

AD_____

Award Number: W81XWH-06-1-0016

TITLE: Calpain-Dependent Proteolysis of the Androgen Receptor

PRINCIPAL INVESTIGATOR: Maria Mudryj, Ph.D.

CONTRACTING ORGANIZATION: University of California, Davis
Davis, CA 95616

REPORT DATE: November 2009

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-11-2009		2. REPORT TYPE Final		3. DATES COVERED (From - To) 15 OCT 2005 - 14 OCT 2009	
4. TITLE AND SUBTITLE Calpain-dependent proteolysis of the androgen receptor				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0016	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Maria Mudryj, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California, Davis Davis, CA 95616				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Previous analysis of the CWR22Rv1 relapsed androgen independent tumor line revealed that it expresses the full length androgen receptor (FL-AR) and an low molecular weight (LMW) that has a deletion of the C-terminal ligand binding domain (LBD). Calpain proteolysis removes the LBD generating a constitutively active molecule. Our studies showed that the LMW AR is present in some prostate tumors. Inhibition of calpain activity by calpain inhibitors in CWR22Rv1 cells prevents AR proteolysis. Calpain inhibition by calpeptin and the HIV protease inhibitors in the absence androgens promotes cell death in cell culture and animal xenograft studies. In Rv1 cells a 39aa insertional mutation of the AR sensitizes AR to calpain proteolysis, while in the related R1 cells the levels and activity of calpain are elevated. In R1 cells LMW AR expression is regulated by the ERK kinase. In studies that address the role of the LMW AR in transcription we found that two CWR22 derived cells lines have a very similar AR binding pattern. Surprisingly the gene expression profile of the two lines is very different and most importantly the cohort of androgen regulated genes is different in the two cell lines. This indicates that AR binding is not sufficient to drive androgen-dependent transcription, and that transcription is dependent on additional factors that are cell specific. An analysis of the LMW AR binding and gene regulation indicates that the LMW AR binds to a subset of sites that are bound by the FL-AR, indicating that the LMW-AR does not bind a distinct set of genes. Expression studies found that the LMW AR is not subject to regulation by Filamin A a AR co-repressor.					
15. SUBJECT TERMS androgen receptor, androgen independence, calpain, proteases, proteolysis, HIV protease inhibitors, CWR22-R1, CWR22- Rv1, HIV protease inhibitors, chromatin immunoprecipitation, expression array					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 76	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	2
Key Research Accomplishments.....	10
Reportable Outcomes.....	11
Conclusion.....	12
References.....	13
Appendices.....	20

Final report- P.I.: Mudryj, Maria
Award Number W81XWH-06-1-0016
Title: Calpain-dependent proteolysis of the androgen receptor.

Introduction:

The CWR22 androgen dependent xenograft model, which mimics human prostate cancer, is a useful tool to study the emergence of androgen independence since the tumors exhibit androgen dependent growth. Following androgen withdrawal, the tumor regresses, and androgen independent tumors emerge. Analysis of CWR22Rv1 (Rv1) cell line, derived from a relapsed tumor revealed that it expresses the full length androgen receptor (AR) as well as an ~80-85 KD truncated form of AR that has a deletion of the C-terminal ligand binding domain (LBD). Analysis of human prostate tumors indicates that several tumors express higher levels of this low molecular weight (LMW) AR than noncancerous prostate tissue. In addition, the CWR22-derived R1 cell line expresses the LMW AR. Our studies focused on 1) the mechanisms that lead to the generation of the LMW AR and 2) on the role of the LMW AR on gene expression. Briefly, we found that the 39 aa insertional mutation in the Rv1 AR (E3DM-AR) that sensitizes this AR to calpain 2 proteolysis. In contrast in CWR22 derived androgen independent cell line R1 activation of calpain 2 by Extracellular Signal-Regulated Kinases 1 and 2 (ERK) promotes AR proteolysis. An analysis of human tumor samples found that LMW-AR levels are higher in tumors that have an increased calpain/calpastatin ratio and/or increased levels of phospho-ERK (pERK). Treatment of Rv1 cells with a calpain inhibitor reduces truncated AR expression, and in the absence of androgen, induces apoptosis. We made the serendipitous discovery that an HIV protease inhibitor inhibits calpain activity and is also effective in inducing apoptosis in the Rv1 cell line in cell culture and animal studies. The Rv1 and R1 were used to define LMW-AR target genes. Expression microarray analysis was used to analyze AR dependent transcription in the presence and absence of androgen. The analysis revealed that although related, R1 and Rv1 had significantly different gene expression profiles in response to androgen. In contrast, AR chromatin immunoprecipitation combined with promoter DNA microarrays (ChIP-on-chip) studies showed R1 and Rv1 cells have a similar AR binding profile. Coupling of the microarray study with ChIP-on-chip analysis identified direct AR targets in R1 and Rv1 cells. Interestingly only 6% of the Rv1 androgen-regulated genes, but 42% of the R1 androgen-regulated genes, bound AR. A screening of transcription factor binding motifs revealed that the glucocorticoid response element, GATA, Sp1 and FoxJ2 most frequently co-present with AR binding motifs in the AR direct target genes. Moreover, the most prominent function of transcripts that were direct AR targets was transcriptional regulation. This study

indicates that AR-dependent gene expression is dependent on factors that vary greatly in the two cell lines. Cellular localization analysis of AR showed that the LMW-AR is the predominant form (~90%) present in the nucleus in Rv1 cells cultured in the absence of androgen, allowing us to identify 128 potential LMW-AR specific chromosomal binding sites. Quantitative RT-PCR analysis of the genes closest to these sites revealed that LMW-AR can regulate certain genes in a different manner than full-length AR.

Body

Hypothesis: calpain cleavage of the AR generates a constitutively active molecule that acts in a dominant manner to confer androgen independence in prostate cells.

Specific Aim 1. Identify the calpain cleavage site in the AR.

Specific Aim 2. Generate cDNA constructs corresponding to the truncated AR (AR-tr) and define the role of the truncated AR in androgen independent proliferation.

Specific Aim 3. Determine if inhibition of calpain expression represses androgen independent proliferation.

1) Identify of the calpain cleavage site, role of LMW AR in transcription, inhibition of calpain activity.

Our preliminary studies suggested that calpain cleaves the AR to generate an ~80 KD truncated LMW AR and a smaller 30 KD cleavage fragment. As our studies progressed it became evident that multiple low molecular forms were expressed in the Rv1 cells, making isolation and characterization extremely difficult. Furthermore, published studies indicated that alternative splicing could also generate LMW- AR forms, although different groups reported different alternative splice isoforms. The one consistent finding was that all of the LMW AP forms lacked the LBD. We therefore took a different approach and focused in the study of the etiology and function of the LMW AR. We found that the LMW AR was expressed in human prostate tumors and that expression in part correlated in increased proteolysis of other calpain substrates supporting the role of calpain in AR proteolysis. Using calpain inhibitors we demonstrated that in the absence of androgen calpain inhibition promoted apoptosis in Rv1 cells. However, the calpain inhibitors that are available are very toxic in animal, and our original strategy was to used cell overexpressing the endogenous inhibitor calpastatin. However, we found reports that HIV protease inhibitors could also inhibit calpain activity. Since these are

drugs that are currently in use we decided that it would be more clinically relevant to determine if these agents altered the expression of LMW AR. We found that the HIV protease inhibited expression of the LMW AR and under androgen depletion promoted apoptosis. The cell culture result was recapitulated in an animal model; administration of the HIV protease inhibitor reduced the growth of the tumors.

Since the salient feature of the LMW AR whether generated by proteolysis of alternative splicing was the absence of the LBD. Therefore we generated a molecule that was missing this domain, and found that it was effective in promoting transcription in transient transfection studies.

The above described studies have been published and experimental details are in the attached manuscript. (S Libertini et al. Evidence for Calpain mediated Androgen Receptor Cleavage as a Mechanism of Androgen Independence and Potential Therapeutic Target in Prostate Tumors. *Cancer Research* **69**, 9001-5). A review on the mechanisms leading to androgen independence was also published. (H Devlin and M Mudryj. Progression of Prostate Cancer: Multiple Pathways to Androgen Independence. *Cancer Letters* 274 (2):177-86).

2) Mechanisms that regulated LMW AR expression.

While the Rv1 model has been extensively used, it is unusual since the AR harbors a duplication of an exon that encodes part of the DNA binding domain, resulting in an insertion of 39aa. In contrast the R1 cell line derived from the CWR22 tumor does not have this insertion, but also expresses the LMW AR. To uncover the etiology of the LMW AR we studied these two cell lines. The 39aa insertion is near the junction of the LBD. Using cDNAs encoding the wildtype and 39aa mutant AR and transfection of these plasmids into PC3 cells we found that the insertion sensitized the AR to proteolysis. This explains in part why the LMW AR is so abundant in the Rv1 cells.

The R1 cells, which were independently derived from the same xenograft do not have the insertion mutation, yet express higher levels of the LMW AR. This model therefore more closely approximates what is found in human tumors. We found that R1 cells express higher levels of calpain 2 and higher levels of phospho-ERK, a kinase that can phosphorylate and activate calpain 2. Using calpain 2 specific siRNA we decreased calpain 2 levels. This resulted in lower levels of the LMW AR. Likewise siRNA mediated decrease of ERK also reduced expression of the LMW AR. We also used an inhibitor of MEK, a kinase that is upstream of ERK, to reduce ERK activity. Inhibition of MEK resulted in a decrease of the LMW AR. In a

converse experiment, ERK activity was activated and there was an increase in the LMW AR. These cell culture studies indicate that, in part, the generation of the LMW AR is regulated by the MAP signaling pathway. Finally, analyses of human tumor samples found that LMW-AR levels are higher in tumors that have an increased calpain/calpastatin ratio and/or increased levels of phospho-ERK. This suggests that a higher calpain/calpastatin ratio collaborates with activated ERK to promote the generation of the LMW-AR.

The above described studies have been published and the experimental details are in the attached manuscript. (H Chen et al. Erk Regulates Calpain 2 Induced Androgen Receptor proteolysis in CWR22 Relapsed Prostate Tumor Cell Lines. *Journal of Biological Chemistry* 285(4):2368-74).

3) Androgen dependent gene regulation and AR binding in R1 and Rv1 cells.

We and others have demonstrated that the LMW AR is expressed in cell lines and in human tumors. However, it is unclear how these LMW AR forms regulated gene expression. To decipher the role of the LMW AR in gene expression we first needed to define androgen regulated genes and define AR binding patterns in the R1 and Rv1 cells. Several lines of evidence indicate that they were derived from a common ancestor. Karyotypes of the two cell lines are very similar; both lines shared the same structural abnormalities, including a reciprocal translocation between chromosomes 6 and 14. Both lines have the same AR (H847Y) mutation that is present in the parental CWR22 cells. Therefore we anticipated that these two cell line would be useful in defining the role of the LMW AR in transcription.

To compare the two CWR22 relapse lines, we used the Affymetrix HG-U133 Plus2.0 Gene Chip microarray. The analysis was conducted in duplicate at the same density in charcoal stripped serum or two hours following addition of 10nM DHT. Comparison of R1 and Rv1 gene expression profiles in castrate levels of androgen identified 1275 genes that were differentially expressed (fold change ≥ 1.5 or ≤ -1.5 ; $P \leq 0.05$) in R1 vs. Rv1 cells. Analysis of the microarray data identified 1941 transcripts that were differentially expressed in R1 vs. Rv1 cells treated with DHT. Of these, 60% were identical to the transcripts that were differentially expressed in the absence of androgen. As expected, R1 cells expressed 4-fold higher levels of calpain 2 mRNA than Rv1 cells. R1 cells also expressed 11.7-fold higher levels of c-MET. Rv1 cells have more neuroendocrine characteristics since the expression of neuronal specific enolase (ENO2) was 12-fold higher in Rv1 cells than in R1 cells, and the expression of chromogranin A and B, and synaptophysin were higher in Rv1, indicating their neuroendocrine nature. The most significant

pathway differences between R1 and Rv1 cells both in the presence and absence of androgen involved metabolic pathways. In summary, although these two lines were derived from the same CWR22 xenograft and have similar morphologies, at the molecular level they are distinct.

Next we analyze genes differentially regulated in the two cell lines in response to a two hours androgen treatment. We found that the expression of 854 transcripts was altered by a two hour DHT treatment in Rv1 cells. The same analysis was conducted using R1 cells and in contrast to Rv1 cells, the expression of only 77 transcripts changed following addition of DHT for 2hr. A comparison of the DHT-responsive R1 and Rv1 transcripts identified only 10 that were commonly regulated in both cell lines, again indicating the large differences between these two lines.

The differentially expressed genes in response to DHT for 2hr were analyzed by Ingenuity System's Pathway Analysis (IPA) to identify most significant associated biological networks and canonical pathways (metabolic and cell signaling) altered in the two cell lines. IPA identified two significant biological networks associated with the differentially expressed genes in R1. The significantly associated functions include gene expression, cellular development, cell cycle and embryonic development. The most significantly associated canonical pathways are notch signaling, clatrin-mediated endocytosis, JAK/Stat signaling, and p53 signaling. In Rv1 cells, a total of 18 biological networks were identified that are significantly associated with the differentially expressed genes. The significantly associated functions include cellular development, visual system development and function, cancer, cell cycle, molecular transport and protein trafficking. The most associated canonical pathways include aminoacyl-tRNA biosynthesis, axonal guidance signaling, DNA damage response, cell cycle, p53 signaling and clatrin-mediated endocytosis.

Since the cohort of androgen regulated transcripts differed in R1 and Rv1 cells we wondered if they were regulated differently because the AR bound to different regulatory regions. The Human Promoter 1.0R Array (Affymetrix) was used to detect AR binding to regulatory regions. A total of 1225 and 2021 AR binding sites ($FDR \leq 0.05$) were identified in R1 and Rv1 cells, respectively, when treated with DHT for 2hr. A comparison of AR binding across chromosomes in R1 and Rv1 cells treated with androgen showed that AR binding pattern was similar, but not identical. Therefore, while the androgen regulated gene profile of the two cell line is different the AR binding pattern is similar.

A motif analysis of the AR binding sites was conducted to determine whether AR binds to the established consensus AR response element (ARE). Previous studies conducted in LNCaP, LNCaP derived cells, or AR transfected PC3 cells reported that only ~10% of the AR

binding regions had a canonical class 1 ARE binding motif when two positions were allowed to vary from the palindromic consensus with 3 nucleotide spacing. They also found that 78% of the binding regions contained the AR binding half-site motif. In this study we found in Rv1 cells only 4% of the sites had the canonical ARE and 35% had the AR half-site motif. Likewise, in R1 cells, 6% of the sites had the canonical ARE and 46% had the AR half-site motif.

By coupling the ChIP-on-chip with microarray expression data, we identified that, of the 854 differentially regulated genes in Rv1 cells in response to DHT for 2hr, AR bound to nearby chromosomal sites of only 53 genes (6%). IPA analysis showed that the biological functions most prominently associated with these 53 genes were transcriptional regulation, cell cycle, and metabolic process. The same analysis was performed in R1 cells. Of the 77 differentially regulated genes after adding DHT for 2hr, AR bound to the nearby chromosomal regions of 32 genes (42%). The major biological functions associated with these 32 genes are transcriptional regulation and metabolic process.

A comparison of R1 and Rv1 revealed that the majority of the AR bound sites near the differentially regulated genes were common. However, only three closest genes [CCAAT/enhancer binding protein delta, claudin 4, and arylamine N-acetyltransferase type I] adjacent to the common AR binding sites in both R1 and Rv1 cells showed correlated transcriptional regulation in both lines. This argues that only a subset of AR chromosomal binding sites exhibit transcriptional regulation. Considering that other transcription factors might play collaborative role in AR function, we used Transcription Element Search System (TESS) to screen for motifs most frequently co-exist with AR binding motifs present in the above differentially regulated genes. The transcription factor motifs that most frequently co-exist with AR binding motifs included GRE, GATA binding protein 1 (GATA-1), Sp-1 and forkhead box J2 (FoxJ2) in both R1 and Rv1 cells.

The extensive difference in gene expression of R1 and Rv1 cells strongly argues that while they are derived from a common xenograft CWR22 tumor, at the molecular levels they are very different.

Previous studies have shown that AR binding sites can be far away from transcription start sites [23, 28]. The coverage of the promoter array used for this study is limited within ~10kb from transcription start sites. Therefore, the actual direct AR targets in R1 and Rv1 cells are most likely higher than what we found. While the number DHT-regulated genes was much higher in Rv1 cells, the number genes that are DHT-regulated and are associated with an AR binding site is more comparable in R1 and Rv1 cells. This suggests that AR binding or AR/DNA complex stability in Rv1 cells is greater or that a large number of the DHT-regulated transcripts

in Rv1 cells are indirect AR targets. Several mechanisms may account for this discrepancy. The presence of a 39aa insertion mutation in the Rv1 AR that results in the duplication of the DNA domain may facilitate DNA binding, or the interactions with other DNA binding protein. Alternatively, the different complement of AR co-regulators in Rv1 and R1 cells may govern AR-dependent gene regulation.

The most common function by far of direct AR target genes in R1 (9 genes) and Rv1 (11 genes) involved regulation of transcription, while the second most common functions were regulation of the cell cycle or metabolism. However, only CEBPD was commonly regulated in both cell lines. If the AR-regulated transcription factors are very different in the two cell lines, then the subsequent indirect AR target transcripts would be different as well. Therefore it is not surprising that the AR-dependent transcription profile of R1 and Rv1 cells is distinct.

The above described studies have been prepared for publication. Please see the attached manuscript for experimental details. (H. Chen et al. Genome-wide analysis of androgen receptor binding and gene regulation in two CWR22-derived prostate cancer cell lines. *Submission pending approval of all co-authors.*

4) The role of LMW AR in gene regulation

Previous studies, including ours found that in transient transfection studies an AR missing the LBD can transactivate expression of AR-regulated promoters in the absence of androgen. However, it is unclear if the LMW AR can bind to and regulate expression of endogenous genes. To address this question we analyzed the cellular location of the LMW-AR in the nuclear and cytosolic fractions of Rv1 and R1 cells proliferating the presence and absence of androgen. In Rv1 cells in the presence of androgen, the FL-AR and LMW-AR are present in the nucleus. The FL-AR is more abundant in the cytosol, whereas the LMW-AR is more abundant in the nucleus. Notably, in the absence of androgen the Rv1 nuclear fraction consists predominantly (~90%) of the LMW-AR (Figure 1A). In R1 cells the predominant form of the AR in the nucleus in the presence of androgen is the full length AR (FL-AR). In the absence of androgen the nuclear fraction contains substantially higher amount of the LMW-AR (~60%). In the cytosolic fraction the AR is almost exclusively full length. Utilizing our finding that the nuclear fraction of Rv1 cells consists predominantly (~90%) of the LMW-AR in the absence of androgen, we performed ChIP-on-chip analysis on Rv1 cells proliferating in androgen-depleted medium to investigate the potential LMW-AR chromosomal binding sites. The same analysis was conducted on R1 cells.

Analysis of the ChIP-on-chip data revealed 128 binding sites ($FDR \leq 0.05$) in Rv1 cells proliferating in androgen-depleted media (Table 1). Furthermore, the 128 sites were also present in the total of 2021 binding sites identified in Rv1 cells treated with 10nM DHT for 2hrs using the same criteria (i.e. $FDR \leq 0.05$) (Figure 1B, C). A closer examination of these 128 binding sites revealed that 20% of the sites showed exactly the same start and end position in the absence or presence of DHT. The remainder (80%) were within the range of ~35-1000bp upstream of the start position or downstream of the end position. Furthermore, the addition of androgen induced modest [1 going to 2] enrichment of AR binding to the sequence adjacent (within 50Kb) to 46 sites ($46/128=36\%$), and high enrichment [3 or more] to the sequence adjacent to 14 sites ($14/128=11\%$) (Figure 1C). Addition of androgen did not cause enrichment in the AR binding to the rest 68 sites ($68/128=53\%$). Addition of androgen also resulted in the AR binding to sites that were not bound in the absence of androgen, indicating the requirement of the FL-AR for binding to specific sequences.

Next we compared AR binding in Rv1 and R1 cells cultured in androgen depleted media. Analysis of the ChIP-on-chip data obtained from R1 cells failed to reveal binding sites that were statistically significant ($FDR \leq 0.05$). Due to the low level of the AR (both FL- and LMW-AR) in the nucleus of R1 cells proliferating in androgen depleted media, the assay may not be sensitive enough to detect binding that reaches the threshold of statistical significance. Nevertheless, the examination of the best potential binding sites provided by Cisgenome showed that 15 binding sites overlapped with the binding sites identified Rv1 cells (Table 1 labeled with *). Furthermore, all these 15 sites were identified as statistical significant ($FDR \leq 0.05$) binding sites in R1 cells treated with DHT for 2hr (Figure 1B).

We analyzed the 128 binding sites to determine whether the LMW-AR binds to the established consensus AR response element (ARE). Similar to FL-AR binding pattern, only 6% ($8/128$) of the LMW-AR binding sites contained the typical ARE and 48% contained the AR half-site motif (Figure 1D).

Further analysis of the 128 binding sites identified a total of 118 genes that were closest to the AR binding site (Table 1). Only 20% of the chromosomal binding sites were located within 2Kb up- or down-stream from the transcription start sites (Table 9). Notably, about 20% of the binding sites were more than 10kb up-stream of the transcriptional start site and 9% of the sites were more than 10kb down-stream of the transcriptional end site. Several genes (CGI-115, EPHX1, RGPD5, LPP, RHOH, MAT2B, CYP3A43, ANKRD20A3, TMEM60 and GOLGA8G) had two sites bound by the LMW-AR, and in all of the cases except (RGPD5) the two binding sites in each gene were within close vicinity (35 up to 4551bp apart).

We then examined the expression profile of the closest genes adjacent to the 128 sites in response to DHT for 2hr. Of the 118 closest genes identified in the ChIP-on-chip study of AR binding in the absence of androgen, we found 6 genes (CDKN1B/p27, FABP7, IL-6R, KRIT1, SMA4 and UGT2B15) showed differential expression in response to DHT (Figure 2A). It has been reported that CDKN1B/p27 and UGT2B15 are androgen-responsive gene. Interestingly, a significant enrichment of AR binding sites was observed at CDKN1B/p27 gene locus (Figure 1C), which corresponded with the up-regulation of its expression 2hr post DHT addition. When the analysis included the neighbor genes near the 128 sites, 13 (13/128=10%) were differentially regulated by androgen. Some of the genes were over 50,000 bp from the AR binding site. When the criteria for identifying androgen-regulated genes were relaxed (fold change ≥ 1.3 ; $P \leq 0.10$), 56 (56/128=44%) of the sites were associated with genes that were androgen regulated. This suggests that for some genes LMW-AR binding in the absence of androgens is not functional or not sufficient and FL-AR is required for their maximal gene regulation.

To further investigate the role of LMW-AR in gene regulation, the expression of several closest genes was analyzed in the absence of androgen following treatment with calpeptin for 48hr. Since treatment with calpeptin reduces LMW-AR expression and the amount of LMW-AR present in the nucleus (Figure 2B), this allows us to study the transcriptional regulation of genes adjacent to the potential LMW-AR binding sites. We examined the effect of LMW-AR inhibition on the expression of the above six closest genes (CDKN1B/p27, FABP7, IL-6R, KRIT1, SMA4 and UGT2B15) that showed differential expression after adding DHT for 2hr. Varied responses to the inhibition of LMW-AR were observed (Figure 2B). The expression of UGT2B15 was down-regulated after adding DHT. Interestingly, inhibition of LMW-AR also significantly reduced their expression, indicating that LMW-AR might function as an activator whereas FL-AR functions as a repressor of the gene. The expression of IL-6R, on the other hand, was elevated after adding DHT. Inhibition of LMW-AR also significantly enhanced its expression, indicating that LMW-AR might function as a repressor while the FL-AR an activator of the gene. Addition of DHT strongly up-regulated the expression of FABP7 and SMA4 (Figure 2A). Inhibition of LMW-AR showed slight reduction of their expression, indicating that LMW-AR acts like FL-AR as an activator of the genes, but much weaker than FL-AR. While modestly up-regulated by FL-AR after adding DHT, no effect was observed on the expression of CDKN1B/p27 and KRIT1 when LMW-AR was inhibited (Figure 2A), indicating that LMW-AR binding might be non-functional.

Since our preliminary studies suggested that at least half of the hinge domain was removed on the LMW-AR (unpublished), we hypothesized that the failure to interact with the hinge region interacting co-regulators contributed to the functional differences between FL-AR and LMW-AR. Filamin A has been reported to repress AR-dependent transcription by interacting with hinge domain. We analyzed the endogenous gene expression of UGT2B15 in DHT for 2hr or in the absence of androgen following siRNA-mediated silencing of filamin A. As shown in Figure 8C, anti-filamin A siRNA reduced its mRNA level to 11%. Silencing of filamin A expression relieved repression of UGT2B15 in the presence of DHT (Figure 2B: 'siRNA control AD-' vs. 'siRNA control DHT 2hr' and 'FLN siRNA DHT 2hr'), indicating that filamin A is the co-repressor of FL-AR in the suppression of the gene. Silencing of filamin A in the absence of androgen, on the other hand, showed no effect on the expression of UGT2B15 (Figure 2C : 'siRNA control AD-' vs. 'FLN siRNA AD-'), indicating that the regulatory activity of LMW-AR is not affected by filamin A. This suggests that the difference in their interaction with co-regulator filamin A contributes to the different transcriptional outcome of FL- and LMW-AR.

It is known that AR-regulation of gene transcription is governed in part by an interaction with multiple binding partners. These interaction occur via the different domains, including the hinge domain and the LBD. Since the LMW forms do not have the LBD and are missing at least a part of the hinge domain, the repertoire of molecules that interact with the FL and LMW-AR would not be identical, which may contribute to their different transcriptional activity on specific genes. Silencing of filamin A, a co-repressor known to interact with the hinge domain of AR, abolished the suppression of UGT2B15 in response to androgen but had no effect on the expression of UGT2B15 in the absence of androgen, supporting our hypothesis that FL- and LMW-AR may interact differently with co-regulators and subsequent transcription activation/repression. This study is almost completed and will be submitted shortly for publication. (M Mudryj et al. Low molecular weight androgen receptor isoform regulated transcripts are refractory to Filamin A repression.)

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of the role calpain in AR proteolysis in two different cell culture lines.
- Demonstrating that an HIV protease inhibitor can mimic calpain inhibitors and reduce the proliferation of Rv1 cell in tissue culture studies and in a xenograft model.
- Defining the role of the MAP signaling pathway in the etiology of the AR-LMW forms.

- Characterization of the androgen regulated gene expression profile in Rv1 and R1 cells.
- Characterization of the AR DNA binding profile in R1 and Rv1 cells.
- Identification of direct AR target genes in R1 and Rv1 cells.
- Identification of genes regulated by the LMW AR form.

REPORTABLE OUTCOMES:

Manuscripts

Stephen Libertini, Clifford G. Tepper, Veronica Rodriguez, David M. Asmuth, Hsing-Jien Kung and **Maria Mudryj**. Evidence for Calpain mediated Androgen Receptor Cleavage as a Mechanism of Androgen Independence and Potential Therapeutic Target in Prostate Tumors. *Cancer Research* **69**, 9001-5. (Manuscript in appendix)

Hong-Lin Devlin and **Maria Mudryj**. Progression of Prostate Cancer: Multiple Pathways to Androgen Independence. *Cancer Letters* 274 (2):177-86. (Manuscript in appendix)

Beolla, RG, Yu Wang, Alfredo Asuncion, Karim Chamie, Salma Siddiqui, **Maria Mudryj**, Javed Siddiqui, Arul M. Chinnaiyan, Rohit Mehra, Ralph W. deVereWhite and Paramita. M. Ghosh Cellular Localization of Filamin A in Advanced Hormone Refractory Prostate Cancer: Immunohistochemical Correlation with Metastases. *Clinical Cancer Research* **15**(3):788-96. (Manuscript in appendix)

Honglin Chen, Stephen J. Libertini, Yu Wang, Hsing-Jien Kung, Paramita Ghosh, **Maria Mudryj**. Erk Regulates Calpain 2 Induced Androgen Receptor proteolysis in CWR22 Relapsed Prostate Tumor Cell Lines. *Journal of Biological Chemistry* published November 28, 2009 as doi:10.1074/jbc.M109.049379 (Manuscript in appendix)

In Preparation

Honglin Chen, Steve Libertini, Cliff Tepper, Hsing-Jien Kung, Michael George, Satya Dandekar, Bushra el-Batain, Paramita Ghosh, **Maria Mudryj**. Genome-wide analysis of androgen receptor binding and gene regulation in two CWR22-derived prostate cancer cell lines. *Submission pending approval of all co-authors*. (Manuscript in appendix)

Stephen Libertini, Honglin Chen, Veronica Rodriguez, Hau Nguyen, Tilak Koilvaram **Maria Mudryj**. Calpain 2 is a direct transcriptional target of E2F3 and the androgen receptor in prostate tumor derived cells.

Maria Mudryj, Wang Y, Honglin Devin, Stephen Libertini, Paramita Ghosh. Genes regulated by the low molecular weight androgen receptor isoform are refractory to Filamin A repression.

Abstracts/Presentations

AACR meeting 2006 "A Novel Mechanism of Androgen Independence: Calpain-dependent Proteolysis of the Androgen Receptor."

IMPACT meeting 2007 "Evidence for Calpain mediated Androgen Receptor Cleavage as a Mechanism for Androgen Independence and Potential therapeutic Target in Prostate Tumors."

Prostate meeting 2009 "Expression of the low molecular weight isoforms in two different CWR22-derived cell lines."

Patents and licenses applied for and/or issued

None

Degrees obtained that are supported by this award

Honglin Chen received her Ph.D. (Genetics)

Development of cell lines, tissue or serum repositories;

None

Infomatics such as databases and animal models, etc.;

Rv1 and R1 androgen regulated expression microarray studies will be deposited in GEO.

Funding applied for based on work supported by this award

Employment or research opportunities applied for and/or received based on experience/training supported by this award

Honglin Chen was offered and accepted a staff position with Genentech based on the studies supported by this award. Veronica Rodriguez (MS student) was offered a position in a biotech company.

CONCLUSION

Our original studies based on the observation that a LMW AR form is present in the Rv1 cells and that the calpain protease can generate this LMW form greatly evolved. During the course of this study it became apparent there were several LMW forms that could be generated by proteolysis, but also by alternative splicing. Since several mechanisms exist to produce a very similar AR isoform, suggests that this form must be important in the tumorigenesis process. This is further buttressed by the detection of the LMW AR in human prostate cancer.

We initially used commercially available calpain inhibitors to reduce LMW AR expression, but in the course of the study found that an HIV inhibitor could also reduce the expression of the LMW AR. Since HIV protease inhibitors are on the market they could be re-positioned for treatment of

prostate cancer. It is noteworthy that currently there is a clinical trial that is recruiting patients to test the efficacy of HIV protease inhibitor Nelfinavir as a chemotherapeutic.

Calpain inhibitors that are currently available are very toxic, therefore we reasoned that if we know how calpain was activated an alternative drug target could be identified. Our studies on the R1 cells line indicated that inhibitors of the MAP kinase signaling pathway may also be effective therapeutic target. MEK and ERK inhibitors are being developed.

One issue that our studies begin to address is to define the importance of the LMW AR in regulating gene expression in prostate tumorigenesis. Rather than focusing on the precise identify of the LMW AR, we realized that the common feature of all the LMW forms is that they are missing the LBD and as such would translocate into the nucleus in the absence of androgen. This provided us with an opportunity to use two cell lines that have the LMW forms and define the AR binding and androgen dependent gene expression profiles. To accomplish this we first need to determine the similarities and difference of the two cell lines. We anticipated that they would be similar, but to our surprise the androgen-dependent gene expression and the gene expression profile in general of the two times is very different. Yet the AR DNA binding profile is similar. This is a crucial finding, since it argues that androgen dependent gene expression is governed by two features: 1) the binding AR to regulatory regions of genes and 2) the subsequence regulation of AR-mediated transcription by AR co-regulator. These co-regulators are different in different cellular contexts. The LMW AR is subject to regulation by the co-factors as well, but since LMW AR is missing a portion of the molecule it's interaction will be different from that of the full length AR. This is what we have seen in our studies.

From a clinical perspective, the AR interacting co-regulators may be therapeutic opportunities. But since the co-regulators are bound to be different in different prostate tumors, the optimal therapeutic treatment would differ from patient to patient. Understanding the AR regulatory mechanisms in specific tumors is the key to defining the most efficacious treatment.

REFERENCES: References are in the manuscripts.

SUPPORTING DATA: All figures and/or tables shall include legends and be clearly marked with figure/table numbers.

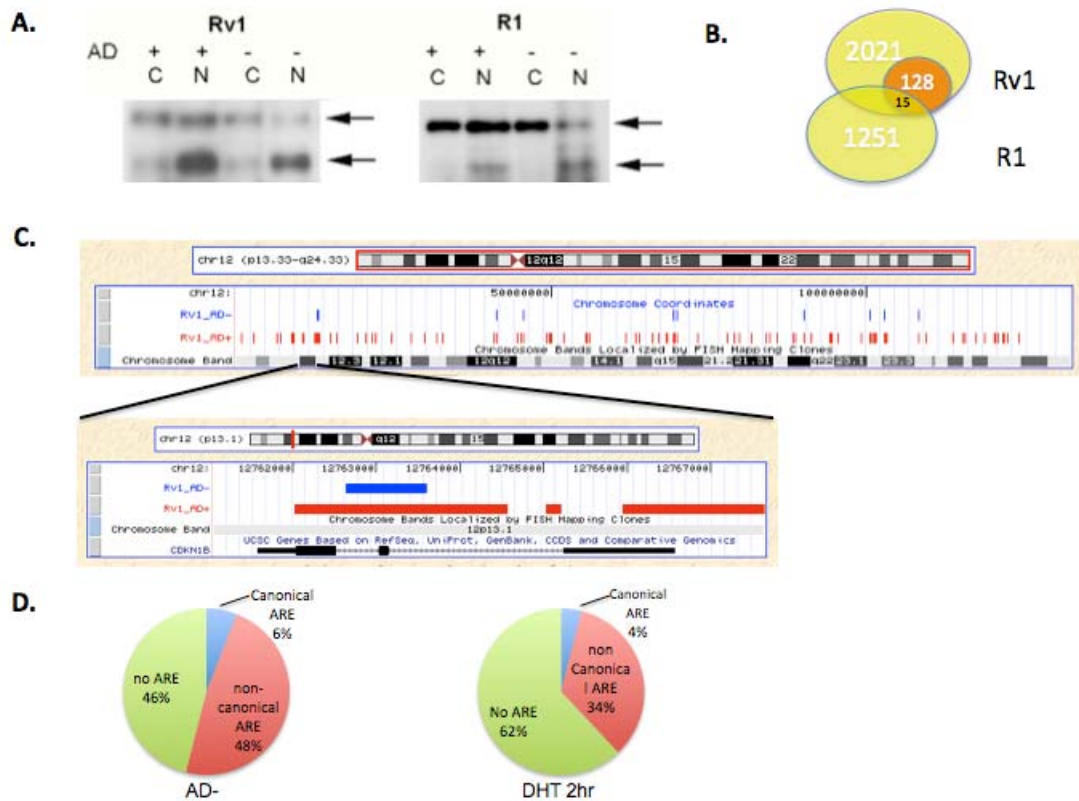


Figure 1. The identification of the potential chromosomal binding sites of LMW AR in Rv1 cells by ChIP-on-chip assay. A. The cytoplasmic and nuclear extracts showed the cellular location of the FL- and LMW-AR in Rv1 and R1 cells in the absence and presence of androgen. B. The diagram showed the number of the overlapping chromosomal binding sites identified in Rv1 cells cultured in the absence of androgen (orange) or stimulated with DHT for 2hr (yellow) and in R1 cells stimulated with DHT for 2hr (yellow). C. An extensive view of the binding sites in chromosome 12 identified in Rv1 cells cultured in the absence of androgen (Rv1-AD-) or stimulated by DHT for 2hr (Rv1_AD+) (top panel). A closer view of the binding sites around the gene CDKN1B in Rv1 cells cultured in the absence androgen or stimulated by DHT for 2hr (bottom panel). The graph was generated using UCSC genome browser. D. A pie chart showing the distribution of the binding motifs of AR in the absence of androgen (AD-) or in response to DHT for 2hr.

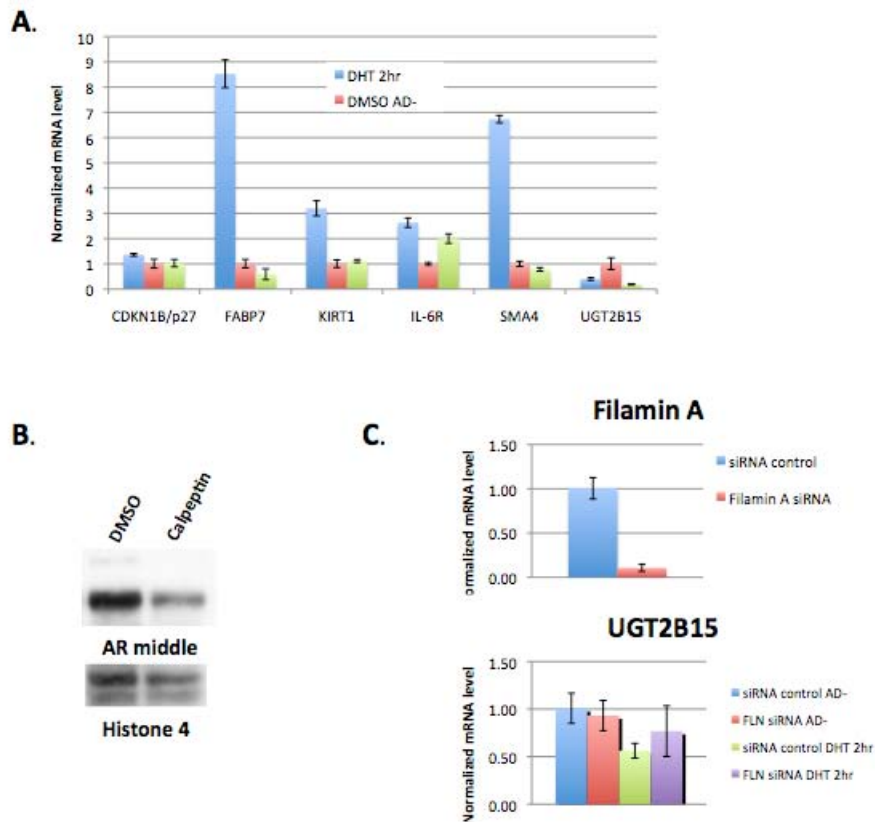


Figure 2. Transcriptional regulation of LMW-AR in Rv1 cells. (A). Real-time PCR analysis of LMW-AR bound genes in response to DHT stimulation or inhibition of LMW-AR expression by calpeptin treatment. Rv1 cells proliferated in the absence of androgen were incubated with 10nM DHT for 2hr or 40uM calpeptin for 48hr. (B). Calpeptin treatment reduced the amount of LMW-AR in the Rv1 nucleus extract. Rv1 cells proliferated in the absence of androgen were treated with 40uM calpeptin for 48hr. Cells were harvested, fractionated and subject to Western blot. The band is the LMW-AR and FL-AR is invisible on this blot. (C). Real-time PCR analysis of Filamin A and UGT2B15. Rv1 cells proliferated in the absence of androgen were treated with anti-filamin A siRNA or control siRNA for 72hr, then harvested for RNA extraction, or treated with 10nM DHT for 2hr and then harvested for RNA extraction.

Table 9. The list of potential LMW-AR chromosomal binding sites identified by ChIP-on-chip in Rv1 cells cultured in androgen depleted medium.

chromosome	start	end	FDR	Closest gene	distance_to_TSS	location
chr1	16864805	16865837	0.05	NBPF1	-52753	TSS_upstream
	22136758	22137152	0.00	HSPG2	-622	TSS_upstream
	* 42578210	42578867	0.05	FOXJ3	-5049	TSS_upstream
	85111676	85111897	0.05	EDG7	-7357	TSS_upstream
	152657272	152657555	0.04	IL6R	13121	intron
	* 159635314	159635709	0.05	C1orf192	-31224	TSS_upstream
	161110301	161110770	0.05	C1orf110	-5307	TSS_upstream
						TES_downstream
	205563333	205563694	0.00	CD55	234307	m
	208025311	208026089	0.04	C1orf74	-1188	TSS_upstream
	216522748	216523246	0.00	CGI-115	-2277	TSS_upstream
	216524107	216524509	0.05	CGI-115	-966	TSS_upstream
	224074724	224075447	0.02	EPHX1	-4513	TSS_upstream
	224076182	224076536	0.03	EPHX1	-3239	TSS_upstream
	226930302	226930964	0.05	RHOU	-6858	TSS_upstream
	227474815	227475660	0.05	RAB4A	1736	intron
	227687485	227687790	0.05	NUP133	23073	intron
chr2	9068246	9068784	0.05	MBOAT2	-7189	TSS_upstream
	* 61096462	61097660	0.00	FLJ32312 LOC9034	1650	5'UTR
	96709406	96710018	0.02	2	-15208	TSS_upstream
	102100298	102100788	0.05	IL1R1	-36290	TSS_upstream
	109906570	109906985	0.02	RGPD7	-846	TSS_upstream
	110771915	110772458	0.02	RGPD5	-282326	TSS_upstream
	111053390	111053866	0.05	RGPD5	-851	TSS_upstream
	* 121756809	121757420	0.05	TFCP2L1	2130	intron
	135313183	135314419	0.00	ACMSD	1146	intron
	217188354	217189045	0.05	IGFBP2	-17672	TSS_upstream
	238433717	238434904	0.00	RAMP1	1385	intron
	241681282	241681890	0.02	MTERFD2	8810	3'UTR
chr3	95228036	95228573	0.05	STX19	1839	5'UTR
	155525085	155526261	0.00	DHX36	-709	TSS_upstream
	170972672	170973372	0.00	MYNN	-524	TSS_upstream
	184090480	184091149	0.00	ATP11B	96830	intron
	189407063	189407672	0.00	LPP	-6047	TSS_upstream
	189411186	189411946	0.00	LPP	-1848	TSS_upstream
chr4	39868356	39869125	0.02	RHOH	-6256	TSS_upstream
	39869633	39869883	0.05	RHOH	-5238	TSS_upstream
	57242033	57242991	0.05	HOP	-191	TSS_upstream
	* 69116609	69117122	0.05	UGT2B17	-26	TSS_upstream
	* 69570641	69571326	0.02	UGT2B15	-5	TSS_upstream
	71417985	71418195	0.05	UNQ689	-796	TSS_upstream
						TES_downstream
	83631386	83632082	0.02	MASA LOC1501	60948	m
	104160587	104161189	0.00	59	-564	TSS_upstream

	116253672	116254863	0.05	NDST4	213	5'UTR
chr5	17358364	17359521	0.00	BASP1 LOC3892	88193	TES_downstream
	43075976	43077397	0.05	89	-589	TSS_upstream
	69043534	69043826	0.05	SMA4	-32839	TSS_upstream
	69174442	69174858	0.05	GUSBP1	-60708	TSS_upstream
	71046170	71046891	0.00	CARTPT	-4219	TSS_upstream
	95003096	95003955	0.00	RFESD	-4816	TSS_upstream
	132412520	132413483	0.00	HSPA4	-2559	TSS_upstream
	133356892	133357581	0.05	VDAC1 MGC2398	11095	5'UTR
*	147271350	147271821	0.05	5	-5328	TSS_upstream
	165140913	165141810	0.05	MAT2B	2278553	TES_downstream
	165145723	165146101	0.00	MAT2B	2283104	TES_downstream
	177409706	177410694	0.00	PROP1	-54352	TSS_upstream
chr6	27555314	27555674	0.02	ZNF184	-6637	TSS_upstream
	32481685	32482038	0.05	BTNL2	1016	intron
	35802763	35803273	0.02	C6orf81	-9818	TSS_upstream
	123134867	123135269	0.05	FABP7	-7276	TSS_upstream
chr7	77258666	77258951	0.00	TMEM60	6874	TES_downstream
	77259297	77259870	0.00	TMEM60	6099	TES_downstream
	86810485	86812436	0.05	CROT	-1486	TSS_upstream
	90173344	90174014	0.05	PFTK1	-2968	TSS_upstream
	91720528	91720823	0.02	KRIT1	-7512	TSS_upstream
	94789986	94790898	0.05	PON1	1337	intron
	99262534	99262861	0.02	CYP3A43	-874	TSS_upstream
	99263324	99263899	0.00	CYP3A43	40	5'UTR
chr8	1908276	1908755	0.04	KBTBD11	-935	TSS_upstream
	1985866	1986424	0.05	MYOM2	5491	5'UTR
	32618295	32618812	0.02	NRG1	93259	intron
*	62764856	62765634	0.02	ASPH	-327	TSS_upstream
	81155377	81156130	0.05	TPD52	-189	TSS_upstream
	92016257	92016819	0.02	EFCBP1	143585	intron
	95635315	95635625	0.05	KIAA1429	-607	TSS_upstream
	104842195	104842405	0.02	RIMS2	-58375	TSS_upstream
	134408137	134408683	0.05	NDRG1	-29731	TSS_upstream
chr9	458522	459780	0.00	DOCK8 MGC2188	196104	TES_downstream
	42014216	42015102	0.00	1	-69629	TSS_upstream
	42473311	42473672	0.05	ANKRD20	115193	TES_downstream
	43008156	43008569	0.05	A3	650064	TES_downstream
	83490582	83491814	0.05	ANKRD20	650064	TES_downstream
	83490582	83491814	0.05	A3	650064	TES_downstream
	83490582	83491814	0.05	TLE1	2217	intron
*	127099076	127099337	0.05	GAPVD1	35275	5'UTR
	130683610	130683946	0.05	CCBL1	396	5'UTR

chr10	75005198	75007594	0.00	USP54	-23293	TSS_upstream
	88842056	88843108	0.04	GLUD1	2020	intron
	111752538	111753211	0.05	ADD3	-2841	TSS_upstream
	114110373	114110898	0.02	ACSL5	-13270	TSS_upstream
chr11	76424867	76425442	0.04	B3GNT6	2047	5'UTR
	101688751	101689123	0.05	BIRC3	-4466	TSS_upstream
	110679133	110682583	0.00	FLJ45803 PAFAH1B	-5110	TSS_upstream
	116515530	116515766	0.00	2	-4601	TSS_upstream
chr12	12762638	12763601	0.05	CDKN1B	1544	exon
	12921346	12922113	0.05	GPRC5A PRICKLE	-13493	TSS_upstream
	41273916	41274369	0.05	1	-4398	TSS_upstream
	45472464	45473769	0.00	SLC38A4	32885	exon
	69324316	69324768	0.02	PTPRR	110089	intron
	* 69842894	69844074	0.02	TSPAN8	452	intron
	89919368	89919774	0.05	DSPG3	3362	intron
	* 100393303	100394366	0.00	SPIC	-953	TSS_upstream
	102787842	102789518	0.00	NT5DC3	-29576	TSS_upstream
	108233254	108234362	0.00	FOXN4	-2401	TSS_upstream
chr13 *	27089576	27090358	0.00	LNK2	2571	5'UTR
	31417733	31418816	0.02	FRY	-85162	TSS_upstream
	48871900	48872719	0.05	CAB39L	1190	5'UTR
chr14	52082233	52082434	0.05	KIAA1344	6629	5'UTR
	57128860	57129691	0.05	C14orf105	323896	TES_downstream
	67066319	67067360	0.00	PLEKHH1	-2921	TSS_upstream
	77235294	77235817	0.02	ALKBH1	8553	intron
chr15	20286282	20286797	0.04	TUBGCP5	-98403	TSS_upstream TES_downstream
	21131612	21131948	0.00	FLJ36144 GOLGA8	111702	TES_downstream
	26277642	26277943	0.05	G GOLGA8	-19612	TSS_upstream
	26596571	26596904	0.05	G	-19580	TSS_upstream
	40350094	40350898	0.02	TMEM87A	2426	intron
	* 66901192	66901763	0.02	ANP32A	-1201	TSS_upstream
	* 89205500	89205991	0.00	FURIN	-7143	TSS_upstream
chr16	53518414	53519450	0.02	IRX5	-3679	TSS_upstream
chr17 *	7998727	7999908	0.00	PER1	-2891	TSS_upstream
	19486254	19486717	0.05	ALDH3A2	-6170	TSS_upstream
chr18	19518446	19519169	0.00	LAMA3	-4752	TSS_upstream
	46811264	46812019	0.02	SMAD4	1031	5'UTR
chr20	31242786	31243701	0.00	C20orf70	23623	TES_downstream
	57966260	57966718	0.05	CDH26	-387	TSS_upstream
chr22	14653344	14654277	0.00	ACTBL1	14126	3'UTR TES_downstream
	19829248	19829710	0.05	FLJ42953	42175	TES_downstream

	19949347	19950008	0.05	GGT2	-39096	TSS_upstream
	43964814	43965717	0.00	C22orf9	21745	TSS_downstream
chrX	106756502	106757108	0.02	PRPS1	-1609	TSS_upstream
	128623040	128623977	0.00	APLN	-6914	TSS_upstream

APPENDICES:

Evidence for Calpain-Mediated Androgen Receptor Cleavage as a Mechanism for Androgen Independence

Stephen J. Libertini,¹ Clifford G. Tepper,² Veronica Rodriguez,¹ David M. Asmuth,³ Hsing-Jien Kung,² and Maria Mudryj^{1,4}

¹Department of Medical Microbiology and Immunology, ²Division of Basic Sciences, Cancer Center and Department of Biochemistry and Molecular Medicine, and ³Division of Infectious Disease, Department of Internal Medicine, University of California Davis, Davis, California and ⁴Veterans Affairs-Northern California Health Care System, Mather, California

Abstract

Prostate carcinoma is the most commonly diagnosed cancer in men and the second leading cause of death due to cancer in Western civilization. Androgen ablation therapy is effective in treating androgen-dependent tumors, but eventually, androgen-independent tumors recur and are refractory to conventional chemotherapeutics. Hence, the emergence of androgen independence is the most challenging problem in managing prostate tumors. We report a novel mechanism of androgen independence: calpain cleaves the androgen receptor (AR) into an androgen-independent isoform. *In vitro* and *in vivo* analyses show that calpain removes the COOH-terminal ligand binding domain generating a constitutively active molecule. Analysis of human prostate tumors indicates that several tumors express higher levels of this truncated AR than noncancerous prostate tissue. In transient transfection studies, the truncated AR is three to five times more potent than the full-length receptor in transactivating transcription. The androgen-independent Rv1 cells express high levels of the truncated AR, and treatment of these cells with a calpain inhibitor reduces truncated AR expression. In the absence of androgen, inhibition of calpain activity induces apoptosis. The HIV protease inhibitor amprenavir inhibits calpain activity and is also effective in inducing apoptosis in the Rv1 cell line. The cell culture studies were reproduced in a mouse xenograft model, where, in the absence of androgens, amprenavir significantly reduces tumor growth. Together, these studies indicate that calpain-dependent proteolysis of the AR may be a mechanism of androgen independence. The calpain inhibition studies suggest that inhibiting this activity may be a potential treatment for some androgen-independent prostate tumors. [Cancer Res 2007;67(19):9001–5]

Introduction

Most prostate cancers initially present as androgen-dependent neoplasms and therapy relies on androgen ablation aimed at blocking androgen receptor (AR) cell signaling. Although initially successful, androgen-independent tumors that are refractory to such treatments eventually emerge (1). Many androgen-independent prostate cancers continue to express the AR and exhibit reinstatement of its function. Several mechanisms may account for

AR activation in low levels of androgen: (a) AR mutations that require low levels of androgen; (b) activation of AR by nonsteroid ligands, such as growth factors and cytokines (2); (c) overexpression/amplification of AR or its coactivators (3); (d) locus-wide histone transcriptional activation at some, but not all, AR targets (4); and (e) proteolytic processing of the AR to an androgen-independent isoform.

The AR consists of four functional domains: an NH₂-terminal regulatory region, a DNA-binding domain, a hinge domain, and a COOH-terminal ligand binding domain (LBD; ref. 5). The binding of hormone to the LBD allows for translocation of the receptor into the nucleus and recruitment of proteins to the transcription complex (6). Previous reports show that deletion of the LBD generates an androgen-independent transcriptional activator (7).

Calpains, calcium-dependent proteinases, are ubiquitously expressed. In general, calpains cleave proteins at a limited number of sites to generate large polypeptides (8). Substrate specificity is based on sequence and substrate conformation (9). Calpain activity is regulated by multiple mechanisms, including calcium modulation, autoproteolysis, phosphorylation, intracellular distribution, and inhibition by calpastatin (8). Interestingly, calpain 2 levels are elevated in invasive prostate tumors and are highest in metastatic neoplasms (10).

Materials and Methods

Cell culture. LNCaP, Rv1, PC3, and MCF-7 cells were obtained from American Type Culture Collection and propagated in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C and 5% CO₂.

Western immunoblot analysis. Cells were lysed in ice-cold radioimmunoprecipitation assay buffer containing E64, leupeptin, and calpeptin (Calbiochem), and protease inhibitor cocktail P8340 (Sigma). Proteins (30–50 µg) were separated on 10% SDS-PAGE gels, transferred to BA-83 membrane (Schleicher & Schuell), and blocked with 5% nonfat dry milk in PBS/0.1% Tween. The following antibodies were used: AR (central) 441 (Ab-1; Lab Vision Corp.), AR (COOH-terminus) C-19 (Santa Cruz Biotechnology, Inc.), AR (NH₂-terminus) PG21 (Upstate), PSA-ER-PR8 (NeoMarkers), and focal adhesion kinase (FAK; clone 4.47; Upstate). Proteins were detected using chemiluminescence (Amersham Pharmacia).

***In vivo* calpain induction.** LNCaP cells were plated, cultured overnight, pretreated with 40 µmol/L of calpeptin or DMSO for 15 min, and then treated with 10 µmol/L ionomycin (Calbiochem) for 20 min. Cells were harvested, lysed, and assayed as described above.

***In vivo* calpain inhibition.** Rv1 cells (2×10^5) were plated in 35-mm plates and cultured overnight. Cells were treated with DMSO, 40 µmol/L calpeptin, or 30 µg/mL amprenavir, for 24 or 48 h, washed with cold PBS, and harvested. Amprenavir (GlaxoSmithKline) was provided by D.M.A. For analysis of calpain inhibition in the presence or absence of androgens, cells were plated at 10^5 in 35-mm plates and propagated in androgen-containing or androgen-depleted media (phenol red-free media/charcoal-stripped

Requests for reprints: Maria Mudryj, Department of Medical Microbiology and Immunology, University of California Davis, Tupper Hall, Davis, CA 95616. Phone: 530-754-6090; Fax: 530-753-8692; E-mail: mmudryj@ucdavis.edu.

©2007 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-1072

serum) for 48 h before addition of calpeptin, amprenavir (at 15 or 30 $\mu\text{g/mL}$), or DMSO. Cells were refed daily for 3 days. Floating and adherent cells were harvested, washed in PBS, and fixed in 4% paraformaldehyde/PBS (pH 7.4) overnight at 4°C. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; 0.2 $\mu\text{g/mL}$ in 1% Triton X-100/2% paraformaldehyde) for 30 min at 4°C in the dark, washed with PBS, spotted onto slides, dried, coverslipped, and examined by fluorescence microscopy.

In vitro calpain assay. LNCaP cells were resuspended in calpain assay buffer [50 mmol/L HEPES buffer (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100]. Calpain was activated with CaCl_2 , and reactions were incubated at 25°C for 60 min. Alternatively, LNCaP or Rv1 extracts were treated with increasing concentrations of calpain 2 (Calbiochem) for 60 min at 25°C. Reactions were terminated by boiling.

Plasmid construction. The truncated form of AR (tr-AR) was generated by PCR amplification of the sequences encoding amino acids 1 to 648 using the primers, 5'-GGATGAAGTGCAGTTAGGGC-3' and 5'-GGTGCTGGAA-GCCTCTCCTT-3', followed by cloning into pCR 2.1 TOPO (Invitrogen). Cloned sequence was excised by *Xba*I and *Bam*HI and cloned into the *Xba*I and *Bam*HI sites of pcDNA3 (Invitrogen).

Transfection and luciferase assays. PC3 cells were propagated in control or androgen-depleted media. Cells were transfected using Effectene (Qiagen) and analyzed as described previously (11).

Cell proliferation assay. Cellular proliferation was assessed using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) following manufacturer's recommendations.

Human prostate specimens. Tumor tissue samples, flash frozen in liquid nitrogen, were obtained from the University of California Davis Cancer Center Tissue Bank. Cellular extracts were prepared as described previously (11).

Animals and xenograft studies. Rv1 xenograft studies were done as described previously (12). Three days after castration, 4-week-old athymic mice (Harlan Sprague-Dawley) were injected with 1.5×10^6 Rv1 cells in Matrigel (1:1). Mice were randomized into control and treatment groups. Once tumors were measurable, mice were treated with amprenavir (160 mg/kg in peanut oil), or vehicle daily. Animal studies were Institutional Animal Care and Use Committee approved.

Statistics. Analyses using a two-tailed Student's *t* test were used to compare two groups. $P < 0.05$ was considered statistically significant. Error bars represent SE.

Results

Calpain cleavage of the AR. To investigate the link between calpain and prostate tumorigenesis, we examined the AR for potential cleavage sites. Theoretically, the consensus calpain cleavage site (9) between residues 648 and 649 of the human AR would generate two polypeptides: an ~80-kDa polypeptide consisting of the transactivation, DNA binding, and hinge domains and a ~30-kDa LBD. To test this possibility, LNCaP cell extracts were treated with increasing concentrations of CaCl_2 to activate calpain. Addition of calcium promoted AR proteolysis to an ~80-kDa truncated form (tr-AR; Fig. 1A). Addition of calpain inhibitors calpeptin or EGTA prevented proteolysis. Calpain activation was confirmed by analysis of FAK (8). Addition of calcium promoted FAK cleavage from a 120- to a 90-kDa form (Fig. 1A). The high calcium levels required for proteolysis implicated calpain 2 (8). Indeed, addition of calpain 2 resulted in the appearance of the tr-AR and a disappearance of the full-length AR (FL-AR; Fig. 1B). In addition, analysis using antibodies directed against the LBD showed that calpain proteolysis caused the disappearance of FL-AR and appearance of a 30-kDa fragment (Fig. 1B), confirming deletion of this region.

Our previous study identified a novel AR mutation in Rv1 cells, a line derived from the relapsed CWR22R-2152 human xenograft. Rv1 cells contain a duplication of the third exon (13) that was not detected in the parental, androgen-dependent CWR22 tumor (14), and express an 80- to 85-kDa tr-AR that is missing the LBD. Treatment of the Rv1 extracts with calpain 2 completely converted the FL-AR to the tr-AR (Fig. 1C).

In vivo inhibition and activation of AR proteolysis. Treatment of LNCaP cells with the calcium ionophore ionomycin activated endogenous calpain resulting in proteolysis of the AR to an ~80-kDa isoform (Fig. 2A). In a complementary experiment, calpeptin treatment of intact Rv1 cells reduced the level of the tr-AR (Fig. 2B). These *in vivo* results establish that the AR is a calpain substrate.

Truncated AR is more efficient in transactivating transcription. An expression plasmid encoding tr-AR was generated to

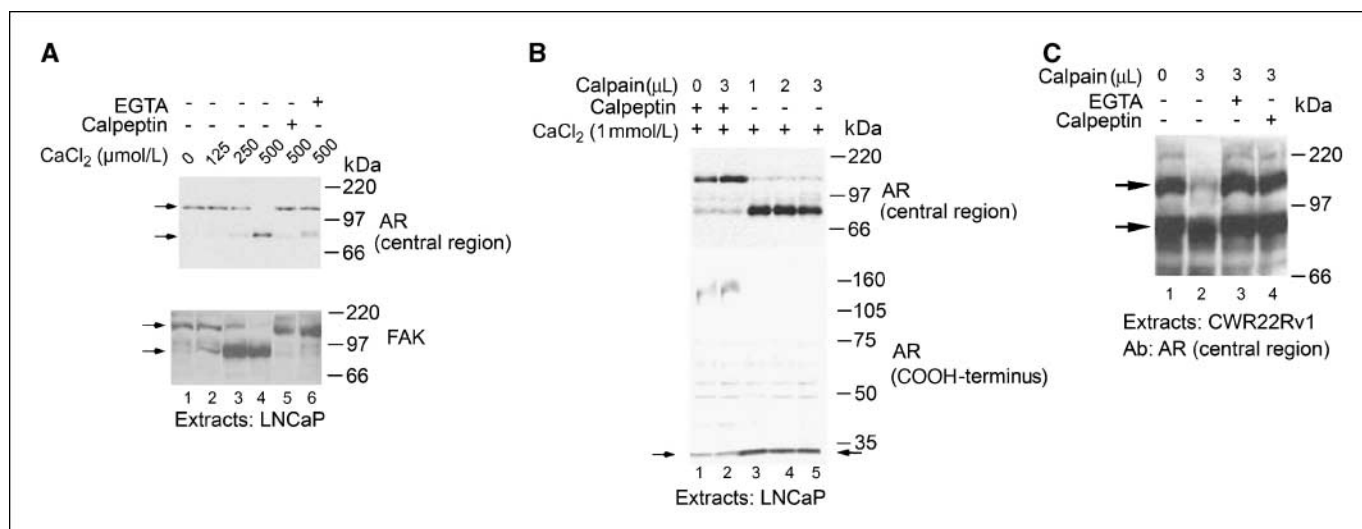
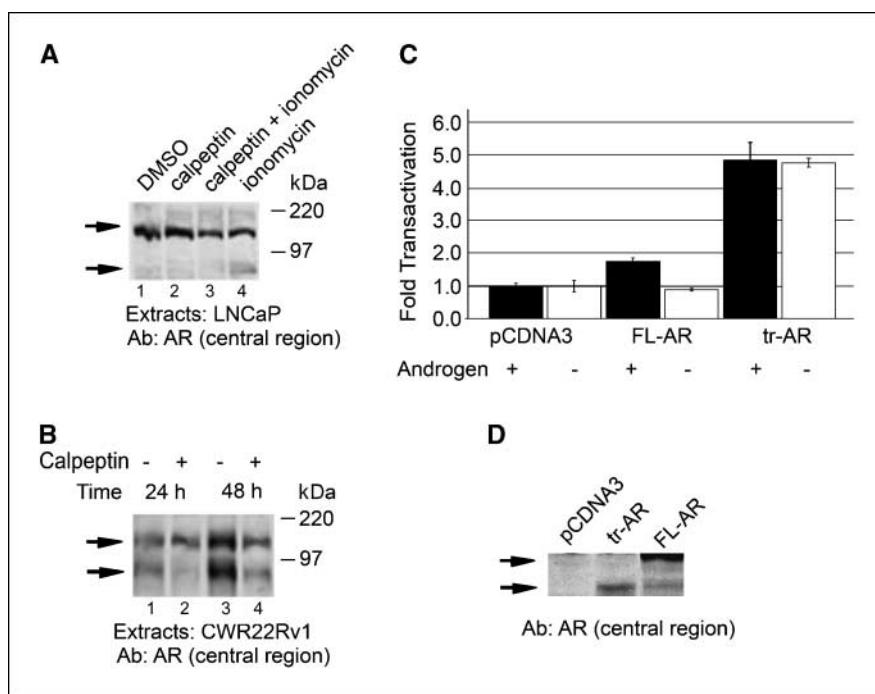


Figure 1. The AR is a calpain substrate. A, Western immunoblot analysis (Ab: AR441) revealed that addition of CaCl_2 to LNCaP extracts resulted in cleavage of the AR to a truncated isoform. Addition of calpeptin or EGTA inhibited AR proteolysis. Addition of CaCl_2 activates proteolysis of FAK to a 90-kDa isoform. B, addition of purified calpain 2 (0.17 units/ μL) to LNCaP extracts promoted AR proteolysis, which was inhibited by calpeptin. An antibody directed against the COOH-terminal region of the AR (C-19) detected the full-length and 30-kDa COOH-terminal fragments. C, treatment of CWR22Rv1 extracts with calpain 2 resulted in the proteolysis of the FL-AR to the truncated isoform.

Figure 2. *In vivo* inhibition of activation of calpain activity. **A**, treatment of LNCaP cells with ionomycin promotes proteolysis of the AR to the truncated isoform. **B**, treatment of CWR22Rv1 cells with 40 $\mu\text{mol/L}$ calpeptin for 24 or 48 h reduces the expression of the tr-AR. **C**, the tr-AR efficiently transactivates transcription of the androgen-responsive PSA promoter. PC3 cells were plated (1.0×10^5 cells/35-mm dish), propagated in control or androgen-depleted media, and transfected with PSA-luciferase 0.03 (μg), 0.050 μg of FL-AR, tr-AR, or vector pCDNA3, and 0.020 μg of the CMV- β -galactosidase plasmids. In the absence of androgens, the tr-AR is ~ 8 -fold more effective than the FL-AR in transactivating transcription. *Columns*, fold transactivation; *bars*, SE. **D**, a Western blot analysis of cells transfected with the control pCDNA3, tr-AR, or FL-AR plasmid along with the luciferase expression plasmid and CMV- β -galactosidase plasmids shows the expression levels of the tr-AR and FL-AR. Loading was normalized to β -galactosidase activity.



determine the efficacy of the tr-AR in transactivating transcription. In transient transfection studies, tr-AR was three to five times more robust than FL-AR in transactivating an AR-dependent prostate specific antigen (PSA) promoter and acted in a ligand-independent manner (Fig. 2C). A Western blot analysis of FL-AR- and tr-AR-transfected cells shows that the levels of AR expression is com-

parable; therefore, the increase in tr-AR activity can be attributed to enhanced transactivation function (Fig. 2D).

Truncated AR expression in prostate tumors. To establish the potential clinical relevance of these observations, the AR was examined in normal and malignant prostate. Nonmalignant tissue (benign prostatic hyperplasia) expressed very low levels of tr-AR

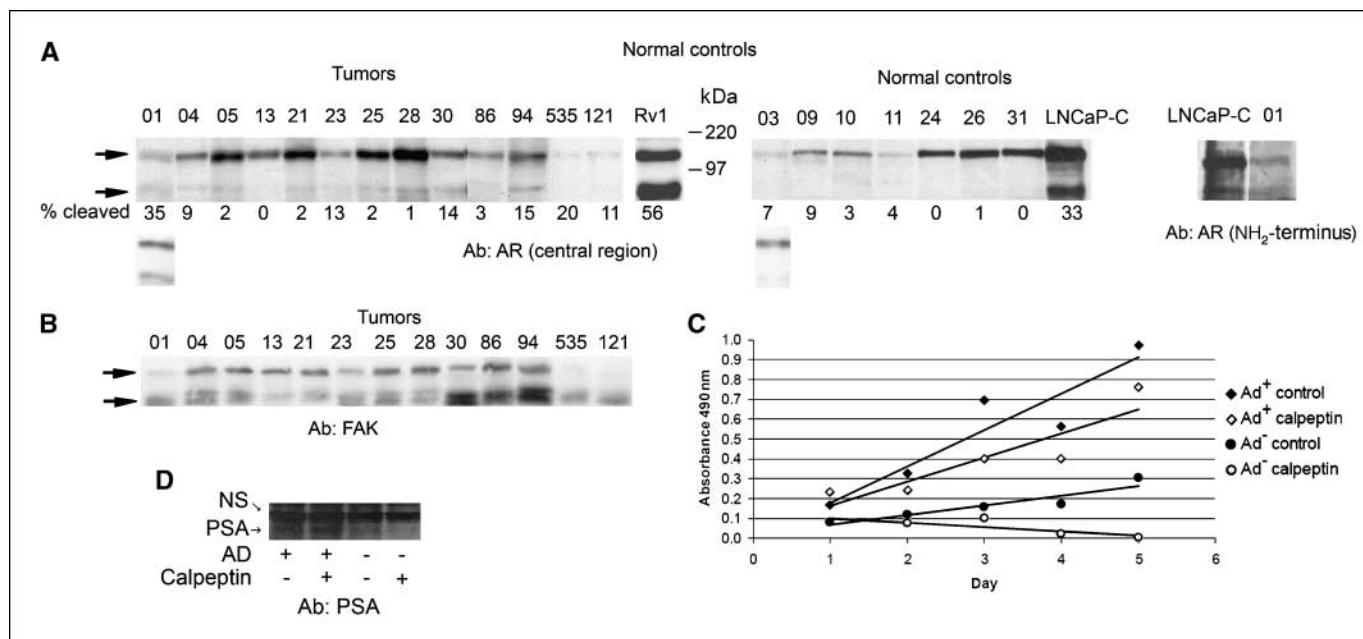


Figure 3. Human prostate tumors express the tr-AR. **A**, tumors 01, 30, and 94 express higher levels of an AR isoform that is similar in size to the isoform detected in CWR22Rv1. *Bottom*, a longer exposure that shows that although the tumor sample and control prostate tissue express similar amounts of the AR, the tumor samples have much higher levels of the truncated isoform. A Western blot analysis using an antibody directed against the NH₂-terminal region of the AR and tumor sample 01 confirms that the truncated form has a COOH-terminal deletion. Partially cleaved LNCaP AR (lighter exposure) served as a marker. Tumor samples were flash frozen in liquid nitrogen within 30 min of tumor resection and stored in liquid nitrogen to minimize proteolytic activity. **B**, expression of cleaved FAK (90-kDa form) is higher in tumor samples that have elevated levels of the tr-AR. **C**, growth curve of Rv1 cells proliferating in AD⁺ media (closed rectangles), AD⁺ media and 40 $\mu\text{mol/L}$ calpeptin (open rectangles), AD⁻ media (closed circles), and AD⁻ media and 40 $\mu\text{mol/L}$ calpeptin (open circles). Cellular proliferation was quantitated using the MTS assay. **D**, Western blot analysis of PSA expression in Rv1 cells proliferating in AD⁺ and AD⁻ media, in the presence or absence of calpeptin. NS, nonspecific band.

(Fig. 3A). However, tumor samples (01, 30, and 94) expressed higher amounts of this isoform. In tumor sample 01, tr-AR accounted for ~30% of the AR. Moreover, tumors with higher tr-AR levels had elevated FAK cleavage, suggesting elevated calpain activity (Fig. 3B).

Inhibition of calpain activity in the absence of androgen promotes apoptosis. Does calpain inhibition in the absence of androgens caused reversion of the Rv1 cells to the parental androgen-dependent phenotype? Cells proliferated in the absence of androgen but more slowly than in androgen-containing media. Calpeptin-treated cells survived well in the presence of androgen, although calpeptin retarded proliferation (Fig. 3C). However, calpeptin treatment in the absence of androgens resulted in a decrease of viable cells. A 48-h calpeptin treatment in the presence of androgens did not reduce the expression of the endogenous androgen-responsive *PSA* gene, whereas calpeptin treatment in the absence of androgens reduced *PSA* expression significantly, suggesting a block in AR-dependent transcription (Fig. 3D). A 72-h calpeptin treatment in the absence of androgens induced apoptosis in ~40% of the cells, supporting the hypothesis that calpain inhibition causes Rv1 cells to revert to an androgen-dependent phenotype (Fig. 4A).

An HIV protease inhibitor inhibits calpain activity. Calpeptin, while effective in cell culture studies, has limited utility for animal studies. A previous report indicated that HIV protease inhibitors inhibit calpain activity (15); therefore, Rv1 cells were treated with amprenavir. The effective amprenavir dose was two or four times the peak plasma level used in anti-retroviral therapy. Amprenavir treatment reduced expression of tr-AR (Fig. 4B). Importantly, treatment of Rv1 cells with amprenavir in the absence of androgens caused the cells to undergo apoptosis (Fig. 4C).

Amprenavir inhibits tumor growth. To test the efficacy of amprenavir in a mouse model, Rv1 tumors were established in castrated nude mice. Amprenavir or vehicle was given daily at a dose that is equivalent to 3.3 times the pediatric dosage used in anti-HIV therapy. Tumor growth was followed for 6 weeks. Amprenavir-treated mice did not exhibit any apparent toxicity and amprenavir treatment resulted in a statistically significant inhibition of tumor growth (Fig. 4D).

Discussion

The role of calpain in the etiology and progression of cancer is supported by reports of increased calpain levels in prostate, renal, and colorectal tumors (10, 16, 17). Calpain cleavage affects various aspects of cell physiology and the consequences of increased calpain activity are determined by cellular context. We and others (18) find that the AR is a calpain substrate. The current study shows that calpain cleaves the AR, removing the LBD to generate a constitutively active molecule that was more robust in transactivating transcription from the *PSA* promoter. One potential explanation for increased tr-AR activity is that the FL-AR has to be activated by ligand, whereas the tr-AR is active immediately after translation, hence exhibits enhanced activity. Alternatively, deletion of a domain that interacts with coactivators and corepressors may prevent recruitment of corepressors into the transcription complex, enhancing transcription. Moreover, the tr-AR and FL-AR may interact with a distinct subset of proteins and potentially transactivate distinct cohorts of genes.

Western blot analysis of AR in tumor tissue detected elevated expression of the truncated AR isoform in some tumors. This analysis provides compelling evidence that expression of this

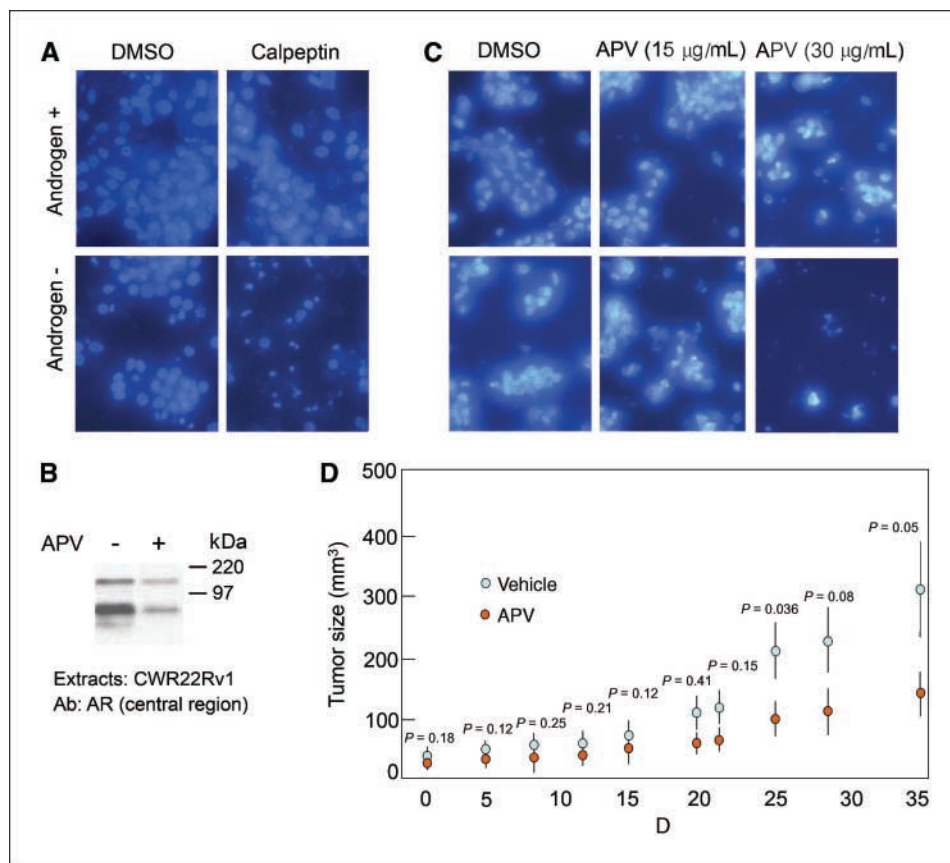


Figure 4. Treatment of Rv1 cells with calpain inhibitors in the absence of androgens promotes apoptosis. **A**, Rv1 cells were propagated in the absence or presence of androgens and treated with DMSO or calpeptin (40 µmol/L) for 72 h. After fixation, cells were stained with DAPI and fluorescence microscopy was used to detect chromatin condensation and pyknotic nuclei. **B**, treatment of Rv1 cells with 30 µg/mL amprenavir (APV; 48 h) reduced the expression of tr-AR. **C**, Rv1 cells growing in the presence or absence of androgen were treated with 15 or 30 µg/mL amprenavir or DMSO for 72 h. DAPI staining was used to detect apoptotic cells. **D**, amprenavir treatment retards the androgen-independent growth of Rv1 xenografts in mice. Rv1 tumors were established in nude athymic mice as described previously. Tumor-bearing mice were randomly assigned to control (8 animals) or experimental (7 animals) groups. When tumors were measurable (~10 d), mice were treated daily with amprenavir (160 mg/kg; 4 mg/25 g mouse, 100 µL) or vehicle (peanut oil) by esophageal gavage. Tumor sizes were measured with digital calipers and volumes were calculated using the formula: tumor volume (mm³) = width × length × height × 0.5236. Results are presented as mean tumor volume for each group at each time point. Red, amprenavir treatment; blue, control. Points, tumor size; bars, SE.

isoform is not a rare event restricted to relapsed cell lines derived from a single tumor (CWR22) but a previously uncharacterized feature of certain prostate tumors. Higher levels of FAK cleavage in these samples suggest higher calpain activity. The role of the truncated AR in the etiology of androgen independence is buttressed by the identification of a Q640 termination mutation in an androgen-independent prostate tumor (19). This mutation results in the expression of a tr-AR missing the LBD.

Rv1 cells, but not the parental CWR22 androgen-dependent xenografts, express high levels of a COOH-terminally truncated AR. During progression of the CWR22 cells to androgen independence, the Rv1 line acquired an additional mutation, resulting in the insertion of sequences near the calpain cleavage site, perhaps sensitizing the molecule to calpain-mediated proteolysis. Treatment of Rv1 cells with calpain inhibitors in the absence of androgens causes the cells to undergo apoptosis possibly by causing reversion to the androgen-dependent phenotype. This argues that androgen-independent growth and survival of Rv1 is in part due to the generation of a constitutively active tr-AR.

Although HIV protease inhibitors were developed to specifically inhibit a HIV protease, they also possess anti-calpain activity (15). We have shown that amprenavir mimics the effects of calpeptin. If increased calpain activity contributes to prostate tumorigenesis, then inhibition of this activity would be an attractive therapeutic target. The results of the current study raise the possibility that HIV protease inhibitors may have efficacy in reducing the growth of certain prostate tumors.

Acknowledgments

Received 3/28/2007; revised 7/25/2007; accepted 8/10/2007.

Grant support: Department of Defense grant pc051049 (M. Mudryj and C.G. Tepper), University of California Davis Cancer Center, and NIH K23 AI01688 (D.M. Asmuth). Studies were conducted in facilities constructed with support from Research Facilities Improvement Program C06 RR-12088-01 (National Center for Research Resources, NIH).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Hongwu Chen for AR and Paramita Ghosh (UC Davis, Davis, CA) for PSA Pro-luc plasmids, Michael Seldin for helpful comments, and Denise W. Law for assistance with animal studies.

References

- Gittes RF. Carcinoma of the prostate. *N Engl J Med* 1991;324:236–45.
- Reddy GP, Barrack ER, Dou QP, et al. Regulatory processes affecting androgen receptor expression, stability, and function: potential targets to treat hormone-refractory prostate cancer. *J Cell Biochem* 2006;98:1408–23.
- Chen CD, Welsbie DS, Tran C, et al. Molecular determinants of resistance to antiandrogen therapy. *Nat Med* 2004;10:33–9.
- Jia L, Shen HC, Wantroba M, et al. Locus-wide chromatin remodeling and enhanced androgen receptor-mediated transcription in recurrent prostate tumor cells. *Mol Cell Biol* 2006;26:7331–41.
- Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *J Cell Biochem* 2004;9:483–90.
- Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Mol Cell* 2002;9:601–10.
- Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol Endocrinol* 1991;5:1396–404.
- Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* 2003;83:731–801.
- Tomba P, Buzder-Lantos P, Tantis A, et al. On the sequential determinants of calpain cleavage. *J Biol Chem* 2004;279:20775–85.
- Rios-Doria J, Day KC, Kuefer R, et al. The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. *J Biol Chem* 2003;278:1372–9.
- Libertini SJ, Robinson BS, Dhillion NK, et al. Cyclin E both regulates and is regulated by calpain 2, a protease associated with metastatic breast cancer phenotype. *Cancer Res* 2005;65:10700–8.
- Nagabhushan M, Miller CM, Pretlow TP, et al. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both *in vivo* and in soft agar. *Cancer Res* 1996;56:3042–6.
- Tepper CG, Boucher DL, Ryan PE, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res* 2002;62:6606–14.
- Sramkoski RM, Pretlow TG II, Giaconia JM, et al. A new human prostate carcinoma cell line, 22Rv1. *In vitro Cell Dev Biol* 1999;35:403–9.
- Gupta AK, Cerniglia GJ, Mick R, McKenna WG, Muschel RJ. HIV protease inhibitors block Akt signaling and radiosensitize tumor cells both *in vitro* and *in vivo*. *Cancer Res* 2005;65:8256–65.
- Braun C, Engel M, Seifert M, et al. Expression of calpain I messenger RNA in human renal cell carcinoma: correlation with lymph node metastasis and histological type. *Int J Cancer* 1999;84:6–9.
- Lakshmikuttyamma A, Selvakumar P, Kanthan R, Kanthan SC, Sharma RK. Overexpression of m-calpain in human colorectal adenocarcinomas. *Cancer Epidemiol Biomarkers Prev* 2004;13:1604–9.
- Pelley RP, Chinnakannu K, Murthy S, et al. Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer Res* 2006;66:11754–62.
- Ceraline J, Cruchant MD, Erdmann E, et al. Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int J Cancer* 2004;108:152–7.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Mini-review

Progression of prostate cancer: Multiple pathways to androgen independence

Hong-Lin Devlin, Maria Mudryj*

Department of Medical Microbiology and Immunology, 3147 Tupper Hall, University of California – Davis, School of Medicine, Davis, CA 95616, USA
 Veterans Affairs-Northern California Health Care System, Mather, CA 95655, USA

ARTICLE INFO

Article history:

Received 28 March 2008

Received in revised form 28 May 2008

Accepted 9 June 2008

Keywords:

Prostate cancer

Androgen receptor

Androgen independence

Calpain

Signaling

ABSTRACT

Prostate cancer remains one of the most commonly diagnosed cancers and a leading cause of cancer death in men. Initially, prostate tumors respond to hormonal therapies, but androgen-independent tumors refractory to these therapies emerge. Identifying the mechanisms responsible for the emergence of androgen independence has been the subject of multiple studies. This article reviews the multiple pathways that have been shown to promote androgen independence, including a recently described mechanism that involves androgen receptor proteolysis to a constitutively active ligand-independent isoform. Identifying the underlying mechanisms of androgen independence is crucial in the design of appropriate therapies for hormonally refractive neoplasms.

© 2009 Published by Elsevier Ireland Ltd.

1. Introduction

Prostate cancer (CaPs) is one of most commonly diagnosed malignancies in the developed world [1]. Most CaPs, as well as normal prostate tissue, are dependent on the presence of androgens for growth and survival. Localized CaPs can be effectively treated with radical prostatectomy or radiation therapy. For more advanced neoplasms androgen ablation therapy aimed at blocking signaling through the androgen receptor (AR), is an effective initial treatment option [2]. Unfortunately, aggressive androgen-independent (AI) cancers refractory to conventional hormonal therapies eventually develop. The majority of AI continue to express AR, which appears to function despite the castrate levels of androgen [3–5].

2. Androgen receptor

The AR mediates the action of androgens, steroid hormones that are essential for the expression of the male phenotype. During development the initial event of male

differentiation, the development of testes from the gonadal ridge, does not require androgens. However, the subsequent differentiation cascade that includes inhibition of the development of female internal genitalia and the induction of differentiation of the Wolffian ducts into the male internal genitalia, depends on the action of testosterone. The development of the prostate, the prostatic urethra and the masculinization of the external genitalia requires the more potent androgen-dihydrotestosterone. During puberty androgens are required for male sexual maturation, while in adulthood they are essential for the maintenance of male reproductive function, muscle mass and bone density [6,7].

The AR is pivotal not only to the initiation and growth of prostate cancers but also in their responses to therapy. Like other members of the steroid hormone superfamily of ligand-activated transcription factors, the AR protein contains several functional domains: an N-terminal regulatory region, a DNA-binding domain (DBD), a hinge domain and a ligand binding domain (LBD) [8] (Fig. 1). The N-terminal region, encoded by the first exon of the AR gene, comprises approximately half of the AR molecule. This domain mediates most transcriptional activity and is an important site for interaction with co-regulators that alter AR tran-

* Corresponding author. Tel.: +1 530 754 6090; fax: +1 530 752 8692.
 E-mail address: mmudryj@ucdavis.edu (M. Mudryj).

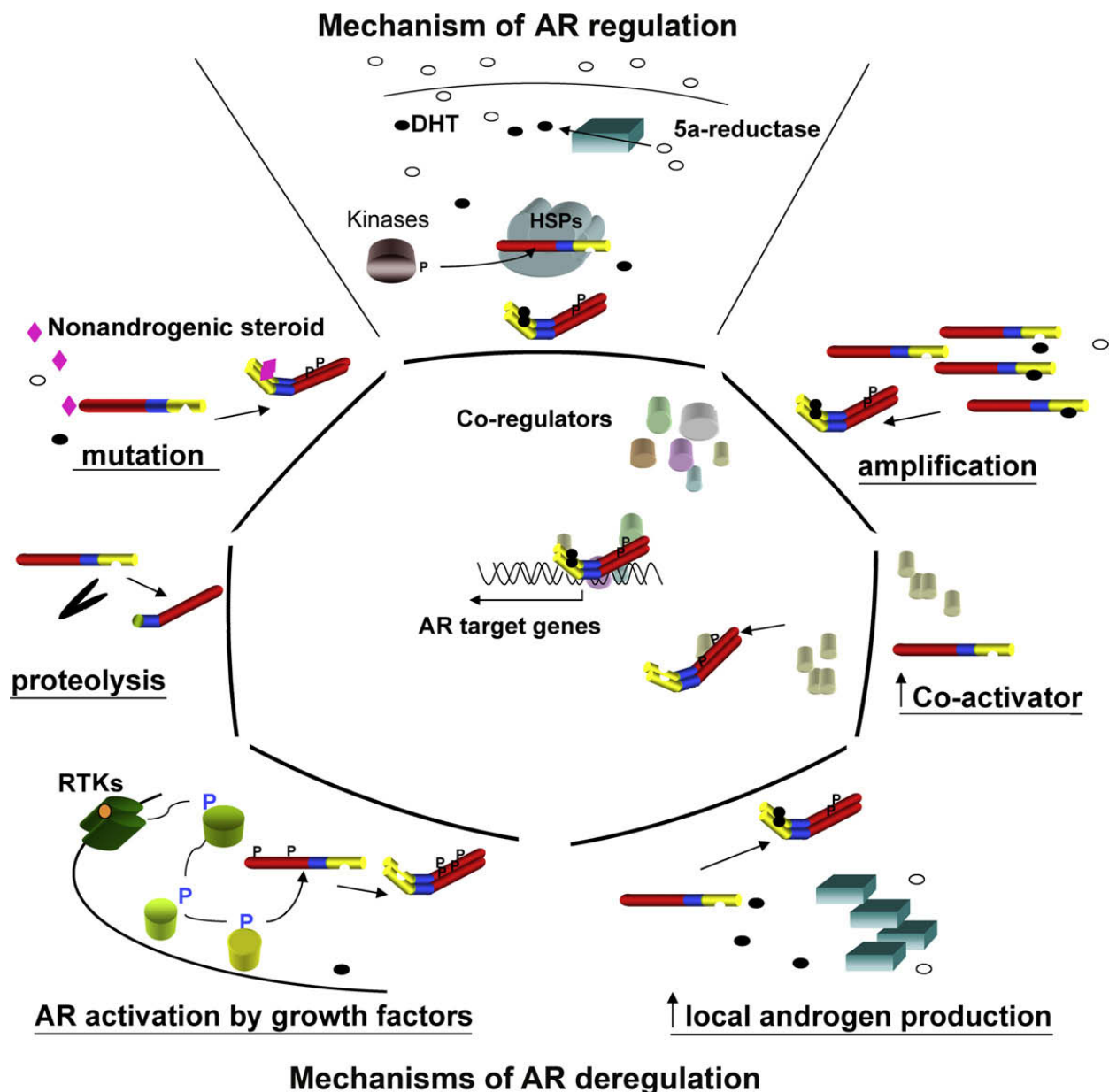


Fig. 1. Mechanisms of androgen receptor deregulation. Normal prostate cells are dependent on the action of the androgen receptor (top of schematic). Androgen-independent prostate cells can employ multiple mechanisms to survive in a low androgen environment. The pathways include AR amplification, mutation, proteolysis, activation by non-steroid growth factors, increase AR co-regulators, and an increase in local production of androgen.

scriptional activity. This domain also contains long polyglutamine and a polyglycine repeats. The polyglutamine repeat differs in length from 14 to 35 amino acids [9], and different polyglutamine repeat lengths have been linked to the modulation of AR activity. Shorter polyglutamine repeats are associated with more aggressive cancer phenotypes, earlier age of onset and a higher probability of recurrence [10–12].

The DBD, the most conserved region of the AR molecule [13], is encoded by the second and third exon. This cysteine-rich region contains two zinc fingers and is essential for recognizing androgen-response elements (ARE), sequences that are usually in enhancer regions of AR-regulated genes. The canonical high affinity ARE sequence consists of two half sites (5'-AGAACA-3') separated by

three nucleotides and binds an AR homodimer [14]. The hinge region, encoded by exon 4, bridges the DBD and the LBD, and includes a nuclear translocation signal, as well as phosphorylation and acetylation sites [15].

The C-terminal sequence, encoded by the distal part of exon 4 and exons 5, 6, 7 and 8, contains the LBD and a second transcriptional activation region. In the absence of hormone, the AR localizes primarily to the cytoplasm bound to heat shock proteins (HSPs). The binding of hormone to the LBD initiates a cascade of events that alters AR conformation, promotes AR phosphorylation, dimerization, dissociation of AR from HSPs, and translocation into the nucleus [16–19]. Interaction of the AR with AR antagonists also modifies receptor conformation but this change favors interaction with transcriptional co-repressors [16].

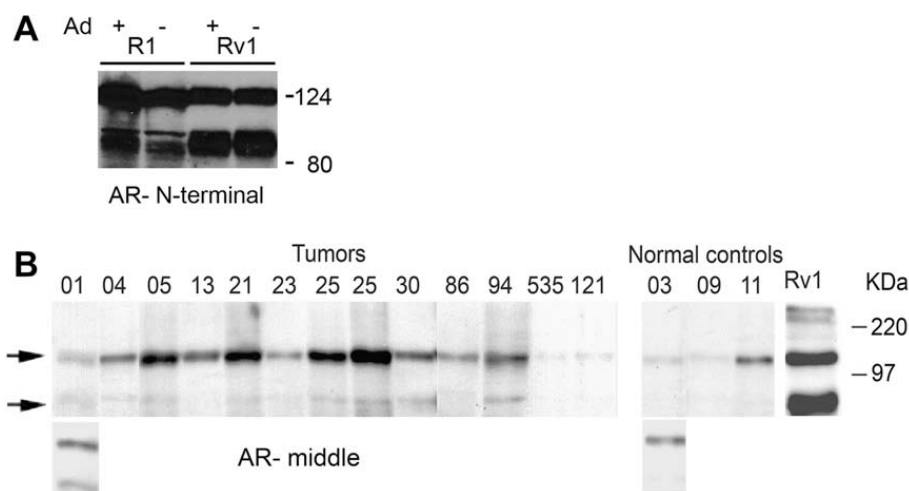


Fig. 2. Expression of truncated AR. (A) Western blot analysis of R1 and Rv1 cells proliferating in the presence (+) and absence of androgen (–). An antibody directed against the N-terminal region of the AR detects both isoforms of the molecule.

3. Multiple paths to androgen independence

The molecular events that drive the transition from an androgen-dependent to androgen-independent state remain unclear. It is unknown whether androgen-dependent cells acquire the ability to proliferate in castrate levels of androgen, or if castrate levels of androgen provide the selective pressure that result in the outgrowth of a minor population of tumor cells that are androgen-independent. Immunohistochemical analyses have shown that most androgen-independent tumors continue to express the AR [3], and the AR responsive PSA protein. This suggests that the AR signaling pathway remains intact but its regulation may be altered. Several mechanisms may account for AR activation in low levels of androgen: (1) Overexpression/amplification of AR; (2) AR mutations that require low levels of androgen or are activated by other steroid ligands; (3) increased local production of androgen by prostate cells; (4) activation of AR by non-steroid ligands such as growth factors and cytokines; (5) altered expression of AR co-activators or co-repressors; and a newly described mechanism; (6) proteolytic processing of the AR to an androgen-independent isoform (Fig. 2).

4. Overexpression/amplification of AR

Amplification of the AR gene is a potent mechanism for increasing expression of the AR. Studies have found that approximately 25–30% of androgen-independent tumors that arise after hormonal therapy have AR amplification [20,21]. The increase in receptor abundance results in sufficient ligand binding for sustained AR signaling in castrate levels of androgens. This is consistent with the reports that patients with AR gene amplification have disease recurrence while on therapy and have a greater likelihood to respond to second line hormonal therapy than patients without AR amplification. Furthermore, an analysis of several xenograft models showed that AR gene expression increased in progression from androgen dependence to androgen independence [22].

5. AR mutations

Mutation of the AR gene to either a hypersensitive receptor or a receptor with expanded ligand specificity would confer androgen-independent properties. The frequency of AR mutations has been controversial. AR mutations in early stages of tumorigenesis appear to be rare (0–4%) [23–25], but are more frequent in more advanced tumors or recurrent tumors [26]. AR mutations were detected in 10–30% of patients with androgen-independent tumors that were treated with anti-androgen therapy [25–28]. Mutations map to all 8 exons. LBD mutations potentially alter ligand-receptor interaction and cluster to three regions – codons 670–678, 701–730, and 874–910 [8,13]. The well studied prostate tumor cell line, LNCaP, has a mutation in the LBD (T877A) that allows for activation by other steroid molecules [29]. An analysis of mutations that arose in cancers treated with anti-androgen found this mutation occurred frequently [30], suggesting that anti-androgen therapy is a selective pressure that drives the proliferation of cells that acquired these mutations. A functional analysis using a colorimetric yeast assay assessed the transcriptional activity of 44 AR mutations in response to ligand binding [31]. The results showed that the mutant receptors had diverse transcriptional activities (summarized in Table 1). Of the 44, 16% exhibited loss of function, 32% had partial function, 7% had wildtype function, and 45% of the tumors (20 tumors) had gain of function mutations. Of the 20 tumors, five had broadened ligand specificity and an additional 7 were partly activated by a combination of two steroid hormones. The importance of the loss of function mutations is unclear, but it is possible that such mutations may confer a growth advantage to cells that have already circumvented the need for androgen.

6. Increased local production of androgen by prostate cells

An increase in local production of highly active androgens has been proposed as a mechanism of androgen inde-

Table 1

AR mutations in prostate tumors studied in a yeast assay system

Mutation	Domain		
<i>Not activated by DHT, DHEA, E2, PG, HC, FL, BI</i>			
A586V	DBD	L830P	LBD
C619Y	DBD	S865P	LBD
M749I	LBD	V866M	LBD
S791P	LBD		
<i>Slightly activated by DHT only</i>			
L574P	DBD	F754L	LBD
G683A	LBD	S759P	LBD
A721T	LBD	F891L	LBD
<i>Moderately activated by DHT only</i>			
K720E	LBD	L880Q	LBD
T755A	LBD	G909E	LBD
<i>Complete (Wt level) activated by DHT only</i>			
Y763C	LBD		
<i>Activity similar to wt AR (activated by DHT and modest activation by DHEA)</i>			
T575A	DBD	R726L	LBD
A587S	DBD	V730M	LBD
R629Q	hinge	V757A	LBD
K630T	hinge	S782N	LBD
S647N	hinge	Q798E	LBD
Q670R	hinge	R846	LBD
I672T	LBD	K910R	LBD
K717E	LBD	Q919R	LBD
<i>Activated by DHT and enhanced activation by DHEA</i>			
A748T	LBD	D890N	LBD
A748V	LBD	A896T	LBD
G750S	LBD		
<i>Activated by DHT, DHEA, PG</i>			
H874Y	LBD		
<i>Activated by DHT, DHEA, PG, E2, FL</i>			
T877A	LBD	most frequently encountered mutation	
<i>Activated by DHT, DHEA, PG, E2, BI</i>			
V715M	LBD		
<i>Activated by DHT, DHEA, PG, E2, FL, BI, HC</i>			
K580R	DBD		
L701H	LBD		

Adapted from Shi et al. [31]. Bold, detected in advanced prostate cancer.

pendence [32]. Analysis of testosterone and dihydrotestosterone in serum and in prostate tumor samples of patients following androgen ablation therapy found that tumor samples had higher dihydrotestosterone levels than serum samples [33]. This suggests that prostate cancer cells, by upregulating 5 α -reductase, may more effectively convert testosterone to the highly active dihydrotestosterone, thus producing sufficient localized hormone to promote AR signaling. Studies of gene expression in tumors resistant to androgen withdraw found expression of several genes important in steroid biosynthesis, squalene monooxygenase, lanosterol synthetase, and HMG-CoA synthetase, were increased in some tumors. The increase in these enzymes suggests an endogenous production of androgenic hormone, rather a conversion of testosterone to a more active form [34]. Thus targeting rate limiting enzymes in this biosynthetic pathway may be a potential therapeutic option.

7. Activation of AR by growth factors and cytokines

Numerous studies indicate that the AR can be activated by interaction with non-steroid molecules or in a

ligand-independent manner. Growth factors that are ligands for receptor tyrosine kinases including epidermal growth factor, insulin-like growth factor, and keratinocyte growth factor, can initiate a signaling cascade that culminates in AR activation [35]. Similarly, overexpression of HER2/neu has been shown to activate AR-dependent gene transcription [36]. Additionally, interleukins (IL6 and 8) [37,38] and neuropeptides [gastrin releasing peptide (GRP) and neurotensin] [39] are able to initiate signaling pathways that lead to activation of the AR in the absence of ligand. While the initiating signaling event differs, all of these mechanisms employ a phosphorylation cascade, including the well known AKT and MAPK pathways [40].

The androgen-independent signaling initiated by neuropeptides is of particular interest since an increase of neuroendocrine cells, and an increase in neuroendocrine cell-derived soluble factors such as GRP, neurotensin, serotonin, IL8, and IL6 in prostate tissue, has been associated with androgen ablation therapy [41–43]. Additionally, receptors for GRP, neurotensin and IL6 are present on prostate tumor derived cells [42]. Thus, neuroendocrine molecules can serve as paracrine factors to stimulate tumor proliferation in an androgen-independent manner [44–46]. IL6 and IL8 mediate inflammatory responses, suggesting that the immune system may play a role in prostate cancer. The latter mechanism underscores the potential importance of the tumor-microenvironment interaction in the development of androgen independence.

8. Altered expression of AR co-activators

Over 130 putative AR interacting co-regulators have been reported in the literature (for review see [47]). It should be noted that some were identified using yeast two hybrid screens with discrete AR domains and their physiologic function is unclear. However, other interactions have been well characterized and shown to regulate AR localization, stability, DNA-binding, and transcriptional activity (Table 2). AR co-regulators can be broadly grouped as (1) Chaperones. These proteins (including heat shock proteins) interact with newly translated AR to promote proper folding, cytoplasmic localization, AR stability and interaction with ligand. (2) Histone and chromatin modifying proteins. (3) Factors that bridge the transcriptional machinery to the AR. Alteration in co-regulator levels has been proposed as a mechanism that contributes to androgen independence.

Some of the best studied cofactors are members of the family of p160 kDa protein steroid receptor co-activators [48]. This family consists of SRC-1, transcriptional intermediary factor 2 (TIF2), and amplified in breast cancer (AIB1/SRC-3) [49–52]. These proteins possess histone acetylase activities, but are also able to recruit other histone acetylases such as the CREB-binding protein p300 and PCAF [18]. An analysis of prostate cancer samples from patients, who failed endocrine therapy, showed that expression of SRC-1 and TIF2 was more intense than in those from patients with benign prostatic hyperplasia or androgen-dependent tumors [53]. Studies also noted that there was

an increase in AIB1 with tumor stage and grade in clinically localized disease [54,55].

The AR interacting protein, Tip60, was originally identified as a co-activator of the HIV encoded TAT protein. This protein, a prominent member of the MYST family of lysine acetyltransferases can acetylate histones and transcription factors including AR, the upstream binding transcription factor (UBF), and c-myc. Tip60 acetylation of AR is essential for Tip60-dependent AR co-activation [56]. Tip60 interacts with the AR in a ligand-dependent manner and has been found concentrated in the nucleus of androgen-independent tumors [57]. However, a further study found that Tip60 expression is decreased in metastatic cells [58].

ARA55 an androgen receptor associated protein also known as Hic-5 (hydrogen peroxide inducible clone 5) is a member of the group III LIM domain protein family. LIM domain proteins localize primarily to focal adhesions, but can also be found in the nucleus. It has been proposed that ARA55 may be an adaptor protein that is important for recruiting or stabilizing histone acetyltransferase-containing complexes at steroid-responsive promoters [59]. ARA55 interacts with AR to increase AR-dependent transcription and has been shown to alter ligand binding specificity [60,61]. Expression of ARA55 is higher in tumor tissue than in non-tumor tissue [62] and increased ARA55 expression in hormone-refractory tumors has been shown to associate with shorter survival [63].

A recent analysis compared gene expression datasets that identified differences in gene expression between normal prostate and tumor tissue using oligonucleotide array technologies [47]. This analysis did not find a consistent elevation of mRNA encoding all of the above mentioned proteins, but did detect some trends. SRC-1 was elevated in two, but decreased in one data set. Two studies found that TIF2 was increased, while one found it to be decreased. One study found AIB1 mRNA to be increased in tumors in comparison to normal controls. However, Tip60 and ARA55 expression was decreased in the majority of the data sets. The lack of consensus across multiple oligonucleotide microarray studies, and between oligonucleotide microarray studies and immunohistochemical (IHC) analyses highlights the difficulty of conducting comparative studies of highly divergent neoplasms to discern critical factors. Although immunohistochemical studies, which identify changes in protein expression of tumor cells and oligonucleotide microarray analyses that identify gene expression signatures of tumors may identify key molecules that contribute to androgen independence, currently it is difficult to reconcile the conflicting results. Hopefully, future studies may further discern the more prominent signaling aberrations.

9. Calpain proteolysis of the AR

Recently, our studies suggest that another mechanism may promote androgen independence in some prostate tumors [64]. Calpain, a calcium dependent protease, can cleave the AR and remove the LBD generating a truncated molecule that retains the transactivation domain and DBD. Original studies of the AR indicate that removal of

the LBD generates a constitutively active molecule that can translocate into the nucleus and function in an androgen-independent manner [65]. Calpains are ubiquitously expressed proteases that, in general, cleave proteins at a limited number of sites to generate large polypeptide fragments [66]. The rules that govern calpain substrate specificity are currently unclear but it appears that calpain recognizes substrate conformation more so than a discrete sequence. However, studies have identified preferred amino acids in certain locations surrounding the cleavage site [67]. Calpain activity is tightly regulated by multiple mechanisms including calcium modulation, autoproteolysis, phosphorylation, intracellular distribution, and inhibition by the endogenous inhibitor calpastatin [66]. Interestingly, recent studies indicate that mRNA levels of the ubiquitous calpain 2 are elevated in invasive prostate tumors and are highest in metastatic neoplasms [68].

Our studies utilized the CWR22 xenograft model of prostate cancer progression that faithfully recapitulates salient features of human prostate tumorigenesis. CWR22 tumors are initially androgen-dependent and following castration regress. However, androgen-independent tumors ultimately emerge. The androgen-independent line 22Rv1, generated from a relapsed tumor, expresses high levels of a C-terminally truncated AR [69]. These cells are androgen-independent, and can proliferate in the absence of androgens. However, they are also androgen responsive since they proliferate more rapidly in the presence of androgens. Our most recent studies identified differences in transcription profiles of 22Rv1 cells proliferating in the presence and absence of androgen (M. Mudryj, unpublished results). Moreover, the R1 androgen-independent cell line, derived from a different relapsed CWR22 tumor, also has elevated levels of the truncated AR [70]. While both lines are derived from the same xenograft, our analysis has identified differences in gene and protein expression (M. Mudryj, unpublished results).

During the progression of the parental CWR22 tumor to androgen independence, the 22Rv1 cells acquired an additional AR mutation, a duplication of exon 3 encoding the second zinc finger of the DNA-binding domain. The insertion of 39 additional amino acids near the putative calpain cleavage site may sensitize the molecule to calpain-mediated proteolysis. Treatment of Rv1 cells with a calpain inhibitor, reduces the expression of the truncated AR and, in the absence of androgens, causes the cells to undergo apoptosis, possibly by causing reversion to the androgen-dependent phenotype of the parental xenograft [64].

A Western blot analysis of AR in tumor tissue detected elevated expression of the truncated AR isoform in some tumors [64, Fig. 2]. This analysis provides evidence that expression of the truncated isoform is not a rare event restricted to relapsed cell lines derived from a single tumor (CWR22), but a previously uncharacterized feature of certain prostate tumors. Higher levels of focal adhesion kinase cleavage in these samples suggest higher calpain activity. The role of the truncated AR in the etiology of androgen independence is supported by the identification of a Q640 termination mutation that would generate a molecule missing the LBD in an androgen-independent prostate tumor [71]. Since at this time the expression of the trun-

Table 2

A partial list of androgen receptor co-regulators

Co-activators	Description/comments
AIB3/ASC2	Rb required for ASC2 activation of AR
ANPK (PKY)	Serine/threonine kinase; does not phosphorylate AR; enhances AR protein stability
ARA24 (Ran)	Contains polyglutamine repeat. Expansion of the AR polyglutamine tract results in reduction in AR-ARA24 interaction
ARA54	Contains a RING finger and B-box domain
ARA55 (Hic-5)	Contains a LIM domain. The mouse homolog is inducible by TGF- β 1
ARA70 (RFG, ELE1)	May function as a bridging factor to p/CAF and TFIIB
ARA160 (TMF)	Shows a greater than additive interaction with ARA70
ARA267 (NSD1)	Contains SET and PHD domains.
ARIP3 (PIAS_x)	Member of PIAS family, SUMO E3 ligase
ARIP4	Member of the SNF2-like family of proteins; has active ATPase activity
ART-27 (UXT/STAP1)	Possibly α -class prefoldin; may be component of centrosome
BAG-1L	Also functions to regulate hsp70
β -Catenin	Transcription factor; Activity regulated by WNT pathway.
BRCA1	Breast cancer susceptibility gene. Interacts with CBP.
ACRM1(PRMT4)	Protein arginine methyl transferases
Caveolin-1	Membrane protein associated with caveoli membrane structures
CBP	Possesses acetyltransferase activity. Interacts with members of the SRC family. Co-activates multiple transcription factors.
Cdc25B	Dual-specificity phosphatases
Cyclin E	Regulates CDK2 activity.
DAXX	Death domain-associated protein
E6-AP	Ubiquitin E3 ligase; contains separable co-activation and ubiquitin ligase domains.
FHL2 (DRAL)	LIM only protein without an LXXLL motif
Gelsolin	Also functions as an actin filament severing and capping protein
HMG-1/-2	Abundant chromatin-associated protein that does not bind a specific DNA recognition sequence; represents separate gene products with extensive sequence identity.
hsp40 (dnaJ, ydj1p)	Member of the chaperone heterocomplex.
p300	Histone acetyltransferase
PGC-1 (LEM6)	General nuclear receptor co-activator.
PIAS1	Protein Inhibitor of Activated STAT (transcription factor)
PRMT1	Protein arginine methyltransferase
RAF (IDE)	Enhances AR and GR DNA-binding
Rb	Tumor suppressor; interacts with E2F1-3 to repress transcription
RIP140	Co-activator at low receptor–co-activator ratio, repressor at a high ratio
SNURF (RNF4)	RING finger protein; may recruit the chromatin remodeling factor HMG1(Y)
SRA	Functions as a RNA transcript and associates with a SRC-1 containing co-regulator complex
SRC-1 (NCoA-1)	Interacts with CBP/p300. General nuclear receptor co-activator. Possesses weak acetyltransferase activity.
SRC-3 (Rac3, ACTR, AIB1, p/CIP, TRAM1)	Interacts with CBP/p300. Possesses acetyltransferase activity
STAT1	Transcription factor
STAT3	Transcription factor
Supervillin	Actin-binding protein
TIF2 (GRIP1, NCoA-2, SRC-2)	General nuclear receptor co-activator.
Tip60	Member of the MYST/SAS family of histone acetyltransferases
Ubc9	SUMO conjugating enzyme
Zac-1	Transcription factor; co-activator of AR in HeLa cells, corepressor in 1471.1 cells
Zimp10	PIAS-like protein
<i>Co-repressors</i>	<i>Description/comments</i>
AES	Member of the Groucho/TLE family of co-repressors
ARA67/PAT1/APPBP2	Promotes a cytoplasmic retention of AR in the presence of androgen
Calreticulin	Endoplasmic reticulum Ca ²⁺ binding chaperone inhibits AR binding to its DNA response elements
Cyclin D1	Regulates CDK4/6 kinase activity
DAX-1	Nuclear receptor; can sequester AR in the cytoplasm, DAX-1 expression is strongly reduced in benign prostatic hyperplasia
DJBP	DJ-1-binding protein; recruits corepressor complex containing HDAC1 and mSin3A
FLNa	Filamin A binds actin filaments; calpain cleavage fragment of filamin A binds AR
GSK3b	Protein kinase; Phosphorylates the AR, suppresses transactivation
HBO1	Histone acetyltransferase
HDAC1	Possess histone deacetylase activity
hRad9	hRad9 colocalize to regions containing DNA double-strand breaks; The FXXLF motif within the C-terminus of hRad9 interrupts the DHT-induced interaction between the AR N-terminus and C-terminus
NCoR	Transcription factor binding protein that facilitates the assembly of a corepressor complex
PAK6	Protein kinase; active PAK6 inhibited nuclear translocation of the ligand-bound AR
PATZ	Interacts with RNF4, (RNF4 is an AR co-activator) to repress AR activity
PTEN	Phosphatase and tensin homologue; tumor suppressor; suppresses AR activity via a PI3K/Akt pathway in the early passage LNCaP cells
RACK1	Receptor for activated protein kinase C 1; an adaptor molecule, promotes the PKC-mediated inhibition of AR
SHP	Regulatory nuclear receptor; interacts with HDACs
Smad4	Tumor suppressor; TGF- β signaling pathway;

Table 2 (continued)

SMRT	Transcription factor binding protein that facilitates the assembly of a corepressor complex
SRY	Contains a high-mobility group (HMG)-box DNA-binding domain characteristic of the SOX family of transcription factors; Y chromosome encoded determinant of male phenotype
TGIF	Transcriptional repressor, recruits sin3A and HDAC1

cated isoform can only be detected by Western immunoblot analysis, this feature of some tumors would not have been detected by other more routine immunohistochemical techniques.

The differences in activity of the full length and truncated AR are currently being investigated but based on previous analyses certain hypotheses can be formulated. The AR interacts with HSPs via the LBD, therefore a C-terminally truncated AR would not interact with these proteins that are retained in the cytoplasm, but rather would translocate into the nucleus. Analysis of 22Rv1 cells found that even in androgen-depleted media the truncated AR localizes to the nucleus [69]. Furthermore, previous studies reported a LBD truncation could bind ARE, albeit with lower efficiency [72]. Therefore, the truncated AR, once in the nucleus could bind to an ARE site to promote AR-dependent transcription. Our studies also found that the truncated AR transactivated transcription from a promoter harboring ARE efficiently, and in an androgen-independent manner. It is however unclear, if in the context of a chromosomal ARE, the wildtype and truncated receptors bind the same sequences and transactivate transcription with the same efficacy. Moreover, the LBD is instrumental for the interaction with several co-regulators, suggesting that deletion of this domain would alter recruitment of co-activators thus altering the AR-dependent transcription profile. The LBD is also required for the binding of anti-androgens, promoting the assembly of co-repressors rather than co-activators. Indeed, treatment of R1 and Rv1 cells with the anti-androgen bicalutamide did not inhibit cell growth (P. Ghosh, personal communication).

Elevated expression of calpain would increase AR proteolysis, but additionally would increase proteolysis of other calpain target proteins, including proteins that are important for adhesion and migration. Therefore, elevated calpain activity may not only promote prostate tumor survival in castrate levels of androgens, but alter other physiological properties that promote tumorigenesis. Studies have shown that inhibition of calpain activity in androgen-independent DU145 cells limits their adhesion, migration and metastatic potential [73]. These studies suggest that inhibition of calpain activity is a potential therapeutic option for some prostate tumors.

10. Androgen-independent, AR-negative tumors

Numerous studies show that most primary and advanced stage cancers express the AR regardless of stage and grade, or hormone sensitivity [74–77]. However, the majority of reports have noted significant heterogeneous staining of the AR in many prostate tumor specimens in contrast to the homogeneous AR staining in normal prostate epithelium. These findings suggest that the variability in AR content increases with the progression of the disease

and might in part account for hormone resistance [74–77]. Absence of AR mRNA and protein expression has been shown in the androgen-independent human prostate tumor cell lines DU145 and PC3 [78,79]. The loss of AR expression in these androgen-independent cells appears to occur at the transcriptional level through CpG methylation of the AR promoter and histone deacetylation, instead of at the genomic level through either deletion or mutation [6,80–83]. This epigenetic alteration of AR may play a role in the development of hormone independence in a subset of prostate cancers that do not express AR. Hypermethylation of the AR promoter is more frequently found in hormone-refractory prostate cancer tissues (29%) compared with untreated primary tissues (10%) [84–86]. Introduction of the AR into AR-negative PC3 cells markedly inhibits cell growth both *in vitro* and *in vivo* [87]. In contrast to cells and tissue that are dependent on the AR for proliferation and/or survival, in this cellular context AR functions as a growth suppressor. There are many plausible explanations for this drastically different AR affect including interaction with a different cohort of AR binding proteins that alters the specificity and amplitude of AR transcriptional activity. It is noteworthy that the androgen-dependent LNCaP cell line has a biphasic response to androgens. At physiological concentrations androgens promote LNCaP growth, but very high levels are growth inhibitory [88], raising the possibility that at inappropriately high levels AR suppresses proliferation, possibly by activating transcription of genes involved in growth suppression. These studies suggest that if cells have circumvented the need for AR, continued signaling may be detrimental to cell growth.

11. Role of stroma cells

While the expression of AR in prostate cancer cells has been the focus of most studies, several have reported that loss of AR expression in stroma cells surrounding malignant epithelial cells also correlates with the progression to hormone resistance [89–91]. Because prostatic stroma mediates the transmission of androgen-induced signaling to the adjacent epithelium, altered AR expression in tumor-associated stroma may lead to abnormal interactions with malignant epithelial cells and hence serve as another potential mechanism driving androgen-independent prostate epithelial cell proliferation. The mechanism leading to the loss of AR expression in stroma cells remains to be investigated. One study suggests that the up-regulation of receptor tyrosine kinase expression may play a role [91]. A strong correlation between loss of the AR protein in stroma cells and the expression of a constitutively active variant EGF receptor (EGFRvIII) in the adjacent epithelium has been identified [91]. The importance of stroma in androgen independence is supported by mouse prostate cancer models and lines derived from mouse prostate tumors [92]. *In*

vitro the CASP 2.1 androgen-dependent lines derived from prostate tumors in *Nkx3.1*; *Pten* mice undergo a growth arrest when deprived of androgens. In contrast when the identical cells are implanted and propagated *in vivo* as renal grafts cell undergo apoptosis following castration. The different behavior of the cells *in vivo* and *in vitro* indicated that the tumor environment is responsible for the apoptosis of the cells. Moreover, studies have shown that stromal AR, not epithelial AR is important for the apoptosis response of androgen deprivation. Together these studies indicate that an AR-dependent paracrine factor derived from the stroma promotes apoptosis of the epithelial tumor cells. The role of stroma may explain the curious outcome of a clinical trial of the 5 α -reductase inhibitor Finasteride [93]. The drug reduced the incidence of prostate cancer, but individuals that developed tumors, developed higher grade neoplasms. This raises the possibility that the reduction of androgen in stromal and epithelial cells may have promoted the survival of epithelial cells that could continue proliferation in the presence of low levels of androgen, but at the same time inhibited the anti-tumorigenic effect of the stroma. These studies underscore the importance of stromal/epithelial cell interactions in the etiology of androgen independence.

12. Conclusions

Tumor cells employ multiple pathways to survive and thrive in castrate levels of androgens. While not mentioned in the above discussion, modulations affecting the RB and p53 tumor suppressor pathways have also been shown to contribute to androgen-independent proliferation [94,95]. Likewise, inhibition of apoptotic responses can promote progression to androgen independence [96,97]. It is noteworthy that a number of the signaling pathways that are involved in bypassing the requirement for androgens interact with each other. Therefore, modulations of several signaling pathways may synergize to confer androgen independence. The importance of some of the well known pathways in the etiology of androgen independence is difficult to discern, since their activation has pleiotropic effects on cell physiology. If multiple alterations are required for the development of androgen independence, targeting one pathway may be sufficient to inhibit tumor growth. The challenge is to identify the altered pathways in individual tumors to design the most effective therapeutics.

Conflict of interest

The authors have no conflict of interest.

Funding sources

M. Mudryj is supported by a VA Merit award and a Department of Defense grant pc051049.

Acknowledgements

We are grateful to Dr. Michael Seldin and Stephen Libertini for critical review of the manuscript.

References

- [1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, CA: A Cancer Journal for Clinicians 57 (2007) 43–66.
- [2] J. Javidan, A.D. Deitch, X.B. Shi, R.W. de Vere White, The androgen receptor and mechanisms for androgen independence in prostate cancer, Cancer Investigation 23 (2005) 520–528.
- [3] J.A. Ruizzeveld de Winter, J. Trapman, M. Vermey, E. Mulder, N.D. Zegers, T.H. van der Kwast, Androgen receptor expression in human tissues: an immunohistochemical study, The Journal of Histochemistry and Cytochemistry 39 (1991) 927–936.
- [4] G.W. Chodak, D.M. Kranc, L.A. Puy, H. Takeda, K. Johnson, C. Chang, Nuclear localization of androgen receptor in heterogeneous samples of normal, hyperplastic and neoplastic human prostate, The Journal of Urology 147 (1992) 798–803.
- [5] M.V. Sadi, P.C. Walsh, E.R. Barrack, Immunohistochemical study of androgen receptors in metastatic prostate cancer. Comparison of receptor content and response to hormonal therapy, Cancer 67 (1991) 3057–3064.
- [6] C.A. Quigley, A. De Bellis, K.B. Marschke, M.K. el-Awady, E.M. Wilson, F.S. French, Androgen receptor defects: historical, clinical, and molecular perspectives, Endocrine Reviews 16 (1995) 271–321.
- [7] H.V. Heemers, D.J. Tindall, Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex, Endocrine Reviews 28 (2007) 778–808.
- [8] M.E. Taplin, S.P. Balk, Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence, Journal of Cellular Biochemistry 91 (2004) 483–490.
- [9] O. Sartor, Q. Zheng, J.A. Eastham, Androgen receptor gene CAG repeat length varies in a race-specific fashion in men without prostate cancer, Urology 53 (1999) 378–380.
- [10] E. Giovannucci, M.J. Stampfer, K. Krithivas, M. Brown, D. Dahl, A. Brufsky, J. Talcott, C.H. Hennekens, P.W. Kantoff, The CAG repeat within the androgen receptor gene and its relationship to prostate cancer, Proceedings of the National Academy of Sciences of the United States of America 94 (1997) 3320–3323.
- [11] D.O. Hardy, H.I. Scher, T. Bogenreider, P. Sabbatini, Z.F. Zhang, D.M. Nanus, J.F. Catterall, Androgen receptor CAG repeat lengths in prostate cancer: correlation with age of onset, The Journal of Clinical Endocrinology and Metabolism 81 (1996) 4400–4405.
- [12] R.K. Nam, Y. Elhaji, M.D. Krahn, J. Hakimi, M. Ho, W. Chu, J. Sweet, J. Trachtenberg, M.A. Jewett, S.A. Narod, Significance of the CAG repeat polymorphism of the androgen receptor gene in prostate cancer progression, The Journal of Urology 164 (2000) 567–572.
- [13] E.P. Gelmann, Molecular biology of the androgen receptor, Journal of Clinical Oncology 20 (2002) 3001–3015.
- [14] P.J. Kallio, J.J. Palvimo, M. Mehto, O.A. Janne, Analysis of androgen receptor–DNA interactions with receptor proteins produced in insect cells, The Journal of Biological Chemistry 269 (1994) 11514–11522.
- [15] A. Haelens, T. Tanner, S. Denayer, L. Callewaert, F. Claessens, The hinge region regulates DNA binding, nuclear translocation, and transactivation of the androgen receptor, Cancer Research 67 (2007) 4514–4523.
- [16] C.A. Berrevoets, A. Umar, A.O. Brinkmann, Antiandrogens: selective androgen receptor modulators, Molecular and Cellular Endocrinology 198 (2002) 97–103.
- [17] Y. Shang, M. Myers, M. Brown, Formation of the androgen receptor transcription complex, Molecular Cell 9 (2002) 601–610.
- [18] M.C. Louie, H.Q. Yang, A.H. Ma, W. Xu, J.X. Zou, H.J. Kung, H.W. Chen, Androgen-induced recruitment of RNA polymerase II to a nuclear receptor–p160 coactivator complex, Proceedings of the National Academy of Sciences of the United States of America 100 (2003) 2226–2230.
- [19] X.B. Shi, L. Xue, J. Yang, A.H. Ma, J. Zhao, M. Xu, C.G. Tepper, C.P. Evans, H.J. Kung, R.W. de Vere White, An androgen-regulated miRNA suppresses Bak1 expression and induces androgen-independent growth of prostate cancer cells, Proceedings of the National Academy of Sciences of the United States of America 104 (2007) 19983–19988.
- [20] T. Visakorpi, E. Hyytinen, P. Koivisto, M. Tanner, R. Keinänen, C. Palmberg, A. Palotie, T. Tammela, J. Isola, O.P. Kallioniemi, In vivo amplification of the androgen receptor gene and progression of human prostate cancer, Nature Genetics 9 (1995) 401–406.
- [21] M.J. Linja, K.J. Savinainen, O.R. Saramaki, T.L. Tammela, R.L. Vessella, T. Visakorpi, Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer, Cancer Research 61 (2001) 3550–3555.

- [22] C.D. Chen, D.S. Welsbie, C. Tran, S.H. Baek, R. Chen, R. Vessella, M.G. Rosenfeld, C.L. Sawyers, Molecular determinants of resistance to antiandrogen therapy, *Nature Medicine* 10 (2004) 33–39.
- [23] J.R. Newmark, D.O. Hardy, D.C. Tonb, B.S. Carter, J.I. Epstein, W.B. Isaacs, T.R. Brown, E.R. Barrack, Androgen receptor gene mutations in human prostate cancer, *Proceedings of the National Academy of Sciences of the United States of America* 89 (1992) 6319–6323.
- [24] H. Suzuki, K. Akakura, A. Komiya, S. Aida, S. Akimoto, J. Shimazaki, Codon 877 mutation in the androgen receptor gene in advanced prostate cancer: relation to antiandrogen withdrawal syndrome, *The Prostate* 29 (1996) 153–158.
- [25] M. Marcelli, M. Ittmann, S. Mariani, R. Sutherland, R. Nigam, L. Murthy, Y. Zhao, D. DiConcini, E. Puxeddu, A. Esen, J. Eastham, N.L. Weigel, D.J. Lamb, Androgen receptor mutations in prostate cancer, *Cancer Research* 60 (2000) 944–949.
- [26] M.E. Taplin, G.J. Bubley, T.D. Shuster, M.E. Frantz, A.E. Spooner, G.K. Ogata, H.N. Keer, S.P. Balk, Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer, *The New England Journal of Medicine* 332 (1995) 1393–1398.
- [27] W.D. Tilley, G. Buchanan, T.E. Hickey, J.M. Bentel, Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence, *Clinical Cancer Research* 2 (1996) 277–285.
- [28] M.E. Taplin, B. Rajeshkumar, S. Halabi, C.P. Werner, B.A. Woda, J. Picus, W. Stadler, D.F. Hayes, P.W. Kantoff, N.J. Vogelzang, E.J. Small, Androgen receptor mutations in androgen-independent prostate cancer: Cancer and Leukemia Group B Study 9663, *Journal of Clinical Oncology* 21 (2003) 2673–2678.
- [29] G. Wilding, M. Chen, E.P. Gelmann, Aberrant response in vitro of hormone-responsive prostate cancer cells to antiandrogens, *The Prostate* 14 (1989) 103–115.
- [30] M.E. Taplin, G.J. Bubley, Y.J. Ko, E.J. Small, M. Upton, B. Rajeshkumar, S.P. Balk, Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist, *Cancer Research* 59 (1999) 2511–2515.
- [31] X.B. Shi, A.H. Ma, L. Xia, H.J. Kung, R.W. de Vere White, Functional analysis of 44 mutant androgen receptors from human prostate cancer, *Cancer Research* 62 (2002) 1496–1502.
- [32] B.J. Feldman, D. Feldman, The development of androgen-independent prostate cancer, *Nature Reviews* 1 (2001) 34–45.
- [33] F. Labrie, A. Dupont, A. Belanger, R. St-Arnaud, M. Giguere, Y. Lacourciere, J. Emond, G. Monfette, Treatment of prostate cancer with gonadotropin-releasing hormone agonists, *Endocrine Reviews* 7 (1986) 67–74.
- [34] J. Holzbeierlein, P. Lal, E. LaTulippe, A. Smith, J. Satagopan, L. Zhang, C. Ryan, S. Smith, H. Scher, P. Scardino, V. Reuter, W.L. Gerald, Gene expression analysis of human prostate carcinoma during hormonal therapy identifies androgen-responsive genes and mechanisms of therapy resistance, *The American Journal of Pathology* 164 (2004) 217–227.
- [35] Z. Culig, A. Hobisch, M.V. Cronauer, C. Radmayr, J. Trapman, A. Hittmair, G. Bartsch, H. Klocker, Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor-I, keratinocyte growth factor, and epidermal growth factor, *Cancer Research* 54 (1994) 5474–5478.
- [36] N. Craft, Y. Shostak, M. Carey, C.L. Sawyers, A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase, *Nature Medicine* 5 (1999) 280–285.
- [37] A. Hobisch, I.E. Eder, T. Putz, W. Horninger, G. Bartsch, H. Klocker, Z. Culig, Interleukin-6 regulates prostate-specific protein expression in prostate carcinoma cells by activation of the androgen receptor, *Cancer Research* 58 (1998) 4640–4645.
- [38] L.F. Lee, M.C. Louie, S.J. Desai, J. Yang, H.W. Chen, C.P. Evans, H.J. Kung, Interleukin-8 confers androgen-independent growth and migration of LNCaP: differential effects of tyrosine kinases Src and FAK, *Oncogene* 23 (2004) 2197–2205.
- [39] L.F. Lee, J. Guan, Y. Qiu, H.J. Kung, Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase, *Molecular and Cellular Biology* 21 (2001) 8385–8397.
- [40] A.G. Papatsoris, M.V. Karamouzis, A.G. Papavassiliou, The power and promise of “rewiring” the mitogen-activated protein kinase network in prostate cancer therapeutics, *Molecular Cancer Therapeutics* 6 (2007) 811–819.
- [41] D. Hirano, Y. Okada, S. Minei, Y. Takimoto, N. Nemoto, Neuroendocrine differentiation in hormone refractory prostate cancer following androgen deprivation therapy, *European Urology* 45 (2004) 586–592. discussion 92.
- [42] G.P. Amorino, S.J. Parsons, Neuroendocrine cells in prostate cancer, *Critical Reviews in Eukaryotic Gene Expression* 14 (2004) 287–300.
- [43] J.C. Reubi, S. Wenger, J. Schmuckli-Maurer, J.C. Schaer, M. Gugger, Bombesin receptor subtypes in human cancers: detection with the universal radioligand [¹²⁵I]-[D-TYR(6), beta-ALA(11), PHE(13), NLE(14)] bombesin(6–14), *Clinical Cancer Research* 8 (2002) 1139–1146.
- [44] I. Sehgal, S. Powers, B. Huntley, G. Powis, M. Pittelkow, N.J. Maihle, Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer, *Proceedings of the National Academy of Sciences of the United States of America* 91 (1994) 4673–4677.
- [45] B.B. Moore, D.A. Arenberg, K. Stoy, T. Morgan, C.L. Addison, S.B. Morris, M. Glass, C. Wilke, Y.Y. Xue, S. Sitterding, S.L. Kunkel, M.D. Burdick, R.M. Strieter, Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells, *The American Journal of Pathology* 154 (1999) 1503–1512.
- [46] M. Bologna, C. Festuccia, P. Muzi, L. Biordi, M. Ciomei, Bombesin stimulates growth of human prostatic cancer cells in vitro, *Cancer* 63 (1989) 1714–1720.
- [47] R. Chmela, G. Buchanan, E.F. Need, W. Tilley, N.M. Greenberg, Androgen receptor coregulators and their involvement in the development and progression of prostate cancer, *International Journal of Cancer* 120 (2007) 719–733.
- [48] S.A. Onate, S.Y. Tsai, M.J. Tsai, B.W. O'Malley, Sequence and characterization of a coactivator for the steroid hormone receptor superfamily, *Science (New York, NY)* 270 (1995) 1354–1357.
- [49] A. Takeshita, G.R. Cardona, N. Koibuchi, C.S. Suen, W.W. Chin, TRAM-1, A novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1, *The Journal of Biological Chemistry* 272 (1997) 27629–27634.
- [50] H. Li, P.J. Gomes, J.D. Chen, RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2, *Proceedings of the National Academy of Sciences of the United States of America* 94 (1997) 8479–8484.
- [51] H. Chen, R.J. Lin, R.L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M.L. Privalsky, Y. Nakatani, R.M. Evans, Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300, *Cell* 90 (1997) 569–580.
- [52] S.L. Anzick, J. Kononen, R.L. Walker, D.O. Azorsa, M.M. Tanner, X.Y. Guan, G. Sauter, O.P. Kallioniemi, J.M. Trent, P.S. Meltzer, AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer, *Science (New York, NY)* 277 (1997) 965–968.
- [53] C.W. Gregory, B. He, R.T. Johnson, O.H. Ford, J.L. Mohler, F.S. French, E.M. Wilson, A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy, *Cancer Research* 61 (2001) 4315–4319.
- [54] H.J. Zhou, J. Yan, W. Luo, G. Ayala, S.H. Lin, H. Erdem, M. Ittmann, S.Y. Tsai, M.J. Tsai, SRC-3 is required for prostate cancer cell proliferation and survival, *Cancer Research* 65 (2005) 7976–7983.
- [55] V.J. Gnanapragasam, H.Y. Leung, A.S. Pulimood, D.E. Neal, C.N. Robson, Expression of RAC 3, a steroid hormone receptor coactivator in prostate cancer, *British Journal of Cancer* 85 (2001) 1928–1936.
- [56] V. Sapountzi, I.R. Logan, C.N. Robson, Cellular functions of TIP60, *The International Journal of Biochemistry & Cell Biology* 38 (2006) 1496–1509.
- [57] M.E. Brady, D.M. O'Zanne, L. Gaughan, I. Waite, S. Cook, D.E. Neal, C.N. Robson, Tip60 is a nuclear hormone receptor coactivator, *The Journal of Biological Chemistry* 274 (1999) 17599–17604.
- [58] J.H. Kim, B. Kim, L. Cai, H.J. Choi, K.A. Ohgi, C. Tran, C. Chen, C.H. Chung, O. Huber, D.W. Rose, C.L. Sawyers, M.G. Rosenfeld, S.H. Baek, Transcriptional regulation of a metastasis suppressor gene by Tip60 and beta-catenin complexes, *Nature* 434 (2005) 921–926.
- [59] M.D. Heitzer, D.B. DeFranco, Mechanism of action of Hic-5/androgen receptor activator 55, a LIM domain-containing nuclear receptor coactivator, *Molecular Endocrinology (Baltimore, MD)* 202006 56–64.
- [60] N. Fujimoto, S. Yeh, H.Y. Kang, S. Inui, H.C. Chang, A. Mizokami, C. Chang, Cloning and characterization of androgen receptor coactivator, ARA55, in human prostate, *The Journal of Biological Chemistry* 274 (1999) 8316–8321.
- [61] M. Kasai, J. Guerrero-Santoro, R. Friedman, E.S. Leman, R.H. Getzenberg, D.B. DeFranco, The Group 3 LIM domain protein paxillin potentiates androgen receptor transactivation in prostate cancer cell lines, *Cancer Research* 63 (2003) 4927–4935.
- [62] C. Mestayer, M. Blanchere, F. Jaubert, B. Dufour, I. Mowszowicz, Expression of androgen receptor coactivators in normal and cancer

- prostate tissues and cultured cell lines, *The Prostate* 56 (2003) 192–200.
- [63] Y. Miyoshi, H. Ishiguro, H. Uemura, K. Fujinami, H. Miyamoto, Y. Miyoshi, H. Kitamura, Y. Kubota, Expression of AR associated protein 55 (ARA55) and androgen receptor in prostate cancer, *The Prostate* 56 (2003) 280–286.
- [64] S.J. Libertini, C.G. Tepper, V. Rodriguez, D.M. Asmuth, H.J. Kung, M. Mudryj, Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence, *Cancer Research* 67 (2007) 9001–9005.
- [65] Jenster, H.A. van der Korput, C. van Vroonhoven, T.H. van der Kwast, J. Trapman, A.O. Brinkmann, Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization, *Molecular Endocrinology* (Baltimore, MD) 51991 1396–1404.
- [66] D.E. Goll, V.F. Thompson, H. Li, W. Wei, J. Cong, The calpain system, *Physiological Review* 83 (2003) 731–801.
- [67] P. Tompa, P. Buzder-Lantos, A. Tantos, A. Farkas, A. Szilagyi, Z. Banoczy, F. Hudecz, P. Friedrich, On the sequential determinants of calpain cleavage, *The Journal of Biological Chemistry* 279 (2004) 20775–20785.
- [68] J. Rios-Doria, K.C. Day, R. Kuefer, M.G. Rashid, A.M. Chinnaiyan, M.A. Rubin, M.L. Day, The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells, *The Journal of Biological Chemistry* 278 (2003) 1372–1379.
- [69] C.G. Tepper, D.L. Boucher, P.E. Ryan, A.H. Ma, L. Xia, L.F. Lee, T.G. Pretlow, H.J. Kung, Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line, *Cancer Research* 62 (2002) 6606–6614.
- [70] C.W. Gregory, B. He, E.M. Wilson, The putative androgen receptor-A form results from in vitro proteolysis, *Journal of Molecular Endocrinology* 27 (2001) 309–319.
- [71] J. Ceraline, M.D. Cruchant, E. Erdmann, P. Erbs, J.E. Kurtz, B. Duclos, D. Jacqmin, D. Chopin, J.P. Bergerat, Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer, *International Journal of Cancer* 108 (2004) 152–157.
- [72] C.I. Wong, Z.X. Zhou, M. Sar, E.M. Wilson, Steroid requirement for androgen receptor dimerization and DNA binding. Modulation by intramolecular interactions between the NH2-terminal and steroid-binding domains., *The Journal of Biological Chemistry* 268 (1993) 19004–19012.
- [73] A. Mamoune, J.H. Luo, D.A. Lauffenburger, A. Wells, Calpain-2 as a target for limiting prostate cancer invasion, *Cancer Research* 63 (2003) 4632–4640.
- [74] C. Magi-Galluzzi, X. Xu, L. Hlatky, P. Hahnfeldt, I. Kaplan, P. Hsiao, C. Chang, M. Loda, Heterogeneity of androgen receptor content in advanced prostate cancer, *Modern Pathology* 10 (1997) 839–845.
- [75] M. Masai, H. Sumiya, S. Akimoto, R. Yatani, C.S. Chang, S.S. Liao, J. Shimazaki, Immunohistochemical study of androgen receptor in benign hyperplastic and cancerous human prostates, *The Prostate* 17 (1990) 293–300.
- [76] J.A. Ruizvelde de Winter, P.J. Janssen, H.M. Sleddens, M.C. Verleun-Mooijman, J. Trapman, A.O. Brinkmann, A.B. Santerse, F.H. Schroder, T.H. van der Kwast, Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer, *The American Journal of Pathology* 144 (1994) 735–746.
- [77] G.S. Prins, R.J. Sklarew, L.P. Pertschuk, Image analysis of androgen receptor immunostaining in prostate cancer accurately predicts response to hormonal therapy, *The Journal of Urology* 159 (1998) 641–649.
- [78] W.D. Tilley, C.M. Wilson, M. Marcelli, M.J. McPhaul, Androgen receptor gene expression in human prostate carcinoma cell lines, *Cancer Research* 50 (1990) 5382–5386.
- [79] J. Trapman, C. Ris-Stalpers, J.A. van der Korput, G.G. Kuiper, P.W. Faber, J.C. Romijn, E. Mulder, A.O. Brinkmann, The androgen receptor: functional structure and expression in transplanted human prostate tumors and prostate tumor cell lines, *The Journal of Steroid Biochemistry and Molecular Biology* 37 (1990) 837–842.
- [80] T. Nakayama, M. Watanabe, H. Suzuki, M. Toyota, N. Sekita, Y. Hirokawa, A. Mizokami, H. Ito, R. Yatani, T. Shiraishi, Epigenetic regulation of androgen receptor gene expression in human prostate cancers, *Laboratory Investigation* 80 (2000) 1789–1796.
- [81] D.F. Jarrard, H. Kinoshita, Y. Shi, C. Sandefur, D. Hoff, L.F. Meisner, C. Chang, J.G. Herman, W.B. Isaacs, N. Nassif, Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells, *Cancer Research* 58 (1998) 5310–5314.
- [82] H. Kinoshita, Y. Shi, C. Sandefur, L.F. Meisner, C. Chang, A. Choon, C.R. Reznikoff, G.S. Bova, A. Friedl, D.F. Jarrard, Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer, *Cancer Research* 60 (2000) 3623–3630.
- [83] J.L. Dai, C.A. Maiorino, P.J. Gkonos, K.L. Burnstein, Androgenic up-regulation of androgen receptor cDNA expression in androgen-independent prostate cancer cells, *Steroids* 61 (1996) 531–539.
- [84] A. Hobisch, Z. Culig, C. Radmayr, G. Bartsch, H. Klocker, A. Hittmair, Androgen receptor status of lymph node metastases from prostate cancer, *The Prostate* 28 (1996) 129–135.
- [85] A. Hobisch, Z. Culig, C. Radmayr, G. Bartsch, H. Klocker, A. Hittmair, Distant metastases from prostatic carcinoma express androgen receptor protein, *Cancer Research* 55 (1995) 3068–3072.
- [86] T.H. Van der Kwast, B. Tetu, Y. Fradet, A. Dupont, J. Gomez, L. Cusan, P. Diamond, F. Labrie, Androgen receptor modulation in benign human prostatic tissue and prostatic adenocarcinoma during neoadjuvant endocrine combination therapy, *The Prostate* 28 (1996) 227–231.
- [87] H. Suzuki, N. Nihei, N. Sato, T. Ichikawa, A. Mizokami, J. Shimazaki, Inhibition of growth and increase of acid phosphatase by testosterone on androgen-independent murine prostatic cancer cells transfected with androgen receptor cDNA, *The Prostate* 25 (1994) 310–319.
- [88] J. Tshilias, W. Zhang, N. Bhattacharya, M. Flanagan, L. Klotz, J. Slingerland, Involvement of p27Kip1 in G1 arrest by high dose 5 alpha-dihydrotestosterone in LNCaP human prostate cancer cells, *Oncogene* 19 (2000) 670–679.
- [89] S.M. Henshall, D.I. Quinn, C.S. Lee, D.R. Head, D. Golovsky, P.C. Brenner, W. Delprado, P.D. Stricker, J.J. Grygiel, R.L. Sutherland, Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer, *Cancer Research* 61 (2001) 423–427.
- [90] E.O. Olapade-Olaopa, E.H. MacKay, N.A. Taub, D.P. Sandhu, T.R. Terry, F.K. Habib, Malignant transformation of human prostatic epithelium is associated with the loss of androgen receptor immunoreactivity in the surrounding stroma, *Clinical Cancer Research* 5 (1999) 569–576.
- [91] E.O. Olapade-Olaopa, D.K. Moscatello, E.H. MacKay, D.P. Sandhu, T.R. Terry, A.J. Wong, F.K. Habib, Alterations in the expression of androgen receptor, wild type-epidermal growth factor receptor and a mutant epidermal growth factor receptor in human prostate cancer, *African Journal of Medicine and Medical Sciences* 33 (2004) 245–253.
- [92] M.M. Shen, C. Abate-Shen, Pten inactivation and the emergence of androgen-independent prostate cancer, *Cancer Research* 67 (2007) 6535–6538.
- [93] I.M. Thompson, P.J. Goodman, C.M. Tangen, M.S. Lucia, G.J. Miller, L.G. Ford, M.M. Lieber, R.D. Cespedes, J.N. Atkins, S.M. Lippman, S.M. Carlin, A. Ryan, C.M. Szczepanek, J.J. Crowley, C.A. Coltman Jr., The influence of finasteride on the development of prostate cancer, *The New England Journal of Medicine* 349 (2003) 215–224.
- [94] N.J. Nesslinger, X.B. Shi, R.W. deVere White, Androgen-independent growth of LNCaP prostate cancer cells is mediated by gain-of-function mutant p53, *Cancer Research* 63 (2003) 2228–2233.
- [95] Z. Zhou, A. Flesken-Nikitin, D.C. Corney, W. Wang, D.W. Goodrich, P. Roy-Burman, A.Y. Nikitin, Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer, *Cancer Research* 66 (2006) 7889–7898.
- [96] T.J. McDonnell, P. Troncoso, S.M. Brisbay, C. Logothetis, L.W. Chung, J.T. Hsieh, S.M. Tu, M.L. Campbell, Expression of the protooncogene bcl-2 in the prostate and its association with emergence of androgen-independent prostate cancer, *Cancer Research* 52 (1992) 6940–6944.
- [97] P. Westin, P. Stattin, J.E. Damber, A. Bergh, Castration therapy rapidly induces apoptosis in a minority and decreases cell proliferation in a majority of human prostatic tumors, *The American Journal of Pathology* 146 (1995) 1368–1375.

ERK REGULATES CALPAIN 2 INDUCED ANDROGEN RECEPTOR PROTEOLYSIS IN CWR22 RELAPSED PROSTATE TUMOR CELL LINES

Honglin Chen¹, Stephen J. Libertini¹, Yu Wang², Hsing-Jien Kung³, Paramita Ghosh^{2,4}, Maria Mudryj^{1,4}

From Department of Medical Microbiology and Immunology¹, Department of Urology², Division of Basic Sciences³, Cancer Center and Department of Biochemistry and Molecular Medicine, University of California, Davis, California 19616 and Veterans Affairs-Northern California Health Care System⁴, Mather, California 95655

Running Head: ERK and Calpain regulates AR-LMW levels

Address correspondence to: Maria Mudryj, Ph.D., Department of Medical Microbiology, 3147 Tupper Hall, University of California, Davis, Davis CA 95616. Fax: 530-752-8692; E-mail:

mmudryj@ucdavis.edu

Androgen ablation therapy is effective in treating androgen-dependent prostate tumors however, tumors that can proliferate in castrate levels of androgen eventually arise. We previously reported that in CWR22Rv1 (Rv1) cells, the protease calpain 2 can cleave the androgen receptor (AR) into a constitutively active ~80 KDa low molecular weight (LMW) form. In this study, we further dissect the mechanisms that produce the AR LMW forms using Rv1 cells and the related CWR22-R1 (R1) cells. The 39 a.a. insertional mutation in the Rv1 AR (E3DM-AR) sensitizes this AR to calpain 2 proteolysis. R1 cells encode the same AR molecule as the parental CWR22 xenograft. Using calpain 2 siRNA and calpeptin, we find that calpain 2 plays a role in the generation of the LMW-AR in R1 cells. Furthermore, LMW-AR expression is regulated by the activation of calpain 2 by Extracellular Signal-Regulated Kinases 1 and 2 (ERK). Inhibition of ERK phosphorylation or siRNA-mediated decrease of ERK expression reduces LMW-AR levels in R1 cells. Conversely, activation of the MAPK pathway results in increased ERK phosphorylation and increased levels of LMW-AR. Finally, analyses of human tumor samples found that LMW-AR levels are higher in tumors that have an increased calpain/calpastatin ratio and/or increased levels of phospho-ERK (pERK). This suggests that a higher calpain/calpastatin ratio collaborates with activated ERK to promote the generation of the LMW-AR.

Prostate cancer is a commonly diagnosed malignancy that is treated with hormonal therapy aimed at blocking signaling through the androgen receptor (AR). Initially androgen ablation therapy is effective, but eventually, this treatment leads to

the development of aggressive relapsed tumors that thrive in the absence of androgens. Analysis of clinical samples revealed that over 90% of the relapsed tumors express AR (1-4). The AR, a member of the steroid hormone superfamily of ligand-activated transcription factors (5, 6) is central to the initiation and growth of prostate tumors and their responses to therapy. In the absence of ligand, the AR is retained in the cytoplasm. The binding of hormone alters AR's conformation to promote translocation of the AR into the nucleus where it regulates gene transcription (6-8).

Aberrant AR activity has been postulated to promote proliferation of tumor cells in reduced levels of androgen. Studies have shown that 25-30% of androgen-independent tumors that arose following androgen ablation have AR gene amplification (9, 10). AR mutations are more commonly observed in androgen-independent tumors (11, 12) and usually broaden ligand specificity (13). The AR present in CWR22 xenograft cells has a mutation in the ligand binding domain (LBD) (H847Y) that enhances responsiveness to estradiol and progesterone (14). Structure function analysis of the AR showed that deletion of the LBD generates a constitutively active AR molecule (15). A subsequent study identified a nonsense mutation at Q640 that results in a truncated constitutively active AR in a tumor refractory to androgen ablation therapy (16). We and others previously reported that calpain cleaves the AR molecule to produce various LMW isoforms (17-19), including an ~80 KDa C-terminally truncated AR. We found that the ~80KDa LMW-AR is present in some human prostate tumors (18). Using the androgen-independent Rv1 cell line that expresses high levels of the LMW-AR, we demonstrated that

inhibition of calpain activity induces apoptosis in cells cultured in the absence of androgen. These studies implied that calpain-dependent proteolysis of the AR may play an important role in conferring androgen-independence in a subset of prostate cancer cases (18). In this study, we show that calpain 2 and ERK collaborate in the generation of the LMW-AR.

Experimental Procedures

Cell culture and pharmacological agents- LNCaP, Rv1, PC3, and DU145 cells were obtained from American Type Culture Collection. R1 cells were provided by Dr. Elizabeth Wilson (University of North Carolina). Rv1, PC3, DU145 and R1 cells were propagated in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C and 5% CO₂. LNCaP cells were propagated in 10% FBS. RWPE, PRNS-1-1 and PZ-HPV-7, obtained from Dr. Ralph deVere White, were maintained in a keratinocyte serum-free medium supplemented, with 50 mg/ml bovine pituitary extract, and 5 ng/ml epidermal growth factor (Invitrogen). All cell lines were incubated at 37 and 5% CO₂. For *in vivo* inhibition of calpain activity, 2 x 10⁵ cells were plated in 35-mm plates and cultured in androgen-containing or androgen-depleted media (phenol red-free media/charcoal-stripped serum) for 48 h. Bicalutamide (Casodex) was from AstraZeneca, Cheshire, UK. For calpain inhibition studies, cells were treated with DMSO or 60 µmol/L calpeptin (Calbiochem) for 24 or 48 h, washed with cold PBS, and harvested. For MEK inhibition studies, cells were treated with 20 µM U0126 (Cell Signaling) or DMSO for 24 and 48 h. PKC activity was stimulated by treatment with 10 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (LC Laboratories) dissolved in DMSO.

Western immunoblot analysis- Cells were placed in a 4°C radioimmunoprecipitation lysis buffer that contained calpeptin, and a protease inhibitor cocktail (Sigma). Thirty micrograms of protein were separated on 8%, 10%, or 12% SDS-PAGE gels and transferred to BA-85 membrane (Schleicher & Schuell) and blocked with 5% nonfat dry milk in PBS and 0.1% Tween-20. The following antibodies were used: AR (central) clone 441 (Ab-1; Lab Vision Corp.), AR NH2-

terminus (N-20; Santa Cruz Biotechnology), Calpain 2 (Domain III, Sigma), calpastatin (1F7E3D10, Calbiochem), ERK (Cell Signaling), pERK (Thr202/tyr204, Cell Signaling), and FAK (clone 4.47; Upstate), GAPDH (clone 6C5, Santa Cruz). Proteins were detected using Enhanced chemiluminescence (GE Healthcare).

RNA interference- 2 x 10⁵ Rv1 and R1 cells were plated in 60 mm dishes. 24 h later, the cells were transfected with 130nM calpain 2 siRNA ONTARGET plus smartpool or ERK 1 and 2 siRNA ONTARGET plus smartpool (Dharmacon Research Inc.) with lipofectamine 2000 (Invitrogen). The ONTARGET Plus non-targeting siRNA was used as negative control. Cells were harvested for RNA analysis 72 h post transfection (RNeasy mini kit) (Invitrogen).

In vitro calpain assay- Cells were resuspended in calpain assay buffer [50 mmol/L HEPES buffer (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100]. Calpain was activated with addition of CaCl₂ to 1 mM. The reactions were incubated at 25°C.

Transfection- Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Cells were harvested 48 h after transfection, and subjected to analysis as previously described (18).

Cell proliferation assay- Cellular proliferation was assessed using the 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (Promega) following manufacturer's recommendations.

Real time PCR- Total cellular RNA was prepared from cells (RNeasy) and cDNA was synthesized from 1µg RNA using QuantiTect (Qiagen) reverse transcription kit. cDNAs were diluted 1:4 in ddH₂O and 2µl of cDNA was added to 5µl of EXPRESS SYBR® GreenER qPCR supermix (Invitrogen) and 200 nM of each primer, for a total volume of 10µl. GAPDH was used as the standard. PCR conditions were: 20-sec initial denaturation step at 95°C, 40 cycles at 95°C for 3 s, 60 °C for 30 sec, followed by melt curve at 95 °C for 15 sec, 60 °C for 15 sec, increase to 95 °C over 20 min. additional 95 cycles starting at 60 °C with 0.5 °C increase per cycle for melt curve analysis. The study used the Eppendorf Mastercycler ep Realplex. Primer sequences: GAPDH: 5'TGCACCACCAACTGC TTA3' and

5'AGAGGCAGGGATGATGTTTC3'; CLDN4:
5'AACCCTGACTTTGGGATCTG3'
and 5'AGATGCAGGCAGACAGAGTG3';
HPRT1: 5'TGACACTGGCAAAACAATGCA3'
and 5'GGTCCTTTTCACCAGCAAGCT3'.

Statistics. Analyses using a two-tailed Student's t test were used to compare two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the Rv1 and R1 cell lines. Two castrate resistant cells lines, R1 and Rv1, were derived from two independent CWR22 relapsed tumors. The cellular phenotypes of the Rv1 and R1 cells are similar. In the presence of androgen the cells tend to grow in clusters, while in the absence of androgens they tend to be more scattered and less adhesive (Figure 1A). The AR in both lines has the same LBD mutation as the CWR22 xenograft (20, 21). As previously reported, R1 and Rv1 cells express the LMW AR forms (20, 21) (Figure 1B). Western immunoblot analysis indicated that R1 cells expressed higher levels of AR than Rv1 cells, but the ratio of the LMW to full length (FL)-AR was higher in Rv1 cells. The size of the FL-AR in the R1 cells is smaller than the FL-AR in the Rv1 cells, since R1 cells do not have the 39 amino acid duplication of exon 3. Closer inspection revealed that the ~80 KDa LMW forms could be resolved into several discrete bands (Figure 1B). The MTS proliferation assay confirmed that the R1 and Rv1 cell proliferation rates were only slightly slower in androgen-depleted media compared to cells grown in the presence of androgen (Figure 1C). The proliferation assay conducted in the presence of 10 uM Casodex indicated that R1 and Rv1 cells were refractory to the effects of this AR inhibitor (Figure 1D). While all three lines are responsive to androgen, only LNCaP cells are dependent on androgen to sustain growth.

Generation of the LMW-AR involves calpain. We have reported previously that the inhibition of calpain activity by calpeptin reduces the expression of the LMW-AR in Rv1 cells (18). Likewise, treatment of R1 cells, proliferating in the presence or absence of androgen (Ad), with calpeptin reduced the levels of LMW-AR in R1 cells (Figure 2A). We previously showed that proteolysis of the calpain substrate focal adhesion

kinase (FAK) is a good indicator of calpain activity (22). Calpeptin treatment of R1 cells reduced the levels of LMW-FAK (Figure 2A). To further analyze the role of calpain in the generation of LMW-AR, calpain 2 expression was analyzed in several tumor derived, as well as immortalized, prostate cell lines. R1 cells expressed much higher levels of calpain 2 than Rv1 and LNCaP cells (Figure 2B). Interestingly, the two AR negative and highly metastatic cells lines, PC3 and DU145, expressed the highest levels of calpain 2. Given that calpain activity is regulated by its endogenous inhibitor calpastatin, we analyzed calpastatin levels as well, and found that expression was comparable in all the cell lines (Figure 2B). R1 cells had higher amounts of proteolyzed FAK, indicating greater calpain activity (Figure 2C). The extent of FAK cleavage was greater in the absence of androgen, suggesting that calpain activity may be higher under androgen-depleted conditions. To further confirm the involvement of calpain 2 in the generation of the LMW-AR forms in R1 cells, we used calpain 2 siRNA to reduce calpain 2 expression. A previous study reported that calpain 2 has a very long half life of 5 days (23). A 6-day treatment resulted in a ~60% reduction of calpain 2 protein levels in R1 cells (Figure 2D) and reduced levels of the LMW-AR forms (Figure 2D). This treatment also reduced FAK proteolysis indicating that calpain 2 activity was reduced. This analysis indicates that calpain 2 plays a role in the generation of the LMW-AR in R1 cells.

In R1 cells, the expression of claudin 4 (CLDN4) is highly repressed by the addition of androgen (Figure 2E). If calpeptin treatment reduces the levels of LMW-AR, then in the absence of androgen the expression of androgen repressed genes may be further activated. In the absence of androgen calpeptin treatment of R1 cells further increased the expression of CLDN4, thus arguing the LMW-AR has a role in transcription of certain genes.

The exon 3 duplication sensitizes E3DM-AR to calpain proteolysis. Rv1 cells express higher levels of the LMW-AR but have low expression of calpain 2 protein and calpain activity (Figure 2). We hypothesized that the exon 3 duplication sensitizes the E3DM-AR to calpain cleavage. The AR-null PC3 cells expressing high levels of calpain 2 were transfected with cDNA plasmids

encoding either the wildtype or E3DM-AR. As expected, the E3DM-AR was slightly larger than the wildtype receptor (Figure 3A). Additionally, the LMW forms generated in cells transfected with the E3DM-AR were larger than the LMW forms generated from the wildtype AR cDNAs. To test the hypothesis that the E3DM-AR is more sensitive to calpain-dependent proteolysis, extracts prepared from the transfected cells were treated with CaCl_2 to activate endogenous calpain activity. As shown in Figure 3B, the AR was progressively cleaved into the smaller forms by the addition of CaCl_2 . The amount of FL-AR remaining was quantitated and indicated that the E3DM-AR was degraded more rapidly than the wt-AR. The inclusion of calpeptin retarded proteolysis, indicating that proteolysis was calpain dependent (figure 3B). While the ~80 KDa forms were present initially and throughout the time course, as proteolysis progressed, the LMW-AR was further proteolyzed to smaller peptides. In vivo, the ~ 80 KDa LMW-AR forms that are generated by proteolysis can translocate into the nucleus, where they would be less susceptible to further proteolysis. In vitro, as was previously observed (17) activated calpain proteolyzes the AR to still smaller forms. The mutant E3DM-AR was cleaved more rapidly than the wildtype FL-AR, resulting in the disappearance of the FL-AR (compare lanes 4 and 9).

The expression of the LMW-AR is regulated by ERK. Calpain activity is tightly regulated by various mechanisms, including phosphorylation. Previous studies have shown that ERK can phosphorylate calpain 2 to stimulate protease activity (24). ERK expression was analyzed in immortalized (RWPE-1, PZ-HPV-7 and pRNS-1-1) and tumor derived (PC3, LNCaP, Rv1, R1 and DU145) cell lines. All of the tumor derived cells had higher levels of ERK in comparison to the immortalized cell lines (Figure 4A). A comparison of R1 and Rv1 cells proliferating in the absence and presence of androgen showed that R1 cells had higher levels of the active form of the protein (pERK) under both conditions (Figure 4B).

ERK is phosphorylated and activated by MEK, a dual threonine and tyrosine kinase (24). Treatment of R1 cells with the MEK inhibitor U0126 for 24 or 48 h reduced ERK phosphorylation (Figure 4C). An analysis of the

AR in the same extracts (Figure 4C) indicated that inhibition of ERK activity reduced the levels of LMW-AR. Similar results were found in Rv1 cells (data not shown). To confirm that LMW-AR expression is dependent on ERK, cells were treated with control siRNA and ERK siRNA. Inhibition of ERK expression resulted in decreased levels of LMW-AR (Figure 4D). This analysis established that ERK activation has a role in the etiology of the LMW-AR forms.

Since the PKC activator phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) can result in ERK phosphorylation (25), Rv1 and R1 cells were treated with TPA in the absence of androgen for 1 or 2 hours to stimulate ERK activity. This treatment promoted an increase in levels of the LMW-AR indicating that activation of this pathway resulted in enhanced AR proteolysis (Figure 5A). TPA treatment of Rv1 cells also resulted in decreased levels of the FL-AR; after a 2 hour TPA treatment the FL-AR was barely discernable, arguing that in vivo, as in vitro, the Rv1 AR is more sensitive to proteolysis.

To test our hypothesis that an increase in calpain 2 and ERK activity collaborate in promoting LMW-AR expression we examined calpain 2, calpastatin and pERK levels in 6 of 13 tumor samples previously analyzed for the expression of the LMW-AR. Three of the thirteen samples that had the highest levels LMW-AR (01, 31 and 94) and three that had low levels of LMW-AR (21, 25, and 28) were used in the analysis (Figure 5B). The expression of LMW-AR was defined as percent of total. Interestingly, the levels of the endogenous calpain inhibitor calpastatin was variable. It was higher in sample 21 and 25, which have lower levels of LMW-AR and lowest in Sample 01. Samples 01 and 31 had high levels of pERK (Figure 5C). The remaining samples had low pERK levels. Therefore, the three samples that had the highest LMW-AR had high levels of pERK or a high amount of calpain 2. Conversely, samples that had low LMW-AR levels had little pERK and had elevated calpastatin levels. This limited analysis suggests that in human tumors an increased ratio of calpain to calpastatin and increased ERK activity, work in concert contribute to increased LMW- AR expression.

DISCUSSION

R1 and Rv1 cell lines were derived from relapsed CWR22 tumors and express the FL-AR as well as LMW-AR forms. However, the FL and LMW-AR forms expressed in Rv1 cells is larger than those in R1 cells due to a 39 a.a. insertional mutation at the junction of the DBD and hinge region (21). Transient expression of the E3DM-AR cDNA in PC3 cells also results in the expression of slightly larger LMW forms than transfection of the wt-AR cDNA. Activation of calpain AR-transfected PC3 extracts indicates that the E3DM-AR is more susceptible to proteolysis than the wt AR. *In vivo* activation of calpain activity through activation of ERK also promotes a more rapid proteolysis of the E3DM-AR. Early studies reported that a serine protease can proteolyze the AR to generate a ~30 or ~40 kDa fragment containing the LBD (26). More recently, an independent study found that *in vitro*, calpain proteolyzes the AR to smaller amino-terminal fragments; those fragments include a ~75 KDa polypeptide (17). Our data suggest that the junction between the DBD and LBD might be especially sensitive to proteolysis. Therefore, it is not unexpected that the insertion of 39 additional a.a. near this region would alter AR structure and further sensitizes the molecule to calpain proteolysis (27, 28). Unlike Rv1 cells, R1 cells have an AR that is identical to the AR in the parental CWR22 xenograft. Therefore, we postulated that other molecular alterations must account for the increased expression of the LMW-AR. The current study shows that R1 cells express higher levels of calpain 2 and pERK than Rv1 cells. These two features collaborate to elevate calpain activity and promote proteolysis of the AR and FAK. The role of calpain in the degradation of AR is substantiated by the reduction of LMW-AR caused by inhibition of calpain by calpeptin or a decrease of calpain 2 by siRNA. A comparison of R1 and Rv1 cells indicated that R1 cells had higher levels of ERK and p-ERK. The participation of ERK in AR proteolysis was demonstrated by an siRNA-mediated decrease of ERK and by the inhibition of ERK phosphorylation by the MEK inhibitor U01286. Therefore a decrease of ERK levels or ERK activity reduces LMW-AR expression. Activation of ERK by TPA in Rv1 and R1 cells results in a

time-dependent increase in the generation of LMW-AR. The short interval required for increased LMW-AR generation is consistent with activation of a signaling cascade that results in the activation of a protease. The MAP kinase phosphorylation cascade which leads to ERK activation has been well studied and is considered a target for cancer therapeutics (29). Since ERK activation in prostate tumors has been previously reported (30) this is a potential mechanism that could contribute to the expression of LMW-AR in human tumors. Likewise, increased calpain 2 expression has been observed in prostate tumors. Since the activity of calpain 2 is partly regulated by calpastatin, the ratio of calpain/calpastatin affects calpain 2 activity. The expression of calpastatin has not been previously studied in prostate tumors. However, an increase in the calpain/calpastatin ratio has been reported in a study of colorectal cancer (31), which showed that calpastatin levels are high in normal mucosa, but decreased in tumor tissue. Moreover, increased expression of calpain 2 was detected in colorectal tumors and polyps suggesting that the increase of calpain 2 levels may be an early event in the tumorigenesis process. At this point we cannot rule out that calpain 1 contributes to the generation of the LMW-AR. Interestingly, calpains 1 has been shown to activate ERK (32) and therefore all of these molecules may be components of a regulatory pathway. The importance of the calpain/calpastatin equilibrium and the activation of the MAP kinase signaling pathway in prostate tumorigenesis remains to be defined.

Recent studies reported that the LMW-AR forms expressed in Rv1 cells are derived from an alternatively spliced AR mRNA (33-35). However, the studies do not agree on the identity of the spliced forms that give rise to the LMW-AR forms. Our analysis shows that several LMW-AR forms are expressed in Rv1 and R1 cells. Since we did not completely eliminate the expression of the LMW-AR by inhibiting calpain 2 and pERK, some of the LMW-AR forms could be derived from alternatively spliced AR mRNA. This is analogous to results obtained from studies of cyclin E. In transformed cells several LMW cyclin E forms can be detected (36). Studies have shown that some of the LMW cyclin E forms are derived from alternatively spliced mRNAs, while

others are generated by proteolysis of cyclin E protein (22, 37-39). The LMW cyclin E forms have altered cellular localization and are associated with higher kinase activity (40, 41). We agree with the interpretation of Guo *et al.* (35) that several mechanisms can be employed to generate LMW-AR forms. These LMW-AR forms may not be identical, but they would share critical features including the presence of the activation and DBD domains and a deletion of the LBD. Such AR molecules would be able to translocate into the nucleus in an androgen independent manner, bind to DNA and activate or repress gene transcription. Furthermore, the interaction of the LMW-AR and FL-AR with various AR-interacting proteins may differ and therefore if the LMW-AR and the FL-AR bind to identical DNA

sequences, they may have differential effects on gene transcription.

Multiple calpain substrates have been previously implicated in cellular transformation. This suggests that an alteration of the calpain/calpastatin equilibrium, which is observed in some tumors, would affect multiple pathways that drive tumor progression. The modulation of calpain activity could result in a constellation of changes that would be difficult to ascribe to any individual molecule. This feature of calpain-driven deregulation of cell physiology also provides a therapeutic opportunity. The inhibition of calpain activity, even partially, could be sufficient to modify multiple tumor survival and proliferative pathways, which, in synergy with other therapeutics, could be effective in halting tumor progression.

REFERENCES

1. Chen CD, Welsbie DS, Tran C, *et al.* Molecular determinants of resistance to antiandrogen therapy. *Nature medicine* 2004;10(1):33-9.
2. Gregory CW, He B, Johnson RT, *et al.* A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer research* 2001;61(11):4315-9.
3. Gregory CW, Kim D, Ye P, *et al.* Androgen receptor up-regulates insulin-like growth factor binding protein-5 (IGFBP-5) expression in a human prostate cancer xenograft. *Endocrinology* 1999;140(5):2372-81.
4. Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND, van der Kwast TH. Androgen receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* 1991;39(7):927-36.
5. Xia L, Robinson D, Ma AH, *et al.* Identification of human male germ cell-associated kinase, a kinase transcriptionally activated by androgen in prostate cancer cells. *The Journal of biological chemistry* 2002;277(38):35422-33.
6. Shang Y, Myers M, Brown M. Formation of the androgen receptor transcription complex. *Molecular cell* 2002;9(3):601-10.
7. Berrevoets CA, Umar A, Brinkmann AO. Antiandrogens: selective androgen receptor modulators. *Mol Cell Endocrinol* 2002;198(1-2):97-103.
8. Louie MC, Yang HQ, Ma AH, *et al.* Androgen-induced recruitment of RNA polymerase II to a nuclear receptor-p160 coactivator complex. *Proceedings of the National Academy of Sciences of the United States of America* 2003;100(5):2226-30.
9. Visakorpi T, Hyytinen E, Koivisto P, *et al.* In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nature genetics* 1995;9(4):401-6.
10. Linja MJ, Savinainen KJ, Saramaki OR, Tammela TL, Vessella RL, Visakorpi T. Amplification and overexpression of androgen receptor gene in hormone-refractory prostate cancer. *Cancer research* 2001;61(9):3550-5.
11. Marcelli M, Ittmann M, Mariani S, *et al.* Androgen receptor mutations in prostate cancer. *Cancer Res* 2000;60(4):944-9.
12. Tilley WD, Buchanan G, Hickey TE, Bentel JM. Mutations in the androgen receptor gene are associated with progression of human prostate cancer to androgen independence. *Clin Cancer Res* 1996;2(2):277-85.

13. Taplin ME, Balk SP. Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. *Journal of cellular biochemistry* 2004;91(3):483-90.
14. Tan J, Sharief Y, Hamil KG, *et al.* Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells. *Molecular endocrinology* (Baltimore, Md 1997;11(4):450-9.
15. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Molecular endocrinology* (Baltimore, Md 1991;5(10):1396-404.
16. Ceraline J, Cruchant MD, Erdmann E, *et al.* Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer. *Int J Cancer* 2004;108(1):152-7.
17. Pelley RP, Chinnakannu K, Murthy S, *et al.* Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer research* 2006;66(24):11754-62.
18. Libertini SJ, Tepper CG, Rodriguez V, Asmuth DM, Kung HJ, Mudryj M. Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. *Cancer research* 2007;67(19):9001-5.
19. Yang H, Murthy S, Sarkar FH, Sheng S, Reddy GP, Dou QP. Calpain-mediated androgen receptor breakdown in apoptotic prostate cancer cells. *Journal of cellular physiology* 2008;217(3):569-76.
20. Gregory CW, He B, Wilson EM. The putative androgen receptor-A form results from in vitro proteolysis. *Journal of molecular endocrinology* 2001;27(3):309-19.
21. Tepper CG, Boucher DL, Ryan PE, *et al.* Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer research* 2002;62(22):6606-14.
22. Libertini SJ, Robinson BS, Dhillon NK, *et al.* Cyclin E both regulates and is regulated by calpain 2, a protease associated with metastatic breast cancer phenotype. *Cancer research* 2005;65(23):10700-8.
23. Zhang W, Lane RD, Mellgren RL. The major calpain isozymes are long-lived proteins. Design of an antisense strategy for calpain depletion in cultured cells. *The Journal of biological chemistry* 1996;271(31):18825-30.
24. Glading A, Chang P, Lauffenburger DA, Wells A. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *The Journal of biological chemistry* 2000;275(4):2390-8.
25. Lee HW, Ahn DH, Crawley SC, *et al.* Phorbol 12-myristate 13-acetate up-regulates the transcription of MUC2 intestinal mucin via Ras, ERK, and NF-kappa B. *The Journal of biological chemistry* 2002;277(36):32624-31.
26. de Boer W, Bolt J, Kuiper GG, Brinkmann AO, Mulder E. Analysis of steroid- and DNA-binding domains of the calf uterine androgen receptor by limited proteolysis. *Journal of steroid biochemistry* 1987;28(1):9-19.
27. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. *Physiol Rev* 2003;83(3):731-801.
28. Tompa P, Buzder-Lantos P, Tantos A, *et al.* On the sequential determinants of calpain cleavage. *The Journal of biological chemistry* 2004;279(20):20775-85.
29. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* 2007;26(22):3291-310.
30. Price DT, Della Rocca G, Guo C, Ballo MS, Schwinn DA, Luttrell LM. Activation of extracellular signal-regulated kinase in human prostate cancer. *The Journal of urology* 1999;162(4):1537-42.
31. Lakshmikuttyamma A, Selvakumar P, Kanthan R, Kanthan SC, Sharma RK. Overexpression of m-calpain in human colorectal adenocarcinomas. *Cancer Epidemiol Biomarkers Prev* 2004;13(10):1604-9.
32. Sawhney RS, Cookson MM, Omar Y, Hauser J, Brattain MG. Integrin alpha2-mediated ERK and calpain activation play a critical role in cell adhesion and motility via focal adhesion kinase signaling: identification of a novel signaling pathway. *The Journal of biological chemistry* 2006;281(13):8497-510.

33. Dehm SM, Schmidt LJ, Heemers HV, Vessella RL, Tindall DJ. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer research* 2008;68(13):5469-77.
34. Hu R, Dunn TA, Wei S, *et al.* Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer. *Cancer research* 2009;69(1):16-22.
35. Guo Z, Yang X, Sun F, *et al.* A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth. *Cancer research* 2009;69(6):2305-13.
36. Wingate H, Zhang N, McGarhen MJ, Bedrosian I, Harper JW, Keyomarsi K. The tumor-specific hyperactive forms of cyclin E are resistant to inhibition by p21 and p27. *The Journal of biological chemistry* 2005;280(15):15148-57.
37. Porter DC, Keyomarsi K. Novel splice variants of cyclin E with altered substrate specificity. *Nucleic Acids Res* 2000;28(23):E101.
38. Porter DC, Zhang N, Danes C, *et al.* Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms. *Molecular and cellular biology* 2001;21(18):6254-69.
39. Wang XD, Rosales JL, Magliocco A, Gnanakumar R, Lee KY. Cyclin E in breast tumors is cleaved into its low molecular weight forms by calpain. *Oncogene* 2003;22(5):769-74.
40. Bacus SS, Gudkov AV, Lowe M, *et al.* Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. *Oncogene* 2001;20(2):147-55.
41. Delk NA, Hunt KK, Keyomarsi K. Altered subcellular localization of tumor-specific cyclin E isoforms affects cyclin-dependent kinase 2 complex formation and proteasomal regulation. *Cancer research* 2009;69(7):2817-25.

FOOTNOTES

This work was supported by a DOD grants PC051049 (MM), VA Merit (MM) and DOD predoctoral award PC073557 (HC). This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR-12088-01 from the National Center for Research Resources, NIH.

The abbreviation used are: Rv1, CWR22Rv1; AR, androgen receptor; LMW, low molecular weight; R1, FL, full length; CWR-R1; ERK, Extracellular Signal-Regulated Kinases 1/2; pERK, phospho-ERK; TPA, phorbol ester 12-O-tetradecanoylphorbol-13-acetate; MTS, 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; wt-AR, wild-type AR; E3DM-AR, exon 3 duplication mutation AR; FAK, focal adhesion kinase; DBD, DNA binding domain; LBD, ligand binding domain; PKC, protein kinase C; Ad, androgen.

ACKNOWLEDGEMENTS

We thank Dr. Elizabeth Wilson for the CWR-R1 cells, Dr. Clifford Tepper for the E3DM-AR expression plasmid and Dr. Hau Nguyen for critical reading of the manuscript.

FIGURE LEGENDS

Fig.1. Rv1 and R1 cells proliferate in castrate levels of androgen. A. R1 and Rv1 cells proliferating in the presence of androgen (Ad⁺) are less refractile than cells in androgen depleted media (Ad⁻). B. AR expression is greater in R1 than in Rv1 cells, but the FL and LMW-AR expressed in R1 cells is slightly smaller than that expressed in Rv1 cells. C. R1 and Rv1 cells proliferate in castrate levels of androgen, but

proliferation is slightly greater in the presence of androgen. Androgen depletion inhibits LNCaP proliferation. D. Rv1 and R1 cells proliferate in the presence of 10uM Casodex.

Fig 2. Calpain expression and activity in prostate derived cells. A. Inhibition of calpain activity in R1 cells with calpeptin (40uM) for 48 h decreases the expression of the LMW-AR (relative to FL-AR) by 55% in the absence of androgen and 43% in the presence of androgen. B. Top panel: Western blot analysis of calpain 2 levels in non-transformed and tumor prostate cells. Bottom panel: Western blot analysis of calpastatin levels in non-transformed and tumor cells. GAPDH served as a loading control. C. Calpain-dependent proteolysis of FAK from a 120 KDa form to 90 KDa and ultimately smaller forms is indicative of calpain activity. FAK proteolysis is greater in R1 than in Rv1 cells and is greater in both cells in the absence of androgens. D. Calpain 2 siRNA down-regulated calpain 2 protein levels 144 h post transfection in R1 cells. The down-regulation of calpain 2 expression by calpain 2 siRNA reduced the LMW-AR (relative to FL-AR) by 54% in the absence of androgen and 39% in the presence of androgen. Calpain-dependent proteolysis of FAK was also decreased. E. Expression of CLDN4 in R1 cells culture in androgen depleted media, following a 2 h stimulation with DHT and a 24 h treatment with 60uM calpeptin was assessed by real time PCR. CLDN4 expression was standardized to GAPDH. Error bars represent standard deviation, $p < 0.05$.

Fig 3. Transient expression of wt and E3DM-AR cDNA in PC3 cells. A. Transfection of PC3 cells with wt or E3DM-AR cDNA results in the expression of FL and LMW (denoted by arrows and brackets) forms of AR. The 3 nonspecific (NS) bands at ~ 80 KDa present in the non-transfected PC3 cells serve as markers (denoted by dots). The FL and LMW forms expressed in cells transfected with the E3DM-AR are slightly larger. B. Extracts prepared from PC3 cells transfected with wt or E3DM-AR were treated with 1mM CaCl_2 to activate calpain activity. The E3DM-AR is degraded more rapidly than the WT AR. (Compare lane 1 and 6; 2 and 7; 4 and 9).

Fig 4. Inhibition of ERK phosphorylation reduces the expression of the LMW-AR. A. Western blot analysis of ERK expression in non-transformed and tumor-derived cell lines. B. The pERK levels are higher in R1 than Rv1 cells in the presence or absence of androgen. C. R1 cells were treated with 20 uM of the MEK inhibitor U0126 (I) or vehicle (C) for 24 or 48 hours. The top portion of the blot shown in top panel was used to detect AR. Inhibition of ERK phosphorylation reduced the expression of the LMW-AR relative to FL-AR by 32% in 24 h and 51% in 48h. The arrows denote the FL and ~ 80 KDa LMW-AR. D. ERK specific siRNA reduced the expression of pERK and the levels of LMW-AR relative to FL-AR to 51.8% in the presence of androgen and 21% in the absence of androgen.

Fig 5. ERK activation and calpain/calpastatin ratios collaborate to promote expression of the LMW-AR. A. Treatment of R1 and Rv1 cells with TPA (10 nM) for 1 and 2 hours increases the expression of the LMW-AR forms (top panel). Control cells were treated with DMSO. The bottom panel shows that TPA treatment increases pERK levels. B. Higher calpain/calpastatin and pERK levels together correlate with higher expression of LMW-AR in tumor samples. C. Quantitation of the protein levels in panel B. The calpain/calpastatin ratios multiplied by levels of pERK were calculated for tumors that express high levels of LMW-AR (01, 30, 94) and samples that had low levels of LMW-AR (21, 25, 28). The average calpain/calpastatin x pERK levels are significantly higher in samples with elevated levels of LMW-AR. Error bars represent standard deviation, $p < 0.05$.

Figure 1

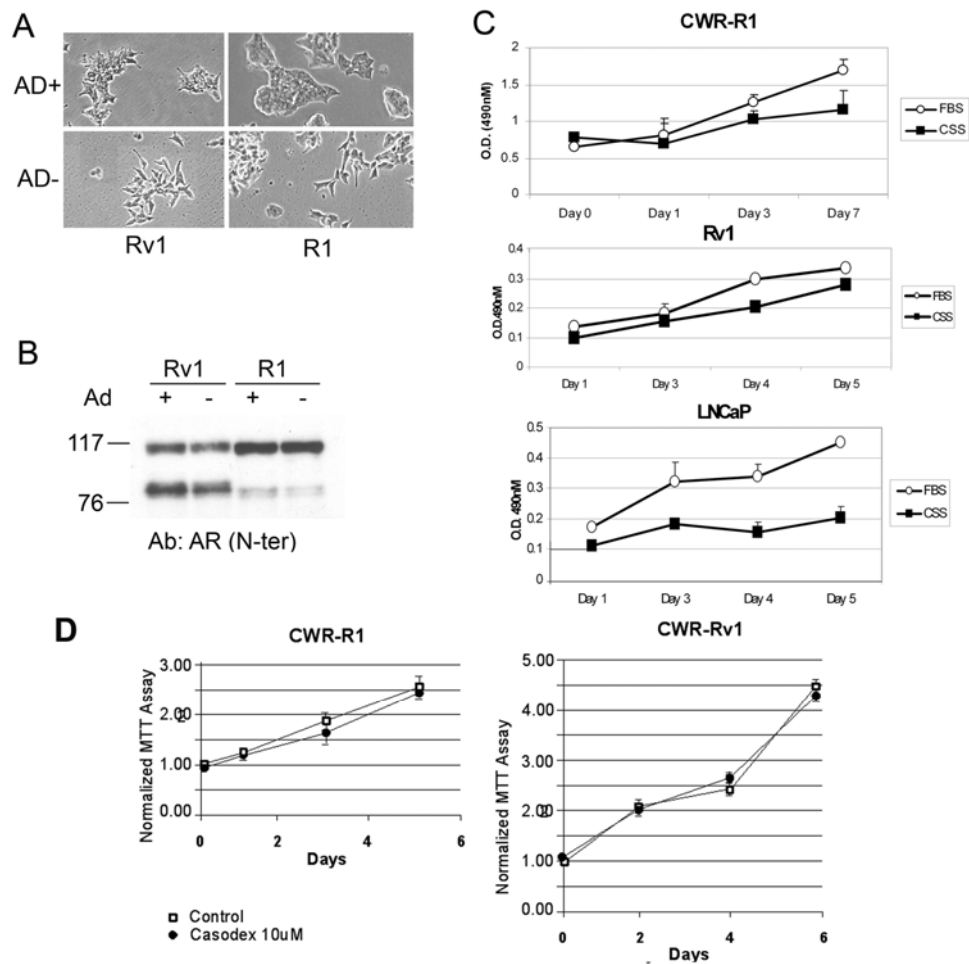


Figure 2

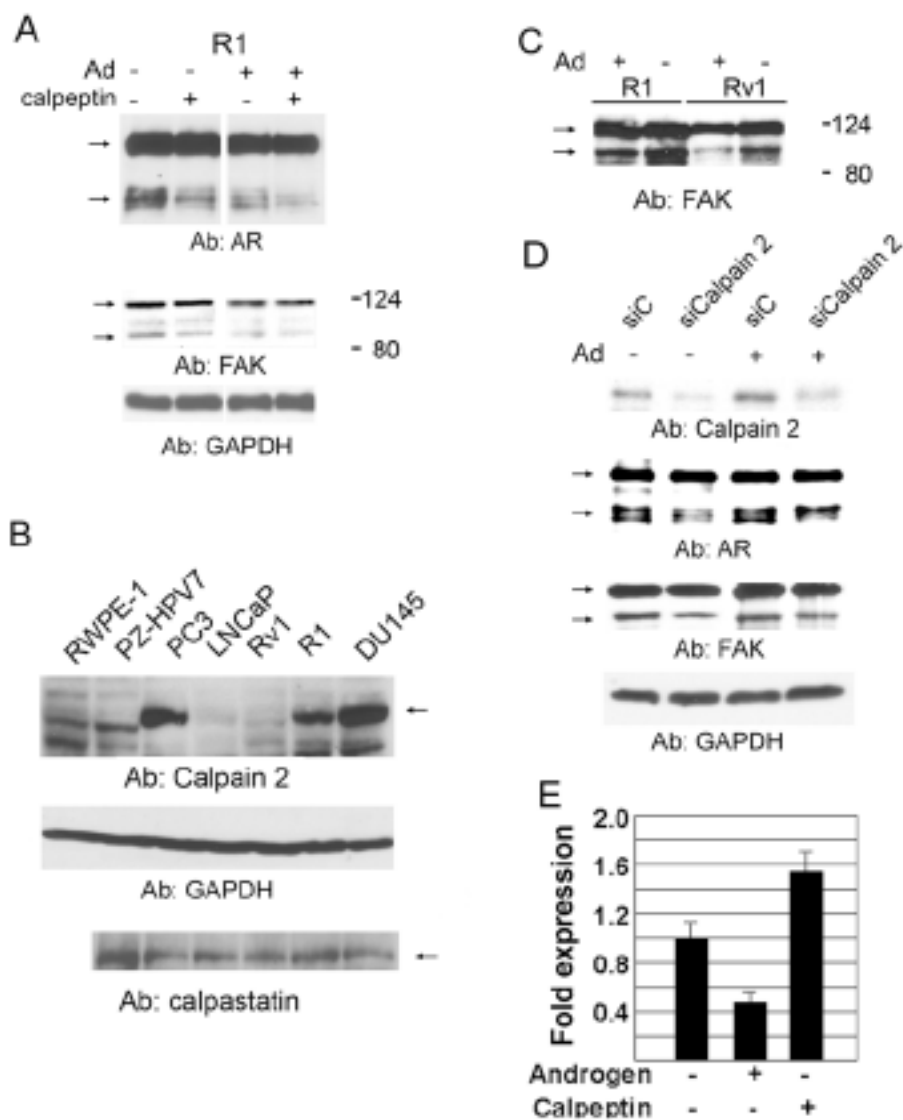


Figure 3

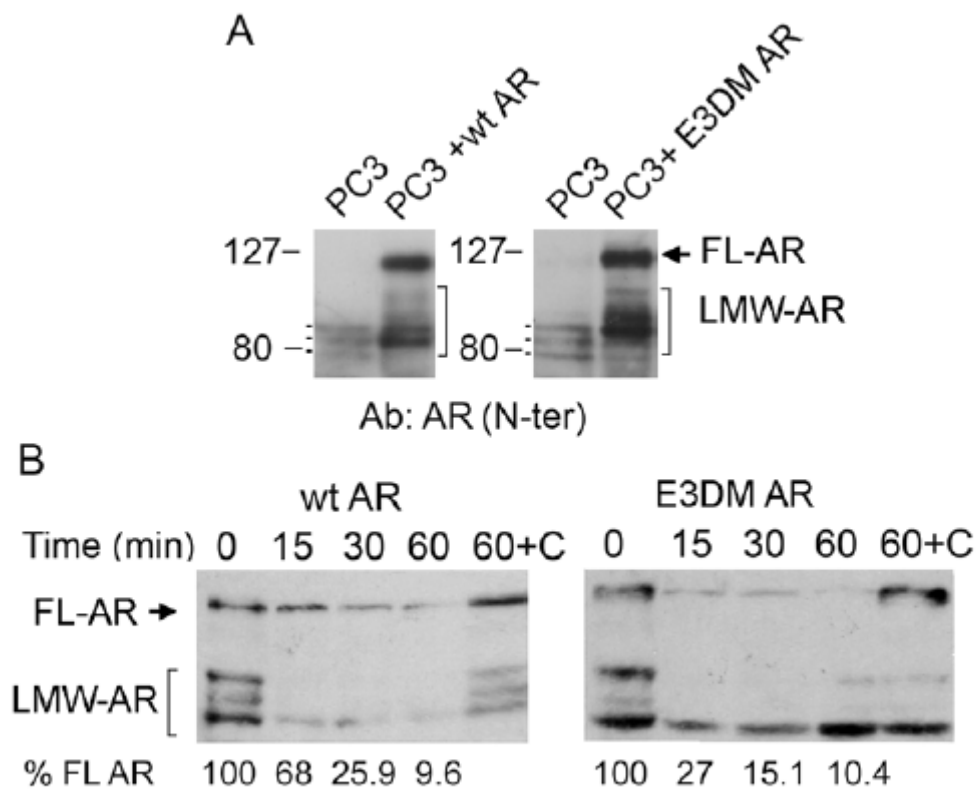
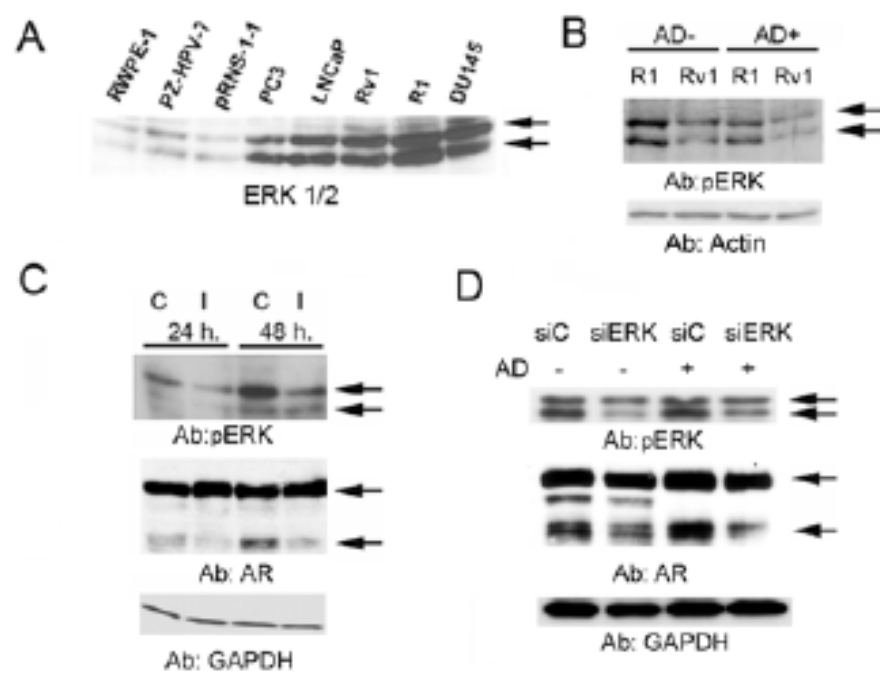


Figure 4



A

TPA

R1 - 1h 2h

Rv1 - 1h 2h

Ab: AR

Ab: pERK

Ab: GAPDH

B

Tumors

01 21 25 28 30 94

Ab: AR

Ab: Calpain 2

Ab: Calpastatin

p-ERK

Ab: Actin

C

Sample	01	21	25	28	30	94
pERK	1.29	0.28	0.79	0.22	1.93	2.15
Calpastatin	0.90	1.70	2.02	1.36	0.93	2.01
Calpain	0.75	0.76	1.13	0.66	0.81	3.06

pERK x $\frac{\text{Calpain}}{\text{Calpastatin}}$

1.07 0.12 0.44 0.11 1.68 3.27

pERK x canp2/cast

arbitrary units

high levels of LMW-AR

low levels of LMW-AR

Genome-wide analysis of androgen receptor binding and gene regulation in two CWR22-derived prostate cancer cell lines.

Honglin Chen¹, Stephen J. Libertini^{1,4}, Michael George¹, Satya Dandekar¹, Cliff Pepper², Bushra Al-Bataina¹, Hsing-Jien Kung^{2,3}, Paramita Ghosh^{2,3}, Maria Mudryj^{1,4}

¹Department of Medical Microbiology and Immunology, ²Department of Biochemistry and Molecular Medicine, Division of Basic Sciences, Cancer Center, ³Department of Urology, University of California, Davis, Davis, California 95616 and ⁴Veterans Affairs-Northern California Health Care System, Mather, California 95655

Running Head: Genome-wide analysis of AR binding in R1 and Rv1 cells

Corresponding author: Maria Mudryj, Ph.D., Department of Medical Microbiology, 3147 Tupper Hall, University of California, Davis, Davis CA 95616. Fax: 530-752-8692; E-mail: mmudryj@ucdavis.edu

Abstract

The emergence of castrate-resistant prostate neoplasms is the most challenging problem in managing prostate cancer. The androgen receptor (AR) continues to have a prominent role in these recurrent tumors, but the receptor's targets have not been well defined. In this study, we investigated AR binding and AR-dependent transcription in two related cell lines derived from androgen-dependent CWR22 relapsed tumors: CWR22Rv1 (Rv1) and CWR-R1 (R1). Both lines are androgen-independent yet androgen sensitive. Expression microarray analysis revealed that although related, R1 and Rv1 had significantly different gene expression profiles in response to androgen. Addition of androgen altered the expression of significantly more transcripts in Rv1 cells than in R1 cells. The analysis of androgen regulated transcripts identified only ten that were commonly regulated in the two cell lines. In contrast, AR chromatin immunoprecipitation combined with promoter DNA microarrays (ChIP-on-chip) studies showed R1 and Rv1 cells have a similar AR binding profile, but AR binding is more extensive in Rv1 cells than in R1 cells. Coupling of the microarray study with ChIP-on-chip analysis identified direct AR targets in R1 and Rv1 cells. The extent of AR binding was not linked to the amplitude of gene expression. The extent of AR binding was not linked to the amplitude of gene expression. Interestingly only 6% of the Rv1 androgen-regulated genes, but 42% of the R1 androgen-regulated genes, bound AR. A screening of transcription factor binding motifs revealed that the glucocorticoid response element (GRE), GATA, Sp1 and FoxJ2 most frequently co-present with AR binding motifs in the AR direct target genes. Moreover, the most prominent function of transcripts that were direct AR targets was transcriptional regulation. However, only one transcriptional regulator, CEBPD, was commonly regulated in both cell lines. This study indicates that in addition to AR binding, AR-dependent gene expression is dependent on factors that vary greatly even in related cell lines.

Introduction

Prostate carcinoma (CaP) is the most commonly diagnosed cancer in men, and the second leading cause of death due to cancer in Western civilization [1]. Most CaPs initially present as androgen-dependent neoplasms and androgen ablation therapy is an effective treatment which blocks androgen receptor (AR) cell signaling. While this therapy is initially successful, androgen independent tumors that are refractory to hormonal therapeutic interventions emerge [2, 3]. Androgen independent CaPs continue to express the AR and androgen-regulated genes. Thus, a better understanding of the action of AR is a pivotal issue in defining the molecular events that lead to the progression of CaP.

As a member of nuclear receptor superfamily that functions as a ligand-dependent transcription factor, AR mediates androgen-regulated gene expression. Androgen bound AR is stabilized and translocated into the nucleus to regulate the expression of target genes by binding to androgen response elements (AREs), or by interacting with other transcription factors bound to their specific recognition sites. The role of AR in CaP progression is to promote expression of specific target genes. For example, prostate specific antigen (PSA), the best studied AR target gene, has been reported to contribute to CaP progression through its protease activity and its ability to induce epithelial-mesenchymal transition and cell migration [4, 5]. Other AR target genes implicated in CaP progression include FGF8, Cdk1 and Cdk2, PMEPA1, TMRPSS2 and amyloid precursor protein [6-10].

Since last decade, microarray techniques have been applied extensively in searching for genes that are AR regulated specifically in prostate tumors. Although gene expression profiling is a powerful technique for depicting the global function of the androgen receptor in a specified model, it does not distinguish whether alteration of gene expression is dependent on a direct or indirect action of AR. Moreover, despite the well-characterized AREs in the promoter and enhancer, little is known about AR *cis*-regulatory sites across the human genome. ChIP-on-chip technology has been used for the identification of chromosomal binding sites of transcription factors to identify novel targets [11, 12]. Therefore, coupling microarray studies with ChIP-on-chip allows the identification of *bona fide* AR target genes.

The CWR22 androgen dependent xenograft model, which mimics human prostate cancer, has been used to study the emergence of androgen independence [13]. In male nude athymic mice this xenograft exhibits androgen dependent growth and secretes PSA. Following androgen withdrawal, the tumors regress and PSA levels plummet. Importantly, the model simulates the clinical course of prostate cancer in that PSA levels eventually increase and androgen independent tumors emerge [14]. Like most androgen independent tumors, CWR22 recurring tumors continue to express the androgen receptor [7], which contains a mutation (H847Y) in the ligand binding domain (LBD) of the molecule [15]. Since this model recapitulates salient features of human prostate tumors in has been used extensively to study the emergence of androgen independent neoplasms.

Two cell lines, R1 and Rv1 [16] were isolated in separate laboratories from CWR22 relapse tumors. Several lines of evidence indicate that they were derived from a common ancestor. Karyotypes of the two cell lines are very similar; both lines shared the same structural abnormalities, including a reciprocal translocation between chromosomes 6 and 14 [16]. Both lines have the same AR (H847Y) mutation that is present in the parental CWR22 cells [16]. The Rv1 AR also contains a duplication of exon 3 [that encodes the DNA binding domain (DBD)], which results in an insertion of 39 additional amino acids [17]. Additionally, we and others found that R1 and Rv1 express an ~80KDa LMW form of AR with a deletion of the C-terminal LBD [17, 18]. Both cell lines have the p53 Q331R (exon 9) mutations, and both are heterozygous for a polymorphism in p53 intron 3 [16]. However, R1 cells have an additional p53 codon R273H mutation, a result that is consistent with increased p53 levels [16]. Furthermore, DNA profiling studies showed additional differences. Of the 21 alleles detected in

each cell line, only 67% were identical [16]. Therefore, while the cell lines have significant similarities, they also exhibit differences.

In the current study, microarray analysis was used to investigate the gene expression profile in response to hormone stimulation in R1 and Rv1 cells. Surprisingly, the changes in the gene expression were greater in Rv1 than in R1 cells and very few transcripts were regulated commonly. ChIP-on-chip analysis using a promoter array revealed that AR binding pattern was similar between R1 and Rv1. By coupling microarray analysis with ChIP-on-chip study, direct AR target genes in R1 and Rv1 cells were identified. This analysis, along with previous studies helps establish the rules that govern androgen-dependent gene expression in prostate cancers.

Materials and Methods

Cell culture and pharmacological agents. Rv1 cells were obtained from American Type Culture Collection. CWR-R1 cells were provided by Dr. Elizabeth Wilson (University of North Carolina). Rv1 and R1 cells were propagated in RPMI 1640 () supplemented with 5% fetal bovine serum (), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C and 5% CO₂. For studies in androgen depleted conditions cells were propagated in phenol-red free RPMI 1640 () supplemented with 5% charcoal-stripped fetal bovine serum (), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C and 5% CO₂.

Western immunoblot analysis. Cells were directly placed in a radioimmunoprecipitation assay lysis buffer that contained the Sigma protease inhibitor cocktail (AEBSK, Aprotinin, E64, leupeptin and peptatin as well as ??? µM calpeptin (Sigma). Thirty micrograms of protein were separated on 8%, 10%, or 12% SDS-PAGE gels and transferred to 0.22µM nitrocellulose supported membrane (GE). The membrane was blocked with 5% nonfat dry milk in PBS and 0.1% Tween 20 before the addition of specific antibodies. The following antibodies were used: AR (central) 441 (Ab-1; Lab Vision Corp.), AR NH₂-terminus N-20 (Santa Cruz Biotechnology, Inc.), Calpain 2 (Sigma), calpastatin, ERK and phosphoERK (Cell Signaling), and focal adhesion kinase (FAK; clone 4.47; Upstate). Proteins were detected using chemiluminescence (GE Healthcare).

Microarray analysis. Labeling of samples, hybridization to U133A GeneChips (Affymetrix, Santa Clara, CA), staining, and scanning were done as described in the Affymetrix Expression Analysis Technical Manual. Fluorescence intensity values (.CEL files) generated from hybridized, stained GeneChips were analyzed with R statistical software (v.2.01, and “affy” BioConductor package) and BRB Array Tools to identify genes that were differentially expressed. The settings used for Robust Multichip Analysis in R included Microarray Suite 5.0-based background correction, quantile normalization, and Robust Multichip Analysis–based algorithms for calculation of expression values using perfect match only fluorescence intensities. A $P \leq 0.05$ and a mean fold change of ≥ 1.5 -fold were used as criteria for filtering genes for clustering analyses. Hierarchical clustering and comparative fold-change analysis were used to identify and group similar patterns of gene regulation. Assignment of genes to functional categories was done by annotation of gene lists with the program, Database for Annotation, visualization, and Integrated Discovery (<http://apps1.niaid.nih.gov/david>) and literature-based classification was done by hand. Statistically overrepresented (Fisher exact probability score < 0.05) biological processes within clusters were identified using Expression Analysis Systematic Explorer v.1.0 analysis software.

Quantitative Real-time PCR. Total cellular RNA was prepared from Rv1 cells utilizing RNeasy® mini kit (Qiagen Inc. CA) based on manufacturer's protocol. cDNA was synthesized from 1µg RNA using QuantiTect® reverse transcription kit based on manufacturer's protocol. cDNAs were diluted 1:4 in ddH₂O and 2µl of diluted cDNA was added to 5µl of EXPRESS SYBR® GreenERTM qPCR supremix (Invitrogen Life Science, CA) and 200 nM of each primer. GAPDH, HPRT or RPL13A were used as the endogenous expression standards. PCR conditions were: 20-sec initial denaturation step at 95°C, 40 cycles at 95°C for 3 s, 60 °C for 30 sec, followed by additional 95°C 15sec, 60°C to 95°C over 20 min ramp for melt curve analysis. Primer sequences used in the study are in Supplementary Methods. Data was collected by the Mastercycler® ep Realplex (Eppendorf AG, Hamburg).

Ingenuity pathway analysis (IPA) The microarray expression data was uploaded into IPA software using Reference sequence (RefSeq). A total of 2322 genes were mapped utilizing the IPA database. Fold change of 1.5 and p value of ≤ 0.05 were applied as the cutoff criteria. Gene

networks were algorithmically generated based on their connectivity and assigned a score. A score of 3 or higher indicates a 99.9% confidence level that the network was not generated by chance alone. Canonical pathways analysis identifies the pathways, from the IPA library of canonical pathways, which are most significant to the input data set. The significance of the association between the data set and the canonical pathway is determined based on two parameters: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a *P* value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.

ChIP-on-chip assay and analysis. R1 and Rv1 cells were cultured in phenol-red free RPMI and 5% charcoal-stripped serum for 72 hours before that addition of 10nM dihydrotestosterone (DHT) for 2 hours (identical condition used for the expression array studies). Briefly, soluble chromatin was prepared by sonication of formaldehyde-fixed treated or untreated cells. AR-associated DNA was enriched by immunoprecipitation with anti-AR antibody directed against the N-terminal domain, followed by reversal of the crosslinks and DNA purification. The ChIP DNA was amplified by random priming using the GenomePlex Whole Genome Amplification (WGA) kit. Briefly, the initial random fragmentation step was eliminated and amplicons were labeled by incorporation of biotinylated ddATP with terminal deoxytransferase (TdT). The entire ChIP DNA amplicons were applied to the Human Promoter 1.0R Array (Affymetrix). A cutoff of $FDR \leq 0.05$ was used as criteria for filtering AR binding sites.

Results

Comparison of the gene expression profiles of R1 and Rv1 cells

To compare the two CWR22 relapse lines, we used the Affymetrix HG-U133 Plus2.0 Gene Chip microarray to identify differences in gene transcription. The analysis was conducted in duplicate for each line proliferating in identical conditions, at the same density in charcoal stripped serum or two hours following addition of 10nM DHT. The two hour time point was chosen to minimize the number of transcripts that were not direct transcriptional target. The 10nM DHT concentration, while higher than the optimal physiocological concentration, was chosen to ensure detectable DHT-mediated transcription within 2 hours. Comparison of R1 and Rv1 gene expression profiles in castrate levels of androgen identified 1275 genes that were differentially expressed (fold change ≥ 1.5 or ≤ -1.5 ; $P \leq 0.05$) in R1 vs. Rv1 cells. Analysis of the microarray data identified 1941 transcripts that were differentially expressed (fold change ≥ 1.5 or ≤ -1.5 ; $P \leq 0.05$) in R1 vs. Rv1 cells treated with DHT. Of these, 60% were identical to the transcripts that were differentially expressed in the absence of androgen (Figure 1A and Supplementary Table 1). As expected, R1 cells expressed 4-fold higher levels of calpain 2 mRNA than Rv1 cells, but the levels of the calpain inhibitor calpastatin were similar in both cell lines (Figure 1B) [19]. put in expression results. R1 cells also expressed 11.7-fold higher levels of c-MET (Figure 1B). Notably, Rv1 cells have more neuroendocrine characteristics than R1 cells since the expression of neuronal specific enolase (ENO2) was 12-fold higher in Rv1 cells (Figure 1B) than in R1 cells, and the expression array analysis indicate that Rv1 cells expressed higher levels of chromogranin A and B, and synaptophysin (Supplementary Table 1). ENO2 expression was not altered by androgen (data not shown). The most differentially expressed genes between R1 and Rv1 are listed Figure 1C. The most significant pathway differences between R1 and Rv1 cells both in the presence and absence of androgen involved metabolic pathways (Figure 1D). In summary, the gene expression profiles of R1 and Rv1 indicates that although these two lines were derived from the same CWR22 xenograft and have similar morphologies, at the molecular level they are distinct.

Comparison of androgen regulated transcripts in R1 and RV1 cells

Next we analyze genes differentially regulated in the two cell lines in response to a two hours androgen treatment. Using a cutoff of fold change ≥ 1.5 or ≤ -1.5 and $p \leq 0.05$, we found that the expression of 854 transcripts was altered by a two hour DHT treatment in Rv1 cells (Figure 2A, Supplementary Table 2). The same analysis was conducted using R1 cells and in contrast to Rv1 cells, the expression of only 77 transcripts changed following addition of DHT for 2hr (Figure 2A, Supplementary Table 2). Therefore, the transcriptional response to DHT was greater in Rv1 cells than in R1 cells. A comparison of the DHT-responsive R1 and Rv1 transcripts identified only 10 that were commonly regulated in both cell lines (Figure 2B), again indicating the large differences between these two lines. DHT-dependent regulation of six transcripts was validated by realtime PCR (Figure 2C). Interestingly the expression of HES1 was DHT regulated in both cell lines, but expression was repressed in R1 cells and activated in Rv1 cells. The expression of two well-known androgen responsive genes KLK3 (PSA) and TMPRSS2 was not significantly altered by DHT in either cell line, thus confirming previous reports that the transcripts are not androgen regulated in these cell lines [9, 20, 21].

SUBHEADING

The differentially expressed genes in response to DHT for 2hr were analyzed by Ingenuity System's Pathway Analysis (IPA) to identify most significant associated biological networks and canonical pathways (metabolic and cell signaling) altered in the two cell lines. The Fisher's exact test was used to determine the probability that the association between the dataset and a given pathway is due to chance alone. IPA identified two significant biological networks associated with the differentially expressed genes in R1 cells (Figure 3A,

Supplementary Figure 1). The significantly associated functions include gene expression, cellular development, cell cycle and embryonic development (Figure 3B). The most significantly associated canonical pathways are notch signaling, clatrin-mediated endocytosis, JAK/Stat signaling, and p53 signaling (Figure 3C). In Rv1 cells, a total of 18 biological networks were identified that are significantly associated with the differentially expressed genes (Figure 3A and Supplementary Figure 2). The significantly associated functions include cellular development, visual system development and function, cancer, cell cycle, molecular transport and protein trafficking (Figure 3B). The most associated canonical pathways include aminoacyl-tRNA biosynthesis, axonal guidance signaling, DNA damage response, cell cycle, p53 signaling and clatrin-mediated endocytosis (Figure 3C).

AR chromosomal binding sites in R1 and Rv1 cells in response to DHT

Since the cohort of androgen regulated transcripts differed in R1 and Rv1 cells we wondered if they were regulated differently because the AR bound to different regulatory regions. The Human Promoter 1.0R Array (Affymetrix) was used to detect AR binding to regulatory regions. This oligonucleotide (25-mer)-based, high-density tiling array covers 25,500 promoters with probe sets spanning at least 10 kb of genomic content per gene (~7.5 kb upstream and ~2.45 kb downstream of the Transcriptional Start Site [TSS]) and at a resolution of 35 bp. A total of 1225 and 2021 AR binding sites ($FDR \leq 0.05$) were identified in R1 and Rv1 cells, respectively, when treated with DHT for 2hr. Figure 4A shows the distribution of the binding sites along chromosomes in two cell lines. A comparison of AR binding across chromosomes in R1 and Rv1 cells treated with androgen showed that AR binding pattern was similar, but not identical (Figure 4B). Certain sites were AR bound only in Rv1 cells while others were AR bound only in R1 cells. We focused on the binding pattern for three well-known androgen responsive genes KLK3 (PSA) [20], NKX3.1 [22] and TMPRSS2 [21] in R1 and Rv1 cells, to determine if their lack of AR regulation of KLK3 and TMPRSS2 was due to a lack of AR binding. In Rv1 cells sequences near the KLK3 (PSA) genes bound AR (-4603, -3484 and -2499 upstream of its TSS), while there was no AR binding near or in the KLK3 gene in R1 cells (Figure 4C). In R1 cells AR bound in the 3'-UTR (2149 downstream of TSS) of the NKX3.1 gene, whereas in Rv1 cells AR bound not only in the 3'-UTR (2059 downstream of TSS) but also in the intron (1164 downstream of TSS) of NKX3.1 (Figure 4C). AR binding in the 5'-UTR (two sites: 6382 and 7179 downstream of TSS) of the androgen regulated gene TMPRSS2 was detected in Rv1 cells, but no binding near or in the TMPRSS2 gene in R1 cells (Figure 4B). This analysis indicated that AR binding following addition of DHT was more extensive in Rv1 than in R1 cells and most of the R1 AR bound sites were also AR bound in Rv1 cells. Therefore, while the androgen regulated gene profile of the two cell line is different the AR binding pattern is similar. We further analyzed AR binding to the sites identified in our study using ChIP analysis. Following addition 10nM DHT for 2 hours in Rv1 and LNCaP AR binding was detected in Rv1 cells, further confirming our results (Figure 4D).

Motif analysis of AR binding sites

A motif analysis of the AR binding sites was conducted to determine whether AR binds to the established consensus AR response element (ARE). Previous studies conducted in LNCaP, LNCaP derived cells, or AR transfected PC3 cells [23] reported that only ~10% of the AR binding regions had a canonical class 1 ARE (AGAACA_nTTGTTCT) binding motif when two positions were allowed to vary from the palindromic consensus with 3 nucleotide spacing. They also found that 78% of the binding regions contained the AR binding half-site motif (AGAACA). In this study we found in Rv1 cells only 4% (86/2021) of the sites had the canonical ARE and 35% (700/2021) had the AR half-site motif. Likewise, in R1 cells, 6%

(76/1225) of the sites had the canonical ARE and 46% (568/1225) had the AR half-site motif (Figure 5A).

The expression profile of genes closest to the AR binding sites in R1 and Rv1 cells in response to DHT for 2hr

Further analysis of the AR binding sites identified 965 and 1518 genes that were closest to the AR binding site in R1 and Rv1 cells, respectively (data not shown). Notably, while some closest genes only contained one AR binding site, many others had more than one AR binding sites. By coupling the ChIP-on-chip with microarray expression data, we identified that, of the 854 differentially regulated genes in Rv1 cells in response to DHT for 2hr, AR bound to nearby chromosomal sites ($FDR \leq 0.05$) of only 53 genes (6%). The location of the AR binding sites include intron (15 genes), exon (2 genes), 5'-UTR (9 genes), 3'-UTR (3 genes) and within 5Kb upstream from the TSS (25 genes) (Figure 5B and supplementary Table 3A). Additionally, two genes had AR binding sites that were more than 10Kb upstream of TSS, whereas three genes had AR binding sites that were more than 10Kb downstream of the transcriptional end site (TES). IPA analysis showed that the biological functions most prominently associated with these 53 genes were transcriptional regulation, cell cycle, and metabolic process (Figure 5C). The same analysis was performed in R1 cells. Of the 77 differentially regulated genes after adding DHT for 2hr, AR bound to the nearby chromosomal regions ($FDR \leq 0.05$) of 32 genes (42%). The AR binding sites identified include intron (4 genes), 5'-UTR (3 genes), 3'-UTR (2 genes), within 5Kb upstream of the TSS (8 genes), more than 10Kb upstream of the TSS (14 genes) and more than 10Kb downstream of the TES (8 genes) (Figure 5B and supplementary Table 3). Sites that are far upstream or downstream of the AR regulated gene reside in putative enhancers of other annotated genes. However, the genes closed to the site are not AR-regulated. The major biological functions associated with these 32 genes are transcriptional regulation and metabolic process (Figure 5C).

A comparison of R1 and Rv1 revealed that the majority of the AR bound sites near the differentially regulated genes were common. However, only three closest genes [CCAAT/enhancer binding protein delta (CEBPD), claudin 4 (CLDN4) and arylamine N-acetyltransferase type I (NAT1)] adjacent to the common AR binding sites in both R1 and Rv1 cells showed correlated transcriptional regulation (fold change 1.5 and $p < 0.05$) in both lines. This argues that only a subset of AR chromosomal binding sites exhibit transcriptional regulation. Of these three common AR direct targets, CEBPD and NAT1 have been reported to be androgen responsive genes [24, 25] and CLDN4 has been reported to be deregulated in both primary and metastatic prostate cancer [26]. Considering that other transcription factors might play collaborative role in AR function, we used Transcription Element Search System (TESS) to screen for motifs most frequently co-exist with AR binding motifs present in the above differentially regulated genes. TESS identifies transcription factor motifs using site or consensus strings and positional weight matrices from the TRANSFAC, JASPAR, IMD, and our CBIL-GibbsMat database. The transcription factor motifs that most frequently co-exist with AR binding motifs included GRE, GATA binding protein 1 (GATA-1), Sp-1 and forkhead box J2 (FoxJ2) in both R1 and Rv1 cells (not shown).

Our analysis of direct AR target genes in R1 cells revealed that ~25% of genes (7 genes- NAT1, NKX3.1, CEBPD, HEY, POP1, PHF20L1, NDRG1) mapped to chromosome 8 (Figure 6A) and all were positively regulated by androgen. Hey and Hes are transcription factors that are the downstream targets of the Notch signaling pathway. Furthermore, one of the AR sites has a single half ARE, while all of the other sites have more than one half ARE. In Rv1 cells 15% of the genes (8 genes- NAT1, CHRNA2, CEBPD, RB1CC1, ZBTB10, PLEKHF2, LAPTM4B, MTDH) mapped to chromosome 8. Seven were positively regulated by androgen,

while one was repressed. Five of the eight AR binding site contained at least one ARE half site. Two of the genes were commonly regulated in both cell lines- NAT1 and CEBDP, while the others were not. Next we asked if AR bound near transcripts that were androgen regulated in R1 cells, but not in Rv1 cells and vice versa. In the presence, but not in the absence of androgen, AR bound to the same or similar sites. The high percentage of direct AR target genes on one chromosome suggests that chromosome 8 is exceptionally rich in AR-regulated genes.

Discussion

The extensive difference in gene expression of R1 and Rv1 cells strongly argues that while they are derived from a common xenograft CWR22 tumor, at the molecular levels they are very different. However, since both cell lines have the same chromosomal translocations and harbor the same AR and p53 mutations [16] they were derived from a common progenitor. It is unclear if the parent xenograft is composed of a single precursor cell type that develops into distinct lineages following androgen ablation, or if the xenograft is already composed of several morphologically indistinguishable, but molecularly distinct, cell types that originally emerged from a precursor cell. We favor the hypothesis that the xenograft is composed of several distinct cells and that the androgen independent cells constitute a small component of the morphologically unidentifiable cells that expanded following the selective pressure of androgen ablation. The current study indicated that tumors which appear to be homogenous may be composed of several cell types, further complicating gene expression analysis.

The analysis of androgen-dependent gene transcription in R1 and Rv1 cells revealed that very few transcripts were commonly regulated. In R1 cells the most regulated pathway is the Notch signaling pathway and the most common function of androgen regulated transcripts involves gene expression. Two downstream effectors of Notch signaling, the Hes1 and Hey1 transcriptional repressors, are androgen regulated but in opposite direction. Hes1 expression is androgen repressed, while Hey1 expression is elevated. Hey1 has been shown to be a negative co-regulator of AR, therefore, transactivation of Hey1 may serve as a negative feedback loop to limit AR-dependent transcription.

In Rv1 cells, the Notch pathway is not androgen regulated, while the most prominent androgen regulated pathways includes Aminoacyl-tRNA biogenesis, DNA damage response, axonal guidance signaling, and JAK/Stat signaling. The most common functions of androgen-regulated transcripts are cellular development, cell cycle, development, and vision system development. An analysis of the networks, function and pathways that are androgen regulated in R1 and Rv1 cells indicates that androgen controls a different gene expression program in the two cells lines.

Thus far, the genome-wide studies of AR chromosomal binding have utilized the androgen-dependant LNCaP cell line, androgen independent LNCaP derived cell lines [27, 28] [23, 27] and PC3 cells transiently expressing AR [29]. Our analysis studied two related CWR22 derived androgen independent, but androgen responsive, cell lines. The study used the human promoter array with coverage of ~10Kb up/down-stream of the TSS, thus scanned regions proximal to the TSS of known genes throughout the genome. Studies of AR binding in PC3 cells transiently transfected with AR using chromatin immunoprecipitation followed by sequencing (ChIP-seq) identified AR binding sites associated with androgen dependent gene regulation. The AR binding sites were varying distances from the TSS but were preferentially located near the TSS of genes that were androgen regulated. 22.4% of the AR sites mapped to within 2Kb of the TSS and ~40% were within 12 Kb of the TSS. Therefore, while our analysis could not identify all AR binding sites, it focused on known transcripts throughout the genome. The majority of AR binding sites were located more than 2Kb upstream of the TSS in both R1 and Rv1 cells, and more AR binding was detected in Rv1 cells than in R1 cells. This correlates with our result that Rv1 cells have a greater number of DHT regulated transcripts than R1 cells. Most of the AR sites in R1 cells were identical or similar to the sites in Rv1 cells. Consistent with previous findings, the majority of the AR binding sites did not contain the canonical AREs. However, a significant number of the sites contained an AR half-site motif and in many cases had more than one half-motif. Therefore all of the studies thus far indicate that the AR half-site is associated with AR binding, while the canonical ARE is rare. As previously reported a

number of AR binding sites have neither a canonical or half-site ARE. AR may directly bind previously unidentified sequences as has been proposed by Lin et al [29], or alternatively, the AR may be binding indirectly by interacting with another DNA binding protein.

An analysis of motifs co-present with AR identified several transcription factors including GRE, GATA, Sp-1 and forkhead box J2 (FoxJ2) in both R1 and Rv1. The GATA motif has been identified by all previous AR binding studies [23, 27, 29]. Sp1 is a very common transcription factor binding site found in many promoter sequences. Moreover, previous studies have shown that Sp1 and the AR interact to promote transcription [30] therefore the presence of Sp1 may serve to enhance AR-dependent gene expression. Studies by Jie et al [27] also found that GRE sites were co-present with AR binding sites. The FoxJ2, a member of the forkhead family of transcription factors, has a core sequence that is common to other family members, including FoxA1. Therefore, all of the AR binding studies indicate that GATA and Forkhead transcription factor binding sites are co-present with AR binding sites. The importance of the Forkhead factor in AR-dependent gene expression is further substantiated by a recent report that a single nucleotide polymorphism that is associated with an increased prostate cancer risk resides in a FoxA1 site and this polymorphism facilitates stronger androgen responsiveness [31]. Previous studies have suggested that the forkhead and GATA proteins may act as 'pioneer' factors that are capable of initiating chromatin opening [32]. The major role of these proteins may be to open the chromatin and allow AR binding, rather than to specifically promote AR binding. Subsequence events, such as stabilizing AR/DNA interaction and recruiting appropriate co-factors to regulate gene transcription may rely on additional factors.

A closer analysis of well studied androgen-regulated genes identified AR binding to sequences near the PSA and TMPRSS2 genes in DHT-treated Rv1 cells, but not in DHT-treated R1 cells. However, DHT-treatment of Rv1 cells did not transactivate transcription of either gene. AR binding to the 3'UTR of the NKX3.1 gene was detected in DHT-treated R1 and Rv1 cells. A recent report showed that the androgen responsive element of this gene resides in the 3'UTR [33]. AR binding to this site was more extensive in Rv1 than in R1 cells, yet NKX3.1 transcription was transactivated only in R1 cells. This indicates that while AR binding is required, it is not sufficient for AR-androgen-dependent gene expression and that increased binding does not ensure increased gene expression.

However, we identified differences in AR binding between Rv1 and LNCaP cells. In Rv1 cells AR binding near the TMPRSS2 gene was identical in the presence and absence of androgen. Since the TMPRSS2 gene is located on chromosome 21. We compared our AR binding results with that obtained by Wang et al [23]. The comparison is imperfect since Wang et al [23] used a tiling array covered the entire non-repetitive regions of chromosomes 21 and 22, different DHT concentrations and different duration of DHT exposure. However, all of the sites that were present in human promoter 1.0R array were present in the tiling array. Our analysis found that while AR bound to a region near the TMPRSS2 TSS in both studies, the site that was bound by AR Rv1 cells was not identical to the site bound by AR in LNCaP cells. The analysis of AR binding in Rv1 and R1 cells, and this very limited example of TMPRSS2 AR binding in LNCaP cells, suggest that AR binding to some sites is cell context dependent.

By coupling gene expression profiles with ChIP-on-chip analysis, we found that 46% of the differentially expressed transcripts identified in R1 contained AR binding sites, indicating they are most likely direct AR targets. In contrast only 6% of the transcripts identified in Rv1 cells had AR binding sites. Previous studies have shown that AR binding sites can be far away from transcription start sites [23, 28]. The coverage of the promoter array used for this study is limited within ~10kb from transcription start sites. Therefore, the actual number of direct AR

targets in R1 and Rv1 cells are most likely higher than what we found. While the number DHT-regulated genes was much higher in Rv1 cells, the number of genes that are DHT-regulated and are associated with an AR binding site is more comparable in R1 and Rv1 cells. This suggests that AR binding or AR/DNA complex stability in Rv1 cells is greater or that a large number of the DHT-regulated transcripts in Rv1 cells are indirect AR targets. Several mechanisms may account for this discrepancy. The presence of a 39aa insertion mutation in the Rv1 AR that results in the duplication of the a portion of the DNA domain may facilitate DNA binding, or the interactions with other DNA binding protein. Alternatively, the different complement of AR co-regulators in Rv1 and R1 cells may govern AR-dependent gene regulation.

The most common function by far of direct AR target genes in R1 (9 genes) and Rv1 (11 genes) is regulation of transcription, while the second most common functions were regulation of the cell cycle or metabolism. However, only CEBPD was commonly regulated in both cell lines. If AR-regulated transcription factors are very different in the two cell lines, then the subsequent indirect AR target transcripts would be different as well. Therefore it is not surprising that the AR-dependent transcription profile of R1 and Rv1 cells is distinct.

We noted that in R1 cells ~25% of the genes that contained AR binding sites and were androgen regulated mapped to chromosome 8. All of the transcripts are transactivated. Multiple studies have suggested a link between chromosome 8 and prostate cancer [34] including 8p deletion, 8q amplification [35] and susceptibility loci a 8q24 [36, 37]. One susceptibility locus maps to a Foxa1 site within a sequence that functions as an AR-responsive enhancer, but is not adjacent to any identifiable gene [31]. This enhancer may have a long range effect on the expression of multiple genes on the same chromosome.

In summary our study of androgen independent but androgen sensitive cells lines that were derived from a common progenitor exhibit similar AR binding profiles. The GATA, GRE, Foxj2 and Sp1 binding motifs are co-present with AR binding sites in both cell lines. However, the DHT-dependent gene expression profile of the two cell line is completely different. The AR is regulating a different program in the two cell lines. This was surprising since these cells have a vary similar appearance. Since the two cell lines descended from a common progenitor it is possible that this progenitor had stem cell-like characteristics. Alternatively, it is possible that all cancer cells may retain some elements of plasticity and can evolve their gene expression program. The combined ChIP-on-chip with microarray analysis also revealed that only a subset of closest genes adjacent to AR binding sites showed differential expression in response to DHT arguing that 1) Binding of AR to the vicinity of these genes is insufficient in transcriptional regulation in certain cell context or under the specific experimental conditions applied; or 2) The binding sites are indeed nonfunctional. Similarly, other groups have reported that only a subset of binding sites by AR in LNCaP [23] or by ER [38] are functional, as there are many more binding sites identified than differentially regulated genes. It is apparent that the presence of a half-ARE or AR binding is not sufficient for androgen dependent gene regulation and that AR co-regulators are at least as important in controlling AR-mediated transcription as As more studies of AR binding couple with expression microarray analysis are conducted in different cellular contexts the factors that rules that govern AR-dependent gene expression will become more apparent.

Acknowledgements

We thank Dr. Elizabeth Wilson for the CWR-R1 cells. This work was supported by a DOD grants PC051049 (MM), VA Merit (MM) and DOD predoctoral award PC073557 (HC). This investigation was in part conducted in a facility constructed with support from Research

Facilities Improvement Program Grant Number C06 RR-12088-01 from the National Center for Research Resources, NIH.

Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legends

Figure 1. Differences in gene expression of R1 and Rv1 cells in the presence and absence of a DHT. A. Venn Diagram of the number of genes differentially expressed in R1 and Rv1 cells. B. Western blot analysis of several proteins that are differentially expressed in R1 and Rv1 cells. C. The most differentially expressed transcripts in R1 and Rv1 cells. D. The functions of genes differentially regulated in R1 and Rv1 cells.

Figure 2. Differences in AR-dependent gene expression of R1 and Rv1 cells. A. Venn Diagram of the number of AR-regulated transcripts in R1 and Rv1 cells and transcripts that are commonly regulated in the two cell lines. B. Real time PCR validation of several AR-regulated transcripts.

Figure 3. comparison of biological networks, pathways and function of R1 and Rv1 DHT regulated transcripts. A. The most prominent DHT-regulated network in R1 and Rv1 cells. Red indicates transactivation, green indicates repression. B. The most common functions of transcripts regulated by DHT in R1 and Rv1 cells. C. The most common DHT regulated canonical pathways regulated in R1 and Rv1 cells.

Figure 4. Distribution of AR binding sites in R1 and Rv1 cells. A. Number of AR binding sites detected on individual chromosomes in R1 and Rv1 cells treated for 2 hours with 10 nM DHT. B. More detailed mapping of AR binding on chromosome 1. C. Precise location of AR binding to PSA, NKX3.1 and TMPRSS2 genes in R1 and Rv1 cells. D. ChIP analysis of AR binding to sites in the TMPRSS2 gene in Rv1 and LNCaP cells.

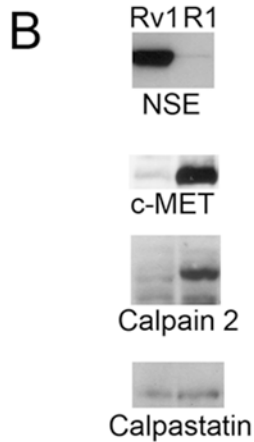
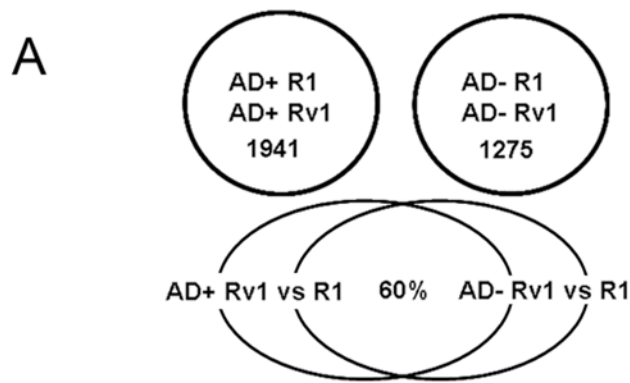
Figure 5. Characteristics of AR binding sites and direct AR transcriptional target genes. A. The half ARE is present in many AR binding sites, while the canonical ARE is not. B. ARE located near the TSS are preferentially located in intron sequences. C. The most significant function of AR-regulated transcripts in R1 and Rv1 is transcriptional regulation.

Figure 6. AR binding pattern on chromosome 8 in R1 and Rv1 cells. Top panel- all of the AR binding sites. Lower panel- AR sites associated with transcripts that are AR regulated in R1 and Rv1 cells. Genes in Red are androgen transactivated, genes in Green are repressed.

Reference:

1. Jemal, A., et al., *Cancer statistics, 2002*. CA Cancer J Clin, 2002. **52**(1): p. 23-47.
2. Huggins, C. and C.V. Hodges, in *Cancer Res*. 1941. p. 293-297.
3. Gittes, R.F., *Carcinoma of the prostate*. N.Engl.J.Med., 1991. **324**(4 AD - Scripps Clinic and Research Foundation, La Jolla, CA 92037 UR - PM:1985245): p. 236-245.
4. Borgono, C.A. and E.P. Diamandis, *The emerging roles of human tissue kallikreins in cancer*. Nat Rev Cancer, 2004. **4**(11): p. 876-90.
5. Whitbread, A.K., et al., *The role of kallikrein-related peptidases in prostate cancer: potential involvement in an epithelial to mesenchymal transition*. Biol Chem, 2006. **387**(6): p. 707-14.
6. Gnanapragasam, V.J., et al., *Regulation of FGF8 expression by the androgen receptor in human prostate cancer*. Oncogene, 2002. **21**(33): p. 5069-80.
7. Gregory, C.W., et al., *Androgen receptor expression in androgen-independent prostate cancer is associated with increased expression of androgen-regulated genes*. Cancer Res, 1998. **58**(24): p. 5718-24.
8. Xu, L.L., et al., *PMEPA1, an androgen-regulated NEDD4-binding protein, exhibits cell growth inhibitory function and decreased expression during prostate cancer progression*. Cancer Res, 2003. **63**(15): p. 4299-304.
9. Lin, B., et al., *Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2*. Cancer Res, 1999. **59**(17): p. 4180-4.
10. Takayama, K., et al., *Amyloid precursor protein is a primary androgen target gene that promotes prostate cancer growth*. Cancer Res, 2009. **69**(1): p. 137-42.
11. Bernstein, B.E., et al., *Genomic maps and comparative analysis of histone modifications in human and mouse*. Cell, 2005. **120**(2): p. 169-81.
12. Cawley, S., et al., *Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs*. Cell, 2004. **116**(4): p. 499-509.
13. Wainstein, M.A., et al., *CWR22: androgen-dependent xenograft model derived from a primary human prostatic carcinoma*. Cancer Res, 1994. **54**(23): p. 6049-52.
14. Nagabhushan, M., et al., *CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar*. Cancer Res, 1996. **56**(13): p. 3042-6.
15. Tan, J., et al., *Dehydroepiandrosterone activates mutant androgen receptors expressed in the androgen-dependent human prostate cancer xenograft CWR22 and LNCaP cells*. Mol Endocrinol, 1997. **11**(4): p. 450-9.
16. van Bokhoven, A., et al., *Molecular characterization of human prostate carcinoma cell lines*. Prostate, 2003. **57**(3): p. 205-25.
17. Tepper, C.G., et al., *Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line*. Cancer Res, 2002. **62**(22): p. 6606-14.
18. Gregory, C.W., B. He, and E.M. Wilson, *The putative androgen receptor-A form results from in vitro proteolysis*. J Mol Endocrinol, 2001. **27**(3): p. 309-19.
19. Chen, H., et al., *ERK regulates calpain 2-induced androgen receptor proteolysis in CWR22 relapsed prostate tumor cell lines*. J Biol Chem. **285**(4): p. 2368-74.
20. Riegman, P.H., et al., *The promoter of the prostate-specific antigen gene contains a functional androgen responsive element*. Mol Endocrinol, 1991. **5**(12): p. 1921-30.
21. Tomlins, S.A., et al., *Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer*. Science, 2005. **310**(5748): p. 644-8.

22. He, W.W., et al., *A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer*. Genomics, 1997. **43**(1): p. 69-77.
23. Wang, Q., et al., *A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth*. Mol Cell, 2007. **27**(3): p. 380-92.
24. Yang, G., et al., *Differential expression of CCAAT/enhancer-binding protein-delta (c/EBPdelta) in rat androgen-dependent tissues and human prostate cancer*. J Androl, 2001. **22**(3): p. 471-80.
25. Butcher, N.J., et al., *Induction of human arylamine N-acetyltransferase type I by androgens in human prostate cancer cells*. Cancer Res, 2007. **67**(1): p. 85-92.
26. Landers, K.A., et al., *Identification of claudin-4 as a marker highly overexpressed in both primary and metastatic prostate cancer*. Br J Cancer, 2008. **99**(3): p. 491-501.
27. Jia, L., et al., *Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity*. PLoS One, 2008. **3**(11): p. e3645.
28. Takayama, K., et al., *Identification of novel androgen response genes in prostate cancer cells by coupling chromatin immunoprecipitation and genomic microarray analysis*. Oncogene, 2007. **26**(30): p. 4453-63.
29. Lin, B., et al., *Integrated expression profiling and ChIP-seq analyses of the growth inhibition response program of the androgen receptor*. PLoS One, 2009. **4**(8): p. e6589.
30. Lu, S., G. Jenster, and D.E. Epner, *Androgen induction of cyclin-dependent kinase inhibitor p21 gene: role of androgen receptor and transcription factor Sp1 complex*. Mol Endocrinol, 2000. **14**(5): p. 753-60.
31. Jia, L., et al., *Functional enhancers at the gene-poor 8q24 cancer-linked locus*. PLoS Genet, 2009. **5**(8): p. e1000597.
32. Cirillo, L.A., et al., *Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4*. Mol Cell, 2002. **9**(2): p. 279-89.
33. Thomas, M.A., D.M. Preece, and J.M. Bentel, *Androgen regulation of the prostatic tumour suppressor NKX3.1 is mediated by its 3' untranslated region*. Biochem J. **425**(3): p. 575-83.
34. Bova, G.S. and W.B. Isaacs, *Review of allelic loss and gain in prostate cancer*. World J Urol, 1996. **14**(5): p. 338-46.
35. El Gammal, A.T., et al., *Chromosome 8p deletions and 8q gains are associated with tumor progression and poor prognosis in prostate cancer*. Clin Cancer Res. **16**(1): p. 56-64.
36. Yeager, M., et al., *Identification of a new prostate cancer susceptibility locus on chromosome 8q24*. Nat Genet, 2009. **41**(10): p. 1055-7.
37. Al Olama, A.A., et al., *Multiple loci on 8q24 associated with prostate cancer susceptibility*. Nat Genet, 2009. **41**(10): p. 1058-60.
38. Carroll, J.S., et al., *Genome-wide analysis of estrogen receptor binding sites*. Nat Genet, 2006. **38**(11): p. 1289-97.
39. Ceraline, J., et al., *Constitutive activation of the androgen receptor by a point mutation in the hinge region: a new mechanism for androgen-independent growth in prostate cancer*. Int J Cancer, 2004. **108**(1): p. 152-7.
40. Jenster, G., et al., *Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization*. Mol Endocrinol, 1991. **5**(10): p. 1396-404.
41. Chmelar, R., et al., *Androgen receptor coregulators and their involvement in the development and progression of prostate cancer*. Int J Cancer, 2007. **120**(4): p. 719-33.



C

Gene	Fold elevated in R1	P value
IGFBP5	66.27	0.05332
SNAI2	42.47	0.02586
MSX2	34.64	0.00710
SERPINB5	28.21	0.01473
SHOX2	27.29	0.00619
ASS1	23.22	0.02328

Gene	Fold elevated in Rv1	P value
TARP	89.8	0.04694
STEAP1	52.97	0.02472
NMNAT2	44.2	0.02818
GJA1	40.0	0.04728
HPGD	34.64	0.03241
KRT19	28.22	0.04901

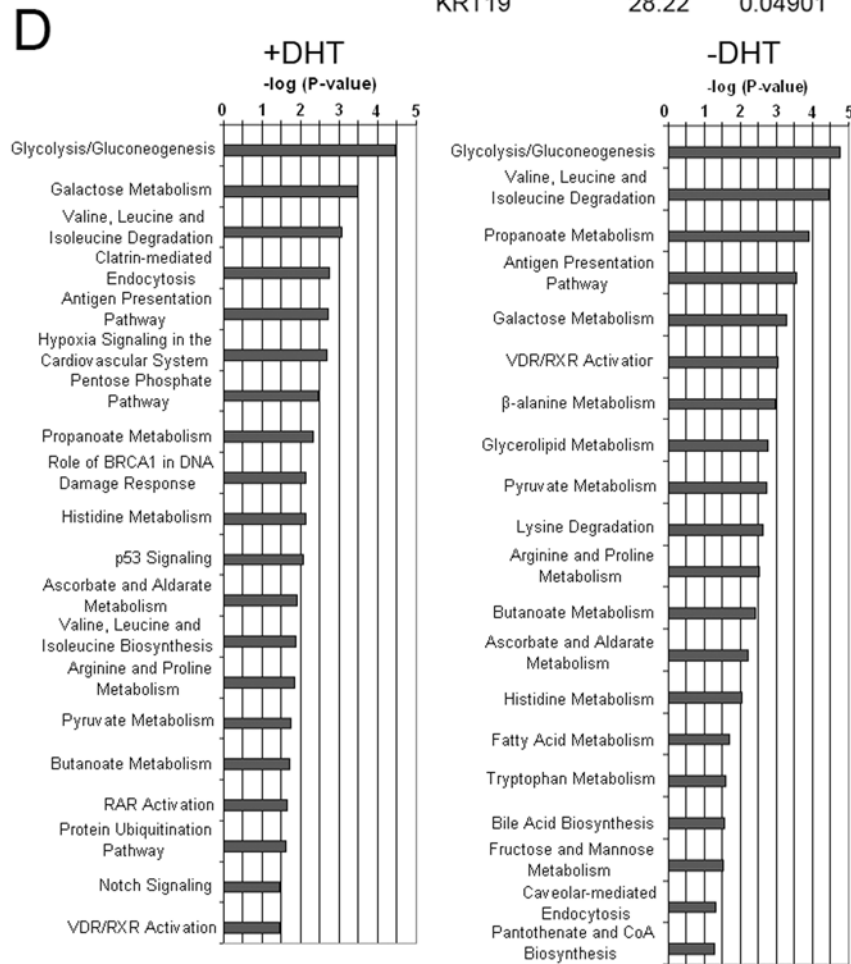


Figure 2

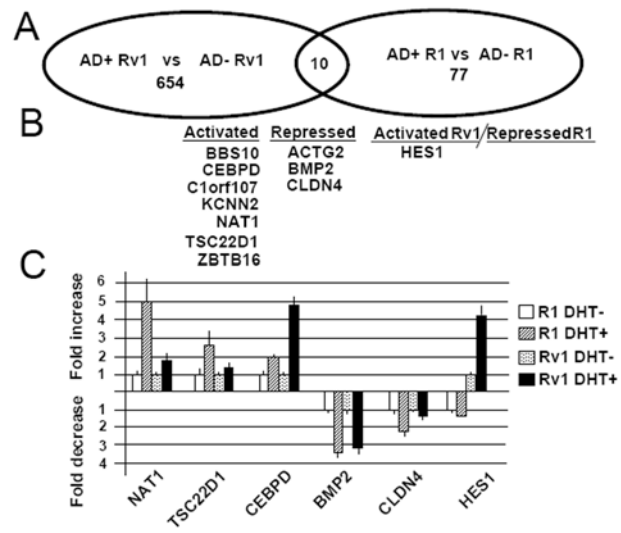


Figure 3

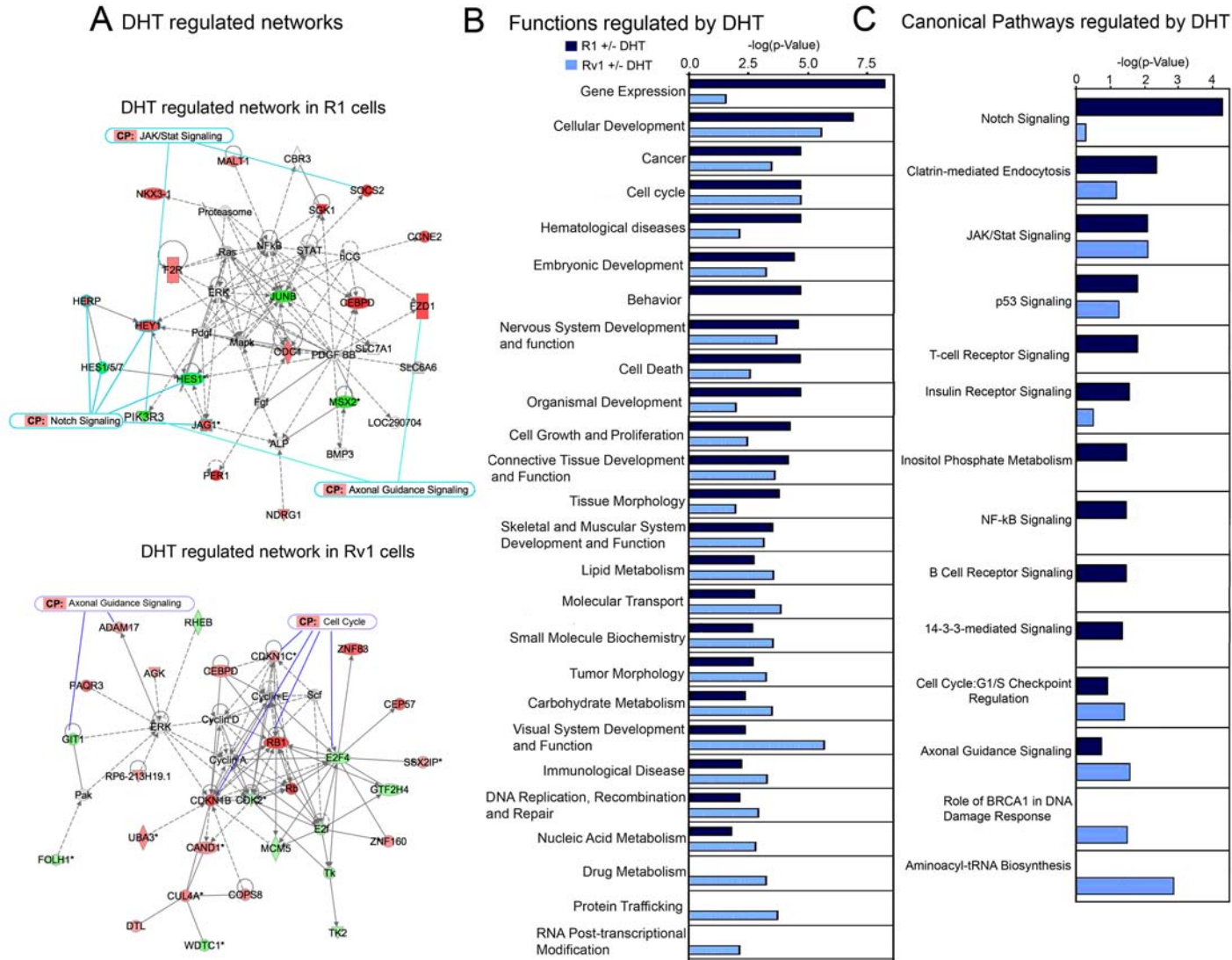


Figure 4 AR chromosomal binding sites

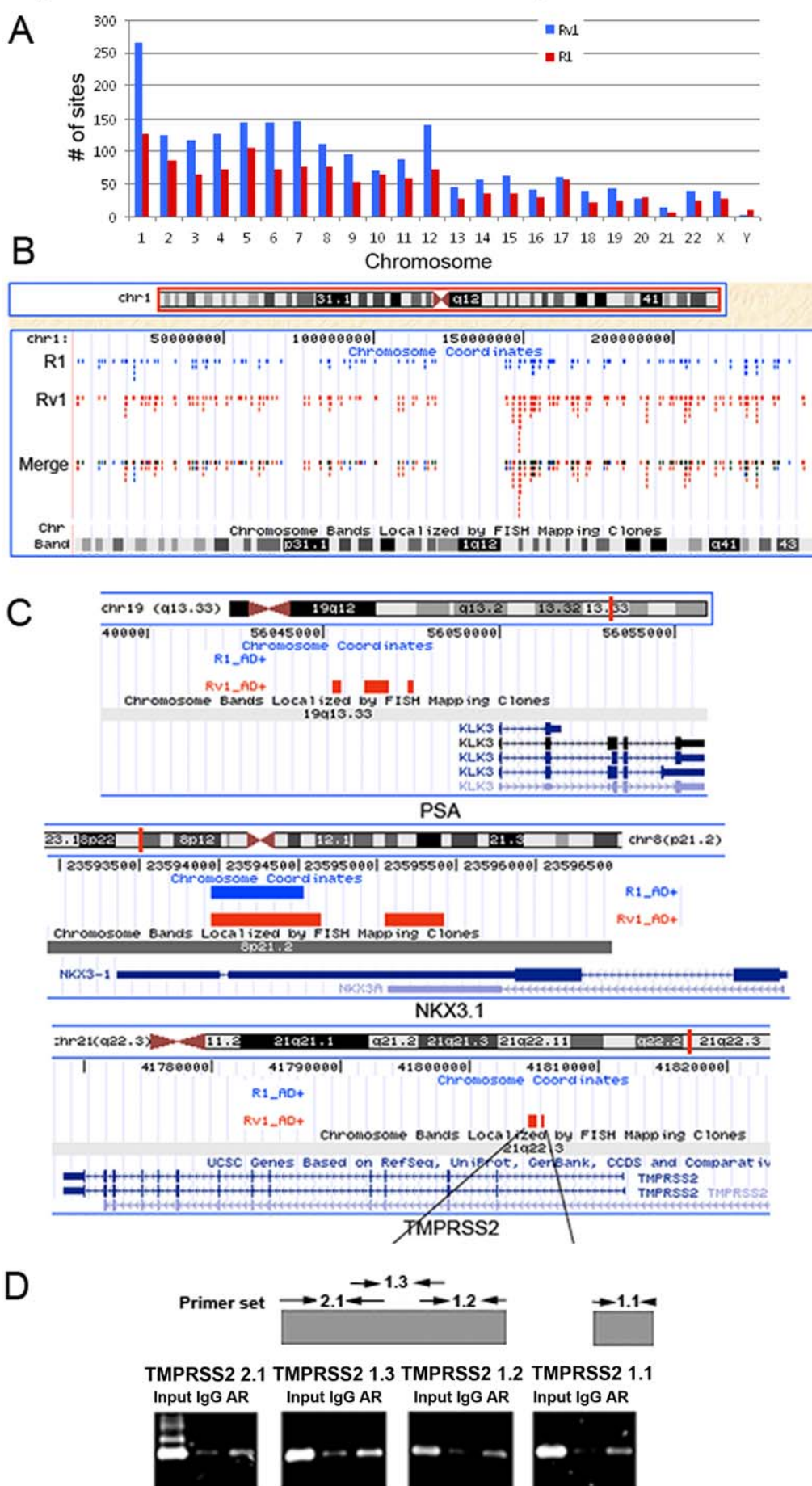
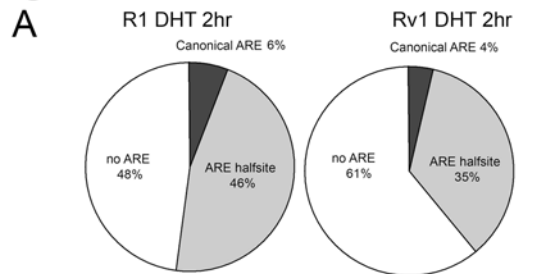


Figure 5



B AR bound and DHT regulated genes

	R1	Rv1
# of DHT regulated gene bound by AR	32	53
% of DHT regulated genes bound by AR	42	6
<u>Location of ARE</u>		
intron	12.5%	28%
exon	0	3.7%
5'UTR	9.4%	17%
3'UTR	6.25%	5.6%
within 5KB of TSS	25%	47%
over 10KB from TSS	68%	9.4%

C Most significant functions regulated by direct AR target genes

Function	# of genes	
	R1	Rv1
Transcriptional regulation	9 (28%)	11 (20%)
Cell cycle		5 (9.4)
Metabolic Process	4 (12.5%)	3 (5.7)

Figure 6

