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CHARACTERIZATION OF ANDROGEN RECEPTOR AND NUCLEAR RECEPTOR CO-REGULATOR EXPRESSION IN HUMAN BREAST CANCER CELL LINES EXHIBITING DIFFERENTIAL REGULATION OF KALLIKREINS 2 AND 3

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Accumulating evidence indicates that androgens and the androgen receptor modulate the development and progression of breast adenocarcinoma; however, the precise role and actions remain poorly defined. We examined previously the steroid hormone regulation of 2 known androgen-regulated kallikreins, *KLK3* (encoding PSA) and *KLK2* (encoding human kallikrein 2 or hK2) in BT-474, T-47D, ZR75-1, MCF-7, MFM-223 and BT-20 human breast cancer cells and found that they were differentially regulated, with the cells showing variable responses to androgen. To determine if this variable response was reflected by differences in androgen receptor, we characterized the expression of androgen receptor in these cells by Western blot analysis and saturation binding analysis. In addition, we sequenced androgen receptor cDNA from each of these cell lines to check whether any androgen receptor mutations were present. The expression of 11 nuclear receptor co-regulatory factors (SRC-1, AIB1, ARA24, ARA54, ARA55, ARA70, ARA160, FHL2, PDEF, NCoR1, SMRT) was compared in these cell lines by semi-quantitative RT-PCR to determine if the pattern of receptor co-activators or -repressors expressed in these cells might explain the differential regulation of *KLK2* and *KLK3*. The levels of androgen receptor varied among the cell lines, but did not correlate with hK2 and PSA secretion determined previously. No mutations within the coding regions of the receptor were detected. With the exception of receptor expressed by MCF-7 cells, the polymorphic CAG repeat length was in the normal range. Every breast cancer cell line exhibited a distinct expression pattern of the nuclear receptor co-regulators examined raising the possibility that the relative levels of these co-activators/-repressors might differentially modulate androgen receptor transcriptional activity within the promoter/enhancer region of *KLK2* and *KLK3* of these cells.

Key words: *androgen receptor; co-activators; co-repressors; prostate-specific antigen; human glandular kallikrein; breast cancer*

Prostate specific-antigen (*KLK3* or *PSA*) and human glandular kallikrein (*KLK2*) are 2 androgen regulated members[1,2] of the human kallikrein gene family[3] of serine proteases. The serum PSA level is a well-established indicator for the detection of prostate cancer[4] and for monitoring disease progression. The potential value of hK2, the protein product of *KLK2*, as a further indicator is strongly suggested by recent studies.[5–7] Although both kallikreins are highly expressed in prostate, they have been detected in other tissues as well, particularly in female breast and breast cancer cells.[8–10]

The prognostic and biological role of PSA in breast cancer is not fully known; however, several studies indicate a correlation between PSA levels and early stage disease and longer relapse-free survival,[11–13] suggesting PSA is a positive prognostic indicator. Recent studies suggest that PSA may function as an endogenous antiangiogenic protein,[14] perhaps by converting plasminogen to biologically active angiostatin-like fragments.[15] PSA levels in recurrent disease correlate with a poor response to tamoxifen therapy and a shorter overall survival period.[16] hK2, which acts to cleave the proPSA zymogen and thus liberate active PSA, has also been detected in breast tumour cytosols, where its expression was directly proportional to PSA as well as to estrogen and progestin receptor levels.[17] Further study into the regulation of *KLK2* and *KLK3* expression and function are necessary to clarify their role in the pathophysiology of breast cancer and to better define their utility as prognostic indicators of this disease.

Androgen receptor (AR) expression is frequently found in primary breast cancer tumors,[18–20] but the precise role of androgen in this disease is not well established. Recent findings suggest that breast cancer cell lines may differently respond to androgen treatment, with proliferation stimulated in MCF-7 and MDA-MB-453 cells and inhibited in T-47D, ZR-75-1[21] and MFM-223[22] cells. Despite the divergence of androgen effects, these results are consistent with an involvement of androgen in regulating breast cancer cell growth via an interaction with the AR.

We examined a number of androgen receptor positive human breast cancer cell lines for expression of *KLK2* and *KLK3* mRNA and protein.[23] Significant amounts of both kallikreins were secreted by BT-474, T-47D and MFM-223 breast cancer cells after androgen stimulation, but not by MCF-7, ZR-75-1 or MDA-MB-453 cells. BT-474 cells produced 500–1,000-fold more hK2 than T-47D cells. Unlike T-47D and MFM-223 cells, BT-474 cells produced much more hK2 than PSA. The differential expression of these kallikreins in only certain cell lines might reflect differences in androgen receptor function or signaling in these cells, the elucidation of which could shed some light on the molecular mechanisms underlying the role of androgen as well as kallikreins in breast cancer.

Two of the most common factors that might modulate androgen receptor function are the cellular levels of the receptor and mutations within the receptor coding sequence that affect receptor interactions and function. In addition, it is now clear that transcriptional control by steroid receptors is a multistep process involving a diversity of nuclear receptor co-regulatory proteins (co-activators and co-repressors) that the activated receptor recruits to the gene promoter region.[24] The activity of a steroid receptor in a particular cell line thus depends not only on the expression levels of the

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receptor itself, but may also be influenced by which co-regulatory proteins are expressed.

We compare the expression of androgen receptor in 6 human breast cancer cell lines (BT-474, ZR-75-1, T-47D, MCF-7, MFM-223 and BT-20) showing differential regulation of *KLK2* and *KLK3* by androgen. In addition, cDNA prepared from androgen receptor mRNA from these cells was sequenced to establish whether mutations in the coding region are present. Lastly, the expression of 11 nuclear receptor co-regulatory factors was compared in these cell lines to determine if the pattern of receptor co-activators or -repressors expressed in these cells might explain the differential regulation of *KLK2* and *KLK3*.

MATERIAL AND METHODS

Cell culture

All breast cancer cell lines were purchased from American Type Culture Collection with the exception of MFM-223 cells, which were a gift from Dr. R. Hackenberg (Philipps University, Marburg, Germany). All cells were maintained in DMEM (without phenol red) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere, with the exception of T-47D cells, which were grown in RPMI 1640 with the addition of 0.25% insulin. Cells were plated in medium supplemented with FBS treated with charcoal to remove steroids, 48 hr before beginning experiments.

Western blot analysis

Cytosol extracts were prepared as described previously[25] and the total protein concentration was determined by the Bradford method (Pierce, IL). Aliquots of extract (10 μg protein) were run on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) and transferred to Hybond-C Extra membrane (Amersham Life Sciences, Piscataway, NJ), which was incubated overnight with blocking solution (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4, 0.2% Tween 20 and 5% powdered skim milk) at 4°C. After washing, the membrane was incubated for 1 hr at room temperature with 1 μg/ml PAR-1 affinity-purified polyclonal antibody to the human androgen receptor,[26] followed by a 1-hr incubation with horseradish peroxidase-labeled goat anti-rabbit antiserum (Kirkegaard Perry Laboratories, Gaithersburg, MD) diluted 1:10,000 in blocking solution. Immunoreactive bands were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL) and exposure of the membrane to Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ) according to kit instructions.

Ligand binding analysis of the AR in cytosols

Cytosols extracted from cell lines were used in saturation ligand binding analyses as described previously.[25] Briefly, aliquots of the cytosol preparations were incubated with varying concentrations of [^{3}H-methyl]trienolone ([^{3}H]R1881; New England Nuclear, Boston, MA), in the presence or absence of 1 μM dihydrotestosterone (Sigma Chemical Co., St. Louis, MO). Triamcinolone acetonide was added to all incubants to prevent possible binding of [^{3}H]R1881 to the progesterone receptor.[27] Bound [^{3}H]R1881 was separated from unbound by gel filtration on Sephadex LH-20 mini-columns and radioactivity was measured at 50% efficiency in a LS6500 (Beckman Coulter, Hialeah, FL) liquid scintillation spectrophotometer. Specific binding was calculated as the difference between total binding (measured in the absence of DHT) and non-specific binding (measured in the presence of DHT). The resulting data were analyzed by the method of Scatchard[28] using a computer-assisted nonlinear curve fitting method.[29] For each cell line the analysis was done in triplicate.

RNA extraction and RT-PCR

Cells were grown until 80% confluent, harvested and total RNA was extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD) as directed by the manufacturer. Total RNA yield and purity were determined spectrophotometrically by measuring the absorbance of aliquots at 260 and 280 nm. Two micrograms of total RNA were reverse transcribed using oligo dT primers and Superscript II reverse transcriptase (Gibco BRL) in a final volume of 20 μl. One μl of cDNA was amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM dNTPs (deoxynucleoside triphosphates), 2.5 U of HotStar*Taq* DNA polymerase (Qiagen, Valencia, CA) and 100 ng of the appropriate primers in an Eppendorf thermal cycler. The cycling conditions were 95°C for 15 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and a final extension step at 72°C for 10 min, unless listed otherwise.

Androgen receptor cDNA sequencing

Four sets of primers were used for the amplification of exon 1 of the androgen receptor (Table I). For primers A7–A8, 45 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 3 min were carried out. The primers used for the amplification of exons 2–8 have been described previously[31] and are listed in Table II. The primers used amplify overlapping regions of the whole AR cDNA sequence. Equal amounts of PCR products were electrophoresed on 1% agarose gels and visualized by ethidium bromide staining. The visible bands were cut out, purified using a Gel Purification Kit (Qiagen) and the purified DNA was directly sequenced by the Sanger method using the corresponding forward PCR-primer. The PCR product was sequenced again in the opposite direction if sequencing results showed a departure from the published androgen receptor mRNA sequence deposited in GenBank (accession number XM 010429).

Semi-quantitative RT-PCR

For quantification of nuclear receptor co-regulator mRNA expression levels using relative RT-PCR,[32] a range of dilutions (0.35–350 ng) of reverse transcribed cDNA from the cell lines were subjected to PCR using the primers and conditions indicated in Table III. PCR products were isolated by electrophoresis on 1% agarose gels and were stained with SYBR-gold (Molecular Probes, Eugene, OR) and visualized on a Epi Transilluminator (UVP, Upland, CA). Images were analyzed densitometrically by software incorporated in the transilluminator. Data were plotted as the log of densitometric units against the log of equivalent RNA concentration to identify a linear range of exponential amplification. Linear regressions were obtained using SigmaPlot Scientific Graphing Software (version 2.00, Jandel Corporation, San Rafael, CA). For comparisons within a particular cell line, measurements were corrected for β-actin. A 28-cycle PCR for both actin and the target gene was carried out and the β-actin primers were F: 5′-ACAATGAGCTGCGTGTGGCT-3′ and R: 5′-TCTCCTTAATGTCACGCACGA-3′. For comparisons between cell lines, measurements were corrected for 18S rRNA, as we found β-actin expression varied considerably between cell lines. In this case, random hexamers were used during the reverse transcription instead of oligo dT primers and the linear range of amplification of the 18S rRNA was determined at 16 cycles. The primers used for the amplifica-

TABLE I – PRIMERS USED FOR THE AMPLIFICATION AND DIRECT SEQUENCING OF AR EXON 1[1]

Primer name	Sequence (5′→3′)	PCR product length (bp)
A1	cgcctgttgaactcttctgagc	
A2	gctgtgaaggttgctgttcct	427
A3	cacaggctacctggtcctgg	
A4	ctgccttacacaactccttggc	416
A5	gctcccacttcctccaaggacaattac	
A6	cgggttctccagcttgatgcg	528
A7	ccagagtcgcgactactacaactttcc	
A8	ggactgggatagggcactctgctcacc	477

[1]See reference 30.

TABLE II – PRIMERS USED FOR THE AMPLIFICATION AND DIRECT SEQUENCING OF AR EXONS 2–8[1]

Primer name	Sequence (5′→3′)	PCR product length (bp)
AR1F	gtcaaaagcgaaatgggcccc	
AR2R	gtcgtccacgtgtaagttgcg	642
AR3F	actgaggagacaacccagaag	
AR4R	gcgtcttgagcaggatgtggg	612
AR5F	acccccccaggaattcctgtgc	
AR6R	ctgcagaggagtagtgcagag.	450

[1]See reference 31.

tion of 18s rRNA were F: 5′-CTACCACATCCAAGGAAG-GCA-3′ and R: 5′-TTTTTCGTACTACCTCCCCG-3′. The relative levels of the co-factors are expressed as the percentage of β-actin or 18S RNA concentration that gave the same optical density.

RESULTS

Androgen receptor protein expression

Cytosol extracts from 6 breast cancer cell lines were analyzed for AR protein by Western blot analysis. LNCaP and DU-145 prostate cancer cells were included as a positive and negative control respectively. A single immunoreactive band of approximately 110 KDa was detected in all cell lines, except for BT-20 and DU-145 cells, which are known to be devoid of androgen receptor (Fig. 1). MFM-223 cells had the highest content of androgen receptor protein among the breast cancer cell lines, similar to that of LNCaP cells. Second in rank were ZR-75-1 cells, with significantly lower AR levels than the MFM-223. BT-474 and T-47D cells appeared to have equal amounts of receptor, whereas an immunoreactive band was barely detectable for MCF-7 cells.

For the comparison of androgen receptor capable of binding ligand, saturation binding assays using [^{3}H]R1881 as radioligand were carried out on cytosol extracts prepared from the various cell lines. Figure 2 illustrates representative data obtained for BT-474 cells. The results from all cell lines are summarized in Table IV. A single class androgen binding site of high affinity (K_d = 0.1–0.2 nM) was detected in all breast cancer cell lines examined except BT-20. In concordance with our Western blot analysis, MFM-223 and ZR-75-1 cells had the highest concentrations of androgen binding and MCF-7 cells had the lowest level. T-47D cells exhibited <25% of the androgen binding measured in BT-474 cells, even though the 2 cell lines appeared to have equivalent amounts of receptor protein by Western blot analysis.

Androgen receptor sequence

RT-PCR was used to amplify androgen receptor cDNA from the breast cancer cells lines. The PCR products were purified and directly sequenced. To validate our ability to detect mutations androgen receptor cDNA from LNCaP cells was also analyzed as a control. The known mutation at codon 877 of the receptor[33–34] of these cells was detected. No mutations were detected in any of the androgen receptor cDNA amplified from the breast cancer cell lines; however, differences within the polymorphic CAG repeat region of exon 1 were noted. ZR-75-1 and MFM-223 cells had 26 CAG repeats, BT-474 cells had 25 and T-47D cells had 22, whereas MCF-7 cells had only 14 CAG repeats (these sequences have been deposited in GenBank accession numbers AF321914–AF321917). LNCaP cells analyzed in parallel had 26 repeats. Comparison of the second polymorphic region within exon 1 of the androgen receptor, the GGN repeat region, indicated that all examined cell lines had 23 repeats, except for ZR-75-1 cells, which had 24 repeats. In addition to full-length receptor, a splice variant isoform lacking exon 3 was detected in both MCF-7 and BT-474 cells, as has been reported previously.[31]

Expression of the nuclear receptor co-regulators

The expression of 11 transcriptional co-regulators was examined in 6 breast cancer cell lines (BT-474, T-47D, ZR-75-1, MCF-7, MFM-223 and BT-20) by RT-PCR. In our study, we included 2 co-activators (SRC-1 and AIB1) and 2 co-repressors (SMRT and NCoR1) that are known to interact with several members of the thyroid and steroid hormone receptor superfamily,[35–38] a number of androgen receptor-associated proteins (ARA24, ARA54, ARA55, ARA70 and ARA160)[39–43] and 2 co-activators that are reported to interact specifically with the androgen receptor (FHL2 and PDEF).[44–45] Our results showed that most of the co-factors tested were expressed in all cell lines at various levels. ARA24 was ubiquitously expressed at very high levels, which was not surprising because it was first identified as a nuclear G-protein (RAN, Ras-related nuclear protein) involved in multiple nuclear functions.[46] ARA55 was present in relatively high amounts only in BT-20 cells and was barely detectable after 35 cycles of amplification in T-47D, MCF-7 and ZR-75-1 cells. No expression of ARA55 was detected in either the BT-474 or MFM-223 cells. FHL2 and PDEF were not detected in the BT-474 and BT-20 cell lines respectively (data not shown).

To obtain a more accurate profile of the co-regulator expression in the AR positive cell lines, we used relative RT-PCR for semi-quantitative analysis of transcript levels. The expression levels of the different co-regulators within a given cell line were normalized using β-actin as the reference gene. Representative results obtained with T-47D cells are shown in Figure 3. A summary of the data comparing co-regulator expression within each cell line tested is presented in Figure 4. ARA24 mRNA was confirmed to be highly abundant and ubiquitously expressed, whereas ARA54, ARA55 and SMRT transcripts were present at <1% of β-actin expression in all cell lines and were therefore omitted from the plots.

In BT-474 cells (Fig. 4*a*), all co-regulators analyzed were expressed at relatively low levels, with PDEF, AIB1 and NCoR1 mRNAs being the more abundant. FHL2 mRNA was not detected. In T-47D cells (Fig. 4*b*), PDEF was the highest expressed co-regulator transcript, followed by FHL2 and ARA70. PDEF mRNA was also highly expressed in ZR-75-1 cells (Fig. 4*c*), as were ARA70 and AIB1. In MCF-7 (Fig. 4*d*), the predominant co-activator transcript was AIB1. ARA70 was the highest expressed co-activator mRNA in MFM-223 cells, where NCoR1 mRNA was not detected (Fig. 4*e*).

The expression of β-actin was quite variable across cell lines; therefore, we used 18S rRNA as an internal control to compare the expression of specific co-regulators across the cell lines. The results are presented in Figure 3 as ratios of the relative optical density of the PCR product of the target transcript to that of 18S rRNA. SRC-1 and AIB1 mRNA (Fig. 5*a*,*b*) were highly expressed in the MCF-7, BT-474 and ZR-75-1 cells. The levels of ARA70 mRNA were similar among the cell lines except for MCF-7 cells, which had lower amounts (Fig. 5*c*). ARA160 mRNA levels were high in T-47D cells (Fig. 5*d*) and PDEF mRNA levels were high in both T-47D and ZR-75-1 cells (Fig. 5*f*). T-47D cells also appeared to have the highest expression of FHL2 mRNA, in contrast to BT-474 cells, which were negative for this androgen receptor specific co-activator (Fig. 5*e*).

DISCUSSION

The results of these studies indicate that the differential androgen induction of PSA and hK2 levels observed in established breast cancer cell lines is not directly related to the level of androgen receptor expression or to mutations within the coding regions. Our Western blot analysis showed that MFM-223 cells, which produce low levels of PSA and hK2 in response to androgen,[23] had the highest levels of androgen receptor protein expression of all the breast cancer cell lines examined. Similarly, ZR-75-1 cells, which fail to express these kallikreins even after

TABLE III – PRIMERS USED FOR THE AMPLIFICATION OF AR CO-FACTORS

AR co-factor	Sequence (5′→3′)	Annealing temperature	Coordinates
SRC-1	F: tctgcctctgctaactcttca	58°C	912–932
	R: tcaaggtcagctgtaaactgg		1351–1330
ARA24	F: acgaccttcgtgaaacgtcat		184–204
	R: acaggtcatcatcctcatccg		763–743
ARA160	F: ctctggcagcaggtacttcat		488–508
	R: aggagttccacttcgtccact		1206–1186
AIB1	F: ggcagaatggaacctatgaat	56°C	2982–3002
	R: agggaattcctaagaagaggc		3302–3282
ARA54	F: ggaagagaccctagcatactt		432–452
	R: tctctgcagtcaccttacatg		1105–1085
ARA55	F: tctgtgagctagatcggttgc		218–238
	R: aaggcggtcaccatcttgtgt		854–834
ARA70	F: gctttgcagagtgtgtgtgtg		1235–1255
	R: ggggaagttatgttcctccg		1743–1725
NCoR1	F: tgatgggctctctgagcagga	60°C	1392–1412
	R: tgctgcccagttacgaccatg		2187–2167
PDEF	F: gaccagtgaggagagctggaccga		1107–1130
	R: tgaccttgggctctggaaggtcag		1558–1535
SMRT	F: taggtgccatctcccaaggaa		794–814
	R: gacgcaggtagtcctcctgt		1546–1527
FHL2	F: cggctgtgactgcaaggactt		141–161
	R: agcagtcgttatgccactgcc		725–745

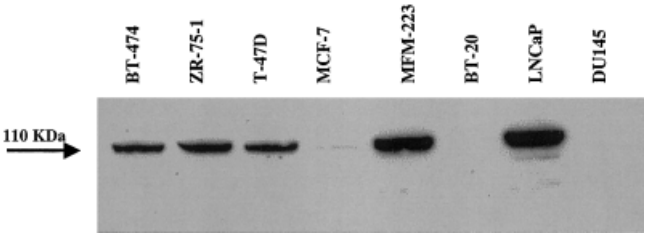

FIGURE 1 – Western blot analysis of AR protein expression in 6 breast cancer cell lines. LNCaP and DU145 prostate cancer cell lines were included as a positive and negative control, respectively. An 110-KDa immunoreactive band was detectable in all cell lines (there is a faint band in the MCF-7) except in BT-20 cells.

TABLE IV – MAXIMUM BINDING CAPACITY AND DISSOCIATION CONSTANT OF ^{3}HR1881 BINDING TO CYTOSOL EXTRACTS OF HUMAN BREAST CANCER CELL LINES

Cell line	[^{3}H]R1881 binding to the AR	
	Bmax	Kd (nM)
BT-474	75 ± 4	0.1
T-47D	17 ± 2	0.1
ZR-75-1	101 ± 9	0.1
MCF7	<10	
MFM-223	145 ± 15	0.2
BT-20	0	0

[1]Ligand binding analysis was performed to determine the concentration of the androgen receptor. Values for Bmax (saturation of binding) and Kd (binding affinity) represent the mean (± SE) of 3 independent experiments. All concentrations are in fmol per mg of total protein.

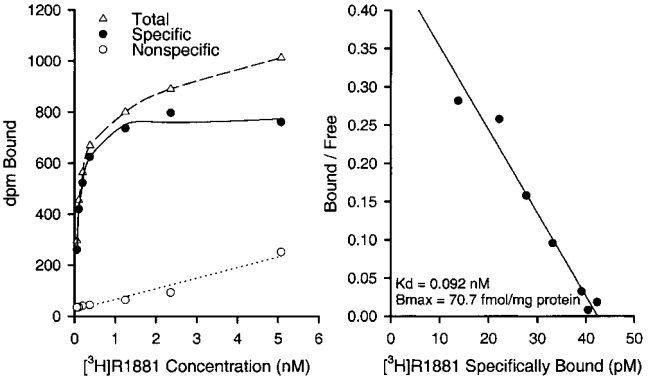

FIGURE 2 – Saturation binding data (*a*) and Scatchard analysis (*b*) of [^{3}H]R1881 binding in cytosolic extracts of BT-474 cells. Aliquots of the cytosol preparations were incubated with varying concentrations of [^{3}H]R1881 in the presence or absence of 1 μM unlabeled DHT.

androgen treatment,[23] also expressed high levels of androgen receptor protein.

Because immunoreactive receptor protein may not always reflect functional receptor levels, we also evaluated the capacity of the receptor to bind ligand. Results from saturation binding assays were in general agreement with Western blot analysis: MFM-223 cells and ZR-75-1 cells exhibited the highest levels of androgen binding. In contrast, BT-474 and the T-47D cells, which produce high amounts of PSA and hK2 upon androgen stimulation, exhibited markedly lower levels of androgen receptor protein and androgen binding. These data indicate that the differences in kallikrein expression cannot be ascribed to differences in androgen receptor availability for ligand binding. Interestingly, T-47D cells expressed levels of androgen receptor protein similar to BT-474 cells, but only 25% the level of androgen binding. This suggests that there may be differences in post-translational processing of the androgen receptor in these cells that might partially explain the difference in the levels of kallikrein production induced in these 2 cell lines.[23] MCF-7 cells expressed very low levels of androgen receptor protein and androgen binding, which could account for

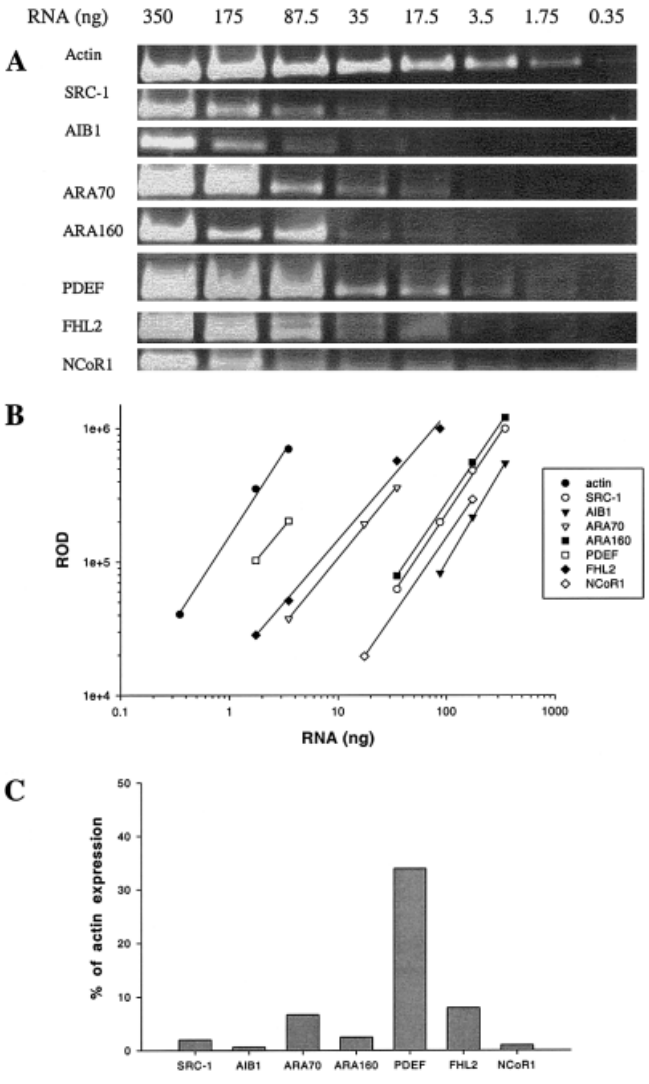

FIGURE 3 – Androgen receptor co-factor mRNA expression in the T-47D breast cancer cell line. Total RNA was extracted and subjected to reverse transcription. PCR, using specific primers, was carried out for different dilutions of cDNA, so that a linear range of amplification was obtained. The PCR products were fractionated on 1% agarose gels, stained with SYBR Gold (*a*) and analyzed densitometrically; the relative optical density (ROD) was plotted against an equivalent RNA concentration (*b*). We determined the relative RNA amount of every co-factor needed to obtain similar ROD units and expressed it as a percentage of the value obtained for actin (*c*).

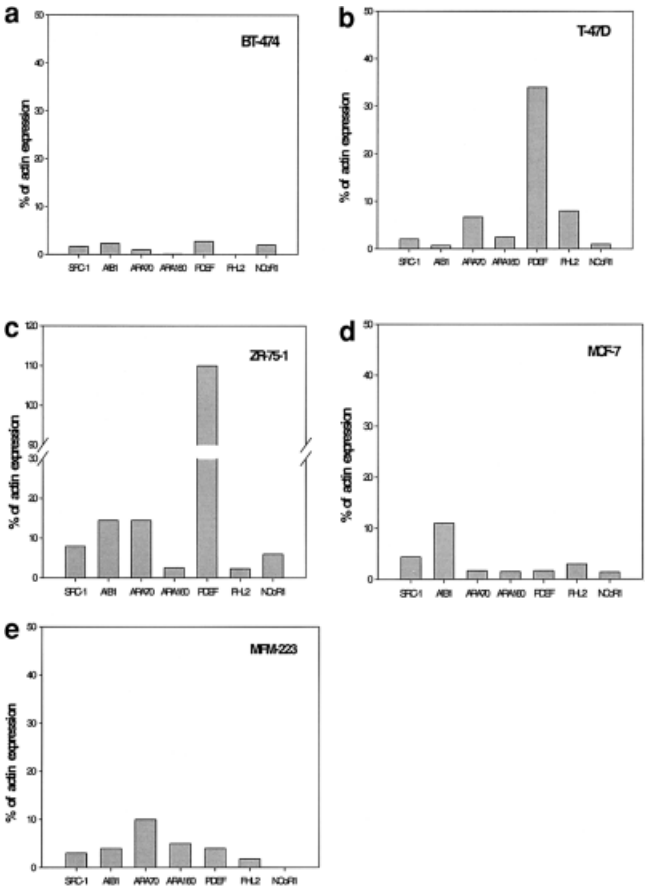

FIGURE 4 – Androgen receptor co-factor mRNA expression in BT-474, T-47D, ZR-75-1, MCF-7 and MFM-223 breast cancer cell lines cell lines (the data presented here were obtained as described in Fig. 3).

the inability of the receptor to drive the transcription of *KLK2* and *KLK3*.

Numerous mutations have been described for the androgen receptor, many of which have been shown to affect its ability to regulate target gene expression. For example, substitutions in the DNA-binding zinc finger domain of the receptor can impair its capacity to bind to specific DNA sequences and to modulate the expression of responsive genes without affecting its ability to bind ligand.[47] Thus androgen receptor mutations in these breast cancer cell lines, if present, could have provided an explanation for differential *KLK2* and *KLK3* expression in these cells, particularly ZR-75-1 and MFM-223 cells. No mutations were detected in androgen receptor cDNA generated from any of the breast cancer cells examined. Although slight differences in the polymorphic regions of the N-terminal domain (exon 1) were noted, these alone cannot account for the differences in kallikrein induction. *In vitro* experiments have demonstrated an inverse relation between the number of CAG and activation of androgen responsive reporter constructs,[48,49] indicating that longer CAG tract lengths encode less active receptors. Thus, one would expect the MCF-7 AR, with only 14 CAG repeats, to be more active as compared to the receptor expressed by the other breast cancer cells with tract lengths of 22–26 CAG repeats. Androgen fails to activate *KLK2* and *KLK3* gene expression in MCF-7 cells.

A large variation in the expression of nuclear receptor co-regulators was observed. A plethora of receptor co-regulators have been cloned in recent years and it is likely that more will be identified. The reason for such a large number of these factors is unknown but it has been postulated that specific co-activators may be required for different target genes or that they may be selectively involved in some but not all receptor actions.[50] The action of a steroid receptor at a given promoter may be modulated by the pool of co-activators that are present in a cell and may compete to interact with a particular receptor type. Of the co-regulatory factors examined in our study, results obtained with SRC-1 were most interesting. SRC-1 has been demonstrated to bind and co-activate the activation function 1 (AF-1) domain of the androgen receptor as well as other steroid hormone receptors.[36,37] Our results show that the SRC-1 mRNA levels correlate with the PSA secretion levels in the PSA-positive cell lines (Fig. 5*a*). The highest amounts of PSA are secreted from BT-474 cells and these cells express high levels of SRC-1 mRNA. T-47D cells express moderately high levels of PSA and exhibit a lower abundance of SRC-1 transcripts. MFM-223 cells secrete less PSA and express lower SRC-1 mRNA levels than T-47D cells. This co-activator may play an important role in the modulating androgen regulation of PSA production.

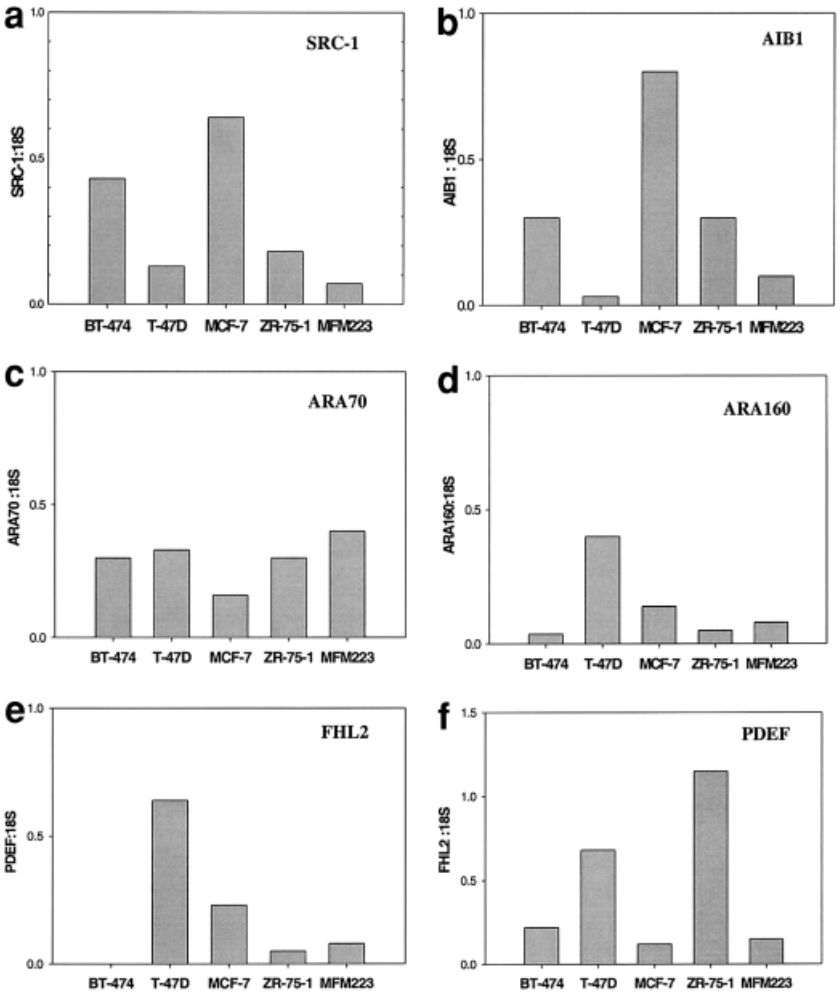

FIGURE 5 – Quantification of mRNA expression of AR co-activators in breast cancer cell lines. Total RNA was extracted from BT-474, T-47D, ZR-75-1, MCF-7 and MFM-223 cell lines and subjected to reverse transcription using random hexamers. For different dilutions of cDNA, a 28-cycle PCR was carried out, using specific primers, so that a linear range of amplification was obtained. A 16-cycle PCR was carried out for 18s rRNA that was used as an internal control. The PCR products were fractionated on 1% agarose gels, stained with SYBR Gold and analyzed densitometrically. The signal obtained for every co-factor was normalized against 18s rRNA and the results are expressed as the ratio of their RODs.

AIB1 was originally cloned as a gene that is amplified in ER-positive BT-474, MCF-7 and ZR-75-1 breast cancer cell lines, whereas it is expressed in low levels in the T-47D cells.[38] Our results confirm these findings and allow us to speculate that it may enhance the transcriptional activation function of the androgen receptor in the BT-474, but less so in T-47D and MFM-223 cells, where its levels are lower (Fig. 5*b*).

A number of androgen receptor associated (ARA) proteins have been recently identified by Chang's group.[39–43] ARA70 was the first protein claimed to interact specifically with the AR,[42] but recent data show that it can also associate with other steroid receptors.[51] We found that among the breast cancer cell lines tested, MCF-7 had the lowest levels of androgen receptor protein and ARA70 mRNA expression. The high level expression of ARA70 in BT-474 cells might explain the ability of estradiol to stimulate PSA production in these cells,[23] because this co-factor has been shown to enhance androgen receptor transcriptional activity in the presence of this hormone.[52] ARA24/Ran is an abundant protein that is involved in nuclear transport of proteins and RNA, cell cycle progression and nuclear structure in mitotic regulation, as well as RNA and DNA synthesis.[46] It was demonstrated recently that it could also interact with the polyglutamine (CAG repeat) region of the AR to enhance transactivation.[39] Both ARA24 and ARA160 could increase androgen sensitivity so that 0.1 nM DHT could efficiently stimulate a reporter gene linked to a PSA promoter sequence in transfection experiments with human prostate PC-3 cells.[39] The strong androgen receptor co-activating properties of ARA160 could only be seen in transfected PC-3 and Chinese hamster ovary cells, whereas it was a relatively weak co-activator in DU-145 and MCF-7 cells, indicating that intracellular environments may modulate the activity of co-activator.[43] It is tempting to speculate that these co-regulators may contribute to the regulation of PSA and hK2 expression, especially in T-47D cells, where ARA160 seems to be relatively elevated. ARA54 and ARA55 were expressed in low levels and thus are less likely to play a major role in modulating androgen action. It is noteworthy, however, that there is significant expression of ARA55 in the BT-20 cells. From all the co-factors tested, ARA55 was the most highly expressed, an unexpected finding because this cell line is devoid of sex steroid hormone receptors; it is possible that this protein is involved in other non-steroid hormonal pathways.

FHL2 (four-and-a-half LIM 2) protein is expressed in the myocardium and in the epithelial cells of the prostate where it co-localizes with the androgen receptor to the nucleus. Thus far, it appears to bind specifically to the androgen receptor and enhances its transcriptional activity.[44] FHL2 mRNA was not detectable in BT-474 cells, thus it is not essential for androgen action in this cell line. On the other hand, FHL2 mRNA was markedly elevated in T-47D cells and thus may be involved in androgen receptor mediated regulation of PSA/hK2 secretion in these cells (Fig. 5*f*).

PDEF (prostate-derived Ets factor) is a novel prostate epithelium-specific transcription factor and co-regulator of the androgen receptor that can act both synergistically with the androgen receptor as well as in an androgen-independent manner to enhance PSA gene expression.[45] It may have such a role in the T-47D cell line, where it was found to be present at relatively high levels (Fig. 5*e*).

Two co-repressors that we included in our study, SMRT (silencing mediator of retinoid and thyroid hormone receptor) and NCoR1 (nuclear receptor corepressor 1). These interact with several nuclear receptors, but their function with regard to the androgen receptor has not been investigated. They are expressed at low levels in all cell lines with NCoR1 mRNA levels being slightly higher in ZR-75-1 cells. We speculate that the relative expression of these co-factors may in part modulate the ability of androgens to stimulate PSA/hK2 production and secretion.

The relative importance of a co-regulatory protein in modulating androgenic regulation of PSA and hK2 secretion in a particular cell line is likely influenced by the composition of the pool of such proteins expressed in those cells. The relative contribution of each co-activator may depend on cell type or co-activator levels in the cells. For example, ARA160, FHL2 and PDEF may play such a role in the T-47D cells, where their mRNA levels were much higher, but not in the BT-474 cells. The fact that the AR gene is intact and the protein is functional and at relatively high amounts in the ZR-75-1 cells, makes attractive the hypothesis that there are more co-factors to be discovered, whose restricted expression may suppress transcription in these cells. Our data provide information on the differential expression of key co-regulatory factors in several breast cancer cell lines. It remains to be determined whether this varied expression pattern is responsible for the variation in the response of androgen treatment on *KLK2* and *KLK3* expression or it other factors, such as the expression of key transcription factors other than the androgen receptor, are involved. Given the potential anti-angiogenic action of PSA, further studies assessing these possibilities may aid in our understanding of the role of androgens in breast cancer.

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REFERENCES

1. Riegman PH, Vlietstra RJ, van der Korput JA, Brinkmann AO, Trapman J. The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 1991; 5:1921–30.
2. Young CY, Andrews PE, Montgomery BT, Tindall DJ. Tissue-specific and hormonal regulation of human prostate specific glandular kallikrein. Biochemistry 1992;31:818–24.
3. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. Trends Endocrinol Metab 2000;11:54–60.
4. Diamandis EP. Prostate-specific antigen-its usefulness in clinical medicine. Trends Endocrinol Metab. 1998;9:310–6.
5. Kwiatkowski MK, Recker F, Piironen T, Pettersson K, Otto T, Wernli M, Tscholl R. In prostatism patients the ratio of human glandular kallikrein to free PSA improves the discrimination between prostate cancer and benign hyperplasia within the diagnostic "gray zone" of total PSA 4–10 ng/mL. Urology 1998;52:360–5.
6. Magklara A, Scorilas A, Catalona WJ, Diamandis EP. The combination of human glandular kallikrein and free prostate-specific antigen (PSA) enhances discrimination between prostate cancer and benign prostatic hyperplasia in patients with moderately increased total PSA. Clin Chem 1999;45:1960–6.
7. Partin AW, Catalona WJ, Finlay JA, Darte C, Tindall DJ, Young CY, Klee GG, Chan DW, Rittenhouse HG, Wolfert RL, Woodrum DL. Use of human glandular kallikrein 2 for the detection of prostate cancer: preliminary analysis. Urology 1999;54:839–45.
8. Diamandis EP, Yu H. Non-prostatic sources of prostate-specific antigen. Urol Clin North Am 1997;24:275–82.
9. Magklara A, Scorilas A, López-Otín C, Vizoso F, Ruibal A, Diamandis EP. Human glandular kallikrein (hK2) in breast milk, amniotic fluid and breast cyst fluid. Clin Chem 1999;45:1774–80.
10. Lovgren J, Valtonen-Andre C, Marsal K, Lilja H, Lundwall A. Measurement of prostate-specific antigen and human glandular kallikrein 2 in different body fluids. J Androl 1999;20:348–55.
11. Yu H, Diamandis EP, Sutherland DJ. Immunoreactive prostate-specific antigen levels in female and male breast tumors and its association with steroid hormone receptors and patient age. Clin Biochem 1994;27:75–9.
12. Diamandis EP, Yu H, Sutherland DJ. Detection of prostate-specific antigen immunoreactivity in breast tumors. Breast Cancer Res Treat 1994;32:301–10.
13. Yu H, Giai M, Diamandis EP, Katsaros D, Sutherland DJ, Levesque MA, Roagna R, Ponzone R, Sismondi P. Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. Cancer Res 1995;55:2104–10.
14. Fortier AH, Nelson BJ, Grella DK, Holaday JW. Antiangiogenic activity of prostate-specific antigen. J Natl Cancer Inst 1999;91:1635–40.
15. Heidtmann HH, Nettelbeck DM, Mingels A, Jager R, Welker HG, Kontermann RE. Generation of angiostatin-like fragments from plasminogen by prostate-specific antigen. Br J Cancer 1999;81:1269–73.
16. Foekens JA, Diamandis EP, Yu H, Look MP, Meijer-van Gelder ME, van Putten WL, Klijn JG. Expression of prostate specific-antigen (PSA) correlates with poor response to tamoxifen therapy in recurrent breast cancer. Br J Cancer 1999;79:888–94.
17. Black MH, Magklara A, Obiezu C, Levesque MA, Sutherland DJA, Tindall DJ, Young CY, Sauter ER, Diamandis EP. Expression of a prostate associated protein, human glandular kallikrein (hK2) in breast tumors and in normal breast secretions. Br J Cancer 2000;82:361–7.
18. Lea OA, Kvinnsland S, Thorsen T. Improved measurement of androgen receptors in human breast cancer. Cancer Res 1989;49:7162–7.
19. Kimura N, Mizokami A, Oonuma T, Sasano H, Nagura H. Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. J Histochem Cytochem 1993;41:671–8.
20. Soreide JA, Lea OA, Varhaug JE, Skarstein A, Kvinnsland S. Androgen receptors in operable breast cancer: relation to other steroid hormone receptors, correlations to prognostic factors and predictive value for effect of adjuvant tamoxifen treatment. Eur J Surg Oncol 1992;18:112–8.
21. Birrell SN, Bentel JM, Hickey TE, Ricciardelli C, Weger MA, Horsfall DJ et al. Androgens induce divergent proliferative responses in human breast cancer cell lines. J Steroid Biochem Mol Biol 1995;52: 459–67.
22. Hackenberg R, Luttchens S, Hofmann J, Kunzmann R, Holzel F, Schulz KD. Androgen sensitivity of the new human breast cancer cell line MFM-223. Cancer Res 51:5722–7, 1991.
23. Magklara A, Grass L, Diamandis EP. Differential steroid hormone regulation of human glandular kallikrein (hK2) and prostate-specific antigen (PSA) in breast cancer cell lines. Breast Cancer Res Treat 2000;59:263–70.
24. McKenna NJ, Xu J, Nawaz Z, Tsai SY, Tsai MJ, O'Malley BW. Nuclear receptor co-activators: multiple enzymes, multiple complexes, multiple functions. J Steroid Biochem Mol Biol 1999;69:3–12.
25. Heisler LE, Evangelou A, Lew AT, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol Cel Endocrinol 1997;126:59–73.
26. Puy L, MacLusky NJ, Becker L, Karsan N, Trachtenberg J, Brown TJ. Immunocytochemical detection of androgen receptor in human temporal cortex: characterization and application of polyclonal androgen receptor antibodies in frozen and paraffin-embedded tissues. J Steroid Biochem Mol Biol 1995;55:197–209.
27. Zava DT, Landrum B, Horwitz KB, McGuire WL. Androgen receptor assay with [^{3}H]methyltrienolone (R1881) in the presence of progesterone receptors. Endocrinology 1979;104:1007–13.
28. Scatchard G. The attraction of proteins for small molecules and ions. Ann NY Acad Sci 1949;51:660–72.
29. Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 1980; 107:220–39.
30. Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS. Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci USA 1989;86:9534–8.
31. Zhu X, Daffada AI, Chan CMW, Dowsett M. Identification of an exon 3 deletion splice variant androgen receptor mRNA in human breast cancer. Int J Cancer 1997;72:574–80.
32. Murphy LD, Herzog CE, Rudick JB, Fojo AT, Bates SE. Use of the polymerase chain reaction in the quantitation of mdr-1 gene expression. Biochemistry 1990;29:10351–6.
33. Harris SE, Rong Z, Harris MA, Lubahn DD. Androgen receptor in human prostate carcinoma LNCaP/ADEP cells contains a mutation

which alters the specificity of the steroid dependent transcriptional activation region. Endocrinology 1990;126:93.
34. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. Biochem Biophys Res Commun 1990;173: 534–40.
35. Xu L, Glass C, Rosenfield MG. Coactivator and corepressor complexes in nuclear receptor function. Curr Opin Genet Dev 1999;9: 140–7.
36. Alen P, Claessens F, Verhoeven G, Rombauts W, Peeters B. The androgen receptor amino-terminal domain plays a key role in p160 coactivator-stimulated gene transcription. Mol Cell Biol 1999;19: 6085–97.
37. Bevan CL, Hoare S, Claessens F, Heery DM, Parker MG. The AF1 and AF2 domains of the androgen receptor interact with distinct regions of SRC1. Mol Cell Biol 1999;19:8383–92.
38. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 1997;277:965–8.
39. Hsiao PW, Lin DL, Nakao R, Chang C. The linkage of Kennedy's neuron disease to ARA24, the first identified androgen receptor polyglutamine region-associated coactivator. J Biol Chem 1999;29:20229–34.
40. Kang HY, Yeh S, Fujimoto N, Chang C. Cloning and characterization of human prostate coactivator ARA54, a novel protein that associates with the androgen receptor. J Biol Chem 1999;274:8570–6.
41. Fujimoto N, Yeh S, Kang HY, Inui S, Chang HC, Mizokami A, Chang C. Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate. J Biol Chem 1999;274:8316–21.
42. Yeh S, Chang C. Cloning and characterization of a specific coactivator, ARA70, for the androgen receptor in human prostate cells. Proc Natl Acad Sci USA 1996;93:5517–21.
43. Hsiao PW, Chang C. Isolation and characterization of ARA160 as the first androgen receptor N-terminal-associated coactivator in human prostate cells. J Biol Chem 1999;274:22373–9.
44. Muller JM, Isele U, Metzger E, Rempel A, Moser M, Pscherer A, Breyer T, Holubarsch C, Buettner R, Schule R. FHL2, a novel tissue-specific coactivator of the androgen receptor. EMBO J 2000; 19:359–69.
45. Oettgen P, Finger E, Sun Z, Akbarali Y, Thamrongsak U, Boltax J, Grall F, Dube A, Weiss A, Brown L, Quinn G, Kas K, et al. PDEF, a novel prostate epithelium-specific Ets transcription factor, interacts with the androgen receptor and activated prostate specific-antigen gene expression. J Biol Chem 2000;275:1216–25.
46. Rush MG, Drivas G, D'Eustachio P. The small nuclear GTPase Ran: how much does it run? Bioessays 1996;18:103–12.
47. McPhaul MJ. Molecular defects of the androgen receptor. J Steroid Biochem Mol Biol 1999;69:315–22.
48. Mhatre AN, Trifiro MA, Kayfman M, Kazemi Esfarjani P, Figlewicz D, Rouleau G, Pinsky L. Reduced transcriptional regulatory competence of the androgen receptor in X-linked spinal and bulbar muscular atrophy. Nat Genet 1993;5:184–8.
49. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. Nucleic Acids Res 1994;22: 3181–6.
50. Katzenellenbogen JA, O'Malley BW, Katzenellenbogen BS. Tripartite steroid hormone receptor pharmacology: interaction with multiple effector sites as a basis for the cell- and promoter-specific action of these hormones. Mol Endocrinol 1996;10:119–31.
51. Alen P, Claessens F, Schoenmakers E, Swinnen JV, Verhoeven G, Rombauts W, Peeters B. Interaction of the putative androgen receptor-specific coactivator ARA70/ELE1alpha with multiple steroid receptors and identification of an internally deleted ELE1β isoform. Mol Endocrinol 1999;3:117–28.
52. Yeh S, Miyamoto H, Shima H, Chang C. From estrogen to androgen receptor: a new pathway for sex hormones in prostate. Proc Natl Acad Sci USA 1998;95:5527–32.