK3	Protease (prostate specific)	−1.0	2.4	5.0	17	1.5	10	16	**57**
HT2352	KLK3	Protease (prostate specific)	1.0	1.0	1.0	1.0	1.0	3.9	15	**36**
U80669	NKX3.1	Transcription	1.0	5.0	6.1	1.8	3.6	4.6	**20**	8.6
D87953	NDRG1	ER stress response	−1.0	1.3	1.5	1.5	1.2	3.8	8.3	**13**
D12686	EIF4G1	Translation	3.9	2.8	1.4	1.0	1.0	1.0	**12**	6.6
Z19002	ZNF145	Transcription	1.0	3.1	4.4	2.6	1.0	9.1	**10**	7.6
D87452	KIAA0263	Unknown	**9.3**	7.4	5.6	4.0	4.7	5.1	8.4	4.1
X02544	ORM1	Immune response	1.0	1.0	1.0	1.0	1.0	1.0	5.9	**8.4**
U62531	SLC4A2	Transporter	2.9	5.4	2.1	1.0	1.0	1.0	**7.9**	1.0
M31606	PHKG2	Metabolic enzyme (glycogen)	1.9	6.5	4.4	3.4	1.0	1.0	**7.6**	7.5
U87972	IDH3B	Energy conversion	4.0	1.0	1.0	1.0	**7.1**	1.6	1.0	1.0
X77567	INPP5A	Signal transduction	1.0	1.0	1.0	1.0	**6.6**	4.4	1.4	1.0
J02947	SOD3	Free radical scavenging enzyme	1.8	3.4	1.0	1.0	1.0	5.8	**6.2**	1.0
L29008	SORD	Catablic enzyme (sorbitol)	−1.4	−1.1	1.1	1.6	−1.2	1.5	2.6	**5.4**
S80437	FASN	Catabolic enzyme (fatty acid)	1.2	1.3	1.4	1.2	1.2	1.2	2.7	**5.2**
D55716	MCM7	DNA replication and repair	5.0	**5.0**	3.2	1.0	1.0	2.7	4.6	1.0
U75276	TAF3B2	Transcription	2.7	**5.0**	3.9	2.4	1.5	1.9	3.6	1.0
X90824	USF2	Transcription	2.8	**4.8**	2.2	−2.1	1.1	2.3	3.0	−2.1
U46569	AQP5	Channel	1.3	4.2	4.2	1.4	**4.5**	3.8	2.9	2.5
U02031	SREBP2	Transcription (lipogenesis)	1.2	3.0	2.0	1.3	−2.9	−1.1	3.1	**4.5**
M28211	RAB4	Exocytosis	1.4	1.7	2.2	1.5	1.5	2.7	**4.5**	3.8
M83667	CEBPD	Transcription	1.0	1.2	2.7	1.6	3.1	**4.4**	2.9	1.0
U37408	CTBP1	Transcription	2.8	2.5	1.3	1.3	**4.4**	3.5	−1.8	−5.2
S83365	Rab5-IP	Exocytosis	1.0	2.4	1.0	1.0	1.0	1.0	**4.4**	3.7
D25215	HERC3	Protein degradation	1.4	2.3	2.0	−1.3	1.0	3.4	**4.3**	3.4
X04828	GNAI2	Signal transduction	2.7	**4.2**	3.3	1.0	1.0	1.0	3.2	1.0
HT3317	NDUFA9	Energy conversion	1.5	1.5	2.3	1.0	1.0	1.0	**4.1**	1.0
U07919	ALDH6	Metabolic enzyme (alcohol)	−1.0	1.2	2.1	2.4	1.1	2.7	3.7	**4.0**
J05448	POLR2C	Transcription	2.1	3.5	3.7	1.0	1.0	1.0	**4.0**	1.0
L25270	SMCX	Unknown	1.4	**3.9**	1.6	1.0	1.0	1.0	3.2	**1.0**
M55210	LAMC1	Cell – ECM communication	1.3	1.7	2.4	1.3	1.4	2.5	**3.9**	2.7
X12433	EST	Unknown	1.3	1.9	1.7	2.9	2.9	3.1	3.9	**3.9**
X99961	EST	Unknown	1.0	1.0	1.0	1.0	1.0	1.0	**3.9**	3.0
L32976	MAP3K11	Signal transduction	**3.7**	3.1	2.2	1.1	1.0	1.0	3.4	3.6
D78335	UMPK	RNA and DNA synthesis	1.1	−1.0	−1.0	−1.0	−1.0	−1.0	2.0	**3.6**
L28821	MAM2A2	Catabolic enzyme (n-glycans)	**3.6**	2.5	2.5	1.0	1.0	1.0	2.7	1.0
D38583	S100A11	Signal transduction	1.4	1.7	1.8	1.2	1.3	1.4	2.3	**3.6**
D83782	SCAP	Protease (lipogenesis)	1.1	1.5	1.2	1.1	1.0	1.1	2.6	**3.5**
L22454	NRF1	Transcription	1.1	−1.2	−1.2	−1.2	2.9	**3.4**	2.1	2.1
U71300	SNAPc3	Transcription	2.0	2.1	**3.4**	1.9	1.6	1.8	2.1	1.1
U58658	EST	Unknown	1.0	**3.4**	1.3	1.0	1.0	1.0	1.0	1.0
D31886	RAB3GAP	Exocytosis	1.1	**3.4**	3.1	1.1	1.2	1.7	2.0	1.2
M24899	THRA2	Transcription	**3.3**	3.2	2.1	1.0	1.0	1.0	1.2	1.0
U73167	HYAL3	Metabolic enzyme (hyaluronic acid)	2.5	3.0	**3.2**	1.0	1.0	1.0	3.1	1.0
X61123	BTG1	Cell cycle	1.0	2.1	2.3	1.8	1.5	2.0	**3.2**	2.0
L31573	SUOX	Metabolic enzyme (sulfite)	1.5	3.1	2.5	1.5	1.8	**2.0**	**3.2**	2.3
U12778	ACADSB	Metabolic enzyme (fatty acid)	1.6	1.9	**3.1**	2.1	1.7	2.2	1.6	1.2
U09877	SKIV2L	DNA replication and repair	**3.1**	2.4	1.6	1.0	1.0	1.0	1.4	1.0
X78338	ABCC1	Transporter	−1.0	1.3	1.5	1.5	1.4	1.8	2.8	**3.1**
U76421	ADARB1	RNA-editing enzyme	1.3	2.0	2.6	1.3	2.4	2.6	**3.1**	2.4
D49490	PDIR	ER stress response	−6.0	−2.7	−1.4	1.2	−1.5	1.2	2.1	**3.1**
X99268	TWIST	Transcription	1.3	1.0	1.0	1.0	1.0	1.7	**3.0**	2.8
L38933	EST	Unknown	1.1	2.4	**3.0**	1.6	1.0	1.0	1.2	1.1
U53174	RAD9	DNA replication and repair	1.4	**3.0**	1.0	1.0	1.0	1.2	2.6	1.8
Down-regulated genes (<−threefold)										
X90846	MAP3K10	Signal transduction	**−9.5**	**−9.5**	−2.0	−1.9	−2.6	−2.7	−3.1	−1.4
Z25884	CLCN1	Channel	−3.9	−3.9	**−7.5**	**−7.5**	**−7.5**	**−7.5**	−4.2	−1.5
U63743	KNSl6	Cell cycle	**−6.5**	−2.6	−1.7	−1.4	**−6.5**	**−6.5**	−4.4	−3.4
HT3059	HLA-E	Immune response	−1.8	−2.0	−2.6	−2.2	−2.0	**−6.1**	−3.4	−2.3
D49490	PDIR	ER stress response	**−6.0**	−2.7	−1.4	1.2	−1.5	1.2	2.1	3.1
U09414	ZNF137	Transcription	**−5.6**	−2.5	−1.4	−1.0	−1.8	−1.7	−1.5	−1.5
D38437	PMS2L3	Unknown	**−5.3**	−3.9	−1.3	−1.1	−1.1	−1.8	−3.2	−2.4
X63629	CDH3	Cell–cell communication	−4.3	**−5.3**	**−5.3**	−3.0	**−5.3**	**−5.3**	**−5.3**	−1.6
U37408	CTBP1	Transcription	2.8	2.5	1.3	1.3	4.4	3.5	−1.8	**−5.2**

Continued

Table 1 (*Continued*)

Accession	gene	Function	0.1 nM treatment 1 h	6 h	12 h	24 h	10 nM treatment 1 h	6 h	12 h	24 h
HT2217	MUC3A	Extracellular matrix	−1.3	−1.8	−2.2	−1.4	**−4.9**	−4.1	−3.5	−1.2
X73113	MYBPC2	Cytoskeleton	**−4.9**	−1.8	−1.5	−1.0	−1.1	−1.3	−1.6	−1.1
X55005	THRA1	Transcription	−1.9	−3.0	−3.9	−2.7	−1.0	**−4.3**	−3.3	1.4
M15059	FCER2	Immune response	−2.5	**−4.1**	−3.0	−1.1	−1.5	−1.9	−2.0	1.2
X14766	GABARA1	Signal transduction	−2.1	−2.1	−2.5	−1.5	−1.7	−2.8	**−4.0**	−1.3
U17743	MAP2K4	Signal transduction	**−3.8**	−1.4	−1.3	−1.2	−1.4	−1.1	**−1.2**	−1.6
U58130	SLC12A1	Transporter	−2.4	−2.0	−1.7	−1.7	−1.4	−2.4	**−3.8**	1.2
X51804	EST	Unknown	**−3.8**	1.0	1.0	1.1	1.1	1.7	1.1	−1.1
D64110	BTG3	Cell cycle	−1.3	−1.1	1.0	1.1	−1.2	−1.3	−1.9	**−3.7**
HT3413	NF2	Cytoskeleton (tumor suppressor)	−1.0	**−3.7**	−2.7	−1.4	−1.1	−1.2	−2.6	1.5
M55422	H-plk	Transcription	**−3.7**	−2.3	−1.4	1.1	−1.0	−1.1	−1.6	−2.7
L10338	SCN1B	Channel	−3.1	**−3.6**	−2.4	−1.3	−1.3	−1.5	−2.5	−1.3
L20815	CDSN	Extracellular matrix	−3.5	−2.0	−2.0	−1.7	−1.5	−2.5	**−3.6**	−1.9
X99101	ESR2	Transcription	−2.2	−2.2	−3.4	−1.2	−1.1	−1.5	**−3.5**	1.3
M18737	GZMA	Apoptosis	−3.4	−3.1	−1.9	1.4	−1.5	−2.0	**−3.5**	1.2
Z11899	POU5F1	Transcription	−3.2	**−3.4**	−2.1	−1.3	−1.4	−2.2	−1.8	−1.4
S78187	CDC25B	Cell cycle	**−3.4**	−2.0	−1.7	−1.4	−1.7	−1.7	−2.0	−1.6
S50017	CNP	RNA metabolism	−1.4	−1.6	**−3.3**	−1.5	−1.3	−2.1	−1.3	−1.0
U77413	OGT	ER stress response	1.1	1.5	1.8	1.3	1.4	1.4	1.2	**−3.3**
D38491	KIAA0117	Unknown	**−3.3**	−2.1	1.2	1.4	1.4	1.2	1.1	−1.2
U11037	SEL1L	Signal transduction	**−3.3**	−2.5	−2.0	−1.1	−1.4	−1.6	−3.0	−1.8
U59423	MADH1	Signal transduction	1.3	1.2	1.1	−1.2	1.1	−1.3	−2.0	**−3.2**
M35878	IGFBP3	Signal transduction	−1.9	**−3.2**	−1.2	−1.1	2.8	3.0	−1.0	−1.7
X94232	MAPRE2	Cytoskeleton	1.2	−1.0	−1.1	−3.1	−1.0	−2.7	**−3.2**	−1.0
U03057	SNL	Cytoskeleton	−1.2	−3.0	−1.3	−1.2	−1.2	−1.2	**−3.2**	1.1
M17754	BN51T	Cell cycle	−1.4	**−3.1**	−2.0	−1.1	−3.1	−2.6	−1.2	2.1
D83646	MMP16	Protease	−1.3	−1.0	1.1	1.2	−1.5	−2.0	**−3.1**	−1.8
M69197	HPR	Immune response	−1.1	−1.4	−1.6	−1.2	−1.2	−1.3	−1.9	**−3.1**
M96859	DPP6	Protease	−2.7	−1.9	−1.8	1.0	−1.6	**−3.0**	**−3.0**	−1.0
J00212	IFNA21	Cytokine	−2.4	−2.6	**−3.0**	−1.2	−1.2	−1.5	**−3.0**	−1.2
L42374	PPP2R5B	Signal transduction	−1.0	−1.2	−1.2	−1.1	−1.0	−1.3	−2.1	**−3.0**
D17532	DDX6	RNA helicase	−2.1	**−3.0**	**−3.0**	−1.9	−2.5	−2.5	**−3.0**	−1.4
Y08265	EST	Unknown	**−3.0**	−2.6	−2.2	−1.3	−1.5	−2.7	−1.2	

1996; Amler *et al*., 2000; Mousses *et al*., 2002). Thus androgen regulation of critical target genes may play a role in normal prostate growth and prostate tumorigenesis.

Therefore, systematic and comprehensive analysis of the ARGs should provide the reporters for androgen signaling in CaP. These reporters may serve as potential biomarkers for predisposition/initiation/progression of prostate cancers (Xu *et al*., 2001; Vaarala *et al*., 2000; Amler *et al*., 2000). Towards this aim, we are analysing the ARG repertoire in prostate cancer cells using high-throughput approaches and evaluating their significance in human CaP (Xu *et al*., 2000a, 2001). The objective of this study is to define biochemical pathway(s) regulated by androgen signaling by analysing coordinated expression of genes linked to specific pathway(s) and further evaluate the role of such pathway(s) in human prostate tumorigenesis. We have determined the temporal expression profiles of ARGs in widely used hormone responsive LNCaP cells (Horoszewicz *et al*., 1983). Our results show coordinated regulation of ARGs in distinct and specific biochemical pathways and this report focuses on our new observations on the coordinated regulation of genes in ER stress response. Further, significantly decreased expression of two selected ER stress responsive genes, NDRG1 and HERPUD1, were noted in cancer cells when laser capture microdissected (LCM) paired normal and cancer cells from prostate cancer patients were analysed. Abrogation of regulation of ER stress responsive genes, e.g., NDRG1 and HERPUD1 may contribute in prostate tumorigenesis.

Results

Androgen regulated gene repertoire as a reporter for probing androgen signaling in LNCaP cells

We analysed the temporal gene expression profiles in LNCaP cells in response to 0.1 nM and 10 nM synthetic androgen R1881 between the 0–24 h time points. LNCaP cells were chosen for this study, because of consistent androgen responsiveness of this cell line in luciferase-reporter assays, when compared with other AR positive immortalized normal epithelial cells or cancer cells (T Segawa and S Srivastava, unpublished) (Segawa *et al*., 1998). Although LNCaP cells harbor mutant ARs these cells are the widely used prostate cancer cell culture model for hormonal regulation until better experimental models are available and validated. When we applied the arbitrary cutoffs of >threefold induction or <threefold repression over the control level of expression in at least one of the eight androgen treatment conditions (1, 6, 12, and 24 h for 0.1 and 10 nM R1881 treatments), 241 up-regulated genes and 341 down-regulated genes qualified as potential ARGs

out of ~3500 expressed transcripts in LNCaP as determined by this GeneChip. Evaluation of ARG profiles in LNCaP cells, showing a pattern of increase/decrease expression over a time period, revealed up-regulation of 56 genes and down-regulation of 42 genes showed (Table 1). Additional data for ARGs can be obtained from our web site, www.cpdr.org/LNCaP/GeneChip. As noted in Table 1, the expression changes of most ARGs were of higher magnitude in 10 nM than in 0.1 nM. Of the known ARGs in the context of prostate, human kallikrein2 (KLK2), Prostate Specific Antigen (PSA or KLK3), and NKX3.1 were among the highly induced genes. Clusterin and PSMA were among the down-regulated genes. These results served to validate the evaluation of ARGs using high-density oligonucleotide array system.

The ARG repertoire identified in this study is anticipated to include both direct as well as indirect targets of AR. Understanding of direct and indirect AR targets is critical as both may influence the overall biologic functions of AR. Nonetheless, preliminary attempts were made to sort out direct AR directs using computational and kinetic approaches, which of course will require future experimental validations. Since genes directly regulated by the AR are expected to be more critical in androgen mediated cell signaling, we have evaluated the presence of cis-regulatory elements in the 5′ promoter regions of ARGs. Consensus DNA sequences to which AR binds (androgen responsive element, ARE), are already characterized. We searched for potential ARE sequences in 5′ promoter regions of ARGs (Claessens *et al*., 2001). For this purpose, we selected early androgen-induced transcription regulators with the assumption that these were more likely to be direct targets of AR. As in Table 1, numerous transcription regulators were found among the early-induced ARGs, which may represent early events of AR signaling, that is, AR-Network. Indeed, a number of early-induced ARGs do contain potential ARE sequences in their 5′ promoter regions (Table 2). Further, experimental documentation for potentially new direct AR targets will be needed.

Temporal expression profile in response to androgen treatments of LNCaP cells revealed coordinated expression changes of functionally related genes

Previous results of microarray experiments have shown that genes with coordinated expressions might have a distinct biological function in common (Iyer *et al*., 1999; Fambrough *et al*., 1999). We applied hierarchical clustering methods to group the ARGs solely based on the expression patterns (Eisen *et al*., 1998) (Figure 1). Functional information of the genes was retrieved with the aid of internet-based gene repository databases, GeneCard, UniGene, and OMIM. The analysis was performed in an unbiased manner. Here we identified two biochemical pathways previously shown to be androgen regulated and one novel androgen regulated pathway i.e., ER stress response.

Table 2 ARE-like sequences in promoter region of ARGs

*Gene**	*Consensus ARE (GGTACAnnnTGTTCT)*	*Position***
KLK3	AGAACAgcaAGTGCT	−0.2 kb
	GGATCAgggAGTCTC	−0.4 kb
	GGAACAtatTGTATC	−4.1 kb
KLK2	GGAACAgcaAGTGCT	−0.2 kb
CEBPD	AAAACAattTGTGCT	−1.9 kb
NKX3.1	AATACAcccTGTTCC	−3.5 kb
	AATAGAttgTGTATT	−3.3 kb
NRF1	GGTATTgctTGTACC	−2.3 kb
POLR2C	AGTACAaacAGTACT	−0.3 kb
SNAPC3	GGAACGgtcTGTGTC	−4.2 kb
TAF3B2	GGAACAcatTGAACC	−1.5 kb
	GGTTCAagtGGTTCT	−1.1 kb
THRA	AGAACAggcTGTTCC	−3.1 kb
SCAP	TGCACAtgtTGTTCT	−0.1 kb
	AGAGCAgagAGTTCT	−0.7 kb
SREBP2	AGTACAaatTGTTTT	−1.6 kb
	AGAACTgctTGAACC	−2.4 kb

*See discussion for gene selection. **Relative to transcription start site. AREs for KLK2 and KLK3 genes have been documented earlier, whereas AREs for other genes suggested here need further experimental validation

Coordinated regulation of genes involved in fatty acid and cholesterol biosynthesis by androgen Coordinated regulation of expression of genes involved in cholesterol and fatty acid synthesis were identified in response to androgen (Figure 1). In agreement with recent reports (Swinnen *et al*., 1997; Heemers *et al*., 2001), many genes involved in fatty acid and cholesterol synthesis: fatty acid synthase (FASN), fatty acid amide hydrolase (FAAH), low density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), and farnesyl-diphosphate farnesyltransferase (FDFT1) were all coordinately up-regulated in response to 10 nM R1881 and to a lesser extent with 0.1 nM R1881. Up-regulation of these genes is believed to be mediated through androgen dependent activation of SREBPs (Heemers *et al*., 2001). In our study androgen also induced expression of SREBP2 as well as SCAP (KIAA0199), which is a chaperone protein, that transports SREBPs from endoplasmic reticulum to Golgi to facilitate the activation of SREBPs. Northern blot and RT–PCR analyses confirmed our GeneChip observations for androgen induction of SREBP2 and SCAP (Figure 1d,e). This finding, as well as the presence of ARE-like sequences in the promoter regions of both SCAP and SREBP2, suggest a potentially direct activation of SREBP2-FASN pathway by androgen (Table 2).

Androgen responsive coordinated regulation of genes associated with polyamine biosynthesis Another expression profile successfully defined by us was coordinated androgen regulation of genes involved in polyamine biosynthesis/metabolism. Although some of these genes were already reported as ARGs, (Fjosne *et al*., 1990) it is striking to note that all five genes coding for enzymes which regulate polyamine synthesis: ornithine decarboxylase 1 (ODC1), S-adenosylmethio-

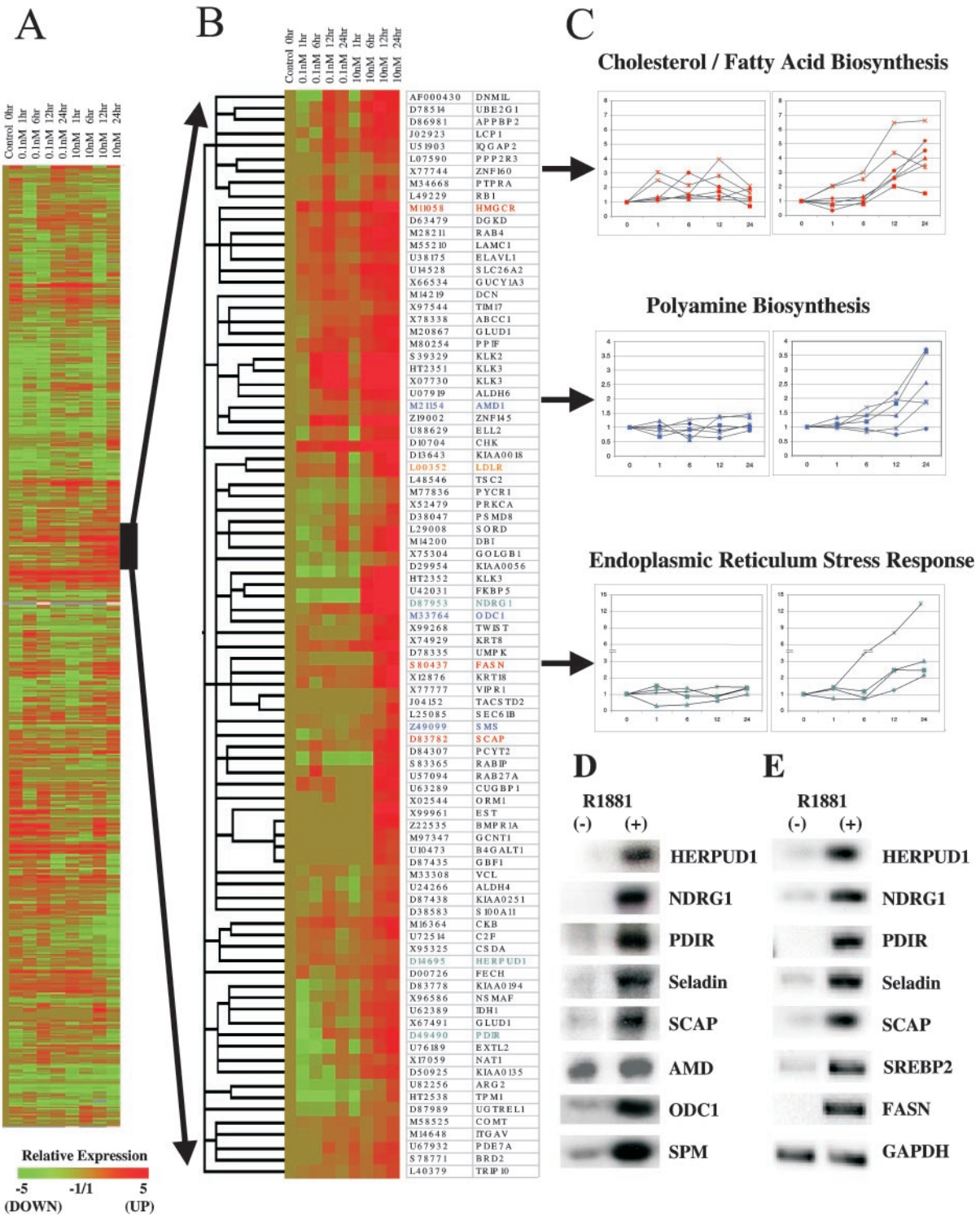


Figure 1 Coordinated gene expression patterns define biochemical pathways in response to androgen signaling in prostate cancer cells. (**a**) Hierarchical clustering using log-transformed relative expression changes over the control condition for all genes called 'Present' by Affymetrix algorithm. Each row and column represents genes and R1881 treatment conditions, respectively, with the representative colors of up-regulation (red), or down-regulation (green). (**b**) Magnified view of a part of the clusters with androgen up-regulated genes. GeneBank accession numbers and abbreviated gene names were shown. Note that genes on the same biochemical pathways were enriched in this cluster: Cholesterol/Fatty acid biosynthesis pathway (in red), Polyamine biosynthesis pathway (in blue), and ER-stress response pathway (in green). (**c**) Coordinated temporal expression changes of the genes on three defined pathways in response to 0.1 nM (left) or 10 nM (right) R1881 treatments: Cholesterol/Fatty acid biosynthesis pathway (■FAAH, ●SREBP2, ◆FASN, ▲LDLR, xHMGCR, *FDFT1, +SCAP, in red), Polyamine biosynthesis pathway (■SMS, ◆ODC1, ▲SAT, xAMD1, *SRM, ●Antizyme, in blue), and ER-stress response pathway (■HERPUD1, ◆ORP150, ▲PDIR, xNDRG1, in green). (**d**) Confirmation of androgen regulation for selected ARGs by Northern blot analysis. (**e**) Confirmation of androgen regulation for selected ARGs by RT–PCR assay

nine decarboxylase 1 (AMD1), spermine synthase (SMS), spermidine synthase (SRM), and spermidine/spermine N1-acetyltransferase (SAT) showed very similar expression patterns and therefore clustered with each other (Figure 1). On the contrary, ornithine decarboxylase antizyme (OAZ), a key molecule known to negatively regulate the accumulation of polyamines in the cells, did not show any expression changes.

Androgen induction of ER stress response genes ER is optimized for synthesizing, folding, and assembling membrane and soluble proteins destined for secretion or trafficking to lysosomes. Environmental changes leading to the accumulation of unfolded or misfolded proteins in the ER constitute a fundamental threat to the cells and therefore trigger a stress response, referred to as the ER stress response. One aspect of the ER stress response is defined as unfolded protein response (UPR) that shows transcriptional induction of genes encoding ER-resident molecular chaperones and folding enzymes (Mori, 2000). As shown in Table 1 and Figure 1 many genes having functions of chaperones and folding enzymes are among ARGs. Of note, ORP150, PDIR, HERPUD1 and NDRG1 known to be involved in ER stress responses were induced by androgen (Figure 1d,e; www.cpdr.org/LNCaP/GeneChip). Oxygen regulated protein 150 kD (ORP150) is an ER-resident molecular chaperone, which suppresses hypoxia-induced apoptotic cell death (Ozawa *et al*., 1999). Protein disulfide isomerase-related (PDIR) is a stress inducible gene with a thioredoxin-like domain, which acts as a catalyst of protein folding in the lumen of the ER (Kanai *et al*., 1998). Besides these typical UPR induced genes, HERPUD1 (KIAA0025), and N-myc downstream regulated gene 1 (NDRG1), which were originally cloned as ER-stress induced genes in HUVEC cells, are also among the ARGs in LNCaP (Kokame *et al*., 1996). An earlier report also showed marked upregulation of NDRG1 in response to androgen (Ulrix *et al*., 1999). In addition to genes involved in ER stress response, number of stress induced genes were among the ARGs (Figure 1). Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA) is a pro-apoptotic gene, which plays a central role in steroid induced anti-inflammation by inactivating NF-kappa-B pathway, which also constitutes a part of ER stress response (Mori, 2000). Cold shock domain protein A (CSDA) is a member of transcription factors with a cold shock domain, which have been implicated in various cellular processes, including adaptation to low temperatures, and nutrient stress (Graumann and Marahiel, 1998). Seladin-1 (KIAA0018) is a recently characterized gene whose expression protected neuron cells from apoptosis caused by oxidative stress (Greeve *et al*., 2000).

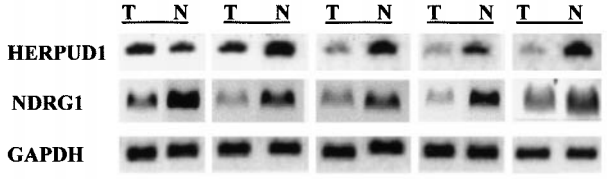
Decreased expressions of ER stress response genes (HERPUD1 and NDRG1) in prostate cancer

To evaluate the modulation of ER stress response genes in CaP, expressions of selected genes (HERPUD1 and NDRG1) were analysed in a test panel of LCM derived paired normal and tumor cells of 20 CaP patients (Figure 2). Decreased expressions of two ARGs in this ER stress response pathway correlated very well with malignant status of the prostate. CaP associated decreased expressions of HERPUD1 and NDRG1 were found in 60 and 70% patient specimens respectively. Prostate cancer associated decreased or lack expression of NDRG1 has been described earlier (Kurdistani *et al*., 1998), however, cancer associated loss of HERPUD1 has not been reported before.

Discussion

In an attempt to define biochemical pathways regulated by androgen signaling in CaP cells, we have analysed temporal gene expression profiles of ARGs in hormone responsive LNCaP cells using Affymetrix oligonucleotide arrays. A proposed model of the AR transcriptional net work derived from this study and other studies is summarized in Figure 3. Our study defined consistent androgen dependent expression changes in groups of genes linking to fatty acid and cholesterol synthesis, and polyamine biosynthesis. Gene expression alterations of many genes in these pathways have been previously noted in CaP (Myers *et al*., 2001; Pizer *et al*., 2001; Epstein *et al*., 1995; Mohan *et al*., 1999; Bettuzzi *et al*., 2000). Evaluation of ARG repertoire from LNCaP cells have also led to identification of novel ARGs, e.g., PART1 (Lin *et al*., 2000), PSDR1 (Lin *et al*., 2001) and PMEPA1 (Xu *et al*., 2000a). Novel observations from this study underscore the androgen modulation of expression of genes in ER stress response and possibly other stress responses. We provide preliminary evidence that two genes involved in ER stress response are down-regulated in CaP.

Among the pathways successfully defined in our study was the fatty acid and cholesterol synthesis



	T<N	T=N	T>N
HERPUD1	12 (60%)	6 (30%)	2 (10%)
NDRG1	14 (70%)	3 (15%)	3 (15%)

Figure 2 Decreased expressions of ARGs on ER stress response pathway (NDRG1 and HERPUD1) in prostate cancers. (**a**) RT–PCR results of NDRG1 and HERPUD1 for five representative matched tumor and normal prostate specimens obtained by LCM. GAPDH serves as an internal control of the input. (**b**) Summary for the expressions of NDRG1 and HERPUD1 in matched tumor and normal prostate specimens

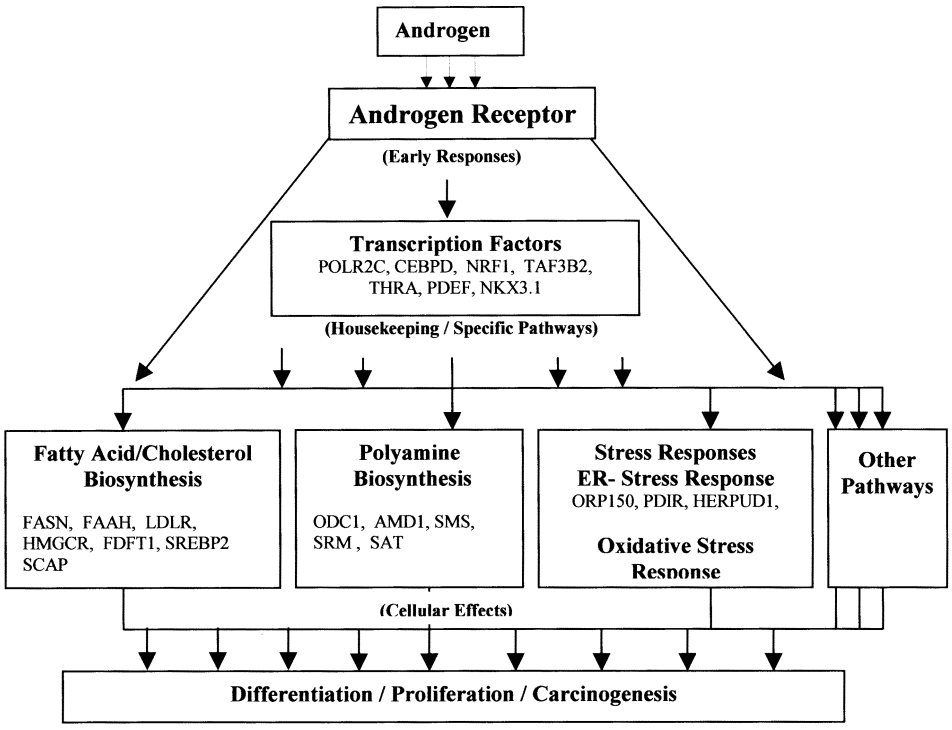


Figure 3 A proposed model of the AR transcriptional net work based on this study and previous reports. Additional experimental validation of new pathways defined here will be needed

pathway. Recently many reports have shown a correlation between FASN overexpression and CaP progression (Myers *et al*., 2001; Pizer *et al*., 2001; Epstein *et al*., 1995). According to these reports, FASN expressions were higher in CaP compared to the normal prostate, and served as a predictor for cancer progression (Epstein *et al*., 1995). While FASN expression is decreased in the normal prostate upon androgen ablation, cancer specimens from patients undergoing androgen ablation showed elevated expression (Pizer *et al*., 2001). We also noted FASN overexpression during androgen independent growth of CWR22 and LNCaP cells, supporting constitutive activation of AR-SREBPs-FASN pathway under androgen withdrawal condition (Myers *et al*., 2001, Segawa T and Srivastava S, unpublished).

Polyamine biosynthesis and metabolism pathways are of physiologic significance in the prostate and prostate cancer, because the amounts of polyamines are highest in the prostate among other organs (Crozat *et al*., 1992). An earlier report had identified spermine as an endogeneous inhibitor of prostate carcinoma cell growth (Smith *et al*., 1995). Spermine was also shown to induce growth arrest in Dunning rat prostate cancer cell lines (Koike *et al*., 1999). In contrast, recent reports have shown positive correlation between CaP progression and the expression of ODC1, AMD, SAT and OAZ (Mohan *et al*., 1999; Bettuzzi *et al*., 2000). Specific ODC1 inhibitor, difluoromethylornithine (DFMO), is being used for chemoprevention or therapeutic trials for CaP (Messing *et al*., 1999; Simoneau *et al*., 2001). Our study emphasizes the androgen regulation of multiple genes in polyamine biosynthesis and metabolism pathways in LNCaP cells. Further studies are warranted to fully understand the role of this pathway in the growth of normal and malignant prostatic epithelial cells.

Androgen modulation of genes involved in the endoplasmic reticulum (ER) stress pathway highlights novel observations of this study. Recently, Travers *et al*. (2000) have reported genome wide analyses of transcriptional responses to ER stress induced by reducing agent dithiothreitol (DTT) and N-linked glycosylation inhibitor tunicamycin in yeast using the high-density oligonucleotide array technology. Surprisingly, their data revealed that many other aspects of secretory pathway functions were also affected by the UPR (Travers *et al*., 2000). These pathways include protein translocation, glycosylation, folding, degradation in ER as well as vesicle trafficking and transport between ER and Golgi to distal secretions. Interestingly, activation of lipid biosynthesis and metabolism seen in our androgen treatment experiments also constitutes a component of UPR under their experimental condition (Travers *et al*., 2000). Furthermore, many genes involved in vesicle trafficking and transport such as Rab proteins are indeed found in our ARGs list (Table 1). Besides the data from LNCaP model, correlation of NDRG1 and HERPUD1 expression with malignant status of the prostate in CaP patients further suggest the involvement of this ER stress pathway in prostate tumorigenesis. NDRG1 is a

member of highly conserved proteins, whose expression is induced by stress responses involving UPR, such as reducing agents, DNA damaging agents and hypoxia (Kokame *et al*., 2000; Park *et al*., 2000). Over expression of NDRG1 in tumor cells decreases the proliferation rate, enhances differentiation, and suppresses the metastatic potency of the cancer cell (Kurdistani *et al*., 1998; van Belzen *et al*., 1997; Guan *et al*., 2000). Androgen induced expression of NDRG1 may represent a response to stress as well as differentiation signals. Like NDRG1, HERPUD1 is also modulated by ER stress response, however little is known about its function. HERPUD1 is a membrane associated ER protein with a ubiquitin-like domain, with homology to DNA repair protein Rad23A (Ulrix *et al*., 1999; van Laar *et al*., 2000). Its induction was also observed in response to other stresses such as DNA damaging agents and osmotic shocks (Ulrix *et al*., 1999).

Although ER stress response appears to be a cellular protective mechanism, there are also studies that suggest mitogenic signals promoting new protein synthesis may also recruit ER stress response proteins (Brewer *et al*., 1997). Thus effect of androgen on this pathway may represent a more complex phenomenon. Biologic implications or the mechanisms of androgen induction of stress response genes including ER stress remains to be defined. Androgen induced oxidative stress in LNCaP cells was originally reported by Ripple *et al*. (1997) and has also been suggested by analysis of ARGs by Xu *et al*. (2001). Stress response induced by androgen possibly reflects a protective response to cope with adverse cellular affects of androgens. Linkage of androgen to cellular stress responses may provide molecular explanation for some of the carcinogenic effects of androgen. Androgens are also reported to induce CaP in rat models (Noble, 1977; Pollard, 1998). Further, men with higher serum androgen show a positive correlation with the risk of CaP (Ross *et al*., 1986). Taken together, androgens, in certain biologic setting may unleash carcinogenic/mutagenic properties. Androgen mediated induction of stress responsive genes including ER stress response may reflect protective functions against androgen induced cellular stress. Reduced/loss of expression of such genes in cancer cells may support pro-carcinogenic effects of androgen. Further studies of ARGs involved in stress response pathways may clarify the mechanisms of AR signaling in cellular stress responses including the ER stress response in CaP cells.

Materials and methods

Cell culture and RNA preparation

Androgen dependent prostate cancer cells: LNCaP were obtained from ATCC (Rockville, MD, USA) and maintained in RPMI1640 with 10% fetal bovine serum (FBS, GibcoBRL, Rockville, MD, USA). For the analyses of temporal expression profiles, cells were cultured in RPMI1640 with charcoal/dextran stripped FBS (cFBS, Gemini Bio-Products, Calabasas, CA, USA) for 5 days, and then treated with 0.1 or 10 nM of synthetic androgen R1881 (NEN, Boston, MA, USA). Poly(A)$^+$ RNAs were prepared at 0, 1, 6, 12, and 24 h after the treatments with FastTrack RNA preparation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Oligonucleotide array analyses

The high-density oligonucleotide array (GeneChip HuGeneFL array; Affymetrix, Santa Clara, CA, USA) represents approximately 5600 human genes/transcripts with some redundancy, roughly one half correspond to known full length genes and the other half correspond to expressed sequence tags (EST) or hypothetical genes. The annotations of genes were regularly updated from EST or hypothetical protein to known genes according to the GeneBank information. Biotin-labeled cRNA was synthesized from 2 microgram of poly(A)$^+$ RNA and hybridized to this high-density oligonucleotide as described previously (Nau *et al*., 2000). Briefly, double stranded cDNA was synthesized using Superscript Choice System (GibcoBRL, Rockville, MD, USA) and reverse transcription primer T7-(dT)24 (GENESET Corp., LaJolla, CA, USA). Biotin-labeled cRNA was synthesized from the cDNA with biotinylated UTP and CTP (ENZO, Farmingdale, NY, USA) using MEGAscript T7 *in vitro* Transcription Kit (Ambion, Austin, TX, USA). The biotin-labeled cRNA was purified using RNeasy spin column (Qiagen, Valencia, CA, USA). Biotinylated cRNA was fragmented, assessed by gel electrophoresis, and placed in a hybridization mixture containing four biotinylated hybridization controls. After 16 h hybridization, GeneChips were washed, stained, and then scanned with GeneArray Scanner (Hewlett-Packard, Santa Clara, CA, USA). The images from the scanned chips were processed using Affymetrix GeneChip Analysis Suite 4.0. The image from each GeneChip was scaled such that the average intensity value for all arrays was adjusted to arbitrary value of 2500, to reliably compare variable multiple arrays. Scaled average difference value (SADV) and Absolute Call data from each GeneChip were exported as flat text files and used for further analysis.

Bioinformatics analyses

GeneSpring version 4.0 (Silicon Genetics, San Carlos, CA, USA) and Excel 2000 (Microsoft, Redmond, WA, USA) were used to derive global trends in expression profiles. In the temporal expression comparisons, ~3500 genes scored as 'Present' in at least one time point were considered. All the imported SADV were divided by the SADV of control condition (0, no treatment) in GeneSpring software, and factor of fold increase or decrease ('fold changes') was determined. SADV values of 500 or less were set to 500 to avoid division by 0 or a negative number. By GeneSpring software, the 'fold changes' were log transformed and hierarchical clustering was performed on ~3500 'Present' genes with Pearson correlation, as initially described by Eisen *et al*. (1998).

Information on functions of the genes was retrieved with the aid of internet-based gene repository databases: GeneCard, UniGene, and OMIM, integrated to GeneSpring software (http://bioinformatics.weizmann.ac.il/cards/; http://www.ncbi.nlm.nih.gov/UniGene/; http://www.ncbi.nlm.nih.gov/Omim/). The linkage of ARGs to biochemical pathways was evaluated in an unbiased manner without a preconceived notion. On the basis of data obtained, decision was made to

follow up on pathway not previously described as androgen regulated. For the prediction of candidate androgen responsive element (ARE) in the promoter regions of ARGs, about 5 kb 5′ sequences of ARGs were obtained through public databases, and homologous sequences for consensus ARE, GGTACAnnnTGTTCT, were searched using GeneQuest 4.0 software (Dnastar, Madison, WI, USA).

Northern blot analysis

LNCaP cells were cultured in RPMI1640 with 10% FBS for 5 days and then stimulated with R1881 at 0.1 or 10 nM for 24 h. Total RNA (10 μg/lane) or poly(A)$^+$ RNA (1 μg/lane) prepared from these cells was electrophoresed through 1% formaldehyde-agarose gel and transferred to a nylon membrane. cDNA probes of representative ARGs were labeled with ^{32}P-dCTP, and Northern hybridization was performed as described before (Xu *et al*., 2000b).

Prostate tissue specimens and laser capture microdissection (LCM)

Matched prostate cancer and normal glands were derived from radical prostatectomy specimens from prostate cancer patients treated at Walter Reed Army Medical Center (Segawa *et al*., 1998). In brief, specimens were obtained by *ex vivo* sextant biopsy of prostates after radical prostatectomy in the operating room, and Tissue-tek OCT-embedded frozen sections were prepared and archived (Miles Inc, Elkhart, IN, USA). One set of slides was stained with H&E to define tumor cells. The frozen sections on slides were dissected using laser captured microdissection according to the modified protocol of the manufacturer (Arcturus Engineering, Mountain View, CA, USA). Total RNA was isolated using MicroRNA kit (StrataGene, La Jolla, CA, USA) and quantified with VersaFluor fluorimeter (BioRad, Hercules, CA, USA) using RiboGreen (Molecular Probes, Eugene, OR, USA).

Reverse transcription-polymerase chain reaction (RT–PCR) assays

Total RNA (1 nanogram) was reverse transcribed into cDNA with a RT–PCR kit (Perkin-Elmer, Foster, CA, USA) and 1/25 of reverse transcribed product was used for PCR to amplify NDRG1, HERPUD1, and a housekeeping gene, GAPDH. The primer sequences for the amplifications were 5′-CTGCACCTGTTCATCAATGCCTAC-3′ : 5′-TGCCATCCAGAGAAGTGACGCTG-3′ for NDRG1, and 5′-GAATGCTGCTCCTCAAGTGGTTG-3′ : 5′-CAGTTTCAGGATCAGTGCCTTCC-3′ for HERPUD1. The optimized PCR conditions for amplifying NDRG1 and HERPUD1 were 38–40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s. The expression of GAPDH was used as an internal control for RNA input. Other controls for the RT–PCR assays included PCR amplification of the RNA samples without reverse transcription. The computer-stored images of agarose gels were analysed by densitometry of the bands. Expression was quantified as fold differences in the normalized expression between tumor (T) and normal (N) tissues with a cutoff of twofold difference. Expressions of representative ARGs were analysed with the same method using LNCaP RNAs with or without R1881 10 nM 24 h treatment.

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