Zhou Wang *Editor*

# Androgen-Responsive Genes in Prostate Cancer

## Regulation, Function and Clinical Applications

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# Preface

Androgens play an important role in the development and progression of prostate cancer, which is the most frequently diagnosed cancer and is the second leading cause of cancer death in US males. Androgen deprivation therapy (ADT) was developed by Dr. Charles Huggins in the 1940s and remains the standard treatment for metastatic prostate cancer. Unfortunately, patients treated with ADT eventually relapse with castration-recurrent or -resistant prostate cancer (CRPC), most within 2 years after ADT. The current treatment for CRPC includes docetaxel combined with prednisone, abiraterone, MDV3100, and provenge. However, these treatments can only prolong the survival of patients by 3–5 months on average. There is an urgent need for new approaches to prevent and/or treat CRPC. The activation of androgen signaling in prostate tumor cells in patients on ADT is a key step leading to castration resistance. Understanding the mechanisms of androgen action and the roles of androgen signaling at different phases of prostate carcinogenesis and progression has significant clinical implications.

Androgen action is mediated through the androgen receptor (AR), a member of the nuclear steroid hormone receptor superfamily, which is an androgen-dependent transcription factor that regulates the expression of androgen-responsive genes. Identification and characterization of androgen-responsive genes and investigation of the mechanisms of their regulation by AR have enriched our understanding of androgen action at the molecular and cellular levels.

With this book, we hope to provide readers with up-to-date information on the regulation, function, and clinical relevance of androgen-responsive genes. Internationally recognized experts have summarized their current research in this volume. Several chapters address the mechanisms regulating the expression of androgen-responsive genes by AR, AR co-regulators, and cell signaling. These chapters also address the importance of androgen-responsive elements, AR binding, chromatin structure, and the dynamic interactions between AR and the nucleosomes. Another important topic in this book concerns the mechanisms of androgen-independent induction of androgen-responsive genes, and the role of AR overexpression and AR splicing variants. In addition, this book addresses the mechanisms of androgen regulations of cell signaling, cell–cell interactions,

epithelial–mesenchymal transition, and prostate cancer cell invasion. This book also describes the application of powerful technologies, such as RNAseq, microarray, and ChIPseq, in the identification and characterization of androgen-responsive coding and noncoding transcripts. Finally, this volume discusses the potential application of androgen-responsive genes in prostate cancer management.

The research on androgen-responsive genes in prostate cancer is moving rapidly, which makes it difficult to provide a comprehensive overview. This book is intended to provide a snapshot of the current status of research of androgen-responsive genes and provide the basis for further exploration of the role of androgen-responsive genes in prostate cancer.

Understanding of androgen signaling in prostate carcinogenesis remains incomplete, despite significant progresses in recent years. Many important questions need to be further addressed. For example, how does androgen stimulate prostate cancer cell proliferation? What are the genes mediating this important process? What are the alterations in androgen signaling during prostate carcinogenesis? Androgens are known to stimulate prostate luminal epithelial cell proliferation via a paracrine mechanism in the normal prostate. However, androgens stimulate prostate cancer cell proliferation by an intracrine mechanism mediated by AR within the prostate cancer cells. What are the mechanisms leading to the transition of androgen action from the paracrine mechanism in the normal prostate to the intracrine mechanism in prostate cancer? The information provided in this book will likely facilitate future research aimed to resolve these questions.

Pittsburgh, PA, USA Zhou Wang

# Contents


**1 Roles of Androgen Receptor Coregulators and Cell Signaling in the Regulation of Androgen-Responsive Genes** ..... 1
Irina U. Agoulnik and Nancy L. Weigel

**2 Selective and Classical Androgen Response Elements in Androgen-Regulated Gene Expression** ..... 13
Frank Claessens, Liesbeth Clinckemalie, Helsen Christine, Lien Spans, Vanessa Dubois, Michaël Laurent, Steven Boonen, and Dirk Vanderschueren

**3 Negative Androgen-Response Elements in Androgen Target Genes** ..... 29
Wei Qi and Zhengxin Wang

**4 Chromatin Looping and Long Distance Regulation by Androgen Receptor** ..... 43
Benjamin Sunkel and Qianben Wang

**5 The Functionality of Prostate Cancer Predisposition Risk Regions Is Revealed by AR Enhancers** ..... 59
Houtan Noushmehr, Simon G. Coetzee, Suhn K. Rhie, Chunli Yan, and Gerhard A. Coetzee

**6 Mechanisms of ARE-Independent Gene Activation by the Androgen Receptor in Prostate Cancer Cells: Potential Targets for Better Intervention Strategies** ..... 85
Manohar Ratnam, Mugdha Patki, Mesfin Gonit, and Robert Trumbly

**7 Androgen Action, Wnt Signaling, and Prostate Tumorigenesis** ..... 101
Zijie Sun and Suk Hyung Lee

**8 Toward Revealing the Complexity of Androgen-Responsive Protein and Noncoding Transcripts in Prostate Cancer** ..... 117
Melanie L. Lehman and Colleen C. Nelson

**9 Androgen-Responsive Gene Expression in Prostate Cancer Progression** ..... 135
Amy H. Tien and Marianne D. Sadar

**10 Androgen Receptor-Regulated Genes in Prostate Cancer Initiation Versus Metastasis** ..... 155
Soo Ok Lee, Chiung-Kuei Huang, Luo Jie, and Chawnshang Chang

**11 Androgen-Independent Induction of Androgen-Responsive Genes by Interleukin-6 Regulation** ..... 177
Zoran Culig

**12 The Effect of AR Overexpression on Androgen Signaling in Prostate Cancer** ..... 187
Alfonso Urbanucci, Kati K. Waltering, Ian G. Mills, and Tapio Visakorpi

**13 The Expression Signature of Androgen Receptor Splice Variants and Their Distinctive Transcriptional Activities in Castration-Resistant Prostate Cancer** ..... 201
Stephen R. Plymate and Jun Luo

**14 Androgen Regulation of the Cell Cycle in Prostate Cancer** ..... 215
Haojie Huang

**15 Androgen Receptor Signaling Interactions Control Epithelial–Mesenchymal Transition (EMT) in Prostate Cancer Progression** ..... 227
Sarah K. Martin, Michael V. Fiandalo, and Natasha Kyprianou

**16 Androgen Receptor Regulation of Serum Response Factor Signaling in Prostate Cancer** ..... 257
Alissa R. Verone and Hannelore V. Heemers

**17 Regulation of Angiogenesis by Androgen-Responsive Gene EAF2** ..... 273
Laura E. Pascal and Zhou Wang

**18 The Role of miR-21, an Androgen-Regulated MicroRNA, in Prostate Cancer** ..... 285
Judit Ribas and Shawn E. Lupold

**19 Androgen-Dependent Oncogenic Activation of ETS Transcription Factors by Recurrent Gene Fusions in Prostate Cancer: Biological and Clinical Implications** ..... 307
Albert Dobi, Taduru Sreenath, and Shiv Srivastava

**20 Clusterin as a Target for Treatment of Castration-Resistant Prostate Cancer** ..... 329
Amina Zoubeidi and Martin Gleave

**Index** ..... 341

# Chapter 1
# Roles of Androgen Receptor Coregulators and Cell Signaling in the Regulation of Androgen-Responsive Genes

**Irina U. Agoulnik and Nancy L. Weigel**

**Abstract** Androgen receptor (AR) is a member of the ligand activated transcription factor family. Its function is regulated by a complex network of coregulatory proteins and cell signaling pathways. AR is a key transcription factor in healthy prostate function and during neoplastic transformation. In normal prostate, it regulates prostate secretory function, stimulates epithelial cell renewal, and maintains the cells in a differentiated state. However, under certain circumstances AR function loses its differentiating thrust and proliferative function becomes dominant. Changes in coregulator expression and alteration of cell signaling pathways may contribute to changes in androgen-dependent AR action and play a major role in castration resistant prostate cancer (CRPC).

**Keywords** Androgen receptor • Coactivator • Phosphorylation • Corepressor

## 1.1 Mechanism of Androgen Receptor Action

Similar to other steroid receptors, AR has four basic domains. Most of the amino-terminal part is an unstructured regulatory region, the N-terminal domain (NTD). It is followed by the DNA binding domain (DBD), hinge region (H), and a ligand

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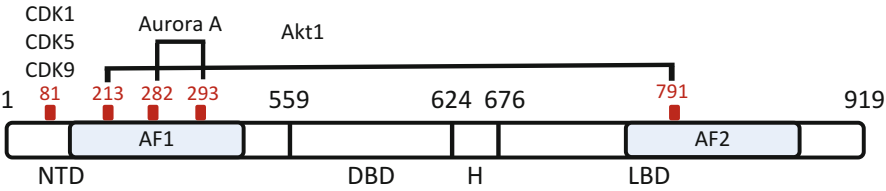


**Fig. 1.1** Structure of AR. The number of amino acids in AR is variable, but the location of specific amino acids and domains is based on an assumed length of 919 amino acids. The variation in length is most frequently due to a variable number of glutamines in the amino-terminal polyglutamine tract. Domain boundaries are indicated by amino acid numbers in *black*. AF1 and AF2 are activation function regions. *Red ticks* and *numbers* are phosphorylated amino acids. Kinases that target these residues are written on *top*. *NTD* N terminal domain, *DBD* DNA binding domain, *H* hinge region, *LBD* ligand binding domain

binding domain (LBD). The LBD consists of 12 helices that have different orientations depending on whether the ligand binding pocket is empty or occupied by an agonist or antagonist. Two major activation functions, AF1 and AF2, are situated in the NTD and LBD of AR, respectively (Fig. 1.1). AF2 activity is regulated by ligand. Binding of the agonist causes a conformational change and closing of helix 12 over the ligand binding pocket, which creates a patch for binding coactivators. Antagonists, such as bicalutamide, prevent helix 12 from closing and expose a groove in the LBD that binds corepressors. AF1 is constitutively active when not hindered by the LBD and also is a major binding site for coactivators.

In the absence of androgens, AR resides in the cytoplasm bound to chaperones that aid in proper folding of the receptor. Hydrophobic androgens penetrate the cell membrane by diffusion and bind to AR resulting in dissociation of cytoplasmic chaperones, AR dimerization, and translocation to the nucleus. Unlike other steroid receptors, there is good evidence that AR dimerizes in an antiparallel orientation forming strong interactions between the amino- and carboxyl-terminal parts of the receptor (N/C interaction) [1]. In the nucleus AR binds to a variety of AR response elements in the promoters and enhancers of its target genes and other sites of unknown functional significance. In the promoter and enhancer regions, AR serves as a scaffold for assembly of promoter- and chromatin context-dependent accessory proteins that activate or repress transcription (Fig. 1.2a, b). It can also bind heterologous transcription factors and regulate their activity similar to other coregulators (Fig. 1.2c). Alternatively, AR can act in the cytoplasm or at the cell membrane, modulating activities of signaling cascades (Fig. 1.2d).

## 1.2 Coactivators

AR has no enzymatic activity. After AR recruitment to the cognate promoter and enhancer regions, it serves as a scaffold for proteins with a variety of enzymatic activities that modify histones and other transcriptional coregulators, facilitating

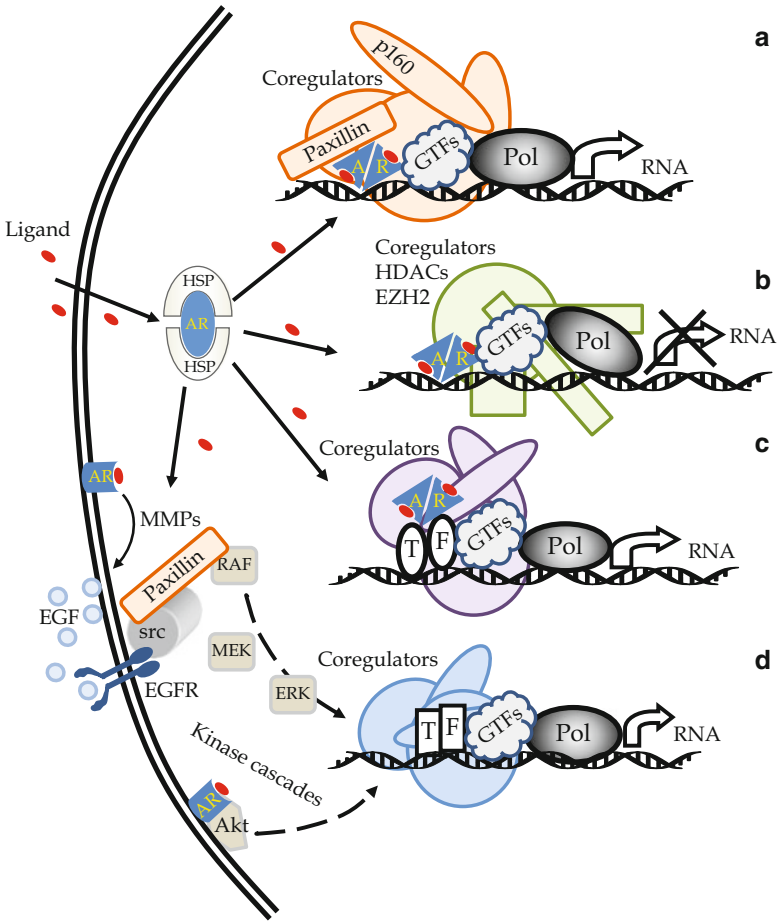


**Fig. 1.2** Model of AR action. Prior to ligand binding AR resides in the cytoplasm bound to heat shock proteins (HSP). Upon treatment with androgens (*red ovals*), AR is activated and regulates transcription in a variety of different ways. (**a**) AR-mediated activation through direct interaction of AR dimers with DNA response elements in promoters and enhancers is regulated by recruitment of coactivators and corepressors, general transcription factors (GTF), and RNA polymerase (Pol). (**b**) AR-mediated repression is poorly understood. There is evidence that androgen bound AR is recruited to the promoters and enhancers of repressed genes and brings with it histone deacetylases (HDACs) and other regulatory proteins such as EZH2 [57]. (**c**) Regulation of transcription by AR with no direct interaction with DNA. AR can be tethered to the gene regulatory regions by heterologous transcription factors. (**d**) Rapid action of AR is mediated by liganded AR in the cytoplasm or on the membrane leading to activation of kinases including Akt and EGFR and its downstream kinases. Activation of these kinases potentially can change expression of genes without direct interaction of AR as well as by modulating the phosphorylation state of AR and its coregulators

recruitment of general transcription factors (GTFs) to create a productive complex for transcriptional activation or repression, and potentially to modulate splicing efficiency. A big part of the pre-initiation complex is represented by coactivators.

The number of proteins that have been shown to regulate AR activity is large and constantly increasing. A few have been shown to change their level of expression in prostate cancer and correlate with patient prognosis. These coactivators will be discussed in this section.

An important feature of the actively transcribed chromatin is elevated acetylation of the histones in these regions. Even at the early stage of low grade prostate cancer, the global histone modification pattern is an independent predictor of recurrence [2]. The first steroid receptor coactivator, SRC-1, was reported in 1995 [3]; it is a member of the p160 family of coactivators, which also includes TIF2/NCOA2/SRC-2 and AIB1/NCOA3/SRC-3. Both SRC-1 and SRC-3 are histone acetyl transferases (HATs) [4, 5] and can recruit additional HATs to AR. p160 coactivators potentiate AR and many other transcription factors by acetylating chromatin to make it more accessible for binding of other proteins. Despite their similar enzymatic activity and domain structure, these three proteins are distinctly regulated and play different roles in prostate cancer.

TIF2 (NCOA2) probably has the best described role in prostate cancer. Along with the well-known oncogene MYC, TIF2 is one of two most frequently amplified or overexpressed genes in prostate cancer [6]. The level of TIF2 is increased at the level of protein, RNA, and gene copy number [6, 7]. In the normal prostate, expression of TIF2 is quite low, but it increases with prostate cancer progression. In primary tumors, high expression of TIF2 is predictive of shorter time to biochemical recurrence only if AR expression also is high, suggesting that TIF2 works in concert with AR at least in the primary prostate cancer [7]. TIF2 is required for AR mediated gene induction but not for repression and is critical for AR activity at low concentrations of androgens. On the other hand, TIF2 expression is suppressed by AR in multiple prostate cancer cell lines in an androgen-dependent manner. Androgen treatment causes AR recruitment to the promoter and intron region of the TIF2 gene and significantly reduces its expression [7]. Consistent with this, TIF2 expression is highest in tumors from patients who have failed androgen ablation therapy [7, 8].

SRC-3 (NCOA3, AIB1) was identified as an oncogene in breast cancer and has since been implicated in multiple malignancies [9]. In prostate cancer, its expression increases with progression, predicts faster recurrence, and correlates with seminal vesicle invasion [10, 11]. SRC-3 is required for optimal induction of AR target genes in prostate cancer cell lines. However, its depletion reduces proliferation of AR expressing prostate cancer cells as well as a subset of AR negative prostate cancer cell lines [10, 12], suggesting that it may stimulate prostate cancer progression in both an AR-dependent and AR-independent manner. Indeed, SRC-3 induces expression of multiple proteins in the IGF and Akt signaling pathways by coactivation of AP-1 transcription factors [11, 13, 14]. The increase in the level of SRC-3 protein with prostate cancer progression may in part be due to frequent mutations in a protein involved in SRC-3 degradation, the E3 ubiquitin ligase SPOP. SPOP binds to SRC-3 phosphorylated on S101/S102 residues and targets it for ubiquitin mediated proteosomal degradation [15]. In a study of 112 prostate adenocarcinomas, Barbieri et. al. reported that 13% of prostate tumors bear mutations of SPOP that all cluster in the SRC-3 binding domain [16].

SRC-1 (NCOA1) expression does not increase with prostate cancer progression; in fact, there is tight correlation between SRC-1 expression in distinct parts of benign and malignant tissue of the same patient. However, high expression of SRC-1 correlates with features of more aggressive types of prostate cancer [17]. Unlike TIF2, SRC-1 is required both for optimal AR-dependent gene activation as well as for repression of some AR target genes, such as maspin. In AR expressing prostate cancer cells, SRC-1 supports cellular proliferation, but in AR negative cells, such as PC3 and DU145, it is dispensable for cell growth [17].

As mentioned above, p160 coactivators can recruit other HATs that are sometimes called cointegrators. Cointegrators, such as p300, CBP, P/CAF, and Tip60, acetylate histones, other proteins in the AR pre-initiation complex, and AR itself [18]. Interestingly, AR acetylation increases its activity due to increased coactivator binding and weakened corepressor interaction, increasing prostate cancer cell viability in mouse xenograft models [19]. Perhaps the most compelling data supports the key role of p300, but not its homolog CBP, in prostate cancer. Almost half of AR target genes in the C4-2 prostate cancer cell line are p300 dependent, compared to less than half of a percent for CBP. In addition, p300 is required for the optimal acetylation of histones H3 and H4, recruitment of TATA box binding protein (TBP) and RNA polymerase II to AR regulated promoters [20] . It also plays a critical role in androgen-independent activation of AR by IL-6, an inflammatory cytokine elevated with prostate cancer progression [21, 22]. The levels of p300 protein increase with prostate cancer progression and signal poor prognosis [23].

A number of AR coactivators play a role in splicing as well as increasing overall AR transcriptional activity. How the coactivators change splicing patterns is still debated but may include assembly of different spliceosome complexes, posttranslational modification of the splicing factors, or by increasing the rate of transcription to favor exon skipping. The DEAD box RNA helicase p68 is an AR coactivator that is recruited to the endogenous PSA promoter. In addition to increasing AR activity, it has been shown to stimulate exon skipping in an MMTV driven CD44 variable exon minigene [24]. The expression of p68 is significantly increased in prostate cancer compared to benign prostate tissue [24].

## 1.3 Corepressors

The best described corepressors of steroid receptors are NCOR and SMRT. Regulation of AR action by these corepressors is distinct from that of other steroid receptors. While other steroid receptors bind NCOR and SMRT in the presence of antagonists, AR can functionally interact with these proteins in an active conformation when bound to agonist [25]. NCoR and SMRT are both recruited to the PSA promoter upon addition of androgen [26]. While catalytically inactive, NCOR and SMRT recruit a number of proteins that mediate their action, such as histone deacetylases. These two corepressors also have unique functions: SMRT plays a role in DNA repair [27] and NCOR can sequester P85α interfering with PI3K signaling [28]. In

a concordant screen of benign prostate, primary, and metastatic prostate cancers for DNA copy number, RNA expression and mutation incidence, Taylor et. al. reported that NCOR expression is lost in 4% of primary tumors and 16% of metastases while SMRT expression is lost in 23% of primary and 21% of metastatic prostate cancer [6]. Based on the findings described in mouse models, loss of these corepressors may increase inflammation [29], a frequently observed phenomenon in prostate cancer that also modulates AR activity.

## 1.4 Cell Signaling

Prostate cancer progression is accompanied by activation of multiple signaling pathways. AR and AR coactivators are among their targets. Over a dozen phosphorylation sites have been described in AR [30]. Some of the sites, such as S213, T282, S293, and S791, are targets of the signaling pathways that are activated with the progression of prostate cancer (Fig. 1.1). Phosphorylation of AR on these sites affects AR transcriptional activity.

Upon addition of androgens, the highest level of AR phosphorylation is observed on S81 [31]. CDK1 and CDK9 phosphorylate AR on this site and, accordingly, S81 level of phosphorylation is increased during M phase of the cell cycle [32]. Phosphorylation on this site is needed for optimal AR recruitment and chromatin binding to the AR regulated gene promoters and enhancers [33].

Levels of Aurora kinases A and B are increased in prostatic intraepithelial neoplasia (PIN) and signal a poor prognosis [34]. Aurora-A phosphorylates AR on T282 and S293 increasing its activity and contributing to androgen-independent proliferation [35].

Akt is a kinase that stimulates cell proliferation, growth, and survival and suppresses apoptosis. Its activity is elevated in many cancers, including prostate. This kinase is activated by recruitment to the membrane by two phosphatidylinositol-polyphosphates, PI(3,4,5)P3 and PI(3,4)P2, with subsequent phosphorylation on T308 and S473 of Akt. Suppression of Akt is achieved by dephosphorylation of either the signaling phospholipids or Akt itself. Akt phosphorylation is low in normal prostate tissue, increased in primary tumors [36], and activated even further in tumors that recur after androgen ablation [37, 38]. There is substantial cross talk between Akt and AR signaling. Akt has been shown to phosphorylate AR on S213 and S791 both in vitro and in vivo [39–42]. The effect of these phosphorylation events on AR activity, stability, and hormone binding is cell context specific. AR localized to membrane lipid rafts interacts with Akt and facilitates local Akt activation in response to androgens [43]. On the other hand an AR primary target gene, inositol polyphosphate 4-phosphatase type II (INPP4B), is a protein that dephosphorylates membrane phospholipids required for recruitment of Akt to the membrane and its activation [44]. Interestingly, INPP4B and another lipid phosphatase PTEN are lost in nearly half of recurrent CRPC [6]. In addition, androgen signaling stabilizes Akt phosphatase, PHLPP, by inducing expression of its chaperone [45].

Multiple members of the Mitogen Activated Protein Kinase (MAPK) cascade are activated with prostate cancer progression. Canonical MAPK signaling is transmitted through the following cascade: growth factor receptors—Ras–Raf–MEK–ERK. HER2 expression was detected in some prostate cancers and was strongly associated with higher tumor grade, increased proliferation index, and poor prognosis [46]. High levels of nuclear MAPK are associated with poor prognosis [47]. Levels of phosphorylated ERK1/2 kinases, undetectable in normal prostate, increase with prostate cancer development and are highest in recurrent castration resistant cancer [48]. Significant cross talk has been reported between the AR and MAPK pathways [47, 49]. Inhibition of MEK, preventing activation of ERK1 and ERK2, reduces AR mediated transcription [47, 50, 51]. ERK1 and ERK2, immediate downstream targets of MEK, have multiple effects on endogenous AR activity and expression. Prolonged treatment with the MEK inhibitor, UO126, results in inhibition of ERK1/2 and a substantial decline in AR protein levels due to a reduction in protein stability [49]. Inhibition of the proteasomal pathway prevents this protein loss. The N/C interaction has been implicated in stabilization of AR protein and inhibition of MEK activity reduces the N/C interaction [49]. Analysis of AR activity at shorter treatment times when AR levels are unchanged shows that inhibition of MEK reduces AR transcriptional activity in a target gene specific manner. Although U0126 reduces AR-dependent induction of PSA and TMPRSS2, it has no effect on AR-dependent repression of PCDH11Y or induction of its primary target gene PMEPA1 [49]. Depleting ERK1 or ERK2 separately using siRNA revealed that only ERK1 was required for optimal PSA expression although both were required for TMPRSS2 expression [49]. Detailed analysis showed that histones in the promoters and enhancers of MEK sensitive genes are acetylated in response to androgen, and this increase in acetylation was inhibited by U0126. In the case of the U0126 resistant PMEPA1, no increase in H3 acetylation was detected during induction of gene expression. The requirement for histone acetylation may explain the finding that the rate of PSA and TMPRSS2 transcription lagged significantly behind PMEPA1 expression after androgen treatment [49]. Both AR and its coactivators contain Ser/Thr-Pro motifs that are often substrates for MAPK and cyclin-dependent kinases. Multiple AR coactivators, including the p160 family of coactivators, have been shown to be targets of these pathways [51–54]. Inhibition of MAPK abolishes phosphorylation of SRC-1 on T1179 and S1185 [49] and decreased AR and SRC-1 functional interaction [49]. Mutation of the MEK phosphorylation site in another member of p160 family, TIF2 (S736), reduces its interaction with AR [51]. In agreement with these data, SRC-1 is not required for PCDH11Y repression by AR, which is insensitive to MEK inhibition [49].

The MAPK pathway in turn is regulated by AR. A portion of agonist bound AR is localized near or in the membrane and stimulates MMP-dependent shedding of EGF, which in turn activates EGF family receptors and their downstream targets, including ERK1/2 [55]. An important component of this process is a scaffold protein paxillin. Depletion of paxillin abolishes the ability of both DHT and EGF to activate ERK1/2 activity in prostate cancer cells [55]. In addition to its role in AR rapid signaling, paxillin mediates AR nuclear translocation and can act as a coactivator on the PSA promoter [56].

## 1.5 Conclusions

Prostate cancer is a heterogeneous disease ranging from an indolent slowly growing disease to aggressive metastatic cancer. Normal prostate and the majority of prostate cancers express AR and are dependent on AR signaling for survival. AR action is determined by hormone levels, by cell signaling, and by the expression/activity of receptor coregulators and other transcription factors. In many cases feedback loops have been described where AR in turn regulates coregulator levels and cell signaling activity. Changes in coregulator and signaling protein levels and activities contribute to the change in AR function. Elevated cell signaling and/or elevated expression of coregulators can reduce the level of hormone required for AR activation. Small molecules that interfere with coregulator interaction or target cell signaling pathways that regulate AR activity may become complementary to currently used AR antagonist treatments and contribute to development of individualized therapies.

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# Chapter 2
# Selective and Classical Androgen Response Elements in Androgen-Regulated Gene Expression

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**Abstract** Once ligand has bound the androgen receptor, it has to find the androgen response elements in the $6.6 \times 10^9$ basepairs of the human genome. Like all other nuclear receptors, the androgen receptor has a specialized domain that consists of two zinc finger modules. One module recognizes a hexameric motif, while the other module serves as a dimerization surface. Surprisingly, the androgen receptor has two types of response elements. Indeed, the two hexamers can either be oriented as direct or inverted repeats. Direct repeats seem to be preferentially involved in androgen responses of the reproductive organs.

The DNA binding by the androgen receptor is not merely the finding of a docking site near the androgen-responsive genes. Indeed, its activity is partly dictated by the type of response element it binds to. Moreover, there is genetic evidence from receptor mutations associated with androgen insensitivity syndrome for an interface between the ligand-binding and DNA-binding domain. This may provide a pathway for allosteric signaling from the DNA sequence to the ligand-binding domain and vice versa.

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## 2.1 Introduction

Androgens control many physiological processes as diverge as development and maintenance of external genitalia, musculoskeletal development, fertility, and sexual behavior. To this end the androgen receptor (AR) that acts as a ligand-inducible transcription factor, has tissue-specific sets of target genes. Also in the prostate, and by extension in prostate cancer cells, the AR has many target genes which can not only be protein coding but also miRNA and even lncRNA-coding genes. In this chapter, we discuss how the AR can locate these genes within the $6.6 \times 10^9$ bp of the human genome and how the DNA sequences to which the AR binds codetermine the activity of the receptor.

## 2.2 The DNA-Binding Domain of the Androgen Receptor

Like all nuclear receptors, the AR has a centrally located DNA-binding domain (DBD). The DBD is the signature domain for the nuclear receptors that can interact autonomously with high affinity and specificity with DNA elements near the androgen target genes (reviewed in [1]). These 15 basepair DNA elements were called Androgen Response Elements (ARE). They will be discussed in more detail in a later section.

### *2.2.1 Comparison of the AR with the Other Steroid Receptors*

The androgen, glucocorticoid, progesterone, mineralocorticoid (GR, PR, and MR) receptors bind to the same sequences, at least in vitro [2]. Obviously, in vivo, the androgen target genes are different from those of glucocorticoids, progestins, or mineralocorticoids. So, while the consensus sequences for the GRE and ARE are identical, and even individual response elements from different genes can be identical [3], the in vivo responses are different. This is most likely a consequence of tissue-specific expression of the receptors, of tissue-specific metabolism of the activating ligands, and also of differences in coregulator expression as well as of tissue-specific chromatin structure and organization (reviewed in [4]).

The DBDs of the GR, PR, MR, and AR resemble each other more than they resemble the DBDs of the other nuclear receptors. These DBDs consists of two so-called zinc fingers, which are zinc-nucleated modules in which four Cysteine residues are coordinated by a Zinc molecule [5].

### 2.2.2 The First Zn Finger

When the 3D structure of the AR-DBD was solved, it was immediately apparent that it is near identical to that of GR and PR [6]. The carboxyterminal part of the first Zn finger is involved in an alpha helical structure which has the ideal dimension to enter the major groove of B-helical DNA and make sequence-specific contacts with a DNA segment of five to six basepairs long. The DNA-contacting residues are conserved between AR, GR, PR, and MR [5].

### 2.2.3 The Second Zn Finger

The second Zn finger module does not make sequence-specific contacts with the DNA, but is involved in dimerization. The classical dimerization surface in the two contacting monomers is oriented antiparallel which explains the high symmetry of the dimer. In contrast to the first zinc finger, the residues involved are not completely conserved between the AR, GR, PR, and MR: the AR is the only steroid receptor which has a Serine in the center of the dimerization surface, where the others have a Glycine, the result being that in the GR, PR, and MR dimerization interface there is a hole called "Glycine hole" [6]. While in theory this would lower the dimerization forces, residue swapping between AR and GR indicates that AR dimerization on DNA is not very different from GR (Verrijdt et al. 2006).

### 2.2.4 The Hinge Region

The DBD and the ligand-binding domain (LBD) of the nuclear receptors are connected via a highly variable hinge region, which was initially thought to function merely as hinge, allowing flexibility in the orientations of the LBD and DBDs. However, later work on the AR revealed that this region has many roles [7, 8]. In case of all steroid receptors, it is known to cover a nuclear localization signal (NLS), it is also the recipient of activity-controlling posttranslational modifications like phosphorylation and acetylation (reviewed in [9]). Moreover, it plays a role in DNA binding, receptor activity, and in intranuclear mobility.

#### 2.2.4.1 Role in Nuclear Translocation

It was first noted in 1993 that a sequence resembling the NLS of the large T antigen of the SV40 virus is present in the AR hinge [10, 11]. NLS mutations affected the intracellular distribution. More recently, a crystal structure of the AR-NLS with importin beta was resolved and this showed that the $^{629}$RKLKK$^{633}$ motif is involved in very specific interactions [12]. Not surprisingly, androgen insensitivity syndrome patients

can have mutations in this NLS, but in seeming contradiction are the observations that AR NLS mutations have also occurred in prostate cancer. Moreover, it was shown that these PrCa mutations increased the activity of the AR, although they had a negative effect on the nuclear translocation [7, 12].

#### 2.2.4.2 Role in DNA Binding

When we produced the AR-DBD for the first time, we included a large part of the hinge region, simply because constructs were based on the presence of restriction sites [13]. Later on, more directed cloning by PCR revealed that an AR fragment which only covers the two Zn fingers has no or very low affinity for DNA. The minimal AR-DBD has to include a carboxyterminal extension for high affinity DNA binding [14]. The twelve most aminoterminal residues of the hinge region, covering the $^{629}$RKLKK$^{633}$ motif, sufficed [15]. Unfortunately, the structure of this so-called carboxyterminal extension (CTE) is unclear. It is striking, however, that it colocalizes with the NLS.

#### 2.2.4.3 Role in AR Activity

Further studies of the role of the CTE in the full size receptor revealed initially puzzling data. Indeed, when the $^{629}$RKLKK$^{633}$ motif was deleted, the AR did not seem to enter the nucleus (based on immunocytochemistry) but the androgen responses increased up to sevenfold [7]. So while the AR was apparently absent in the nucleus, the undetectable nuclear amounts of AR clearly were highly active. This was also true when a heterologous NLS was fused to the aminoterminal end of the AR [8]. This seeming contradiction is in accordance with the observation that AR hinge mutations found in prostate cancer result in a more potent AR even when the nuclear translocation is slowed down [16]. Moreover, Gioeli et al. [17] demonstrated the crucial role of hinge region phosphorylation in AR activity control.

#### 2.2.4.4 Role in Intranuclear Mobility

A possible explanation for the increased AR activity came from the observations of the effects of hinge region mutations on the intranuclear mobility of the AR. With Fluorescent Recovery After Photobleaching technology (FRAP), we observed that not only the distribution of the AR between mobile and immobile fractions but also the residence time of the AR in the immobile fraction was changed [8]. FRAP gives only an overview of the AR population and cannot definitively discriminate between DNA binding and other binding events (e.g., to coactivator complexes). However, the increased mobility and shorter residence times do fit the hypothesis that nuclear receptors, like all transcription factors, cycle on the enhancers, with each cycle having a different function resulting in a chronological recruitment of complexes

involved in histone language writing and reading, recruiting RNA polymerase, RNA pol modifying enzymes, etc. [18].

In conclusion, these data demonstrate that the CTE or $^{629}$RKLKK$^{633}$ motif is a nuclear localization signal and extension of the DBD. In addition, this motif must be recognized by at least one other control mechanism since it determines the intranuclear mobility of the AR. In the spirit of Occam's Razer, we would predict that the same mechanism determines the transactivation potential of the AR, but at this moment it cannot be ruled out that yet other mechanisms are involved. In this respect, it should be noted that the hinge region is also an interaction site for transcription co-regulatory complexes like SWI/SNF [19] or nucleophosmin [20]. Moreover, the N/C interactions are also affected by the hinge region mutations [7, 21].

## 2.3 Androgen Response Elements

Like most transcription factors, the AR has to find back the specific DNA motifs which can be present anywhere in the $6.6 \times 10^9$ bp of, e.g., the human genome. At a first level, the inactive part of the genome is packed into heterochromatin and thus invisible to most transcription factors. The ARE-containing enhancers near the tissue-specific genes that are androgen targets will be in open chromatin. While this reduces the complexity of the search for AREs considerably, it is still unclear how exactly the AR can find them, although growing evidence points at several pioneering factors like FoxA1 and Nkx3.1 that will aid the AR in ARE finding ([22–24]; for more details see chapter by Wang). However, in this section, we will restrict ourselves to the description of the DNA elements that are recognized by the AR DBD, the so-called androgen response elements.

### *2.3.1 Definition of an ARE*

An ARE is a simple DNA motif, able to convey androgen responsiveness to a heterologous reporter gene through direct binding of the AR. Experimentally defining an ARE involves in vitro binding assays like electrophoretic mobility shift assay (EMSA), DNAseI footprinting on the one hand, and transient or stable transfection data on the other (for more experimental details, see [25]). Ultimate proof for an ARE comes from AR binding demonstrated in chromatin immunoprecipitation (ChIP) assays and AR activity shown in, e.g., a transgenic approach in which the ARE is mutated. Unfortunately, the latter demands a long-term investment. Moreover, deleting one ARE is most likely insufficient to affect the androgen responsiveness of a gene that can be controlled by several androgen-responsive enhancers. However, for the enhancers of the PSA, the C3(1) and the mouse vas deferens protein genes, such proof has been provided in transgenic animal models (reviewed in [3]). Nowadays most AR-ChIP data have been derived from the use of

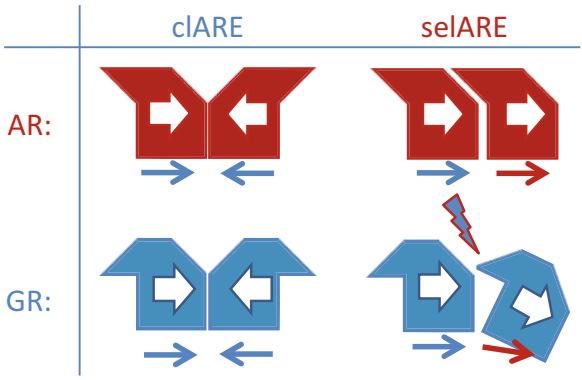


**Fig. 2.1** Sequence logos for the classical ARE and selective ARE, based on AREs for which selectivity was checked in EMSA and in functional analysis

prostate cancer cell lines. However, for epididymis and prostate tissue, AR ChIP data have been reported [26, 27] and can hence be more physiological proof for AR binding. AR ChIP seq data on prostate cancer will no doubt be very informative on DNA binding and how it is modulated by antagonists and other therapeutic strategies against cancer.

#### 2.3.1.1 The Optimal Hexamer Motif for AR Binding

Historically, the consensus high affinity binding sequence for the GR was described to be 5′-AGAACA-3′. After the description of a number of AREs in cellular genes, it became clear that the AR too recognizes this motif (e.g., [28]).

#### 2.3.1.2 AREs Are Hexamer Repeats

It also became clear that most AREs cover a DNA stretch which is extended at its 3′ end beyond the 5′-AGAACA-3′-like motif. A consensus sequence showed that 3′ of the high-affinity binding site, a second binding site is present with a similar consensus, but present in the other strand in the other direction [29]. This is explained by the fact that the AR binds DNA as a symmetrical dimer (see also higher), binding two 5′-AGAACA-3′-like motifs separated by a three nucleotide spacer and organized as an inverted repeat (Fig. 2.1).

#### 2.3.1.3 Selective ARE

The DBD of GR, PR, MR, and AR are very similar, with identity of the residues involved in contacting the DNA and high similarity of the dimerization interface.

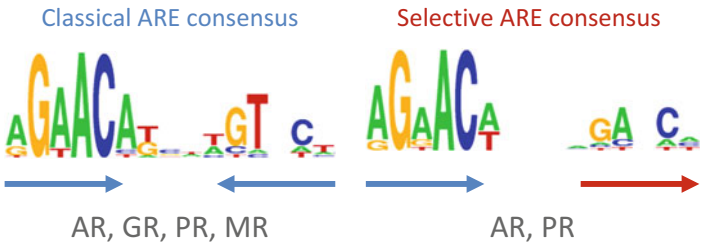


**Fig. 2.2** Schematic presentation of AR- and GR-DBD binding to classical and selective AREs. The orientation of the monomers and the hexamer-DNA sequences are indicated with *arrows*. The structure induced by the carboxyterminal extension of the second zinc finger is represented by a triangular extension. For the GR-DBD, this prevents dimerization on selective AREs

However, since each corresponding hormone has its specific target genes, even in cells where the receptors are coexpressed, efforts have been made to find DNA sequences that are selective for any of the four receptors. DNA motif selections, based on PCR amplifications of DBD-bound oligonucleotides did not reveal selective elements [29]. It was only through the analysis of a series of AREs isolated from androgen target genes that it became apparent that several of these AREs were not recognized by the GR-DBD. These so-called selective AREs (selAREs) consist of a 5′-AGAACA-3′-like hexamer, flanked at three nucleotides downstream by a second hexamer. The similarity of this downstream hexamer to the 5′-AGAACA-3′ is lower compared to that in the classical AREs (clAREs). Mutation analyses indicated that in the selAREs, the two hexamers have a parallel orientation, rather than the inverted orientation seen for clAREs, GRE, and PREs [30, 31]. This was underpinned with experiments like the one described in Fig. 2.3.

The mutational analyses of a series of selAREs revealed which bases are most important for AR binding and which determine selectivity (so prevent GR binding). Despite this information, it is still difficult to predict from its sequence whether an ARE will fall into the selARE or in the clARE group. This is due to several factors: in selAREs as well as clAREs, the guanines and cytosines are at the same positions, the left hexamers have the same orientation and the downstream hexamer can diverge very much from the consensus for clAREs as well as for selAREs (Fig. 2.1). Although for many selAREs, a change of adenine into thymidine at position 3 of the downstream hexamer abolishes selectivity, other selAREs do not have an adenine at this position ([32] and Fig. 2.2). All these reasons explain why one has to do EMSA and functional analyses to determine whether an ARE is selective or not.

#### 2.3.1.4 Role of the Second Zn Finger

For the DBD of the estrogen receptors, residues in the first Zn-finger module dictate higher affinity for 5′-AGGTCA-3′, and in GR, AR, PR, and MR, alternative residues at the same positions dictate high affinity for 5′AGAACA-3′ [33]. Since the two

**Table 2.1** The position-specific probability matrix derived from those AREs for which AR binding as well as androgen responsiveness has been demonstrated. The use of this PSPM in ARE searching is described in section "ARE search with a position-specific probability matrix (PSPM)"

| | | −7 | −6 | −5 | −4 | −3 | −2 | −1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | / | 77 | 0 | 80 | **100** | 0 | 73 | 12 | 23 | 31 | 42 | 4 | 38 | 38 | 12 | 27 |
| C | / | 0 | 0 | 0 | 0 | **100** | 4 | 15 | 39 | 15 | 19 | 11 | 12 | 8 | 73 | 27 |
| G | / | 19 | **100** | 8 | 0 | 0 | 0 | 42 | 19 | 23 | 4 | 77 | 0 | 31 | 0 | 4 |
| T | / | 4 | 0 | 12 | 0 | 0 | 23 | 31 | 19 | 31 | 35 | 8 | 50 | 23 | 15 | 42 |

hexamers that constitute all AREs, selAREs and clAREs alike, resemble the same consensus, it is not surprising that the binding of the AR to selAREs and the nonbinding of the GR to these elements is not determined by the differences in the first zinc finger. Indeed, it was when the second Zn finger was swapped between AR- and GR-DBD that the selectivity was also swapped [14]. We concluded that the second zinc finger of the AR allows dimerization on selective elements, while the second zinc finger of the GR does not (Fig. 2.1). Moreover, the $^{629}$RKLKK$^{633}$ motif is necessary but not sufficient to confer high affinity for selAREs [7].

#### 2.3.1.5 ChIP Data Evaluations: The Consensus Revisited

Genomic AR-binding sites (ARBS) have been described by chromatin immunoprecipitation assays [34–36]. Because of limitations in the software for in silico ARE searches and motif-finding software, it has been hypothesized that the AR not only binds clARE and selARE but also other types of dimeric binding sites in which the two hexamers are organized as direct, inverted, or everted repeats separated by different length spacers. Monomeric AR binding has also been proposed. Careful analysis of six such candidate AREs revealed that they are all either selAREs or clAREs, with three nucleotide spacers ([32] and Table 2.1). The fact that the downstream half-sites can diverge considerably from the 5′-AGAACA-3′ consensus has been confusing. As shown in Fig. 2.3, a selective ARE can be converted into a classical ARE by enhancing its inverted repeat nature at the less conserved hexamer.

The ARBS in the vicinity of the gene encoding the Transmembrane protease, Serine 2 (TMPRSS2) has first been described by ChIP-on-chip [36]. Since the TMPRSS2 upstream sequence is fused to the coding part of oncogenes of the E-twenty six (ETS) family of transcription factors family in over 40% of prostate cancers, the androgen regulation becomes very interesting. The TMPRSS2 enhancer situated 13.5 kb upstream of the gene indeed binds the AR. The DNA motif resembling an ARE and necessary for androgen responsiveness in transient transfection experiments has, however, very low affinity for the AR. Most likely cooperativity with other transcription factors, like the pioneering factors discussed in the chapter by Wang explains how the AR can be recruited to this site. Because of such cooperativity,

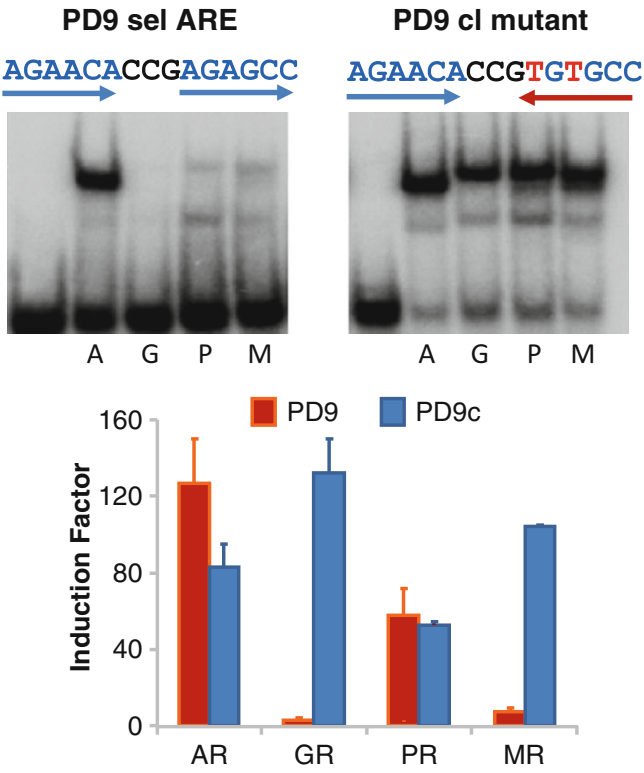


**Fig. 2.3** Change of selectivity of a selective ARE. The ARE is from an AR-binding site near the phosphodiesterase 9 gene [36]. The *left upper panel* shows an EMSA with DBDs from AR, GR, PR, and MR as indicated. The *right upper panel* shows the results of an EMSA with a mutant PDE9-ARE in which the inverted repeat nature was increased. The *lower panel* shows that, while the PDE9 ARE-based reporter is only responsive to androgens and progesterone, the mutant responds to all for steroids. Details on material and methods are described in Denayer et al. [32] and Kerkhofs et al. [39]

the AR will have low affinity for its binding site which can make the traditional way of identifying AREs, i.e., by EMSA and transfections, difficult.

Recently, the group of Olli Jänne discovered in AR ChIP seq data on mouse prostate chromatin, that in some cases, the AR seems to bind DNA elements as a heterodimer with FoxA1 [27]. This is reflected in the sequence of the mixed elements which have one 5′-AGAACA-3′ half site and one FoxA1 half site. It will be interesting to see whether other transcription factors can act similarly as heterodimers with AR. Because of the high relevance of FoxA1 as a pioneering factor and its deregulation in prostate cancer cells, this atypical DNA binding might be an interesting candidate for the development of targeted antagonists for the use in prostate cancer.

#### 2.3.1.6 ARE Search with a Position-Specific Probability Matrix (PSPM)

The differences between selective and classical AREs are so small, and the numbers of known selAREs and clAREs is too limited to device relevant separate matrices. For the time being, we devised a matrix based only on AREs for which direct binding as well as functional data are available (Table 2.1). For searching AREs, we use the matrix scan software [37] available on http://rsat.ulb.ac.be/rsat/. Because of the increased number of false positives with fragment length, the use of this approach is limited to genomic fragments of approximately 500 bp. About 75% of the candidate AREs that are indicated by such in silico searches of genomic AR-binding sites were shown to be positive in band shift and functional analyses [32].

## 2.4 The SPARKI Model

Although the in vitro data were clearly suggesting that the AR has a second type of response elements, it was difficult to assess the in vivo importance of this alternative mode of DNA binding. Based on the in vitro data on the role of the second Zn finger in selARE binding, and the fact that this receptor fragment is encoded by a separate exon in the AR as well as in the GR genes, we developed a transgenic model in which this exon in the AR gene was swapped by that of the GR gene. The resulting model, called SPARKI for "SPecificity affecting AR Knock In" expresses an AR that still binds clAREs with high affinity but has lost high affinity for selAREs [38]. In effect, this model can be considered a knockout of selective AREs. These mice only have a phenotype in the male reproductive organs, which are all reduced in size to approximately 60%. No differences were observed in other androgen target tissues like bone, muscle, kidney, or lacrimal glands, so it seems that selAREs are not involved in the anabolic effects of androgens but have a specific role in reproduction.

### *2.4.1 Role of selAREs in Fertility*

The reduced fertility observed in SPARKI is mainly explained at two sites: in the testis, the number of Sertoli cells is reduced and the spermatogenic process seems to be affected at the second meiotic division; in the epididymis, the sperm maturation is impaired and this correlates with the reduced expression of a subset of the androgen-regulated genes in this tissue. Several of these genes have a known role in sperm maturation and we were able to describe selAREs in two of them [39]. Although the prostates of the SPARKI mice are also reduced in size, gene expression comparison with wild type organs did not reveal significant differences, but this needs further analyses. AR ChIP seq data on SPARKI organs will reveal the importance of the second zinc finger in DNA selectivity of the AR.

### 2.4.2 Role for selAREs in Prostate

Several of the AREs described in AR-binding segments found in human prostate cancer cell lines are selAREs. The fact that the prostate of SPARKI mice is smaller [38] indicates that selAREs have a role in the development of normal prostates, but it still is unclear whether this type of AREs is also involved in the etiology or evolution of prostate cancer.

Interestingly, SRD5A2, the enzyme which converts testosterone in dihydrotestosterone, is a target itself for androgen regulation. Two AR-binding segments in the SRD5a2 gene reported by Hu et al. [26] were demonstrated to contain selective AREs, indicating a possible feedback mechanism [39]. Whether these AREs are also active in prostate and in prostate cancer still remains to be determined.

## 2.5 Allostery

While the cognate ligands of the AR are testosterone and dihydrotestosterone, the DNA elements can also be considered ligands rather than merely AR docking sites near the androgen target genes. There are several lines of evidence that indicate that the DNA sequence indeed can modulate the activity of the binding AR. In this section, we will discuss a possible pathway of allosteric signaling from the DBD to the LBD.

### 2.5.1 Differential Effect of Selective Versus Classical AREs

Several features of the AR have been studied by monitoring the effect of point mutations on the functionality of the receptor in reporter assays involving simple AREs. The effects of disrupting the N/C interactions, the sumoylation of the aminoterminal domain and the role of the glutamine stretch in the control of the overall activity of the human AR have initially all been described on reporter genes controlled by clAREs (reviewed in [40]). However, the same analyses performed with reporter genes based on selAREs gave much less pronounced or no effects [41–43]. Clearly, these data demonstrate that the DNA is not a passive partner of the AR but somehow controls its activity.

### 2.5.2 The DBD–LBD Communications

Many AR mutations have been found in patients with complete or partial androgen insensitivity (AIS) as well as in biopsies of castration resistant metastatic prostate

cancer [44]. Most of these mutations affect the function of the domain they are situated in. However, some DBD mutations do not affect DNA binding and some LBD mutations do not affect ligand binding. Much to our surprise, a DBD mutation can affect ligand binding and vice versa, and an LBD mutation can affect DNA binding. These mutations are situated at the surface of these domains pointing away from the DNA or the ligand. Based on modeling of the AR domains on the DBD–LBD coordinates of the crystal structure of the PPARγ-RXRα, as well as on docking AR DBD against AR-LBD, we propose that indeed, there is a functional interface between these domains, allowing signals from the DNA reaching the LBD and signals from the ligand reaching the DBD [45]. Also in living cells, the AR-LBD stabilizes the DNA binding [46]. Final proof of this allostery might come from structural studies of AR dimers bound to DNA.

## 2.6 Conclusions

The AR was cloned more than 20 years ago. We have learned a lot about its main mechanisms of actions since then. However, we also know that there is still a lot to be discovered, even if we focus on the DNA binding alone:

- How can the DNA-binding domain and the carboxyterminal extension control the different functions of the AR?
- How can different DNA sequences affect the activity of the AR: is there a direct interaction between the DBD and other domains? Despite strong indications, this still needs to be proven in structural analyses.
- Can we exploit the allosteric signals between the DBD and the other domains and translate them in one or more therapeutic targets?
- What is the exact role of selective AREs in prostate cancer, and in the control of the cell cycle in the primary tumor as well as in the metastases, be it hormone sensitive or castration resistant?

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# Chapter 3
# Negative Androgen-Response Elements in Androgen Target Genes

**Wei Qi and Zhengxin Wang**

**Abstract** Genes that are positively regulated by androgen receptor (AR) contain the positive androgen-response element (pARE) in their proximal or/and distal promoter regions. Similarly, genes that are negatively regulated by AR contain the negative androgen-response element (nARE). In this chapter, we review our work on identification and characterization of nARE. We identified seven nAREs in the negative androgen-response region in the TGF-β1 promoter. Each nARE is composed of 15 nucleotides and mediates androgen-dependent inhibition of transcription. The 5′ portions of nAREs are highly conserved and resemble core half-sites in pAREs. The nAREs interacted weakly with AR DNA-binding domain (AR DBD) in gel shift assays. Mutations on the conserved nucleotides in the nARE abolished its interaction with the AR DBD and abolished its inhibition of androgen-driven transcription, suggesting that nARE/AR interaction is essential for nARE-mediated inhibition of transcription. Taken together, these findings indicate that the AR negatively regulates the expression of its target genes via a negative androgen-response region composed of multiple nAREs.

**Keywords** Prostate • Prostate cancer • Androgen • Androgen receptor • Negative androgen-response element

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## 3.1 Introduction

In order to understand androgen receptor (AR) functions in cell survival, differentiation, and proliferation in the prostate gland, as well as in early events leading to prostate cancer, DNA microarray, DNA chip, and SAGE analyses have identified large pools of genes whose expression is affected by androgens [1–5]. Chromatin immunoprecipitation together with microarray analysis (ChIP-chip) characterized the genomic organization of AR-regulated genes [6, 7]. These studies reported a relatively high level of correlation between AR-binding and gene expression and provided the basis for further studies on how AR regulates the target gene expression at the whole genomic scale.

Many features of the androgen-induced expression program appear to be related to activation of the cell's capacity to produce seminal fluid of the prostate gland. Some AR-target genes encode transcriptional regulatory and signaling proteins, which may have important roles in programming the normal prostate cells in response to the androgen. Many genes were modulated similarly in all of prostate cell lines, suggesting conservation of a specific androgen-responsive program, perhaps a vestige of the prostatic epithelial phenotype [1]. Some AR target genes show altered expression in prostate cancer, implying that these genes may be modulated abnormally in cancer cells because of molecular genetic alterations acquired with transformation.

Stimulation of gene expression is only one aspect of the AR signaling. The androgen-repressed genes also appear to play important roles in regulating cell growth, differentiation, survival, and migration, as well as the androgen signaling axis itself [8–10]. The expression of androgen-repressed genes upon ablation therapy is thought to contribute to prostate cancer regression [11]. Despite the importance of repressed genes, most studies have focused on the transcriptionally activated targets. Additionally, researches on gene repression in the context of AR signaling have dealt with mechanisms that antagonize the positive action of AR on unregulated target genes [12, 13]. Relatively little attention has been paid to bona fide AR-mediated gene repression.

Diverse mechanisms of transcriptional repression have been proposed for eukaryotic genes [14]. Most studies of transcriptional repression by nuclear hormone receptors have focused on the nonsteroid, class II receptors such as the thyroid and retinoid hormone receptors. In the absence of ligand, these receptors bind constitutively to their respective response elements to mediate strong active repression, a mechanism that entails the formation of a corepressor complex containing of corepressors (e.g., NCoR, SMRT) and histone modifiers (e.g., HDACs) [15]. Active repression via the recruitment of corepressor complexes has also been observed for the steroidal, class I receptors such as AR [16]. This has mainly been examined in the setting of antagonist-mediated repression. Other studies on negatively regulated (agonist-mediated) AR target genes provoke a mechanism of repression through physical interference with other transcription factors at their cognate promoter elements [8, 9, 14, 17]. One such example was illustrated by the finding of a sequence element (XBE) in the prostate specific antigen (*PSA*) gene [18]. The XBE interacted

with both the AR and the p65 subunit of nuclear factor (NF)-κB. Cross-modulation of the AR and NF-κB p65 mediated inhibition of AR-mediated *PSA* promoter transactivation.

Authors have described regulation of expression of a few AR target genes via negative androgen-response elements (nAREs) [19–21]. The negative ARE was proposed to mediate repression via its direct interaction with AR. But, the nature of the negative ARE and the mechanism by which the negative ARE represses transcription remain elusive.

## 3.2 The Negative Androgen-Response Promoter Element Strongly Inhibits Androgen-Driven Transcription

In order to map negative androgen-response promoter element (nARPE), we made a luciferase reporter (pGL3-4xARE-E4-Luc), which includes four tandem positive androgen-response elements (pAREs) upstream E4 core promoter with a pGL3-basic backbone [22] (Fig. 3.1a). When the pGL3-4xARE-E4-luc reporter was co-transfected with expression plasmid for AR into PC3MM2 cells in the absence or presence of androgen (R1881), AR activated the reporter about 36-fold higher in the presence of androgen than in the absence androgen (Fig. 3.1c). To validate the reporter system, we cloned DNA fragments of various lengths (50, 150, and 250 bp) into pGL3-4xARE-E4-luc reporter between 4xARE and E4 and found that no significant difference between the pGL3-4xARE-E4-luc and the reporter with nonspecific DNA fragments was observed (Fig. 3.1c). Thus, the reporter pGL3-4xARE-E4-luc provided a tool to observe inhibition effect of the nARE, when inserted it into the reporter between 4xARE and E4 (Fig. 3.1b).

Using this reporter system, we identified four nARPEs in TGF-β1 (−673 to −423), CDK2 (−931 to −731), PDEF (−268 to −50), and p21 (−1925 to −1691) promoters (Fig. 3.2), and they dramatically inhibited the androgen-driven luciferase expression when inserted into pGL3-4xARE-E4-luc between 4xARE and E4 [22, 23] (Fig. 3.1c). All identified nARPEs spanned DNA sequences that were 200–250 bp long and mediated strong (16 to 40-fold) inhibition of androgen-driven transcription in close proximity of pAREs. To our surprise, when we used deletion constructors that inserted shorter DNA fragments of these nARPEs into pGL3-4xARE-E4-luc between 4xARE and E4, we could not figure out a short sequence that responses for negative regulation of AR-driven transcriptional activity. Instead, we observed gradual decrease in the activity of nARPEs when truncated from 3′ or 5′ ends. These observations suggest that these nARPEs contain multiple *cis*-elements that function synergistically.

When put four nARPEs in their natural or reverse orientation, all of them showed dramatic inhibition effect on AR-driven transcriptional activities. In contrast, when put four nARPEs about 3 kb upstream the transcription start site, all of them lost inhibition effect on AR-driven transcriptional activities. Identified nARPEs also inhibited transcription activated by ER or Gal4-p53 using pGL3-5Gal4-E4-Luc or

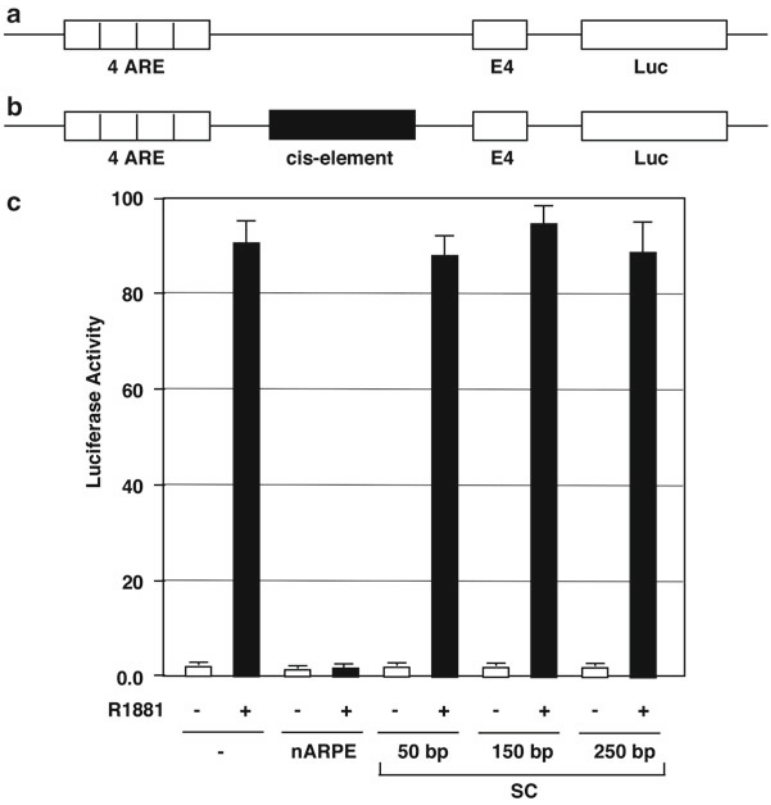


**Fig. 3.1** A luciferase reporter to assay the negative androgen-response element. (**a**) Diagram of the luciferase reporter construct. (**b**) Diagrams of the luciferase reporter construct with a *cis*-element insertion. (**c**) Luciferase assay. PC3MM2 cells were transfected with 5 fmol of pcDNA-AR and 25 fmol of the luciferase reporter constructs that contain no insertion, a negative androgen-response promoter element (nARPE), or scramble control (SC) DNA fragments derived from human PDEF promoter (−3018 to −2775). Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ±SD ($n=3$)

pGL3-2ERE-E4-Luc as the reporter [22]. Enhancer can regulate transcription on both orientations and far away from transcription start site. Thus, identified nARPEs function with a mechanism distinct from the enhancer.

Previous studies suggested that the *TGF-β1* gene was negatively regulated in the prostate gland and positively regulated in prostate cancer cells by androgens [23, 24]. The *TGF-β1* promoter contains multiple positive AREs and a negative ARPE. The positive AREs interact with AR in vitro and in vivo, and both positive AREs and negative ARPE are functional in the synthetic promoters as well as in the integrated *TGF-β1* promoter [23]. These findings indicate that regulation of *TGF-β1* expression in response to the androgen is complicated and could be neutral, positive, or negative

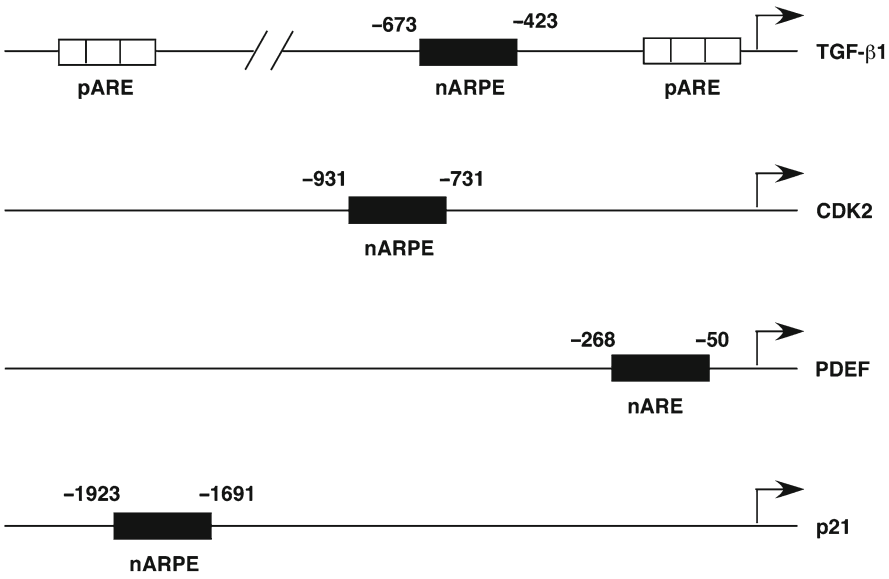


**Fig. 3.2** Diagram shows the negative androgen-response promoter elements in TGB-β1, CDK2, PDEF, and p21 promoters. *pARE* positive androgen-response promoter element, *nARPE* negative androgen-response promoter element

depending on cell types or cell environments. The functional roles of the other three identified nARPEs in the regulation of their corresponding promoters are currently unknown. Two different kinds of *cis*-elements, Ets and hormonal responsive element (HRE), were identified in the maspin promoter [21]. The Ets element is active in regulating maspin expression in normal prostate epithelial cells but inactive in tumor cells. The HRE site is a negative element that is active in both cell types. This negative DNA sequence can repress a heterologous promoter recognized by the androgen receptor. Watt el al. used an enhancer trap approach with randomly cleaved overlapping DNA fragments from an approximately 55-kb P1 cosmid insert encompassing the 5′ half and upstream sequences of the PSMA gene (FOLH1) to isolate an enhancer that strongly activates the FOLH1 core promoter region [20]. The enhancer (PSME) is located in the third intron about 12 kb downstream from the start site of transcription and is characterized by a 72-bp direct repeat within a 331-bp core region. PSME-enhanced expression is repressed in the presence of androgen, mimicking the repression of the endogenous FOLH1 gene. Noss et al. sequenced and analyzed the ability of 5.5 kb of PSMA promoter/leader region to promote transcription [19]. In stably transfected LNCaP cells, the FOLH1 promoter/leader region produced a 21% downregulation in response to androgens, while addition of the enhancer resulted in a 45% downregulation. These results demonstrate significant upregulation of transcription by the PSMA promoter/enhancer, with specificity for the LNCaP prostate cell line, and downregulation of transcription in response to physiological levels of androgen.

## 3.3 The Negative Androgen-Response Element (nARE) Functions Synergistically to Inhibit Androgen-Driven Transcription

Genes that are positively regulated by AR contain the pAREs in their proximal or/and distal promoter regions. The pARE is composed of two short 6-bp sequences known as core half-sites. The primary sequence of the core half-sites is largely limited to two distinct motifs, AGAACA and TGTTCT. The two half-sites can be organized into either spaced palindromic or direct repeats [25]. Active AR homodimer recognizes and binds pARE and subsequently recruits cofactors to regulate transcription of its target genes [26, 27]. In general, one or two pAREs with high affinity for AR-binding localize at the proximal promoter region. On the other hand, the distal androgen-response enhancer region contains multiple low affinity pAREs. In contrast, the promoter structure of genes negatively regulated by AR is still illusive.

Our recent work identified four nARPEs from four androgen-regulated genes, which strongly inhibited androgen-driven transcription [22, 23]. By deletion analysis and sequence alignment, a nARE (5′ CAGACCCTCTTCTCC 3′) was identified from the nARPE in the *TGF-β1* gene [22]. A single copy of the nARE slightly inhibited the androgen-driven transcription, while multiplication of this nARE dramatically enhanced the inhibitory activity (Fig. 3.3).

After identifying one nARE, we built a construct containing a single copy of the identified nARE and a short DNA sequence derived from the negative androgen-response region [22]. The resulting constructs were transiently transfected into PC3 cells with pcDNA-AR, and the transfected cells were grown in the presence or absence of the androgen (R1881). The construct without any sequence insertion between nARE and E4 promoter elements was used as the control. This analysis and further deletion identified six other nAREs in the TGF-β1 nARPE (Fig. 3.4a). Three copies of each identified nARE exhibited strong inhibition of androgen-driven transcription (Fig. 3.4b). One nARE (nARE4) in particular inhibited the basal level of transcription. Six of seven identified nAREs inhibited androgen-driven transcription at a level comparable with that of TGF-β1 nARPE (Fig. 3.4b). These results reveal that the TGF-β1 nARPE contains seven functional nAREs, that together strongly inhibit androgen-driven transcription.

## 3.4 The Negative Androgen-Response Element Functions Through the AR Signaling

Sequence alignment of the seven identified nAREs and the previously identified XBE [18] revealed a highly conserved 5′ half end containing the three most conserved nucleotides (G3, C5, and C7) [22] (Fig. 3.5a). The nucleotides C14 and C15 were also conserved in some nAREs. We found that the 5′ parts of nAREs are similar

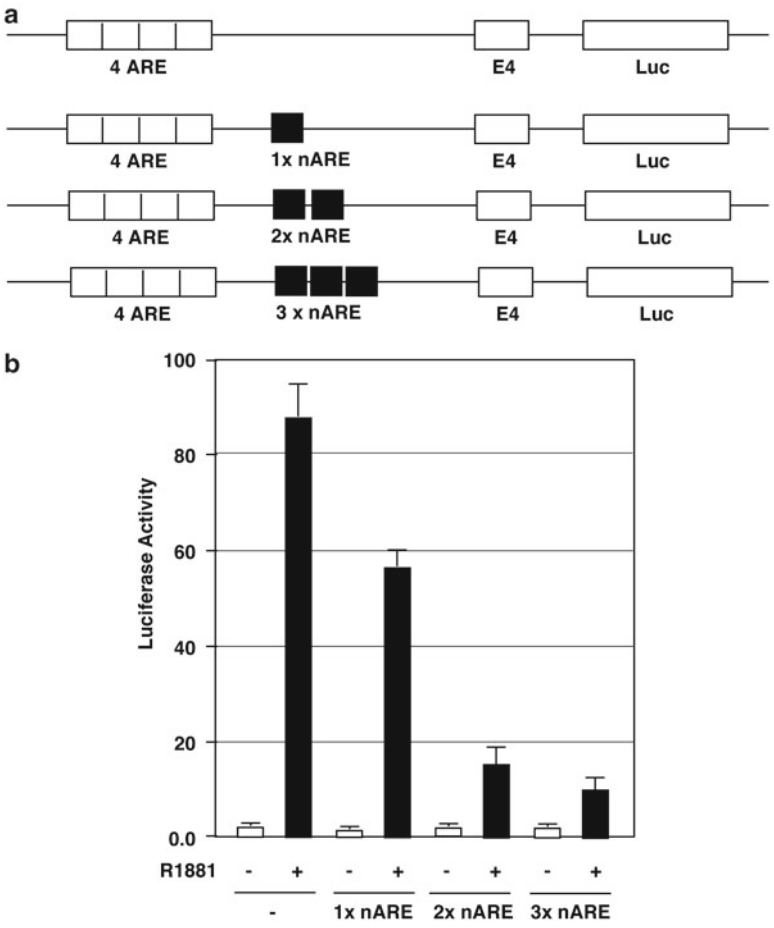


**Fig. 3.3** nAREs function synergistically. (**a**) Diagrams of the luciferase reporter constructs without or with one, two, or three copies of nARE insertion. (**b**) Luciferase assay results show the synergism between nAREs. PC3MM2 cells were transfected with 25 fmol of the luciferase reporter constructs and 5 fmol of pcDNA-AR. Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$)

to the 5′ core half-sites in the consensus pARE. More importantly, the nucleotides involved in AR interaction with pARE (G3 and C5) are conserved in nAREs. We generated the mutations (G3A/C5T) in nARE7 and subcloned two copies of the mutated nARE7 into the pGL3-4xARE-E4-luc reporter. This mutation completely abolished nARE7-mediated transcriptional inhibition [22].

We labeled probes containing two copies of wild-type and mutant nARE7 with $^{32}$P and incubated the probes with the recombinant AR DBD [22]. This AR DBD protein (100 and 200 ng) interacted with the nARE7. The shifted protein–DNA complex was completely abolished by cold nARE7 and pARE probes but not

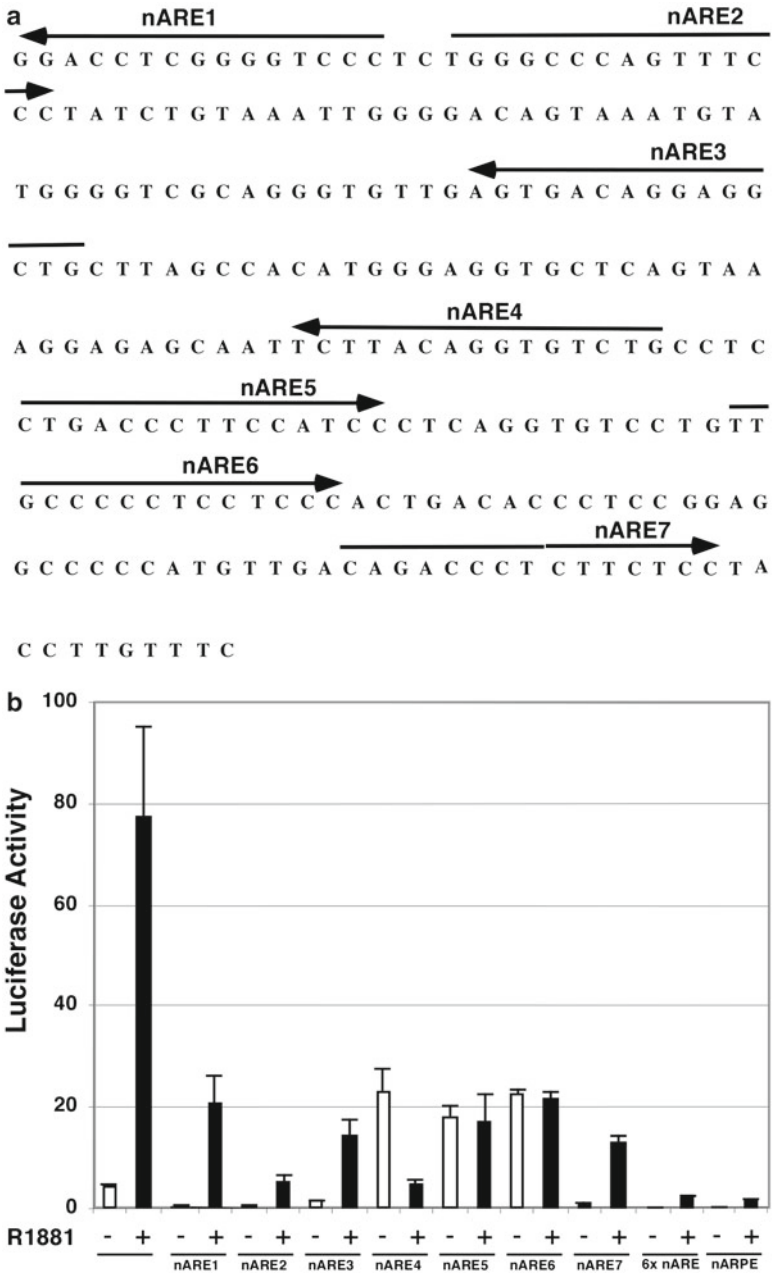


**Fig. 3.4** The TGF-β1 negative androgen-response promoter element contains seven functional nAREs. (**a**) Identified 7 nAREs in the TGF-β1 negative androgen-response promoter element. Sequences and directions of nAREs are indicated by underlines and *arrows*, respectively. (**b**) Luciferase assay results show repression activity of 7 nAREs on androgen-driven transcription. PC3MM2 cells were transfected with 25 fmol of the luciferase reporter constructs and 5 fmol of pcDNA-AR. Transfected cells were allowed to grow in the absence or presence of 10 nM R1881 and harvested for the luciferase assay. The values represent the mean ± SD ($n = 3$)

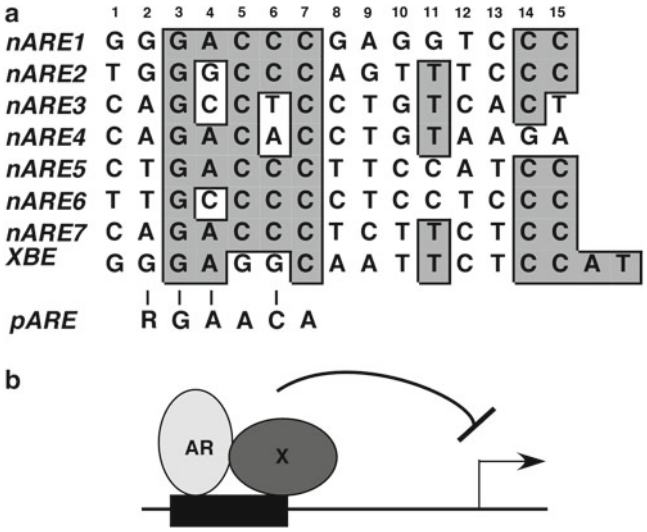


**Fig. 3.5** nAREs resemble core half-sites in pARE (**a**) Sequence alignment of identified seven nAREs and XBE with pARE consensus. The conserved nucleotides are in *shadow*. (**b**) A model for AR function through nARE. *AR* androgen receptor, *X* unidentified transcriptional factor that interacts with AR and the 3′ part of nARE

affected by the mutant pARE (pAREmt) probe, which did not interact with the AR DBD [23, 28]. Mutations on the nucleotides critical for AR/DNA interaction (G3 and C5) abolished the AR DBD/nARE7 interaction. In contrast, low amounts of the AR DBD protein (2.5 and 5 ng) generated much strong shifted binds with pARE probes. These findings suggested that the AR weakly interacts with nAREs and that the AR binds to the nARE, which was consistent with the fact that the nARE contains only a consensus ARE half-site motif. The AR-nARE interaction is also correlated with the function of nAREs. Since gel shift experiments were carried out with the purified AR DBD protein and not a cell extract, it is possible that other proteins may also interact with the nARE and contribute to AR repression of gene expression through the nARE (Fig. 3.5b).

He et al. cloned 860 bp (−765 approximately +95) of masipin promoter sequence from the human genomic DNA and studied the promoter activity [29]. The 860 bp sequence and a series of deletions from 5′ and 3′ ends were inserted into the upstream of luciferase reporter gene, respectively. Results from dual luciferase reporter assay and electrophoretic mobility shift assay indicated that there was a negative androgen-responsive element (5′-GTACTCTGATCTCC-3′) in the region of −277 to −262. It appeared that AR interacted with this nARE. But it is unclear how AR regulates maspin gene expression through this nARE. The nAREs we identified do not resemble those found in the maspin gene but are similar to the 5′ part of the XBE in the *PSA* gene [18]. The XBE interacted with both the AR and the p65 subunit of nuclear factor (NF)-κB [18]. We performed a cotransfection assay and found that

NF-κB p65 did not affect the inhibitory activity of the *TGF-β1* nARPE. However, based on these results, we cannot rule out the competition of other transcriptional factors with the AR in interaction with identified seven nAREs in the *TGF-β1* promoter in transcriptional repression (Fig. 3.5b).

In reports of previous AR-binding analysis of the mammalian genome, authors described the enrichment of many other DNA-binding motifs in AR-binding sites [6, 7]. They found that a consensus AR half-site motif and potential binding sequences for AP-1, ARA, ZNF42n NHF-4a, and epidermal growth factor were linked together. Because the 5′ halves of the nAREs identified in our study are highly conserved and similar to a consensus pARE half-site motif and the 3′ halves are diversified, other transcription factors may participate in nARE function by interacting with the 3′ half of the nARE (Fig. 3.5b). Gel shift experiment was carried out with recombinant and the purified AR DBD protein and showed a weak interaction between nARE and AR DBD. We cannot exclude the possibility that the other transcription factors together with AR synergistically interact with the nAREs to contribute to AR repression of gene expression. Further studies are necessary to identify factors that can bind to the 3′ parts of nAREs to participate in nARE-mediated transcriptional repression.

## 3.5 Future Directions

The synthetic promoters and transient transfection assay are used for characterization and analysis of the nARE. Multiple copies of the construct in a single transfected cell and the poorly chromatinized nonintegrated promoters may render the corresponding transcription factors limiting with respect to the exogenous construct. Thus, it is necessary to further investigate whether the identified nAREs function in the integrated (single copy) chromosomal environment and in their native gene environments.

Distinct histone amino-terminal modifications generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or transcriptionally silent chromatin states [30–32]. With a few exceptions, trimethylations of H3K9, H3K27, and H4K20 are associated with transcriptional repression, whereas methylations of H3K4, H3K36, and H3K79 are associated with transcriptional activation [33, 34]. It is more likely that AR and the other transcription factor (TF) interact with the nARE to recruit corepressors to modify histone tails for suppression of transcription. It is important to investigate whether AR interaction with the nARE recruits corepressors and alters histone modifications (e.g., acetylation, methylation) in the region surrounding the nARE.

It is unknown how AREs function in the regulation of AR-target genes in animals. To fill this gap is to investigate roles of positive and negative AREs in the regulation of *TGF-β1* gene expression and in the development and functions of mouse prostate gland. The positive and negative AREs in the mouse *TGF-β1* promoter can be mutated and the mutated promoters knocked in the *TGF-β1* locus in

the mouse. The consequences of these knock-in mutations on expression of the *TGF-β1* gene and on the development and functions of mouse prostate gland can be investigated.

Expression of maspin is under the influence of both a positive Ets and a negative HRE element. Loss of maspin expression during tumor progression apparently results from both the absence of transactivation through the Ets element and the presence of transcription repression through the negative HRE element recognized by androgen receptor. The PSME activates transcription from its own and heterologous promoters in prostate cell lines; enhancement is greatest in the PSMA-expressing cell line LNCaP (>250-fold). The PSME shows essentially no activity in five non-prostate cell lines. The data demonstrate that both cell-type specificity and androgen regulation are intrinsic properties of the nARPEs. TGF-β1 is a multifunctional cytokine that influences homeostatic processes of various tissues. TGF-β1 expression is inhibited by androgens in the prostate gland, whereas its expression is enhanced by androgens in highly metastatic prostate cancer cells. How androgen signaling positively or negatively regulate TGF-β1 expression in response to various signals or under different environmental conditions is unknown.

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# Chapter 4
# Chromatin Looping and Long Distance Regulation by Androgen Receptor

**Benjamin Sunkel and Qianben Wang**

**Abstract** The importance of the androgen signaling axis to the development and progression of prostate cancer (PCa) is central to our understanding of the disease and the therapeutic strategies currently utilized against it. The androgen receptor (AR) is known to transform the hormone stimulatory signal into an oncogenic gene transcription program required for PCa initiation and progression. Bound by AR primarily at their distal enhancer elements, AR target gene transcription relies on a mechanism known as chromatin looping. Increasingly abundant evidence suggests that changing patterns in AR-mediated chromatin loop formation underlie alterations in gene expression profiles among PCa cases and throughout PCa progression. Defining the role of additional loop-facilitating activities and the impact of genome organization on the patterns of AR-mediated chromatin interactions remains an obstacle to full understanding of transcription regulation by AR.

**Keywords** Androgen receptor • Genome-wide binding • Chromatin looping • Chromatin conformation capture • Interactome • Transcription regulatory architecture • Collaboration factors • Coactivators

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## 4.1 Introduction

That the androgen receptor (AR) plays a central role in prostate development as well as in the initiation and progression of prostate cancer (PCa) is well established. Reduced AR signaling resulting from inherited androgen insensitivity correlates with stunted prostate development and dramatically reduced incidence of cancer [1, 2]. This nuclear hormone receptor mediates androgen-dependent gene transcription upon binding to the activated form of testosterone, dihydrotestosterone (DHT). Androgen binding causes AR dissociation from cytoplasmic heat shock proteins (Hsp), AR dimerization, and nuclear translocation. Within the nucleus, AR regulates transcription via interactions with an ensemble of DNA-binding collaboration factors and with non-DNA-binding activating or repressive coregulators (coactivators and corepressors) at specific genomic locations termed androgen-responsive elements (AREs) [3]. This essential role of androgen signaling, transmitted through AR, in prostate carcinogenesis underscores the implementation of androgen ablation as a primary therapeutic strategy for PCa, and the dependence of most prostate cancers on androgen at the time of diagnosis results in initial regression of the disease [4]. The unfortunate reality is that many prostate cancers will develop androgen independence, rescuing the regulatory activity of AR in what is referred to as castration-resistant prostate cancer (CRPC). In this lethal form of the disease, AR amplification, enhanced androgen sensitivity, AR mutation (resulting in ligand-binding domain loss and constitutive activity), and enhanced androgen synthesis permit a return of AR-mediated transcription differing in many ways from the transcription program observed in the normal prostate and indeed in the early stages of androgen-dependent prostate cancer (ADPC) [5–7]. While an ever-increasing body of evidence has defined many of the epidemiological risks associated with PCa development and understanding of the genetic abnormalities underlying the disease is rapidly growing, the ability of these malignancies to adapt to hormone deprivation and reinitiate AR signaling has confounded attempts to develop effective treatments for CRPC. As such, PCa remains one of the leading causes of cancer-related deaths among American men [8].

Further understanding of AR function in PCa development and its changing role over the course of disease progression from ADPC to CRPC has been greatly enhanced by next generation sequencing methods of the post-genomic era. In search of a more comprehensive view of AR target genes and the carcinogenic pathways they affect, chromatin immunoprecipitation (ChIP) techniques coupled with genome-wide DNA microarray hybridization (ChIP-on-chip) or massively parallel sequencing (ChIP-seq) have identified the changing patterns of AR-binding sites (ARBs) in various PCa cell line models [6, 9–14]. Rather than identifying a list of target genes whose proximal regulatory elements are occupied by AR, these studies uncovered a complex regulatory mechanism typified by distal AR binding to enhancer elements and extensive, yet variable, protein–protein interactions resulting in a repressive or activating state within target gene regulatory regions. Subsequent elucidation of the mechanism by which AR mediates transcription from afar revealed the close spatial localization of AR-bound enhancers with the promoter

regions of androgen-responsive genes. This so-called chromatin looping mechanism has been verified for several androgen-responsive genes and has been demonstrated to play a critical role in AR target gene regulation in CRPC models in the absence of androgen [6, 9, 15–17].

## 4.2 Genome-Wide Analysis of AR-Binding Sites

Early efforts to characterize AR-binding patterns on a broad scale utilized ChIP-on-chip analysis. ChIP-enriched chromatin, precipitated with anti-AR antibodies from untreated or androgen stimulated PCa cells lines, was amplified and hybridized to microarrays covering various portions of the genome. Massie et al. conducted a promoter-specific ChIP-on-chip analysis using microarrays covering ~25,000 known promoter sequences, identifying 1,532 AR-bound promoters in LNCaP cells [10]. Ninety-two of these sites represented regulatory regions of androgen-responsive genes identified in previous gene expression analyses, suggesting a direct, proximal regulatory role for AR in the expression of this subset of genes. Expanding their view beyond proximal promoter elements, Bolton et al. hybridized AR-immunoprecipitated chromatin to tiled microarrays spanning genomic regions ±50 kb from the TSS of 548 hormone-responsive genes and found AR to primarily bind far downstream of androgen-responsive genes [18]. Their results suggested a distal, combinatorial control mechanism as multiple ARBs were often observed within the 100 kb regions centered about androgen-responsive genes. Wang et al. performed ChIP-on-chip in androgen-stimulated LNCaP cells using Affymetrix microarrays representing chromosomes 21 and 22 [9]. 90 androgen-dependent ARBs were identified in this analysis, of which only 34 were found to reside within 500 kb of the transcription start site (TSS) of androgen-responsive genes on these chromosomes. Jia et al. performed a complementary analysis with microarrays spanning chromosomes 19 and 20, corroborating the findings of previous microarrays studies [12].

While these studies were valuable in revealing AR-binding patterns in portions of the genome, a global view of AR binding required the use of genome-wide techniques. Wang et al. endeavored to comprehensively map AR binding and correlate the observed patterns to gene expression profiles in LNCaP (ADPC) and abl (CRPC) PCa models [6]. 8,708 and 6,353 ARBs were identified in LNCaP and abl cells, respectively, using Affymetrix whole genome microarrays. Upon correlation with AR-dependent gene expression profiles, it was observed that the vast majority of ARBs exist more than 10 kb from AR-regulated gene TSS. While many ARBs are conserved between LNCaP and abl, there emerged a large number of cell line-specific binding sites. Interestingly, CRPC-specific AR binding was found to occur near cell cycle and M-phase regulatory genes whose expression was upregulated in abl but not in LNCaP cells.

Utilizing next generation sequencing technology, the Chinnaiyan group identified more than 37,000 AR-bound sites in LNCaP and nearly 13,000 sites in VCaP cells [13].

Nearly all ARBs identified in previous studies were represented in the sequencing data [6], and while there was significant overlap between the two cell lines, cell type-specificity in AR-binding patterns emerged. Importantly, the group correlated the AR-binding patterns with the ChIP-seq data for various AR collaboration factors and histone marks. Interestingly, ERG binding significantly overlapped AR binding in VCaP cells, and it was observed that ERG expression negatively correlated with AR expression in ADPC systems with a consequent attenuation of AR-mediated transcriptional control of target genes. In line with previous findings, only 10.8% and 12.4% of ARBs lay within known gene promoter regions in LNCaP and VCaP cells, respectively, while the remaining binding sites were found at enhancers, within introns, and to a lesser extent within exons [13].

In a recent study from Massie et al. using LNCaP and VCaP cells lines, ChIP-seq analysis similarly identified significantly overlapping AR-binding signatures. As with the Chinnaiyan group, this 2011 study observed a degree of cell type-specificity in AR-binding patterns and found a maximal enrichment of androgen-stimulated genes 25 kb from the observed ARBs [14]. Upon correlation of androgen-stimulated AR binding with RNA Polymerase II (Pol II) ChIP-seq data, the group defined 1,283 androgen-responsive genes characterized by dynamic recruitment of AR and requisite transcriptional machinery. Gene ontology (GO) analysis of this target gene set revealed enrichment in metabolic and biosynthetic pathways as well as dedifferentiation and cell cycle progression pathways observed in previous studies [6, 13, 14].

These genome-wide findings, along with the previous reports described above, depict a scenario in which AR binds to distal enhancers of its target genes, facilitating their upregulation from afar. Additionally, cell type-specific AR binding provides a means for mediating differential gene transcription programs that promote more advanced PCa growth. However, comprehensive identification of AR target genes is hindered by the trend of AR binding distal to the TSS of androgen-responsive genes.

## 4.3 Validating the Looping Hypothesis

With the observation that AR tends to bind to distal enhancers, revealing the mechanism by which AR mediates transcription from afar became central to understanding the regulation of androgen-responsive genes. Chromatin looping, in many ways an extension of the promoter-centric gene regulatory mechanism utilized in prokaryotes, involves the recruitment of regulatory proteins to distal enhancers and subsequent looping out of intervening chromatin as protein–protein interactions with promoter-bound factors facilitate direct enhancer–promoter interactions [19, 20]. This model was proposed to rationalize the observation that enhancers function in a largely position/distance-independent mode to increase the likelihood of gene expression. Rather than relying on random diffusion of distal regulatory elements, loop-facilitating activities such as transcription factors help to overcome the low

probability of an unmediated enhancer–promoter interaction as well as confer specificity to these interactions [19, 20]. Observing these interaction events on a gene-by-gene basis, and more recently on a global scale, has been facilitated by the development of a number of technologies discussed below.

The chromatin conformation capture (3C) assay is capable of identifying physical interactions between loci found at distant regions of the genome [21]. Whether the interaction is intra- or interchromosomal, 3C is capable of demonstrating the close proximity of the two regions at the time of the assay. Briefly, the protocol begins with formaldehyde fixation of protein–DNA and protein–protein interactions followed by restriction enzyme digestion generating complementary overhangs on all chromatin fragments. Addition of ligase under extremely dilute conditions favors intramolecular rather than intermolecular ligation. Crosslinking is then reversed and the chromatin is purified, generating a 3C library consisting of a heterogeneous population of ligation products. Suspicion of a specific long-distance chromatin interaction then informs the selection of PCR primers capable of identifying the desired ligation product [21]. Crosslinking frequency can be measured by performing quantitative PCR (3C-qPCR) of the ligation products as described initially by [22] and presented in detail by [23].

While 3C is a powerful tool for elucidating local chromatin conformations and long-distance interactions, it is often desirable to identify the proteins responsible for facilitating such interactions. Although Splinter et al. demonstrated that CTCF knockdown resulted in reduced chromatin interactions at the *β-globin* locus [22], addition of ChIP to the protocol (ChIP-3C) allows for enrichment of interacting chromatin fragments associated with a single protein. In this way, silencing of the *Dxl5–Dxl6* locus in mouse models of Rett syndrome was found to result from a chromatin loop formed by Methyl-CpG binding protein 2 (Mecp2) that is absent in *Mecp2*-null mice [24].

To inch closer to a comprehensive map of genomic interactions (an interactome), circular chromosome conformation capture (4C) was developed [25]. This method closely resembles the 3C assay; however, ligation proceeds for over a week generating circularized DNA. Whereas 3C requires knowledge of both genomic loci involved in a putative interaction, 4C utilizes primers extending out from a single location to identify all interactions with this locus. Amplified sequences can then be subjected to microarray or sequencing analysis to identify all regions interacting with the location of interest. Zhao et al. utilized primers extending out from the *H19* imprint control region and amplified 114 unique sequences representing loci from each autosomal chromosome [25].

Expanding the pool of primer sets is another approach to increasing the throughput of the 3C assay and is utilized in the chromosome conformation capture carbon copy (5C) assay [26]. Here, a 3C library is generated in the manner described by Dekker and colleagues [21]. A library of primers is then used to identify a network of chromatin interactions. For example, 5C has been utilized to verify the chromatin interactions of the *β-globin* locus control region with nearby genomic sites [27]. Forward and reverse primers contain a hybridization sequence designed

to recognize the regions immediately adjacent to digestion/ligation sites of interest. Addition of ligase results in ligation of the primers to one another, creating a "carbon copy" of the sequence bridging the 3C ligation site. The purified 5C library may then be subjected to microarray or sequencing analysis to identify the cohort of interactions.

To comprehensively map genome-wide chromatin interactions, an unbiased analysis termed Hi-C was developed. In the first report, this protocol revealed an extensive network of interactions across the genome of a human lymphoblastoid cell line, identifying 6.7 million long-distance (>20 kb) interactions at a resolution of 1 Mb [28]. Hi-C begins with fixation of protein–chromatin interactions followed by restriction enzyme digestion. Overhangs left behind are filled in using biotinylated nucleotides. Under dilute conditions, chromatin fragments are ligated to one another, and the chromatin is subsequently fragmented. Streptavidin pull-down allows for enrichment of ligated chromatin fragments, which are then subjected to high throughput sequencing. The sequencing reads are mapped to a reference genome and interactions are characterized. A very recent publication has enhanced the resolution of the original Hi-C protocol. This zoomed-in view of chromatin interactions revealed an even more extensive interaction network divided into self-interacting blocks termed "chromatin domains" [29]. When correlated to ChIP-seq data for a particular transcription factor or chromatin organizer, Hi-C can provide insight into the activities that mediate higher order chromatin structures.

The final iteration of this stepwise march to comprehensive interactome identification was developed [30]. Chromatin Interaction Analysis by Paired End Tag (ChIA-PET) sequencing is capable of identifying all chromatin interactions facilitated in whole or in part by a protein of interest. Beginning as a typical ChIP protocol, enriched chromatin fragments are processed to generate blunt ends. The processed mixture of crosslinked protein–DNA complexes is then divided into two portions, each having a barcode identifiable linker oligonucleotide ligated to the ends of the chromatin. The two uniquely labeled sets of protein-bound chromatin are then mixed together and diluted such that the addition of ligase results in ligation of only those fragments sharing a common protein interaction. In addition to a unique barcode sequence, all linker sequences contain a *MmeI* recognition sequence. Subsequent digestion produces the PET library to be sequenced. In its initial utilization, ChIA-PET analysis was implemented to define the estrogen receptor alpha (ERα)-bound interactome [30]. Interestingly, when correlated to gene expression data, the sites identified by ChIA-PET to be involved in chromatin structuring contained regulatory elements of estrogen-upregulated genes. In addition to identifying singular chromatin loops for ER-regulated genes, the analysis revealed that ER mediated the formation of higher order chromatin architectures, localizing the regulatory regions of multiple estrogen-responsive genes into transcription hubs. The technique has since been applied to elucidate higher order chromatin organization facilitated by the CCCTC-binding factor (CTCF) [31] and to identify transcription regulatory chromatin structures associated with Pol II binding [32].

## 4.4 AR-Mediated Looping

While genome-wide interaction analysis has not been implemented in the study of AR transcriptional regulation in PCa, 3C and its modified version, ChIP-3C, have provided direct evidence that AR-mediated chromatin looping regulates expressed of a subset of AR target genes. Prostate-specific antigen (PSA) is commonly expressed in PCa, and as an AR target gene, *PSA* provides a model for the study of AR-mediated transcription. Using ChIP-3C with anti-AR pulldown, a loop-forming enhancer–promoter interaction stimulated by androgen was observed at the *PSA* locus corresponding to *PSA* expression [15]. Similarly, anti-AR ChIP-3C was used to extensively characterize the androgen-stimulated enhancer–promoter interaction regulating expression at the *TMPRSS2* locus [9], providing a mechanism for the AR-mediated transcription of *TMPRSS2-ETS* fusions, the most frequently observed genomic rearrangements in PCa.

The Wang lab recently revisited the *TMPRSS2* locus, validating the androgen-stimulated interaction between the *TMPRSS2* promoter and an enhancer located 13.5 kb upstream of the TSS by 3C-qPCR. Beyond the originally identified enhancer, three additional AR-bound sites exhibited androgen-dependent crosslinking with the *TMPRSS2* promoter [16]. In contrast to the androgen-stimulated loop formation observed at the *TMPRSS2* locus in LNCaP cells, 3C-qPCR was used to show that androgen-independent interactions occur at the *UBE2C* locus between the promoter and two AR-bound enhancer elements in abl cells [6]. Moreover, these interactions were not observed in LNCaP cells in the presence or absence of androgen, highlighting the important and changing transcription regulatory role played by AR throughout PCa progression. Finally, an important 3C-qPCR result was recently reported by [33] in which one potential mechanism of enhanced PCa susceptibility derived from the 17q24.3 PCa risk locus involves increased AR-mediated looping between this enhancer region and the *SOX9* promoter. Overexpression of *SOX9* has previously been linked to PCa development, and this result reveals that mutations in regulatory regions can lead to *SOX9* deregulation [34–36].

## 4.5 Mechanisms of AR-Mediated Loop Formation

For the genes described above, looping was demonstrated to coincide with AR binding at regulatory regions of AR target genes, resulting in enhanced expression. Combinatorial approaches implemented to fully understand this long-distance regulatory mechanism have revealed a host of additional activities involved in the processes of directing AR to cell-specific binding sites, facilitating enhancer–promoter interactions, and determining the activating or repressive signatures that govern AR target gene expression. Several of these activities have garnered interest based on their observed and proposed role in AR- and general transcription factor-mediated chromatin looping.

### 4.5.1 AR Collaborating Factors

Mounting evidence suggests a crucial role of two particular DNA-binding collaborating factors in the establishment and maintenance of AR-mediated chromatin loops: FoxA1 and GATA2. FoxA1, a member of the winged helix/forkhead family of transcription factors [37] is generally described as a pioneering factor, inducing an open chromatin state through interactions with histones H3 and H4 [38, 39]. FoxA1 interacts with AR at a broad spectrum of enhancer sites, and while FoxA1 plays a role in transcription regulation of some AR targets including *CCND3*, *MYC*, and *CDK6* [40], FoxA1 silencing has no effect on *PSA*, *TMPRSS2*, or *PDE9A* expression [9]. This gene-specific variation in dependence on FoxA1 for AR target expression is partially explained by the recent discovery that FoxA1 serves to enhance AR binding to distal regulatory elements at particular gene loci, resulting in enhanced expression, while preventing binding at others and repressing transcription [40, 41]. AR-mediated chromatin looping at a subset of target genes has been demonstrated to rely partially on FoxA1 colocalization to enhancer elements, as siFoxA1 transfection resulted in a significant reduction in crosslinking frequency between the *UBE2C* enhancers and promoter [16]. Additional evidence for the role of FoxA1 in chromatin loop formation was derived from correlating FoxA1 ChIP-seq data to the ERα-bound interactome revealed by the ChIA-PET protocol. In this analysis, FoxA1 binding was most highly enriched at ERα-bound sites involved in multigene interactions as compared to duplex interaction sites and noninteracting ERα-bound sites [30]. Together these data define one role of FoxA1 as a crucial collaborator in the formation of higher order transcription regulatory chromatin structures.

Members of the GATA family of transcription factors are also implicated in facilitating chromatin loop formation and maintenance. The GATA binding motif, (A/T)GATA(A/G), is enriched at AR-bound enhancer sites and several studies have shown specific GATA family members to play important functional roles in nuclear hormone receptor-mediated transcription programs [42, 43]. Like FoxA1, GATA family transcription factors appear to induce open chromatin conformation upon binding, facilitating the recruitment of additional factors needed for active gene transcription [39]. Specific to PCa, GATA2 shows clear localization to many AR-bound enhancer sites and appears to be essential for *PSA*, *TMPRSS2*, and *PDE9A* expression [9]. With regards to chromatin loop formation, it is known that GATA1, along with its collaborator, FOG-1, are required for regulatory loop formation, inducing transcription of the β-globin locus [44]. While a definitive role for GATA2 in loop formation has not yet been demonstrated, its interactions with AR and the ability of a similarly acting GATA family member to control chromatin looping suggest a central role for GATA2 in AR-mediated loop formation.

### 4.5.2 AR Coactivators

The activating and repressive, non-DNA-binding coregulators (coactivators and corepressors) of AR-mediated transcription serve broad ranging roles in gene

regulation through interactions with various classes of transcription factors. Relevant to the discussion of AR-mediated chromatin looping, the coactivators associate in a cell type- and context-specific manner with AR to facilitate activating chromatin remodeling and interactions with transcriptional machinery at promoter elements [45]. Recent evidence supports the function of several such coactivating proteins in the formation of regulatory chromatin loops and higher order chromatin structures.

Coactivators of the p160 family of acetyltransferases including SRC1, GRIP1, and AIB1 as well CBP/p300 show enhanced localization to AR-bound enhancer regions as demonstrated by Wang and colleagues [15]. A subsequent study utilized fluorescence in situ hybridization (FISH) and 3C to reveal ERα-bound interchromosomal interactions between the *TFF1* and *GREB1* loci are mediated by these coactivators in MCF7 cells [46]. Indeed, siCBP/p300 or siSRC1 transfection resulted in almost complete loss of this interaction. The similarities in activity between ER and AR along with the colocalization of these coactivators with AR at target gene enhancers suggests a common role of these activities in the formation of higher order chromatin interactions between cotranscribed genes. Similarly, BRG1, a chromatin remodeling protein, has been implicated in GATA1-mediated chromatin looping at the β-globin locus. Mutant *Brg1* fetal livers failed to exhibit locus looping and have reduced β-globin expression [47]. The role of GATA2 in AR-mediated chromatin looping has been suggested above, and the overlapping roles of GATA family transcription factors further supports a potential combinatorial loop formation mechanism involving AR, GATA2, and BRG1.

Important to note is the involvement of the Mediator coregulatory complex in chromatin loop formation. This ~30 subunit complement of evolutionarily conserved proteins in conjunction with cohesin has been shown to bridge the gap between enhancer-bound transcription factors and promoter-bound Pol II as a general mechanism for transcription control [48, 49]. The Mediator subunit MED1 colocalizes with AR at the *PSA* enhancer and is essential for *PSA* mRNA expression [15]. A recent study found that PI3K/AKT phosphorylation of MED1 is crucial for the formation of regulatory chromatin loops between *UBE2C* enhancers and the proximal promoter region at this locus. Silencing MED1 resulted in decreased interaction frequency between regulatory sites and consequent reduction in *UBE2C* expression in CRPC cell line models. Thus, the Mediator complex is demonstrated to play an essential role in the formation of regulatory chromatin loops while the cell type-specificity of this role has yet to be fully elucidated.

### *4.5.3 CTCF*

It is increasingly understood that transcription regulation is largely affected by the three-dimensional organization of chromatin within the nucleus. Higher order intrachromosomal looping and interchromosomal interactions mediate developmental- and cell type-specific gene transcription programs. While the role of AR

in looping-dependent transcription has been presented above, recent evidence suggests that localized chromatin interactions such as those involving AR are largely defined by global chromatin organization governed by CTCF. Genome-wide CTCF binding patterns appear to be evolutionarily conserved, suggesting a regulatory role for the chromatin architecture facilitated by this activity. The diverse role of CTCF in genome organization and transcription regulation has been reviewed by [50]. Recent ChIA-PET analysis revealed extensive chromatin interactions facilitated by CTCF, resulting in delineation of chromatin looping classes based on their observed regulatory function. These classes are described as those that sequester transcriptionally active chromatin within a loop (Class I), those that sequester repressive chromatin signatures within a loop (Class II), those that bring loop-associated enhancers and boundary-associated promoters into close proximity (Class III), and those that serve as boundaries between active and repressive tracts of chromatin [31, 51]. Such trends were corroborated by Pol II patterning, indicating regions of active transcription in agreement with the models of CTCF-mediated regulatory looping.

It was further suggested by Handoko et al. that this global chromatin organization could serve to enhance the otherwise random interaction between promoters and distal enhancers. By sequestering the regulatory elements of a gene locus in a single loop or by facilitating direct enhancer–promoter interactions CTCF can enhance the likelihood and specificity of these interactions, providing a basis for cell type-specific gene transcription [31]. Two very recent studies support this logic. Extensive statistical analysis of the combinatorial control of AR target genes revealed a large subset of androgen-responsive genes that reside within the same CTCF block (region flanked on either side by a CTCF binding event) as a site of overlapping FoxA1, AR, and H3K4me2 occupancy [17]. Prostate-specific expression of this subset of genes and their enrichment in PCa pathways suggests tissue-specific gene expression is efficiently regulated by the combinatorial effect of CTCF-mediated genome organization that places active enhancer elements within the same regulatory chromatin structure as the genes they target. Similarly, recent Hi-C data has provided unprecedented resolution of genome-wide chromatin interactions and revealed a general architecture of approximately 1 Mb-sized chromatin domains characterized by extensive interactions separated by CTCF-enriched boundary domains [29]. With tissue-specific genes, such as those regulated by AR, enriched within the chromatin domains and housekeeping genes, CTCF, and Short Interspersed Nuclear Elements (SINE) enriched in chromatin domain boundaries, the model of global chromatin organization impacting AR-mediated looping and transcription regulation is gaining complexity. As the Pol II-bound promoters of highly transcribed housekeeping genes have been found to colocalize in transcription factories [32], this mechanism of chromatin reorganization along with CTCF remodeling and the effect of conserved, noncoding DNA (such as SINE) may all contribute to AR-mediated looping and more generally to tissue-specific gene expression mediated by global chromatin architecture (Fig. 4.1).

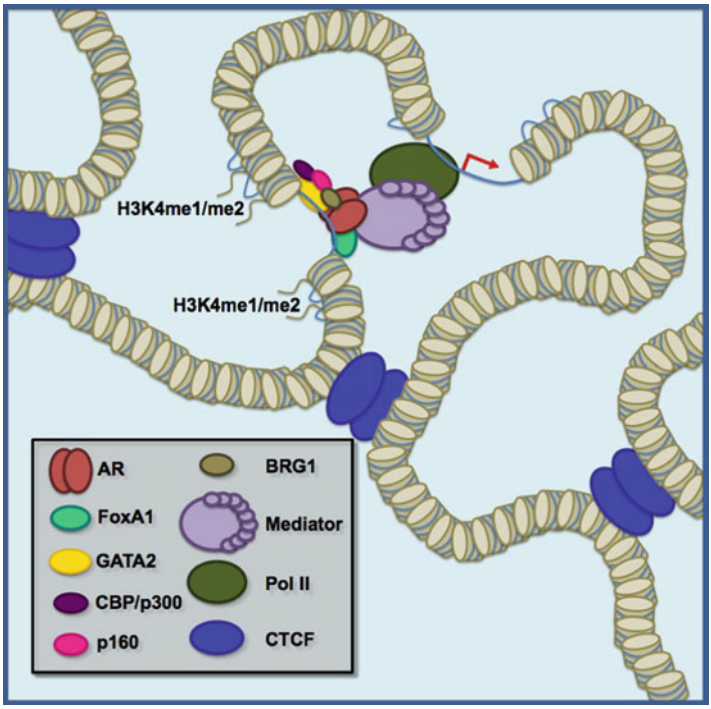


**Fig. 4.1** Proposed model for AR-mediated chromatin looping and transcription within the genome-wide regulatory architecture. CTCF-defined chromatin domains facilitate interactions between distal, AR-bound enhancers, and transcriptional machinery-bound promoters. Permissive histone modifications H3K4me1/me2 mark active enhancer regions, allowing recruitment of collaborating factors and coactivators to further relax and remodel chromatin (FoxA1, GATA2, CBP/p300, BRG1, and p160) and to serve as a scaffold for the regulatory interaction (Mediator)

### *4.5.4 Histone Posttranslational Modification*

Acting in concert with transcription factors and in many cases upstream of them, histone modifications have been found to define transcriptionally active and repressed chromatin regions. Posttranslational modifications to histone residues including acetylation, methylation, phosphorylation, etc., can impact chromatin compaction and permit or prevent transcription factor binding to recognition elements at enhancers and promoters. Global chromatin organization is likely affected by these modifications as CTCF-mediated chromatin interactions divide neatly into classes based on local histone marks [31]. Histone modification signatures also impact genome-wide transcription regulation. Active histone marks were found to correlate with Pol II-bound promoter–promoter and enhancer–promoter interaction sites, correlating with increased expression [32]. Specific to AR-mediated chromatin looping, active histone marks (H3K4me1/me2 and AcH3) overlap with distal

AR-binding sites and define active enhancers [6, 7, 11, 12]. Active histone marks may function upstream of, occur concurrently with, or result from AR, GATA2, or FoxA1 binding, but in all scenarios, permissive histone signatures likely impact recruitment of the AR coregulatory complex that facilitates chromatin looping and target gene expression.

## 4.6 Future Directions

AR activity is under close scrutiny at this time as the cancer field seeks to define its role throughout PCa development and progression. The enigmatic hormone receptor's ability to mediate transcription in the absence of its canonical ligand, from distal binding sites lacking its canonical recognition motif, and in an apparently variable fashion depending on the cell type and disease state under inspection generates many intriguing questions that must be addressed. While the transcription regulatory phenomenon of chromatin looping can explain the distal regulatory function of AR and other nuclear hormone receptors, the requisite list of collaborating transcription factors and coactivators needed to initiate and maintain chromatin loops in various contexts remains to be fully defined. How chromatin looping patterns change in the absence of androgen when AR is observed to bind DNA in the absence of its ligand and mediate a CRPC-specific transcription program has yet to be elucidated. It may be the case that a changing cohort of protein collaborators facilitates the formation of new chromatin loops that enhance the expression of oncogenic genes in advancing PCa. Identification of these activities may inform new therapeutic strategies that prevent the formation of such loops.

The cohort of AR target genes whose transcription depends on the formation of chromatin loops between their regulatory regions has yet to be identified. Such an analysis in various models of PCa is expected to yield unique results identifying genes whose upregulation in CRPC is controlled by the formation of new chromatin structures. Such genes would represent clinically relevant targets as potential facilitators of advanced PCa growth and metastasis. Finally, the impact of global chromatin organization on AR-mediated looping and target gene expression deserves attention. While CTCF-mediated chromatin domain formation appears to be highly conserved, variation does exist. Further analysis may reveal that cancer-associated perturbations to genome-wide organization impact local and interchromosomal AR-mediated chromatin interactions with subsequent alterations to PCa transcription profiles. Ultimately, this model may underlie the high degree of variation in pathways driving PCa between individuals and throughout disease progression.

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# Chapter 5
# The Functionality of Prostate Cancer Predisposition Risk Regions Is Revealed by AR Enhancers

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**Abstract** Prostate Cancer (PCa) genetic risk has recently been defined in numerous genome-wide association studies (GWAS), which revealed more than 50 disease-associated single nucleotide polymorphisms (SNPs), known as tagSNPs, each at a different locus. More than 80% of these tagSNPs are located in noncoding regions of the genome for which functionality remains unknown. We and others hypothesize that at least some of these SNPs affect noncoding genomic regulatory signatures such as enhancers. Many research laboratories including ours have profiled the genomic

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distribution of androgen receptor (AR) and the dynamic state of the PCa genome for active chromatin regions (H3K9,14ac), open chromatin regions (DNaseI), enhancers (H3K4me1/2), and active/engaged enhancers (H3K27ac). In order to identify candidate functional SNPs, which may confer risk associated with PCa, we recently developed an open-source (R/Bioconductor) package, called FunciSNP (Functional Integration of SNPs), which systematically integrates SNPs from the 1000 genomes project with these biologically active chromatin features. Here we report several potential AR enhancers, defined by genome-wide data and from chromatin biofeatures, which may be functionally involved in PCa risk.

**Keywords** Enhancer • Androgen Receptor Occupied Regions (ARORs) • Chromatin • Genome • Single Nucleotide Polymorphism • Post-GWAS function

## Abbreviations

| | |
|---|---|
| AR | Androgen receptor |
| DHT | Dihydrotestosterone |
| ARE | Anrogen responsive element |
| ARORs | Androgen receptor occupied regions |
| GWAS | Genome-wide association studies |
| 1000gp | 1000 genomes project |
| SNP | Single nucleotide polymorphisms |
| FunciSNP | Functional Identification of SNPs |
| LD | Linkage disequilibrium |

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## 5.1 Introduction

Within the past 5 years, prostate cancer (PCa) genome-wide association studies (GWAS) have identified more than 50 risk loci, with more than 80% found in non-coding genomic regions (Fig. 5.1) [1–4]. Integrating data associated with genomic profiles of androgen receptor (AR) occupancy and other enhancers to these known risk regions will inevitably provide an informative genomic landscape that may yield insight and understanding into the etiology and ultimately treatment of PCa. Characterizing and understanding the role of the AR in prostate cancer and the dynamic interactions between AR and nucleosomes is an important area of PCa research [5, 6] and consequently is hypothesized to play "driver" roles at GWAS risk loci. The aim of this chapter is to describe recent research in profiling the AR interactions with genomic features. In addition, we will present and describe the use of a novel bioinformatic tool (developed by our group), which integrates several large-scale data types to identify well-demarcated new genomic risk regions at known risk loci that may have important implications for PCa genetic predisposition.

### *5.1.1 Normal Function of AR in Prostate*

Understanding the role of AR in PCa and the regulation of AR activity remains an intense and important area of research. In normal prostate development and maintenance, AR drives differentiation and regulates the expression of select genes for the production of proteins that mainly function in seminal fluid development, similar to the role estrogen receptor provides in the regulation and production of milk in the breast [7]. Therefore, AR is not normally classified as an oncogene during prostate development and maintenance, whereas its role in prostate tumorigenesis is indisputably as a driver and as such may be viewed as an oncogene during the progression of the disease.

### *5.1.2 AR Significance in PCa*

As early as the 1940s, Dr. Charles B. Huggins (a Nobel laureate) discovered that steroid hormones such as androgen could be used to control the spread of prostate and breast cancer. Orchiectomy, the removal of the male testes, was found to be an effective surgical procedure for the treatment of advanced PCa, later replaced with GnRH agonist [8, 9]. In the 1950s, it was found that patients who relapse after orchiectomy respond better to andrenalectomy, which indicates that the tumors may be stimulated by residual androgens from adrenal glands [8, 9]. From the mid-1990s, multiple two-arm trials looked at the effects of castration versus castration + AR antagonist (such as flutamide, milutamide, cyproterone acetate, and

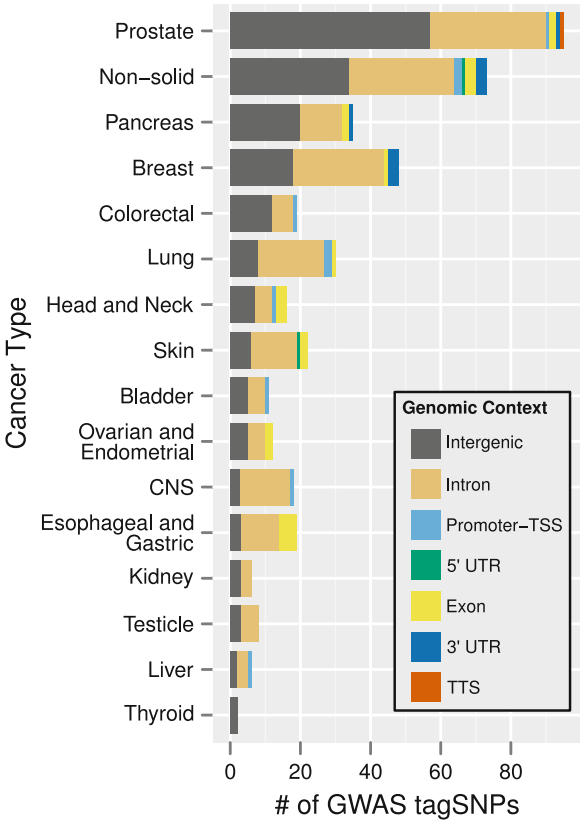


**Fig. 5.1** Summary of all known GWAS SNPs across a number of different cancer types. GWAS SNPs were annotated using known genomic features supplied by HOMER (version 3.9) [43], using build hg19 as reference. GWAS SNPs were extracted from the UCSC Genome table browser, track name "gwasCatalog" with a *P*-value cut-off of 9e–06 [2]. Figure and legend reproduced from Coetzee SG. et al. 2012 [3] with permission from Oxford University Press

bicalutamide) to block effects of residual androgen expression [8, 9]. The hypothesis at the time was to further block AR, which would provide an alternative to castration procedures. After hundreds of millions of dollars invested into researching the effect of these antagonists, it was found that there was minimal improvement, with modest responses to AR antagonists given at relapse after castration, with a general conclusion that more complete AR blockade was no more effective then castration alone [9].

During the same time, basic research labs found that AR and AR regulated genes were highly expressed in castration recurrent/resistant prostate cancer (CRPC). The AR gene was found to be amplified in ~30% of CRPC and AR antagonists selected for AR mutations [7, 10]. By 2000, work by several research groups found that high

intra-tumoral androgen levels in CRPC were synthesized from weak adrenal androgens, and this led to the hypothesis that androgens were being developed in a de novo fashion from cholesterol products [9, 11, 12]. These observations provided important clinical application, and as recent as 2011, survival advantage from post- and pre-chemotheraphy for Abiraterone (CYP17A1 inhibitor, an enzyme for androgen synthesis) in CRPC was found to decrease serum testosterone from ~20–50 ng/dl to <1 ng/dl [11, 12]. These clinical studies provided evidence that AR to some degree is driving PCa progression [11, 12]. In 2012, survival advantage for a novel AR antagonist, MDV3100, was found to be highly selective [13–15]. Despite progressive AR decreasing during tumor progression, tumors have found many genetic means of maintaining AR mediated signaling. We know that most tumors that have become castration resistant remain dependent on AR mediated activity, which has been shown with the advancement of drugs such as MDV3100, which continue to target AR pathway axis [10, 15]. Even more recently, exome sequencing of 50 CRPCs revealed many somatic mutations in the androgen-signaling pathway, DNA repair, and histone/chromatin modifier genes [16]. These and other important findings shed both clinical and biological importance in determining the mechanisms mediating intrinsic and acquired resistance to androgen and AR mediated pathways.

### *5.1.3 Advancement in Sequencing Technology Allows for Comprehensive Profiling of Specific Genomic and Epigenomic Marks*

The last 15 years of biomedical research has been fueled in part by development of new technologies, techniques, and protocols. The recent advent of next-generation sequencing has not only spawned many new exciting applications and opportunities but also challenges for cancer researchers. ChIP-seq is a technique to systematically detect genome-wide protein:DNA interactions and histone modifications. In a given landscape of the genome, there are a variety of diverse proteins that interact with our genome in a sequence and tissue-specific manner. During a typical ChIP-seq experiment, the cells are treated with formaldehyde to stably cross-link all protein:DNA complexes. The chromatin is then sheared and fragmented to approximately 200–1,000 bp by sonication. Generally, such an experiment is done with about 2 million cells to enrich the amount of protein:DNA complex using a specific antibody targeting a protein of interest. Once the purified and enriched DNA fragments are obtained, high-throughput sequencing is performed and fragments of 50–100mers are mapped back to the reference genome. Currently, deep sequencing technology can sequence up to 100 million reads for a given experiment, and this number is very likely to increase exponentially while the overall cost of sequencing will drop over the next 5–10 years [17, 18]. Analyzing deep sequencing data for a particular ChIP experiment requires several important steps, which include initial quality control, peak

calling, peak quality assessment, motif analysis, and large prediction and annotations of the data [17]. There are many different peak calling algorithms available, including MACS2 and FindPeaks [17].

In Sect. 5.2, we will describe the current understanding of AR occupancy regions (ARORs) in prostate cancer cell lines and associated enhancer elements in promoters and non-promoters of genes profiled using deep sequencing technologies. Section 5.3 will introduce the current state of GWAS in PCa and the idea of a novel bioinformatic tool we developed to integrate data profiled from deep sequencing, GWAS, and the 1000 genomes project to identify candidate regulatory elements involved in PCa progression and/or risk.

## 5.2 AROR Identification and Genome-wide Distribution of Enhancer Elements

Myles Brown and his research group was among the first to systematically identify thousands of AR binding sites in PCa cells after DHT treatment by AR chromatin immunopreciptation (ChIP) followed chip hybridization (initially) or by deep sequencing (more recently) (ChIP-seq; described in detail in Sect. 5.1.3) [19]. In addition, we and others profiled AROR in DHT-treated LNCaP cells. We reported that about 20% of histone H3 acetylated at Lys-9 and Lys-14 (H3K9,14ac) enrichment surrounds ARORs [20]. We also observed a high number of AROR peaks distributed in noncoding regions, while a significant enrichment of ARORs was found in promoters of known genes only with overlapping H3K9,14ac (Fig. 5.2a, b). If ARORs overlapping and not overlapping known H3K9,14ac peaks are separated, we observe that the genomic distribution to all known transcription start sites are significantly depleted at promoters for AROR peaks not overlapping H3K9,14ac (Fig. 5.2c, d). Interestingly, AROR peaks overlapping H3K9,14ac are enriched at promoters (Fig. 5.2e). This genomic profile of AROR has given researchers an unprecedented view of the wide distribution of AR binding to the human genome in PCa after DHT stimulation. It has been determined that AR binding sites have AR element (ARE) motif and are generally associated with a consensus motif for FOXA1 [19, 21]. In our own study, we performed de novo motif analysis on our identified AROR peaks. Significantly identified AROR peaks are highly enriched for the known ARE motif, while ARORs located in distant or non-promoters were also enriched for the FOXA1 motif (Fig. 5.3). It has been shown that FOXA1 binds with AR and other steroid receptors in a diverse set of tissues [19]. Among other factors, FOXA1 has been shown to function as a pioneer factor to open the locus prior to AR binding [22]. Further work by the Brown lab and others have found that sites occupied by FOXA1 (and the recruitment of FOXA1) are highly associated with enhancers, since these regions usually have nucleosomes with enhancer histone marks such as H3K4me1 and H3K4me2 [19, 20, 23] (Table 5.1). Prior to androgen stimulation and AR binding, ARE is covered with loosely associated nucleosome that contains a variant of

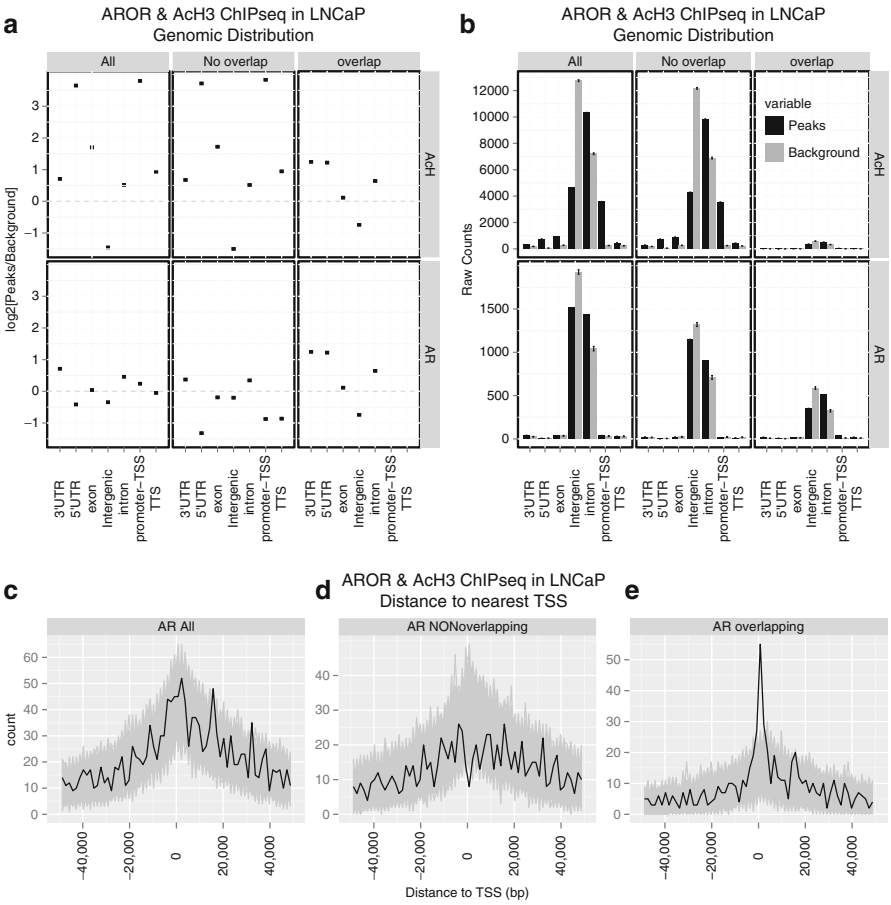


**Fig. 5.2** Complete genomic distribution of AROR and H3K9,14ac. (**a**–**b**) raw counts and log2 ratio (peaks versus background) for H3K9,14ac (AcH) and AR distribution across known genomic regions, 3′UTR, 5′UTR, exon, intergenic, intron, promoter-TSS (promoter transcription start site), TTS (transcription termination site). Each column of plots indicates differing sets of peaks, All—total peaks per type (AcH or AR), No overlap (distinct peak files), overlap (peak files in common between AcH and AR). *Black* color indicates observed peaks, while *gray* colors indicate background as computed 1,000 times (randomly generated regions selected genome wide). (**c**–**e**) Genomic profile of AROR as a function of distance to the nearest canonical gene's TSS. ARORs were divided into (**c**) total, (**d**) AROR not overlapping AcH, (**e**) AROR overlapping AcH. *Red* color indicates observed peaks; while *gray* colors indicate background as computed 1,000 times (randomly generated regions selected genome wide)

H2A.Z which is displaced by AR [24]. At the chromatin level, AR was found enriched at ARE sites associated with H3K4me1 and H3K4me2. These distinct epigenetic marks are highly associated with FOXA1, which provides recruitment in CRPC [25]. This process is reversed by LSD1 (lysine-specific demethylase 1), which mediates demethylation of H3K4me1 and H3K4me2 [25]. Thus it appears the sequence of

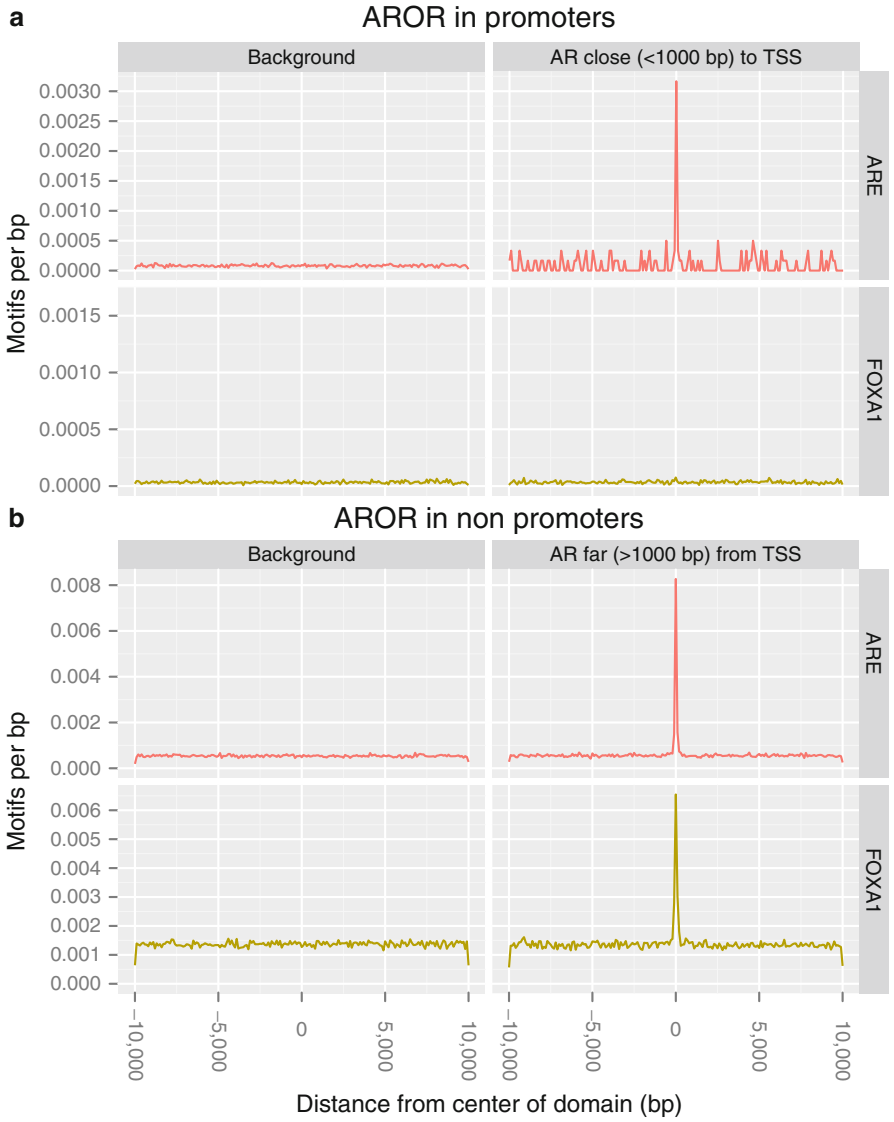


**Fig. 5.3** De novo motif analysis results of ARORs in promoters (<1 kbp from TSS) and non-promoters (>1 kbp from TSS). (**a**–**b**) Genomic profiles are generated using ARORs centered on top two de novo motifs as identified by the ARORs in non-promoters. (**c**) Enrichment table and motif sequence are described

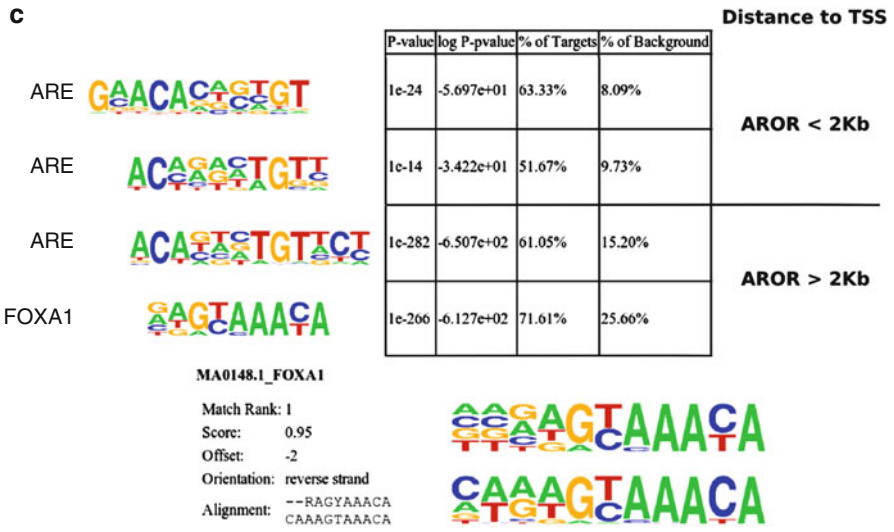


**Fig. 5.3** (continued)

**Table 5.1** Diverse biological features compiled from several different sources. All biofeatures were profiled in LNCaP cells treated with DHT

| Biofeatures | Biological features | Num peaks |
|---|---|---|
| AR [20] | AROR | 3,100 |
| AR [26] | AROR | 6,214 |
| AR [22] | AROR | 205 |
| AR + DNaseI | AROR + Open chromatin | 1,055 |
| AR + H3K9,14ac [20] | AROR + Active/engaged enhancers | 580 |
| AR + HK4me1 | AROR + Enhancers | 456 |
| DNAseI + H3K4me1 + H3K27ac | Open + Active/Enhancers | 5,174 |
| H3K27ac [22] | Active/engaged | 12,578 |
| H3K4me1 [22] | Enhancers | 19,149 |
| H3K9/14ac [20] | Open chromatin | 956 |
| Genomic features (hg19 human build) | | |
| Exons | | 698,453 |
| CTCF Only [30] | | 9,916 |
| DNAseI Only [30] | | 175,507 |
| DNAseI + CTCF [30] | | 4,977 |
| Known promoters (−1000 to +100) | | 39,700 |

events start with the epigenetic alterations of histones, specifically methylation of K4 on the H3 complex, and recruitment of FOXA1, thereby allowing for an open conformational change of the nucleosome, thereby allowing for poised interactions for AR stimulated by DHT to incorporate with ARE, and thereby activate targeted genes responsible for progression and differentiation of the prostate.

Further subsequent findings in advanced castration resistant PCa using LNCaP-abl cells have shown that AR regulates the expression of specific G2/M genes (CDK1, UBE2C, others) in advanced castration resistant but not in androgen sensitive LNCaP cells [23]. In summary, the spectrum of AR regulated genes may change during tumor development and progression.

Recently, it was discovered that FOXA1 suppressive sites are enriched for insulator protein binding sites, which are sites of compact chromatin (CTCF-FOXA1-Groucho family complexes) [26, 27]. They observed, by knocking down FOXA1 followed by ChIPseq of AR binding sites, hundreds of AREs are dependent of FOXA1 while if FOXA1 is deleted, 3,500 AR binding sites are revealed and are independent of FOXA1. These sites are enriched for insulator protein binding sites, CTCF–FOXA1, and set of corepressive elements [26, 27]. The overall physiological importance is unclear, but these studies illustrate that the dynamic integrity of AR binding to the DNA is quite flexible and provides a complex environment with a multitude of diverse functions yet to be fully characterized.

DNaseI hypersensitivity (HS) is a method to assay chromatin accessibility [28]. It allows for a comprehensive profile of distinct genomic regions, which are preferentially unbound by nucleosomes and thus are characterized as open chromatin region. DNaseI sequencing results are aligned to the human genome reference, compared to a background control experiment and peaks are called as described in Sect. 5.1.3, to identify candidate open chromatin regions in LNCaP cells. Most studies have shown that DNaseI hypersensitive sites correlate very well with regulatory elements and enhancer activity in a variety of tissues and cell types [22, 29]. We put together a table, which summarizes the total number of AR and available enhancer peaks across a number of different studies, as well as the overlap with data downloaded from the ENCODE project [30] (Table 5.1).

## 5.3 Identification of Significant Risk Variants Associated with PCa

### *5.3.1 Statistical Analysis and Bioinformatics Tool to Identify Candidate Functional Enhancer Elements*

GWAS have yielded numerous single nucleotide polymorphisms (SNPs) associated with many phenotypes. The main goals for genetic risk factor identification have been to allow clinicians and epidemiologist a factor to predict risk for a disease or treatment such as chemotherapy or surgical prevention. It has also been important to allow researchers an opportunity to understand the biology of cancer by identifying

genes or loci involved in the development of the cancer and finally to identify novel genetic regions or loci for drug targeting and treatment.

The first GWAS study published in 2005 identified a risk region for age-related macular degeneration disease [31], and GWAS have been constantly evolving and expanding their reach in large part due to the advancement of genotyping technology. During the last 6 years, it is apparent that GWAS efforts fostered by these advancements have made significant discoveries, for example, more than 1,449 tagSNPs have been published for association with well over 200 different diseases or traits at a $p$-value of $5 \times 10^{-8}$ [2, 3]. It is also apparent that as new methods and stratifications by populations emerge, these studies could yield even higher numbers of variations associated to a particular disease or trait. Interestingly, approximately 200 genomic variants have been reported for more than 20 different cancer types, and it is expected that this number of associated risk loci will likely exceed 300 by the end of this year [32]. Remarkably, we and others have noted that the vast majority of these variants associated with cancer, in particular with prostate and breast, are enriched in noncoding regions (Table 5.2, Fig. 5.1) [3]. Despite the many novel and insightful biological discoveries GWAS have provided to the scientific community, we have a long way to go before we can fully understand genetic heritability and risk. In the case of breast cancer, there are very rare allele frequencies in a given population with very high effect size, many identified through family-based studies, candidate gene re-sequencing, and regions with candidate risk association. The rare variance contributes on total, over 25% to understanding the familial risk of most cancer whereas only 10–15% contributes to common risk with low to modest effect size [33]. However, the field is currently looking to see if the risk variant for low-to-common allele frequency can be identified with intermediate to large effect size, which is believed to be largely due to genetic factors. Currently, with the advancement in sequencing technology and methodology, we are at the forefront of making discoveries of these low to common variants with measurable effect size with suitable power. And as the advancement of sequencing technology increases and the cost to perform such high-throughput deep sequencing reaches a critical cost–benefit level, the major bottleneck will be computational methods and tools needed in order to tease out the genetic variants and risk associated with many of these critically debilitating human diseases.

At the core of all association studies dominated during the past 6–7 years is the idea of linkage disequilibrium (LD). LD is the observation that two or more alleles in a population segregating together more often than one would expect by chance [3, 34]. What this definition means to researchers interested in identifying genetic basis or identify candidate functional variants in a particular disease is that the identity of the functional variant is not required. As an example, a causal or putative functional variant is located on the ancestral allele, and this variant will segregate successfully through the population over time. If we are able to visualize this variant, then we can measure LD and therefore indirectly identify the variant using markers or tags surrounding the causal or putative functional variant [35, 36].

SNPs are the most common form of variation we have measured in the human population, and therefore SNP markers provide the best opportunity to identify the

**Table 5.2** Reported GWAS tagSNP associated with PCa

| SNP rsID | Chrom | StartPos | Genomic region | Genomic context | Nearest gene | Risk allele freq. | *p*-value | OR | 95% CI | Reference (PMID) |
|---|---|---|---|---|---|---|---|---|---|---|
| rs13385191 | chr02 | 20888265 | 2p24.1 | Intron | C2orf43 | 0.44 | 8.00E-08 | 1.15 | [1.10–1.21] | [44] |
| rs1465618 | chr02 | 43553949 | 2p21 | Intron | THADA | 0.23 | 2.00E-08 | 1.08 | [1.03–1.12] | [45] |
| rs6545977 | chr02 | 63301164 | 2p15 | Intergenic | OTX1—RPL27P5 | – | 5.00E-07 | – | – | [45] |
| rs10187424 | chr02 | 85794297 | 2p11.2 | Intergenic | GGCX—VAMP8 | 0.59 | 3.00E-15 | 1.09 | [1.06–1.12] | [46] |
| rs12621278 | chr02 | 173311553 | 2q31.1 | Intron | ITGA6 | 0.94 | 9.00E-23 | 1.33 | [1.25–1.43] | [45] |
| rs2292884 | chr02 | 238443226 | 2q37.3 | Intron | MLPH | 0.25 | 4.00E-08 | 1.14 | [1.09–1.19] | [47] |
| rs9311171 | chr03 | 37996477 | 3p22.2 | Intron | CTDSPL | – | 2.00E-06 | – | – | [48] |
| rs17181170 | chr03 | 87173324 | 3p12.1 | Intergenic | VGLL3—CHMP2B | – | 3.00E-08 | – | – | [45] |
| rs10934853 | chr03 | 128038373 | 3q21.3 | Intron | EEFSEC | 0.28 | 3.00E-10 | 1.12 | [1.08–1.16] | [49] |
| rs6763931 | chr03 | 141102833 | 3q23 | Intron | ZBTB38 | 0.45 | 2.00E-08 | 1.04 | [1.01–1.07] | [46] |
| rs345013 | chr03 | 145173788 | 3q24 | Intergenic | C3orf58—GM2AP1 | – | 5.00E-06 | – | – | [48] |
| rs10936632 | chr03 | 170130102 | 3q26.2 | Intergenic | SKIL—CLDN11 | 0.52 | 7.00E-22 | 1.11 | [1.08–1.14] | [46] |
| rs17021918 | chr04 | 95562877 | 4q22.3 | Intron | PDLIM5 | 0.65 | 4.00E-15 | 1.11 | [1.08–1.15] | [45] |
| rs7679673 | chr04 | 106061534 | 4q24 | Intergenic | RPL6P14—TET2 | 0.55 | 3.00E-14 | 1.1 | [1.06–1.14] | [45] |
| rs2242652 | chr05 | 1280028 | 5p15.33 | Intron | TERT | 0.81 | 3.00E-24 | 1.15 | [1.11–1.19] | [46] |
| rs12653946 | chr05 | 1895829 | 5p15.33 | Intergenic | IRX4—IRX2 | 0.44 | 4.00E-18 | 1.26 | [1.20–1.33] | [44] |
| rs2121875 | chr05 | 44365545 | 5p12 | Intron | FGF10 | 0.34 | 4.00E-08 | 1.05 | [1.02–1.08] | [46] |
| rs4466137 | chr05 | 82985739 | 5q14.3 | Intron | HAPLN1 | – | 3.00E-06 | – | – | [48] |
| rs37181 | chr05 | 115630003 | 5q22.3 | Intergenic | COMMD10 | – | 0.007 | 1.6 | [1.04–1.30] | [46] |
| rs130067 | chr06 | 31118511 | 6p21.33 | Missense | CCHCR1 | 0.21 | 3.00E-08 | 1.05 | [1.02–1.09] | [46] |
| rs1983891 | chr06 | 41536427 | 6p21.1 | Intron | FOXP4 | 0.41 | 8.00E-08 | 1.15 | [1.09–1.21] | [44] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| rs10498792 | chr06 | 51666631 | 6p12.3 | Intron | PKHD1 | – | 3.00E-06 | – | – | [48] |
| rs339331 | chr06 | 117210052 | 6q22.1 | Intron | RFX6 | 0.37 | 2.00E-12 | 1.22 | [1.15–1.28] | [44] |
| rs9364554 | chr06 | 160833664 | 6q25.3 | Intron | SLC22A3 | 0.29 | 6.00E-10 | 1.17 | [1.08–1.26] | [50] |
| rs12155172 | chr07 | 20994491 | 7p15.3 | Intergenic | RPS26P30—ASS1P11 | 0.2 | 9.00E-06 | 1.05 | [1.00–1.10] | [45] |
| rs10486567 | chr07 | 27976563 | 7p15.2 | Intron | JAZF1 | 0.77 | 2.00E-06 | 1.12 | [1.02–1.25] | [51] |
| rs6465657 | chr07 | 97816327 | 7q21.3 | Intron | LMTK2 | 0.46 | 1.05E-08 | 1.12 | [1.05–1.20] | [45, 50] |
| rs1512268 | chr08 | 23526463 | 8p21.2 | Intergenic | SLC25A37—NKX3-1 | 0.45 | 2.00E-11 | 1.18 | [1.14–1.22] | [44, 45] |
| rs10086908 | chr08 | 128011936 | 8q24.21 | Intergenic | POU5F1B | – | | 1.7 | [1.39–2.07] | [52] |
| rs1447295 | chr08 | 128485038 | 8q24.21 | Intergenic | POU5F1B—MYC | 0.1 | 6.70E-17 | 1.53 | [1.29–1.77] | [49, 53, 54] |
| rs1571801 | chr09 | 124427372 | 9q33.2 | Intron | DAB2IP | – | 0.001 | 1.36 | [1.13–1.65] | [55] |
| rs10993994 | chr10 | 51549496 | 10q11.23 | nearGene | MSMB | 0.4 | 1.26E-06 | 1.19 | [1.04–1.34] | [44, 47, 50, 51] |
| rs4962416 | chr10 | 126696872 | 10q26.13 | Intron | CTBP2 | 0.27 | 2.00E-07 | 1.17 | [1.05–1.30] | [51] |
| rs7127900 | chr11 | 2233574 | 11p15.5 | Intergenic | TH—ASCL2 | 0.2 | 3.00E-33 | 1.22 | [1.17–1.27] | [45] |
| rs10896469 | chr11 | 56309467 | 11q12.1 | TTS | OR5M11 | – | | – | – | [56] |
| rs12418451 | chr11 | 68935418 | 11q13 | Intergenic | TPCN2 | – | 1.20E-06 | – | – | [57, 58] |
| rs731236 | chr12 | 48238756 | 12q13.11 | Exon | HDAC7 | – | 0.05 | – | – | [59] |
| rs10875943 | chr12 | 49676010 | 12q13.12 | Intergenic | TUBA1C—PRPH | 0.31 | 7.00E-12 | 1.07 | [1.04–1.10] | [46] |
| rs902774 | chr12 | 53273904 | 12q13.13 | Intergenic | KRT78—RPL7P41 | 0.17 | 5.00E-09 | 1.17 | [1.11–1.24] | [47] |
| rs12827748 | chr12 | 80088577 | 12q21.2 | Intergenic | PAWR | – | 0.02 | 1.6 | [1.1–2.4] | [59] |
| rs9600079 | chr13 | 73728139 | 13q22.1 | Intergenic | FABP5L1—KLF12 | 0.38 | 3.00E-09 | 1.18 | [1.12–1.24] | [44] |

(continued)

**Table 5.2** (continued)

| SNP rsID | Chrom | StartPos | Genomic region | Genomic context | Nearest gene | Risk allele freq. | *p*-value | OR | 95% CI | Reference (PMID) |
|---|---|---|---|---|---|---|---|---|---|---|
| rs1529276 | chr13 | 103928007 | 13q33.1 | Intergenic | SLC10A2—RPL7P45 | – | 2.00E-06 | – | – | [48] |
| rs11649743 | chr17 | 36074978 | 17q12 | Intron | HNF1B | – | 1.70E-09 | – | – | [60] |
| rs7210100 | chr17 | 47436749 | 17q21.33 | Intron | ZNF652 | 0.05 | 3.00E-13 | 1.51 | [1.35–1.69] | [61] |
| rs1859962 | chr17 | 69108753 | 17q24.3 | Intergenic | CALM2P1—SOX9 | 0.46 | 2.50E-07 | 1.24 | [1.14–1.37] | [45, 47, 50] |
| rs8102476 | chr19 | 38735613 | 19q13.2 | Intergenic | DPF1—PPP1R14A | 0.54 | 2.00E-11 | 1.12 | [1.08–1.15] | [49] |
| rs887391 | chr19 | 41985623 | 19q13 | Intergenic | C19orf69 | – | 9.40E-04 | – | – | [62] |
| rs2735839 | chr19 | 51364623 | 19q13.33 | Intergenic | KLK3—KLK2 | 0.85 | 2.00E-18 | 1.2 | [1.10–1.33] | [50] |
| rs9623117 | chr22 | 40452119 | 22q13.1 | Intron | TNRC6B | 0.21 | 5.00E-07 | 1.18 | [1.11–1.26] | [63] |
| rs5759167 | chr22 | 43500212 | 22q13.2 | Intergenic | RPS25P10—BIK | 0.53 | 6.00E-29 | 1.16 | [1.14–1.20] | [45] |
| rs5919432 | chrX | 67021550 | Xq12 | Intergenic | AR—OPHN1 | 0.81 | 1.00E-08 | 1.06 | [1.02–1.12] | [46] |

ancestral causing or functional allele in a given population exhibiting a shared or common disease or trait. However, as the ancestral causing allele passes through time in a population (over numerous generations), the LD breaks down significantly. This breakdown in LD structure then makes identification of variant causing allele more difficult. Therefore, a systematic identification of all known SNPs or variants is required and to this end, the HapMap project evolved. The HapMap project aim was to identify SNPs using four different human populations to put together a phased haplotype block map and identifiable LD blocks of the entire human genome [37]. This project was successful at the time, identifying 4–5 million SNPs in the profiled population. Although these blocks identified regions of interest, it did not successfully identify the candidate or functional variant [38]. Therefore, the 1000 genomes project (1000 gp) emerged to tackle the issue by harnessing the obvious observation that the world's population is quite diverse than the original four populations profiled from the HapMap project [39]. The 1000 gp set out to sequence more than 1,000 individuals across a larger number of different populations. Currently, the 1000 gp have identified more than 60 million SNPs, indels, somatic mutations, and other genomic variants across the population [39]. This has now allowed us to identify a larger number of variants among a given haploblock containing the identified risk allele. Some have argued this increase in variants coupled with unidentified functional variant provides an even bigger challenge in identifying the causal or functional variant. However, we and others have noted that this increase in number of identified variants provides a deeper resolution of the heritability and thus allows us to harness more informative variants than previously identified [3, 40]. We developed a bioinformatic tool called FunciSNP (Functional Identification of SNP), which allows us to harness the information provided by the 1000 gp, the location of the previously reported risk allele (tagSNP), and the chromatin features to identify the causal and functional regions associated with PCa [3] (Fig. 5.4b) (for results, see Sect. 5.3.3–5.3.4).

### *5.3.2 GWAS PCa*

PCa genetic risk has recently been defined in a number of GWAS, which have revealed 51 disease-associated SNPs, known as tagSNPs, each at a different locus (Table 5.2, Figs. 5.1 and 5.4a). More than 80% of these tagSNPs are in intergenic or intron regions of the genome for which functionality remains unknown. Interestingly, the tagSNPs are distributed among many chromosomes (Fig. 5.4a) with the exception being that chromosomes 1, 14, 15, 16, 18, 20, and 21 do not contain any tagSNPs. They occur at relatively gene rich areas (inner circle of Fig. 5.4a), with the notable exceptions of the tagSNPs at chromosome 3 (middle SNP), 8q, and the distal SNP at 12q. Many tagSNPs track closely with chromatin biofeatures (outer four circles of Fig. 5.4a, and see below).

Taking the 1000 genomes data into consideration, more than 10,000 correlated SNPs are revealed, which each define risk due to linkage disequilibrium (correlated

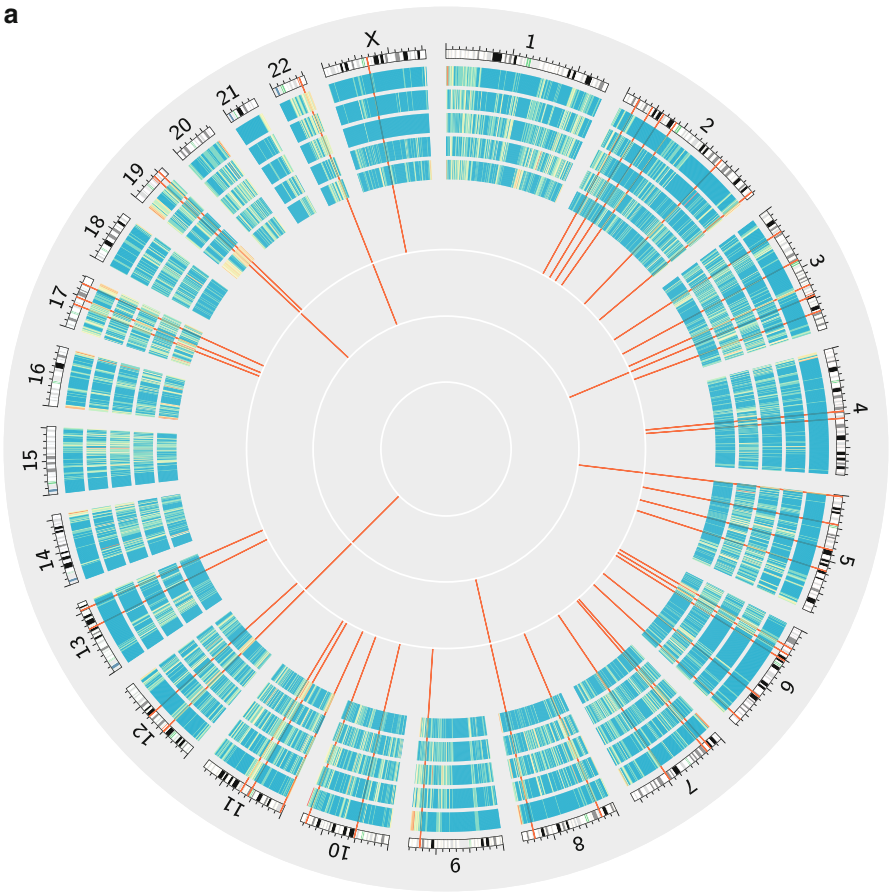


**Fig. 5.4** GWAS integration with biological features. (**a**) Circos plot illustrating genomic distribution of the 51 known GWAS tagSNPs as of June 2012 (*red lines* indicate the position of the tagSNPs with the length reflecting the number of independent tagSNPs within a 10 MB window. The *white concentric circles* are markers for 1, 2, and 3 SNPs; outside to inside). From outermost to innermost, the colored bands describe the concentration (*red, yellow, green, blue*; high to low) DNase1 sensitivity, H3K27ac, H3K4m31, AROR, and genes, respectively. The chromatin marks were obtained from LNCaP cells. On the ideograms the locations of the centromeres are depicted in *green* and tagSNPs in *red*. (**b**) Schematic flowchart to describe FunciSNP. *Purple boxes* represent process before integration with biofeature. *Red boxes* represent information after integration with biofeature (Figure and legend reproduced from Coetzee SG. et al. 2012 [3] with permission from Oxford University Press.). (**c**) Schematic diagram indicating total number of candidate SNPs pooled from the 1000 gp that overlap one or more biological genomic features and which are in linkage disequilibrium to the original GWAS SNP. Total number of SNPs in each section is identified by a *blue box*. (**d**) Overall distribution of $R^2$ values for all identified 1000 gp SNPs overlapping at least one biological feature. Each identified bin reports total number of candidate 1000 gp SNPs

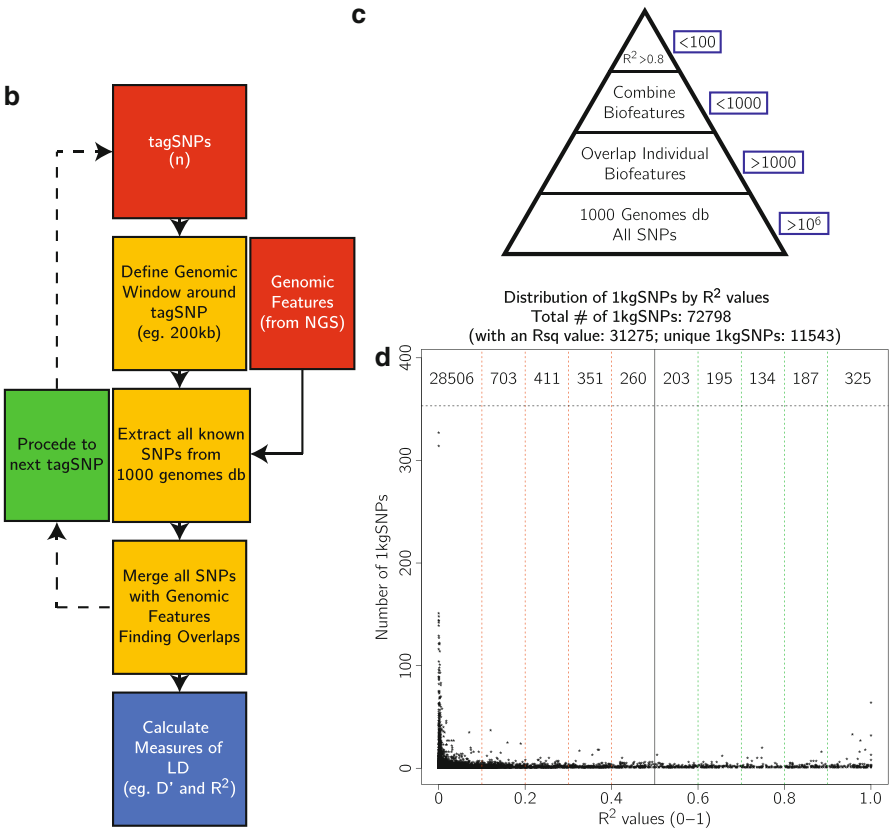


**Fig. 5.4** (continued)

to the tagSNP with an $R^2$>0.8, Fig. 5.4c). The above therefore makes the identification of functional and/or causal SNPs not a trivial task. Therefore, we hypothesize that at least some of these SNPs affect noncoding genomic regulatory signatures, such as enhancers or insulators. As we described in Sect. 6.2, there are distinct regions in the genome profiled in PCa cells, which we and others have identified and have significant biological relevance in PCa.

### 5.3.3 *FunciSNP Integrates AROR, Enhancer, GWAS and 1000gp to Identify Candidate Functional Elements*

In order to reduce the number of candidate functional SNPs profiled in the 1000gp linked to the associated tagSNP in PCa, we developed an open-source (R/Bioconductor) package, called FunciSNP, which systematically integrates the

1000gp SNP data with chromatin features of interest [3]. To define functionality in noncoding DNA associated with PCa risk, we extracted open chromatin and enhancer features generated by next-generation sequencing technologies. The open chromatin state (DnaseI, H3K9,14ac), enhancers (H3K4me1), and active/engaged enhancers (H3K27ac) were generated either by our lab [20], or harvested from the ENCODE project [41], or retrieved from recent publications [22, 26] (Table 5.1 and visualized in Fig. 5.4a). All chromatin features were identified in the same PCa cell line (LNCaP). We identified 113 PCa risk correlated SNPs at androgen receptor occupied regions (ARORs), 1,545 at DNase1 sensitivity sites, and 403 at histone modified regions [H3K4me1 (160 SNPs), H3K9,14 ac (121 SNPs), H3K27ac (122 SNPs)]; all features excluded transcription start sites of known annotated genes (Figs. 5.4d, 5.5, 5.6a). ARORs coinciding with DNase1 sites revealed four novel SNPs correlated with four GWAS tagSNPs. Of the four novel surrogate SNPs, two are located 4 kb upstream from KLK3, one is located within the 3'UTR of NKX3.1, and one is located within an intron of RUVBL1 (Fig. 5.6a; http://goo.gl/cYCl7). DNase1 sites coinciding with any histone modification provided 12 novel risk regions correlated with 10 GWAS tagSNPs (Tables 5.3 and 5.4). Four are located in 8q24 genomic region and each is more than 100 kb away from a known annotated gene.

### *5.3.4 Enhancer Validation in Two PCa Cell Lines and in Primary Prostate Epithelial Cells*

To measure potential enhancer activities in the eight chosen potential enhancer regions, we employed a firefly luciferase reporter (driven by a basal tk-promoter) into which ~1.2 kb potential enhancers coinciding with PCa risk correlated SNPs were cloned. These vectors, along with renilla luciferase controls, were transfected into two PCa cell lines: LNCaP cells, PC3 cells along with an AR expression vector, and normal primary prostate epithelial cells (PREC) along with an AR expression vector and stimulated with DHT. Enhancer activities were measured as previously described [42] and compared with two negative controls (regions with no enhancer histone marks) and a positive control (PSA enhancer). DHT-stimulated enhancer activity for seven of the eight potential enhancers was evident in at least one cell type (the exception is F26), indicating that the identification of enhancers using our protocol is both high and specific (Fig. 5.6b). Further analyses are required to determine the role(s) of these enhancers in PCa risk.

## 5.4 Future Perspective/Discussion

During the past decade three major findings in human genetics/genomics have caused a dramatic change in our appreciation of our genome and how it functions. First, the human genome project revealed that humans have only some 22,000 genes

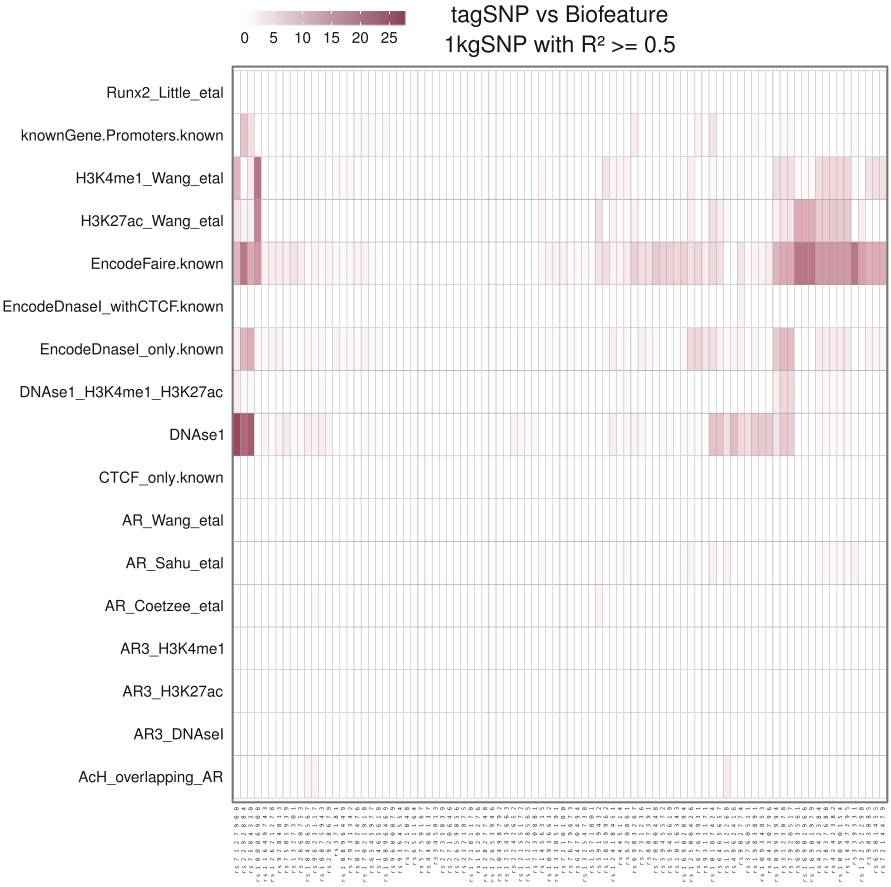


**Fig. 5.5** FunciSNP heatmap of the number of 1000 gp between tagSNP and biofeature for PCa. Total number of candidate SNP is listed by color within each quadrant to represent the number of potential candidate functional SNPs overlapping a biofeature (*y*-axis), which are in linkage disequilibrium to the original GWAS risk SNP (*x*-axis)

within the 3 billion-nucleotide genome. Second, many transcription factors (such as the AR discussed above) do not preferentially occupy gene promoters preferentially but are rather scattered among the many noncoding stretches of DNA in introns and intergenic regions. Third, as pointed out above, GWAS signals for complex diseases are preferentially found in introns and intergenic regions. These three novel insights have pointed to the importance of so-called noncoding DNA (previously even referred to as junk DNA). Transcription factor occupancy, histone modification marks, and nucleosome-depleted regions in chromatin are presently used to annotate functionality within most of our genome. Ultimately this will yield important insight to understand gene expression, genetic risk of complex diseases, and genetic associations with most human phenotypes.

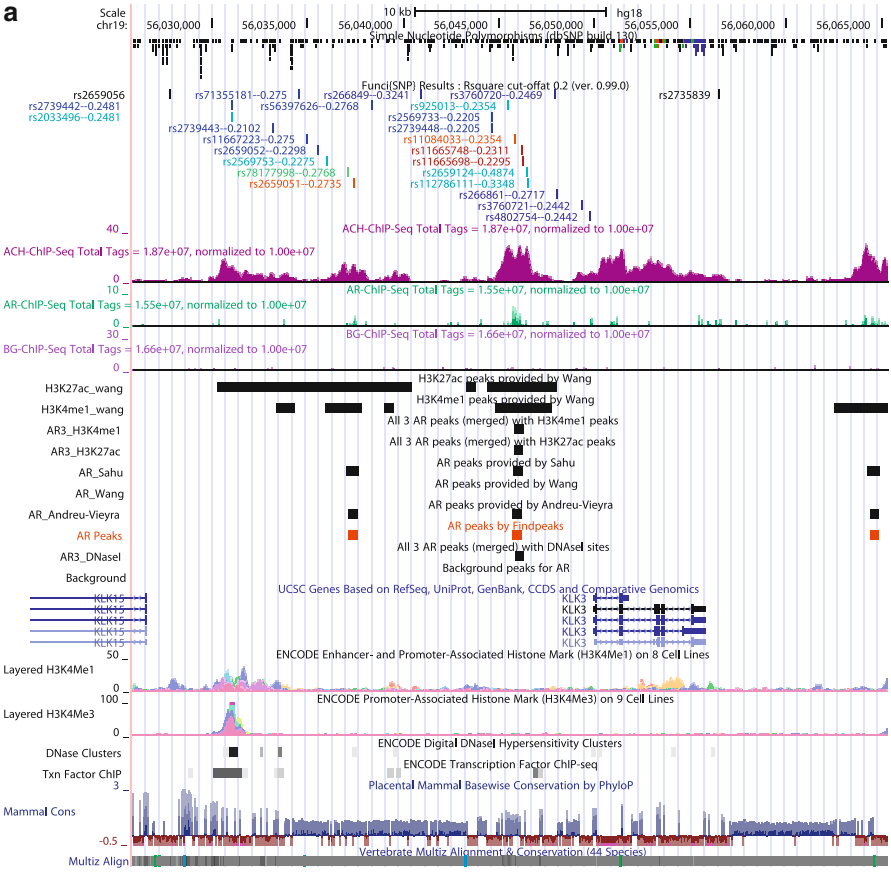


**Fig. 5.6** Genomic plots with FunciSNP results and independent validation on eight enhancer elements harboring a candidate functional SNP, performed in three different cell types. (**a**) UCSC genome browser tracks are ordered in the following manner: dbSNP135, FunciSNP result, biofeatures, refseq genes, and known lincRNA. TagSNP is highlighted in the FunciSNP result track, and each candidate SNP is color coded to reflect the number of biofeatures which it overlaps. The color ranges from *blue* (low number of biofeature overlap) to *red* (high number of overlap). Each candidate function SNP is identified by its known rsID and the calculated $R^2$ value to the known GWAS tagSNP. The results are saved in a UCSC genome session: http://goo.gl/cYCl7. (**b**) Enhancer activities were evaluated using a dual luciferase reporter assay by cloning eight candidate enhancer regions harboring a candidate functional SNP in three different prostate cell types [LNCaP, PC3 (+AR), and PREC (normal primary prostate epithelial cells) (+AR)]. In order to stimulate AR, prostate cells were treated with 10 nM DHT. *Orange–pink* color represents cells treated with DHT and *green–blue* represents cells treated without DHT. Enhancer activities of positive control region (PSA enhancer) ($n=1$) and negative control regions ($n=2$) are presented to the *left*. *Dashed horizontal line* indicates the average enhancer activities of the two negative controls. *Error bar* indicates the standard deviation from the mean of luciferase activity values from four independent experimental replicates

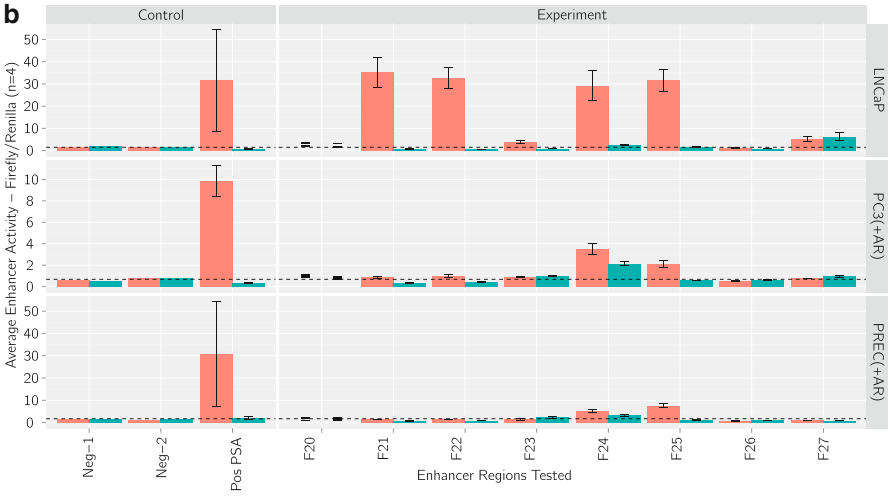


**Fig. 5.6** (continued)

**Table 5.3** Distribution of total number of 1000 gp overlapping at least one biological features as defined in Table 5.1

| | Total SNPs tested | Total number of SNPs coincide with biofeatures (R.squared>0) | Total number of correlated SNPs coincide with biofeatures (R.squared>0.5) |
|---|---|---|---|
| Number of 1000 genome SNPs | 53453 | 20885 | 431 |
| Percent (%) | 100 | 39 | 0.8 |

**Table 5.4** Number of correlated SNPs coincides with biofeatures distributed by each GWAS SNPs for PCa

| Column1 | 1 or more biofeatures | 2 or more biofeatures | 3 or more biofeatures | 4 or more biofeatures | 5 or more biofeatures | 6 or more biofeatures |
|---|---|---|---|---|---|---|
| rs10086908 | 32 | 18 | 4 | 0 | 0 | 0 |
| rs10090154 | 18 | 9 | 6 | 1 | 0 | 0 |
| rs1016343 | 7 | 1 | 0 | 0 | 0 | 0 |
| rs10187424 | 18 | 5 | 0 | 0 | 0 | 0 |
| rs10486567 | 4 | 0 | 0 | 0 | 0 | 0 |
| rs10875943 | 3 | 1 | 1 | 1 | 0 | 0 |
| rs10896449 | 3 | 0 | 0 | 0 | 0 | 0 |
| rs10896469 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs10934853 | 2 | 0 | 0 | 0 | 0 | 0 |
| rs10936632 | 3 | 0 | 0 | 0 | 0 | 0 |

(continued)

**Table 5.4** (continued)

| Column1 | 1 or more biofeatures | 2 or more biofeatures | 3 or more biofeatures | 4 or more biofeatures | 5 or more biofeatures | 6 or more biofeatures |
|---|---|---|---|---|---|---|
| rs10993994 | 19 | 6 | 2 | 1 | 1 | 0 |
| rs11649743 | 2 | 1 | 0 | 0 | 0 | 0 |
| rs12155172 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs12418451 | 7 | 2 | 0 | 0 | 0 | 0 |
| rs12500426 | 12 | 7 | 0 | 0 | 0 | 0 |
| rs12621278 | 5 | 0 | 0 | 0 | 0 | 0 |
| rs13252298 | 17 | 0 | 0 | 0 | 0 | 0 |
| rs13254738 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs1327301 | 4 | 0 | 0 | 0 | 0 | 0 |
| rs13385191 | 2 | 0 | 0 | 0 | 0 | 0 |
| rs1447295 | 18 | 9 | 6 | 1 | 0 | 0 |
| rs1456315 | 1 | 1 | 1 | 1 | 0 | 0 |
| rs1465618 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs1512268 | 6 | 3 | 1 | 0 | 0 | 0 |
| rs1571801 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs16901966 | 27 | 6 | 0 | 0 | 0 | 0 |
| rs16901979 | 27 | 6 | 0 | 0 | 0 | 0 |
| rs16902094 | 8 | 1 | 1 | 1 | 0 | 0 |
| rs17021918 | 5 | 0 | 0 | 0 | 0 | 0 |
| rs1859962 | 9 | 1 | 0 | 0 | 0 | 0 |
| rs1983891 | 2 | 2 | 1 | 0 | 0 | 0 |
| rs2292884 | 37 | 6 | 1 | 0 | 0 | 0 |
| rs2660753 | 4 | 0 | 0 | 0 | 0 | 0 |
| rs2928679 | 4 | 1 | 0 | 0 | 0 | 0 |
| rs3123078 | 24 | 8 | 4 | 2 | 1 | 0 |
| rs339331 | 24 | 0 | 0 | 0 | 0 | 0 |
| rs37181 | 3 | 1 | 0 | 0 | 0 | 0 |
| rs3760511 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs4242382 | 18 | 9 | 6 | 1 | 0 | 0 |
| rs4242384 | 17 | 9 | 6 | 1 | 0 | 0 |
| rs4430796 | 3 | 0 | 0 | 0 | 0 | 0 |
| rs445114 | 4 | 1 | 1 | 1 | 1 | 0 |
| rs4962416 | 2 | 0 | 0 | 0 | 0 | 0 |
| rs5919432 | 9 | 3 | 1 | 0 | 0 | 0 |
| rs5945572 | 7 | 0 | 0 | 0 | 0 | 0 |
| rs5945619 | 7 | 0 | 0 | 0 | 0 | 0 |
| rs620861 | 5 | 1 | 1 | 1 | 1 | 0 |
| rs6465657 | 9 | 1 | 0 | 0 | 0 | 0 |
| rs6470494 | 1 | 0 | 0 | 0 | 0 | 0 |
| rs6501455 | 17 | 2 | 0 | 0 | 0 | 0 |
| rs6545977 | 1 | 1 | 1 | 0 | 0 | 0 |
| rs6763931 | 9 | 4 | 1 | 0 | 0 | 0 |
| rs6983267 | 8 | 4 | 0 | 0 | 0 | 0 |
| rs6983561 | 27 | 6 | 0 | 0 | 0 | 0 |

(continued)

**Table 5.4** (continued)

| Column1 | 1 or more biofeatures | 2 or more biofeatures | 3 or more biofeatures | 4 or more biofeatures | 5 or more biofeatures | 6 or more biofeatures |
|---|---|---|---|---|---|---|
| rs7000448 | 8 | 1 | 0 | 0 | 0 | 0 |
| rs7127900 | 24 | 5 | 3 | 0 | 0 | 0 |
| rs7210100 | 2 | 0 | 0 | 0 | 0 | 0 |
| rs7214479 | 18 | 2 | 0 | 0 | 0 | 0 |
| rs731236 | 6 | 1 | 0 | 0 | 0 | 0 |
| rs7501939 | 3 | 0 | 0 | 0 | 0 | 0 |
| rs7584330 | 29 | 6 | 1 | 0 | 0 | 0 |
| rs7679673 | 2 | 0 | 0 | 0 | 0 | 0 |
| rs7837688 | 18 | 9 | 6 | 1 | 0 | 0 |
| rs7920517 | 24 | 8 | 4 | 2 | 1 | 0 |
| rs7931342 | 3 | 0 | 0 | 0 | 0 | 0 |
| rs8102476 | 4 | 0 | 0 | 0 | 0 | 0 |
| rs887391 | 5 | 1 | 0 | 0 | 0 | 0 |
| rs902774 | 5 | 4 | 0 | 0 | 0 | 0 |
| rs9284813 | 4 | 1 | 0 | 0 | 0 | 0 |
| rs9311171 | 5 | 2 | 0 | 0 | 0 | 0 |
| rs9623117 | 3 | 1 | 1 | 1 | 1 | 1 |
| rs983085 | 16 | 2 | 0 | 0 | 0 | 0 |

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# Chapter 6
# Mechanisms of ARE-Independent Gene Activation by the Androgen Receptor in Prostate Cancer Cells: Potential Targets for Better Intervention Strategies

**Manohar Ratnam, Mugdha Patki, Mesfin Gonit, and Robert Trumbly**

**Abstract** Molecular mechanisms that redirect androgen or AR action to primarily support growth in prostate cancer (PC) cells are not adequately understood. In PC cells in which AR supports robust cell growth in the absence of hormone, AR is localized in the nucleus independent of hormone; still, in these cells androgen is required for activation of its classical target genes that involves AR binding to canonical or noncanonical androgen response elements (AREs). However, following either hormone-dependent or -independent nuclear translocation, AR activates a distinct set of critical growth genes in a ligand-insensitive manner through putative tethered associations of AR with chromatin. Consistent with these observations, splice variants of AR that lack the ligand binding domain support PC growth by activating a transcriptional program distinct from that induced by androgen plus full length AR. Indeed, several studies suggest that specific AR tethering proteins help to redirect AR toward targeting gene sets appropriate to the physiological context. These proteins may also simultaneously suppress the activation of other genes by AR. This review describes how transcriptional signaling by AR is directed by other chromatin bound transcription factors, comprising the AR "tetherome," that could

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work either in concert with AREs or completely independent of them. The potential utility of specific tether-dependent growth signaling mechanisms of AR as tumor-selective drug targets in both early stage and advanced prostate cancer is discussed.

**Keywords** Androgen • Androgen receptor • Androgen response element • Prostate cancer • Elk1 • Ternary complex factor • Tethering protein • Tetherome

## 6.1 The Functional Status of Androgen and Its Receptor in Different Cellular Contexts of Prostate Cancer

A unique feature of prostate oncogenesis is its dependence on stimulation by androgen, which acts by binding to and activating transcriptional signaling by the androgen receptor (AR). Strong preclinical and cumulative clinical evidence support the premise that both early stage and advanced prostate tumors are generally dependent on AR for growth [1–6]. Castrate recurrent prostate cancer (CRPC) has been linked to development of hypersensitivity to castrate levels of androgen, activation of AR without the need for binding androgen, or alternate pathways of intratumoral dihydrotestosterone biosynthesis [7–13]. Growth signaling by AR may be sustained in CRPC through molecular mechanisms that involve amplification or mutation of AR, cross-talk with certain signaling pathways, or through alterations in the AR coregulator complement [11]. An important function of hormone binding to AR is to cause nuclear localization of AR that is essential for its transcriptional activity. However, in PC cells that are adapted to grow in the absence of hormone, the AR apoprotein is localized in an active form in the nucleus, where it is transcriptionally active in the absence of hormone [13, 14]. CRPC cells may acquire the ability to localize adequate AR to the nucleus by the combination of AR amplification and low levels of intratumoral androgen or by completely hormone-independent phosphorylation of AR through hyperactivated signaling pathways. Thus AR may be available for transcriptional signaling in the nuclear compartment under post-androgen ablation conditions and even in the complete absence of hormone.

## 6.2 Interrogating the Role of Classical Response Elements in Ligand-Insensitive Gene Activation by AR

It is generally well accepted that the principal mechanism of androgen/AR signaling that drives tumor growth is transcriptional. Current literature trends indicate that nongenomic effects of androgen, while physiologically significant in several normal

cell types, are relatively minor contributors to prostate tumor growth (in striking contrast to estrogen's effects in breast cancer). In the classical model of gene regulation by AR, the receptor requires bound ligand to homodimerize, enter the nucleus, and bind to well characterized androgen response elements (AREs) associated with target genes [15–19]. Androgen binding also enables phosphorylation of AR that is required for its stabilization and activity [20, 21]. When the bound ligand is an agonist, AR then recruits coactivators; in contrast, when bound to antagonists, corepressors are preferentially recruited [16, 17]. AR contains sites of co-regulator binding that are either ligand dependent or -independent.

The DNA sequence requirements for both canonical and noncanonical AREs have been well established [22]. However, the functional associations of particular ARE enhancer sites with individual androgen target genes have only been definitively established for a limited number of genes. Rather, such associations are based on collective enrichment scores for the occurrence of consensus ARE sequences and AR binding sites within ~50 kb of androgen activated genes. Based on those studies, AR appears to commonly regulate its target gene promoters from multiple AREs located at great distances from the target promoter, generally more than 10 kb [23, 24]. This concept of ARE-mediated gene activation by androgen has been extended a priori to gene activation by AR in different cellular contexts, including (1) cells that are hypersensitized to androgen [12], (2) cells harboring mutated AR with altered ligand specificity [25–27], and (3) cells that are completely independent of androgen for growth. In other words, it has been thought that in all these situations, AR recapitulates the classical mechanism of ARE-mediated gene activation. Indeed, posttranslational modifications and some mutations of AR associated with completely androgen-independent growth of prostate cancer have been presumed to not only allow ligand-independent nuclear localization of the receptor but also its association as a homodimer with target AREs [28–31]; however, there is little direct and unequivocal evidence for a necessary role for AREs in situations in which AR signaling supports the proliferation of prostate cancer cells deprived of hormone.

Several recent studies have offered evidence that ligand-independent gene activation by AR does not involve the binding of AR to AREs. In cells showing AR-dependent growth in hormone depleted media, the well-established AREs of the androgen responsive prostate-specific antigen (PSA) gene enhancer region [32–34] were unoccupied by AR in the absence of hormone [35]. In another study of cells adapted to grow in the absence of hormone [36], specific AREs were assigned to a few selected AR-activated genes based on the observation of hormone-independent binding of AR at those sites. Nevertheless, androgen further substantially increased the binding of AR at those sites, indicating that the binding of AR to those AREs was suboptimal in the absence of androgen.

The question of whether AREs are involved in gene activation by AR in the absence of hormone was more directly addressed in a recent study of variant LNCaP cells that grew robustly in the absence of hormone but whose growth was exquisitely dependent upon the presence of AR [13]. In those cells, whereas AR was localized in the nuclear compartment independent of hormone, the receptor still required androgen to bind to AREs. In the absence of hormone, AR was associated

with chromatin sites that lacked AREs and activated a distinct gene set that was highly and primarily enriched for growth genes. Indeed, following depletion of endogenous AR, a mutant form of AR that was unable to bind to DNA was able to rescue hormone-independent growth. The findings suggested that in prostate cancer cells, AR was capable of supporting growth by activating genes through interactions at nonclassical AR binding sites, presumably by tethered associations with DNA bound transcription factors.

## 6.3 Physiological Significance of ARE-Independent Gene Activation by AR in Early Stage and Advanced Prostate Cancer

As noted above, in prostate cancer cells that are adapted to grow in the absence of hormone, the AR apoprotein is localized in an active form in the nucleus and activates a gene set that is distinct from genes that require androgen for activation in the same cells [13, 36]. This set of genes strikingly overlaps the signature gene overexpression profile of clinical CRPC tumors and is enriched for gene clusters primarily supporting mitotic cell division [13, 36]. In those cells the AR apoprotein can support growth through gene activation that occurs without the direct binding of AR to DNA and likely through tethered associations of the receptor with its target genes [13]. Detailed studies of the interaction of AR with C/EBPα and Elk1 suggested that tethered associations of AR with DNA, and subsequent trans-activation of the target gene, may be insensitive to ligand except in cell contexts in which ligand is needed for nuclear import of AR [14, 37]. As exemplified by the studies of Elk1-AR interactions [37], the same AR tethering mechanisms may support profound genotropic effects of AR in both early stage (hormone-dependent) PC and CRPC cells. Therefore, critical AR tethering proteins may be necessary, though not sufficient, for androgen/AR-dependent growth in early stage as well as advanced prostate cancer cells.

As androgen supports diverse aspects of normal prostate physiology, including development, differentiation, maintenance, and function of the prostate epithelium [38], malignant prostate epithelial cells must selectively support mechanisms that direct androgen/AR signaling to strongly support growth. The precise mechanisms by which prostate cancer cells reprogram AR signaling to primarily support growth have not been well understood. Recent reports have strongly supported the general premise that the pattern of expression of AR tethering proteins during development, differentiation, and malignant transformation of the prostate could redirect AR signaling according to the physiological context. This is illustrated by the ability of several well-established AR tethering proteins to profoundly influence the pattern of gene activation by androgen/AR. Those proteins include HoxB13 (involved in development) [39], C/EBPalpha (involved in terminal differentiation) [14, 40, 41], and Elk1, demonstrated to be obligatory for androgen/AR-dependent malignant growth [37]. As discussed in later sections, AR tethering proteins may interact with

the receptor in a bimodal manner, on the one hand recruiting AR to activate one set of genes and on the other hand binding to AR as a corepressor to inhibit the activation of a different set of AR target genes. Individual AR tethering proteins may thus have critical roles in supporting prostate tumor growth, at various stages of its progression, by diverting AR signaling to critical target genes that are not associated with functional AREs.

## 6.4 Gene Transcription by Major Splice Variants of AR That Lack the Ligand Binding Domain

Prostate cancer cells express splice variants of AR in which carboxyl-terminal regions containing the ligand binding domain are absent [9, 10]. These splice variants have generated recent excitement as an important means by which hormone-independent and antiandrogen-insensitive growth signaling is supported and their upregulation is linked to PC progression [9, 10, 42]. Indeed, small molecules that disrupt interactions of the amino-terminal domain of AR have recently shown promise in PC intervention [43–45]. Interestingly, it was recently demonstrated that the N-terminal A/B domain of AR is alone adequate for association with and transcriptional coactivation of Elk1 [37]. The recruitment of the A/B domain of AR to Elk1 binding elements and transactivation of the target promoter were both insensitive to ligand. Because, as discussed above, the binding of AR to AREs is ligand dependent, it is highly likely that gene activation by the natural AR splice variants requires their association with the target genes through tethering proteins rather than AREs. Consistent with this expectation is a recent report that in CRPC cells, the gene activation program induced by androgen through the full-length AR was distinct from that activated by its splice variants and that the latter primarily activated cell cycle genes [46].

## 6.5 Diversity of Putative Mechanisms of Recruitment of AR to Chromatin: A Genome-wide Perspective

In prostate cancer cells in which AR is localized in the nucleus in the absence of hormone, the AR apoprotein has profound genotropic effects [13]. However, in genome-wide chromatin binding studies using cell line models of advanced prostate cancer, in the absence of hormone, putative tethered associations of AR with chromatin generally give relatively weak signals at best, presumably due to the poor efficiency of immunoprecipitation of AR in such complexes [13, 36]. However, the association of AR with DNA bound transcription factors has been reported by screening a synthetic *cis*-element array of transcription factor binding sites for AR recruitment from a nuclear extract of LNCaP cells [47]; those results suggest that AR may associate with a variety of *cis*-elements by binding to their cognate transcription factors. Notwithstanding the likely technical limitations that could

obscure non-ARE sites of AR binding in the chromatin, a careful examination of the many reports of chromatin binding sites of AR do point to the likely existence of a large proportion of such nonclassical AR binding sites evident from (1) the presence of a significant proportion (~ 50%) of AR binding sites lacking AREs and (2) enrichment for other *cis*-elements including binding sites for ETS, GATA, FKHD, OCT1, AP1, RAR, EGR, forkhead, NF1, and Elk1 [24, 48, 49].

The role of other DNA bound transcription factors occurring at or in the vicinity of AR binding sites may be considered in three major ways. In the first mechanism, the protein may serve as a pioneer factor that alters chromatin structure to direct AR binding. Many studies have highlighted the role of FoxA1 as a pioneer factor for AR, but GATA2 may act as pioneer factor for AR for some classes of genes. A systematic study of the effect of FoxA1 on gene regulation by AR identified three classes of genes: independent of FoxA1, pioneered by FoxA1, and masked by FoxA1 (stimulated upon FoxA1 depletion) [50]. In addition, high FoxA1 protein levels are associated with poor prognosis in clinical prostate cancer and with CRPC [50, 51]. In the second mechanism, the proteins may bind nearby AR and modulate AR activity, by either stimulating or inhibiting AR activation function. An example is Oct1, an important coregulator of AR activity in prostate cancer. Oct1 motifs are one of the most significantly enriched in AR peaks in multiple studies. Moreover, Oct1 can physically interact with AR. Oct1 is not required for AR binding to targets but is required for AR activation of a major class of genes required for cell proliferation of PC cells [23]. In the third mechanism, DNA bound proteins may aid in the recruitment of AR to its binding site. This mechanism is often invoked in the case of ARE half-sites, where the avidity of AR binding may be enhanced by the simultaneous binding of AR to a protein bound at a neighboring site. On the other hand DNA bound proteins may recruit AR to sites completely lacking ARE half-sites. The examples described below represent recent studies of such AR tethering mechanisms, which have profound genotropic and physiologic effects.

## 6.6 Established Mechanisms of Gene Regulation by Interactions of AR with Tethering Proteins

Recent evidence clearly points to a fundamental role for a variable complement of AR tethering proteins, or the AR "tetherome," in directing the androgen/AR signaling program. Among AR tethering mechanisms, the interactions AR with three DNA binding transcription factors have been most thoroughly studied; they are Elk1, C/EBPα, and HoxB13. Additional examples are also noted below.

### *6.6.1 Elk1*

Elk1 is an ETS family transcription factor belonging to the TCF (ternary complex factor) subfamily. TCF proteins including Elk1 are activated by phosphorylation

through MAPK signaling to control growth or to respond to stress [52]. Elk1 is genetically redundant in normal tissues [53], presumably due to functional redundancy within the TCF subfamily [54, 55]. However, standard cell line models of both hormone-sensitive PC and CRPC cells are absolutely addicted to Elk1 for androgen- or AR-dependent growth as well as androgen-stimulated anchorage-dependent and -independent colony formation [37]. Elk1 was partially or fully required for a substantial proportion (27%) of all gene activation by androgen in PC cells [37]; this set of genes was primarily enriched for genes supporting cell growth. Elk-1 was also similarly essential for the AR-dependent growth of CRPC cells in the absence of hormone where it supported induction of many of the same genes by the AR apoprotein [37]. The cooperative gene activation by Elk1 and AR did not involve the classical mechanism of Elk1 activation by phosphorylation [37].

Elk1 binding sites are enriched in relation to chromatin sites of AR binding [49]. Elk1-dependent gene activation by AR occurred by the physical recruitment of AR by Elk1 at Elk1 binding sites in the target genes to produce sustained gene activation. AR bound to Elk1 through its N-terminal A/B domain; this domain of AR, which cannot directly bind to DNA and comprises the ligand-independent activity domain of AR, was by itself capable of inducing Elk1-mediated promoter activation. Interaction with Elk1 enables a distinct and key component of AR signaling in PC that is independent of TMPRSS2 gene fusions [37]. The special role of Elk1 in PC cells is underscored by the observation that Elk3, the closest functional substitute for Elk1, does not interact with AR and is higher in normal prostate epithelial cells and tissues compared to standard models of early stage PC and CRPC as well as clinical prostate tumors [37]. It thus appears that the complement of TCF subfamily proteins or a larger subset of ETS proteins is altered in PC cells to enable critical growth promoting actions of AR through Elk1.

### *6.6.2 C/EBPα*

The CCAAT enhancer binding protein α (C/EBPα) belongs to a family of homo- or heterodimeric basic/leucine zipper transcription factors [56]. C/EBP family proteins are functionally coordinated in inducing the differentiation and function of several tissues [56]; in this role, C/EBPα inhibits cell proliferation [57–59]. C/EBPα exerts antiproliferative effects independent of its ability to bind to DNA [60] by protein–protein interactions that include stabilization of p21 [61, 62], disruption of E2F complexes [63–65], inhibition/degradation of cyclin-dependent kinases 2 and 4 [66, 67], and interaction with the SWI/SNF chromatin remodeling complex [68]. However, dephosphorylation of C/EBPa by activation of the phosphatidylinositol 3 kinase/AKT pathway may abrogate its interactions with cyclin-dependent kinase 2 and E2F complexes [69]; dephosphorylated C/EBPa may also promote proliferation by sequestering retinoblastoma protein [70]. C/EBPα is expressed in prostate epithelial cells, entering the nucleus at the onset of prostate maturation [40]. Although ectopic C/EBPα is antiproliferative in prostate cancer cells [71], C/EBPα is commonly

expressed in malignant prostate tumors in which its relative levels correlate significantly with those of AR, with the highest levels in metastatic tumors [40, 72].

C/EBPα was found to associate with AR as a corepressor of AR and to inhibit ARE-mediated promoter activation by inhibiting coactivator recruitment [71]. Subsequent studies [14] revealed an alternate mechanism of interaction between AR and C/EBPα at C/EBP-binding sites that could override the suppression of classical AR signaling by C/EBPα elsewhere in a gene and also enable AR to activate different genes. This mechanism of recruitment of AR by C/EBPα showed several remarkable features including ligand-independent promoter association and trans-activation per se under conditions that would permit nuclear localization of AR and the absence of a need for AR dimerization. The binding of C/EBPα could not be attributed to a single domain of AR and likely involves multiple AR domains. C/EBPα and AR could also bind synergistically at a composite element comprising an ARE half-site and a C/EBP binding element to activate the target gene [41]. The bimodal interaction of AR and C/EBPα (i.e., the recruitment of AR as a coactivator at C/EBP elements and the recruitment of C/EBPα as a corepressor at AREs) has a profound influence of global gene regulation by androgen/AR in PC cells [14]. The coordinated actions of AR and C/EBPα may support differentiation and function of the normal prostate and may also regulate AR signaling in advanced prostate tumors.

### *6.6.3 HoxB13*

The transcription factor HoxB13 is a homeodomain protein that plays a crucial role in normal prostate development [73, 74]. Endogenous HoxB13 expression is elevated in a fraction of clinical prostate tumors, especially in association with nonresponsiveness to androgen ablation [75]. In hormone-insensitive PC cells, HoxB13 supports cell growth through an androgen-independent pathway by inhibition of p21 expression, resulting in the activation of E2F [75]. HoxB13 also associates with AR as a corepressor of ARE-driven gene activation [39, 76]. In an alternative mode of interaction with AR, HoxB13 bound to homeobox elements recruits AR to support target gene activation by androgen [39]. Gene activation could also occur by the cooperative action of AR and HoxB13 bound to DNA at adjacent sites [39]. Hox B13 and AR associate with each other through their DNA binding domains [39]. In PC cells, the genotropic effects of HoxB13 regulate cell growth and migration as well as lipogenesis. Thus the tethering of AR by HoxB13 may profoundly affect the physiology of both normal and malignant prostate tissues.

### *6.6.4 Other AR Tethering Proteins*

ETS elements are commonly enriched at or in the vicinity of AR binding sites in the chromatin [48, 49]. Prostate oncogenesis is commonly linked to the formation of the

fusion protein formed by the androgen-regulated TMPRSS2 and the ETS protein ERG and less frequently to fusions between TMPRSS2 and other ETS proteins including ETV1, ETV4, and ETV5 [77–79]. TMPRSS2-ERG and AR modulate gene expression in an integrated manner that includes direct interaction between them [80]. However, this interaction leads to suppression of many androgen target genes, possibly suppressing a differentiation program, to enable tumor growth [80]. In contrast, physical association of AR with ETV1 has been demonstrated, resulting in AR and ETV1 reciprocally serving each other as transcriptional coactivators of their respective target promoters; the impact of this interaction on global gene expression and its physiological role in prostate cancer are still unclear, although ectopic overexpression of ETV1 did generate PIN lesions [81]. ETS1 may also directly associate with AR but its physiological significance is yet to be understood [48]. Interactions of AR with other types of tethering proteins resulting in the activation of specific target genes have also been reported—they include the formation of an AR-Sp1 complex [82] and the association of AR with GRIP1 and CBP in coactivator complexes located at non-ARE sites [83].

## 6.7 Salient Features of the Interaction of AR with Tethering Proteins

From the studies of the interactions of AR with several tethering proteins, it is clear that the different mechanisms of AR tethering have many similarities as well as distinctive features, as summarized below. The different modes of interaction of AR with tethering proteins are illustrated in Fig. 6.1.

### *6.7.1 Ligand Sensitivity and AR Dimerization*

In hormone-dependent PC cells nuclear localization of AR can only occur when AR has bound ligand, which could be either an agonist or an antagonist of androgen. In CRPC cells, however AR must be localized in the nucleus either in response to post-ablation levels of androgen or completely independent of hormone. The classical mechanism of ARE-dependent gene activation by AR requires the binding of androgen to AR in hormone-dependent PC cells as well as PC cells in which AR supports growth entirely in the absence of hormone [13]. In hormone-independent cells, binding of AR has been noted in the absence of hormone at a few chromatin sites containing AREs [36] but even at those sites, the hormone-independent binding of AR occurs only at a low basal level and is greatly stimulated by androgen. It is possible that this basal association of AR, observed at a few ARE sites, is the result of a partial tethered association of AR as demonstrated for a composite C/EBPα-ARE element [41]. Experiments using forced nuclear localization of

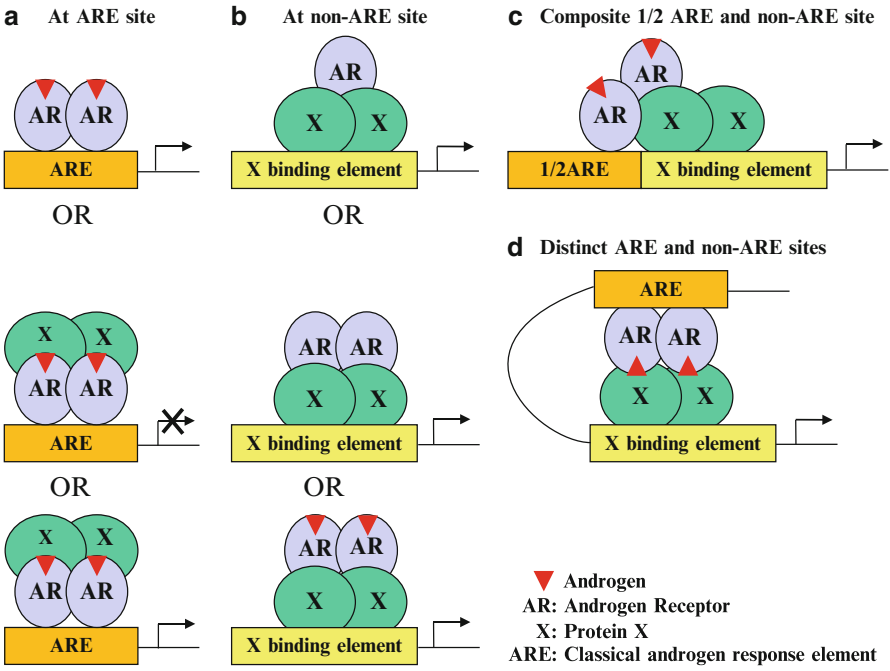


**Fig. 6.1** Schematic models of gene regulation by AR and a hypothetical tethering protein (protein X) in different target gene contexts. (**a**) Androgen induces AR to bind as a dimer to a classical ARE to activate the target gene. Protein X may be recruited to AR as a corepressor or as a coactivator. (**b**) Protein X is bound to its cognate cis-element. AR is recruited to protein X in a ligand-independent manner, as either a monomer or as a dimer. (**c**) Protein X bound to its cognate *cis*-element cooperates with a ½ ARE site to recruit AR in a partially ligand-dependent manner resulting in activation of the target gene. (**d**) AR binds in a ligand-dependent manner to an ARE and is recruited to the target promoter by protein X bound at a distal site

ectopic AR have demonstrated that the binding of AR to tethering proteins and subsequent transcriptional activation by AR may be hormone-independent and insensitive to conventional androgen antagonists [14, 37]. Therefore, in the context of gene activation through tethered association of AR, hormone-dependence may only be due to ligand-dependence of nuclear import of AR. Conventional androgen antagonists support nuclear translocation of AR although they suppress ARE-dependent gene activation; therefore gene activation mediated by tethering of AR is insensitive to the conventional androgen antagonists in both hormone-dependent and CRPC cells [14, 37]. Using a dimerization mutant of AR, it has also been demonstrated that functional recruitment of AR by tethering may not require dimerization of AR [14]. As ligand binding is typically required for AR to dimerize, this observation is also consistent with the ligand-independence of AR-tether interactions upon forced nuclear expression of the AR apoprotein.

### *6.7.2 Bimodal Actions of AR-Tether Complexes at ARE and Non-ARE Sites*

AR is recruited by various tethering proteins at non-ARE sites resulting in target gene activation. In this mode of interaction, AR acts as a coactivator of DNA bound transcription factors. Often, the tethering protein reciprocally associates with AR bound to AREs. In this mode of interaction, the protein may act as either a corepressor or coactivator of AR for classical ARE-driven target genes. Examples in which an AR tethering protein acts as a corepressor of DNA-bound AR are HoxB13 and C/EBPα [14, 39, 76]. On the other hand, ETV1 is an AR-tethering protein that also acts as a coactivator of DNA-bound AR [81]. The reciprocal interactions of AR and its tethering partners at non-ARE and ARE elements in the chromatin add to the diversity of mechanisms by which gene expression could be redirected according to the physiological context through reprogramming of androgen/AR signaling.

### *6.7.3 AR Domains That Bind Tethering Proteins*

Among the examples studied in detail, there is diversity in the domain interface of AR associations with tethering proteins. AR binds to HoxB13 through its DNA binding domain [39] and to Elk1 through its N-terminal A/B domain [37]. C/EBPα, however, did not show a strong AR domain selectivity for binding and its association with AR involved multiple AR domains [14]. The fact that AR has different binding motifs for its interactions with various tethering proteins should enable selective disruption of specific mechanisms of AR action for the novel therapeutic intervention strategies discussed below.

## 6.8 Clinical Significance of AR Tethering Mechanisms

The current clinical paradigm for adjuvant therapy in PC is total and ubiquitous attenuation of AR signaling by androgen ablation and the use of AR ligands that antagonize, sequester, or deplete AR [84]. These drugs have limited efficacy in blocking disease progression because of the ability of prostate tumors to restore androgen/AR signaling by mechanisms that circumvent androgen depletion or that resist antiandrogens. Moreover, androgen ablation is associated with undesirable side effects in a variety of nontarget tissues and organ systems [85–87].

As the majority of advanced prostate tumors that become unresponsive to drugs targeting AR signaling do not “opt out” of their dependence on AR for growth, the resistant cells must retain many essential aspects of the mechanism of action of AR in growth signaling. Therefore, it should be possible to identify and selectively disrupt

a functional arm of AR that is (1) necessary for tumor growth but not for the physiological role of androgen in differentiated normal tissues and (2) preserved as a critical mechanism for supporting growth through progression to CRPC. Targeting such a conserved downstream mechanism of growth signaling by AR should not only confer tumor selectivity but should also evade resistance mechanisms to conventional drugs including inhibitors of androgen synthesis and antiandrogens. In addition, such an intervention should be effective in both early stage and advanced PC without the need for androgen ablation, i.e., without affecting androgen signaling in normal tissues. AR tethering mechanisms offer opportunities for such interventions.

Specific AR tethering mechanisms could be critical for supporting AR-dependent growth both in early stage and advanced prostate cancer. If such interactions do not occur in, or are not critical for, the normal physiological actions of AR, they could be targeted for functionally selective and tumor-specific intervention in prostate cancer. As evident from the preceding discussions, a prototypical example of an AR tethering mechanism that meets virtually all of the above criteria for a desirable target for therapeutic intervention is the Elk1–AR interaction. This and other similar protein–protein interactions of AR could be specifically disrupted by peptide or small molecule agents. This new mechanism-based paradigm for PC treatment promises a broader spectrum of responsive tumors than that of conventional androgen/AR targeted agents with fewer adverse effects on normal tissues.

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# Chapter 7
# Androgen Action, Wnt Signaling, and Prostate Tumorigenesis

**Zijie Sun and Suk Hyung Lee**

**Abstract** Androgen signaling is mainly mediated through AR and plays a critical role in prostate tumorigenesis. Current studies have shown that AR-mediated transcription is facilitated through direct or indirect interactions with different signaling pathways and coregulators. The Wnt signaling pathway and its key component, β-catenin, are critical in embryonic development and tumorigenesis. Emerging evidence suggests a promotional role of the Wnt and β-catenin signaling pathway in prostate cancer development and progression. The discovery of the interaction between AR and β-catenin provides the molecular basis for crosstalk between androgen and Wnt signaling. It has been shown that mutations in adenomatous polyposis coli (APC), β-catenin, and other components of the β-catenin destruction complex are rare in prostate cancer cells. Therefore, the molecular mechanisms underlying β-catenin oncogenic activation in prostate cancer may be different from those observed in human colorectal cancer or other malignancies. Further study of the role and regulation of Wnt signaling and β-catenin should provide fresh insight into our current knowledge of androgen action and prostate tumorigenesis, which may lead to the development of novel therapeutic strategies for prostate cancer patients.

**Keywords** Wnt • β-Catenin • Frizzled • The androgen receptor • Prostate cancer • IGF • TCF/LEF • E-cadherin • PI3K • Akt • PTEN • APC

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## 7.1 Introduction

Prostate cancer affects more than 2,276,000 men and is responsible for more deaths than any other cancers with the exception of lung cancer in this nation (http://seer.cancer.gov/statfacts/html/prost.html). The androgen-signaling pathway mediated through the androgen receptor (AR) and its ligand, testosterone, and 5α-dihydrotestosterone (DHT), is essential for prostate cancer growth [1, 2]. The AR is a nuclear hormone receptor and promotes prostate cancer growth through activation of its downstream target genes. Although the targets of AR activation remain unclear, they are believed to be critical for cellular proliferation because most prostate cancers express AR and are androgen-dependent. Charles Huggins and Clarence Hodges demonstrated that depletion of androgens resulted in significant regression of prostate tumors, heralding the now ubiquitous strategy, androgen deprivation therapy, to treat prostate cancer [3]. Unfortunately, within 2–3 years after initiating therapy, most patients will invariably relapse with a more aggressive form of prostate cancer, known as castration-resistant prostate cancer (CRPC). There is no effective treatment option for CRPC, which is mainly responsible for the mortality of the disease. Several proposed mechanistic models for hormone refractory include *AR* gene amplification, AR mutations, aberrant expression or function of AR cofactors, and abnormal activation of AR and its coactivators through different pathways [1, 4, 5].

The Wnt signaling pathway plays critical roles in embryonic development and tumorigenesis [6–8]. There are 19 closely related Wnt genes that have been identified in humans. Their primary receptors are the seven transmembrane Frizzled proteins, each of which interacts with a single transmembrane LDL receptor-related protein 5/6 (LRP5/6) [9, 10]. A number of different secreted proteins, such as secreted frizzled-related proteins (sFRP), Wnt inhibitory factor-1 (WIF1), and Dickkopf (Dkk) prevent ligand–receptor interactions and thus inhibit Wnt-mediated cellular events [11]. Wnt proteins activate different intracellular targets through either the "canonical" or the "noncanonical" pathways [12]. In the canonical signaling pathways, secreted Wnt ligands bind to Frizzled proteins and regulate the stability of β-catenin, a key component of Wnt signaling [13]. In the absence of a Wnt signal, β-catenin is constitutively downregulated by a multicomponent destruction complex containing glycogen synthase kinase 3β (GSK3β), axin, and APC, which promotes phosphorylation on serine and threonine residues in the N-terminal region of β-catenin following "priming" phosphorylation of Ser45 by Casein Kinase I (CKI), and thereby targeting it for degradation via the ubiquitin proteasome pathway [14–17]. Wnt signaling inhibits this process, leading to the accumulation of β-catenin in the nucleus, in which β-catenin forms transcriptionally active complexes with members of the LEF/TCF family of transcription factors [18]. Noncanonical pathways are alternative modes of Wnt signaling, which do not involve β-catenin. A well-characterized example of noncanonical Wnt signaling is the Drosophila planar cell polarity (PCP) pathway [19]. The c-Jun N-terminal kinase (JNK) pathway and modulation of intracellular calcium have also been suggested to be potential downstream mediators for noncanonical signaling [20–22].

Recent studies have provided multiple lines of evidence demonstrating an interaction between the androgen and Wnt signaling pathways in prostate cancer cells. β-catenin has been shown to be an AR coactivator, substantiating the biological significance of Wnt signaling in prostate tumorigenesis [23–26]. Further investigation of the interaction between androgen and Wnt/β-catenin signaling pathways should contribute to elucidation of the molecular mechanisms underlying prostate cancer development and progression and may lead to identification of new therapeutic targets for the future treatment of prostate cancer.

## 7.2 Roles of Wnt Growth Factors and Frizzled Receptors in Androgen Signaling and Prostate Cells

Expression of Wnt growth factors and their receptors has been examined in prostate cell lines and prostate tissues. Upregulation of Wnt-1 expression has been observed in prostate cancer cell lines and human prostate tumor tissues [27]. The expression of Wnt-1 was positively correlated to Gleason scores, as well as to the cellular level of β-catenin and serum PSA levels. In particular, increased expression of Wnt-1 appeared more significantly in metastatic prostate tumors, such as lymph node and bone marrow specimens [27]. Elevated expression of Wnt-7B was also observed in primary prostate cancer specimens and bone metastases [28]. Elevated expression of Wnt-11 has been observed in prostate cancer samples with Gleason grade 7, or above, in comparison to nonmalignant or low Gleason grade samples [29]. Wnt-11 induces noncanonical pathways, which are alternative modes of Wnt signaling and are independent of β-catenin [12]. Increased Wnt-11 expression also appeared in androgen-independent CWR22 xenograft tumor models [29]. Expression of Wnt-11 in LNCaP cells, which contains a mutated AR (T877A), showed an inhibitory effect on AR-mediated transcription and cell growth. In addition, androgens inhibited Wnt11 expression in a dose-dependent manner in LNCaP cells [29]. Recently, it has been shown that mice with the mutated AR transgene, T877A, have hypertrophic prostates [30]. However, this AR transgene enhances tumor growth in TRAMP mouse model. Importantly, activation of Wnt5a, another ligand for the noncanonical pathway, was shown in prostate tumors. These lines of evidence suggest an important role of the both canonical and noncanonical signaling pathways in prostate cancer cells. More in-depth mechanistic studies are required for defining their regulatory mechanisms in androgen signaling during prostate cancer development and progression.

The Frizzled receptors, including Frizzled-1, -4, -6, and -10, were detected in normal prostate tissues [31–33]. Using cDNA microarray approaches, Wissmann et al. systematically analyzed expression of Wnt signaling components and Wnt targets in prostate cancer cells. Increased expression of sFRP4, Frizzled-4, Frizzled-6, Dishevelled-1, TCF4, and MYC was observed in prostate tumor cells, while expression of Wnt-2, WIF1, β isoform of the catalytic subunit of protein phosphatase 2A (PPP2CB), Cyclin D2 (CCND2), and CD44 was reduced [34].

The critical role for Sfrp1 as a stromal-to-epithelial paracrine modulator in the mouse prostate has been identified in both loss- and gain-of-function mouse models [35]. Forced expression of Sfrp1 in prostatic epithelial cells led to activation of JNK-mediated cell growth, suggesting an involvement of the noncanonical Wnt/JNK pathway in the regulation. In the conditional TGF-beta type II receptor knockout mouse model, the expression of Sfrp-2 can restore the Tgf receptor-associated prostate responsiveness to androgen ablation, providing evidence for the interactions between TGF-beta, androgen, and Wnt paracrine signaling axis in prostatic cell differentiation and survival [36].

Studies in other tissues and organisms have implicated the critical roles of Wnt signaling in the regulation of various cellular events through the canonical and noncanonical pathways [8]. To directly examine the role of Wnt growth factors and their receptors in prostate tumorigenesis, we explored the biological role of Wnt-3a in prostate cancer cells [37]. Using Wnt-3a conditioned medium (Wnt-3a CM) [38], we observed significantly enhanced AR-mediated transcription of both an ectopically expressed 7Kb PSA promoter reporter and the endogenous PSA gene in the absence or presence of low concentration of androgens in LNCaP cells. A similar induction by Wnt3a was also observed on a reporter construct driven by a minimal promoter with two androgen-responsive elements (AREs). Knockdown of AR expression by a specific shRNA and inhibition of AR function by an AR antagonist, bicalutamide, significantly reduced the induction of AR-mediated transcription by Wnt-3a CM, suggesting that the AR is the direct target of Wnt-3a-mediated induction. Most interestingly, Wnt-3a CM also enhanced the growth of prostate cancer cells in an androgen-independent manner. We further demonstrated that the above promotional role of Wnt-3a CM in inducing AR activity is mainly mediated through β-catenin. In addition, purified Wnt-3a showed a similar effect as Wnt-3a CM in enhancing cell proliferation and colony formation of LNCaP cells. These findings provide a direct line of evidence demonstrating that Wnt signaling induces AR-mediated transcription and prostatic cell growth.

## 7.3 Expression and Cellular Localization of β-Catenin in Prostate Cancer

The significance of β-catenin in human tumorigenesis was corroborated by discoveries of mutations in both β-catenin and components of the destruction complex components in tumor cells [7, 39]. These pathological changes further result in an increase in cellular β-catenin. Therefore, many groups have examined β-catenin mutations in prostate cancer samples [40–42]. Approximately 5% of samples revealed mutations at the serine or threonine residues in the NH2-terminal of the β-catenin protein [40–42]. Since the mutations occur focally, it was suggested that alteration of β-catenin may represent a late event in prostate cancer progression. Examination of the β-catenin protein by immunohistochemical assays revealed aberrant localization of the protein in prostate cancer specimens [27, 43, 44]. In one study,

about 20% of hormone-refractory samples showed nuclear localization of β-catenin [43], while the other report observed even higher percentage (38.8%) [44]. Alterations of APC and β-TrCP1, which directly affect the degradation of β-catenin, have also been observed in prostate cancer samples [40].

## 7.4 β-Catenin Interacts with AR Transcriptional Complexes

The biological significance of β-catenin in prostate cancer cells was actually exposed by the discovery of a protein–protein interaction between AR and β-catentin. Truica and colleagues first demonstrated that β-catenin interacts with AR and enhances AR transcriptional activity in LNCaP cells [24]. Our group and others further demonstrated the AR–β-catenin interaction using yeast two-hybrid and other *in vivo* and *in vitro* protein binding assays [23, 26, 45]. Androgens were shown to enhance this interaction, and the ligand-binding domain (LBD) of AR was mapped to be responsible for the binding [26]. An attempt to define the small region and motifs in the AR LBD responsible for this interaction has been made recently [46]. However, since mutations of these motifs within the LBD also diminish the ligand binding activity of the AR, it is difficult to precisely define whether these motifs primarily affect ligand binding or interrupt β-catenin interaction. Results from earlier experiments showed that β-catenin preferentially binds AR over several other receptors, including the estrogen receptor, progesterone receptor, and glucocorticoid receptor [26]. In addition, early experiments suggested that the NH2 terminus and the first six armadillo repeats of β-catenin were involved in the interaction with AR [26]. Deletion of repeat 6 can fully abolish the physical interaction between AR and β-catenin, suggesting a key role of this repeat in the interaction [26]. Posttranslational modifications of β-catenin may affect its affinity and preference for binding partners, including the AR. It was shown that acetylation of β-catenin on lysine K345, located within armadillo repeat 6, decreases the binding affinity of β-catenin for the AR, but enhances its interaction with Tcf-4 [47]. The central armadillo domain of β-catenin, containing five LXXLL motifs, is responsible for binding to a hydrophobic cleft in the activation domain 2 (AF2) of nuclear receptors [48]. However, structural analysis revealed that the leucine residues in these motifs are buried in the hydrophobic core of the armadillo repeats, which is consistent with previous reports that mutation of these motifs does not affect the binding of β-catenin to the AR [26, 46].

To better understand the AR and β-catenin interaction, efforts have also been made to determine the crystal structure of the AR–β-catenin complex(es). The crystal structure for the liver receptor homolog-1 (LRH-1) and β-catenin complex has been resolved [49]. The complex is at 2.8 Å resolution, and the LRH-1 LBD utilizes a novel interaction surface to dock into the positively charged groove at a site that partially overlaps the binding surface for TCF-4. Mutational analysis confirms the interaction and suggests the possibility that LRH-1/β-catenin interactions may be prototypic for other nuclear hormone receptors, including AR. Our preliminary data has shown that truncated AR binds to β-catenin relatively weaker than its other

partners. The interaction between the AR and β-catenin can also be regulated by other factors. Our recent results show that ICAT, inhibitor of β-catenin and T-cell factor, forms a ternary complex with AR and β-catenin in prostate cancer cells and enhances AR-mediated transcription [50]. Intriguingly, whereas full-length ICAT retained the interaction between β-catenin and AR proteins, the truncated ICAT comprising the N-terminal helical domain showed a more pronounced effect on β-catenin binding to AR. These results suggest a novel model for the AR and β-catenin interaction.

## 7.5 β-Catenin Is a Bona Fide Coactivator of AR

The biological consequence of the AR–β-catenin interaction has also been investigated. Several lines of evidence have shown that β-catenin can act as an AR coactivator and enhance its transcriptional activity. Expression of exogenous β-catenin augments AR-mediated transcription on several AR-regulated promoters in both prostate and nonprostate cells [23, 26, 43, 45, 46]. Reduction of cellular levels of β-catenin by antisense or shRNA constructs decreases the expression of the *PSA* gene, a downstream target of AR. In addition, β-catenin-mediated enhancement of AR-transcriptional activity can be completely abolished by deletion of β-catenin armadillo repeat 6 [26], which suggests the enhancement of β-catenin on AR is solely mediated through the interaction between these proteins. The role of endogenous β-catenin in androgen signaling has also been analyzed. It has been shown that β-catenin can be recruited to the endogenous promoter region of *PSA* gene using the chromatin immunoprecipitation assay (ChIP) [51]. More evidence has suggested that the interaction between β-catenin and AR can promote the recruitment of other coregulators to AR-involved transcriptional complexes on its target promoters. Along this line, β-catenin has been shown to interact with GRIP1, a nuclear hormone coactivator, and enhances AR-mediated transcription [51]. In addition, CARM1, a histone methyltransferase, β-catenin, and p300 synergistically enhance AR transcriptional activity [52]. The LIM protein FHL2, an AR coactivator, has also been shown to interact with β-catenin and stimulate AR-mediated transcription in a synergistic manner with p300 [53].

## 7.6 Effects of β-Catenin on Androgen-Mediated Prostate Cell Growth and Tumor Formation

The biological significance of the AR and β-catenin interaction in prostate tumorigenesis has also been examined using different *in vivo* and *in vitro* systems. β-catenin can enhance the sensitivity and the specificity of AR binding to ligands [24]. Expression of exogenous β-catenin in LNCaP cells enhances AR-mediated transcription in the presence of 17β-estradiol or androstenedione, a form of adrenal androgen. In the presence of β-catenin, hydroxyflutamide, an AR antagonist, can act as an

agonist to augment the recruitment and transcriptional activity of the T877A AR mutant in LNCaP cells [54, 55]. The similar enhancement of β-catenin on AR activity was also observed with the W741C AR mutant in a bicalutamide-stimulated LNCaP subline. In contrast, the AR antagonists, L-39 and cyproterone acetate (CPA), which each activates the T877A AR mutant, do not appear to affect the interaction between AR and β-catenin, suggesting that these antagonists may modulate AR activity through different pathways. Mifepristone, an antagonist of steroid receptors, was recently shown to also act as an antiandrogen and inhibit R1881-induced binding of wild type or T877A AR mutant to β-catenin [55]. The significance of the AR and β-catenin interaction has been further examined in castration-resistant prostate cancer (CRPC). Increased expression of endogenous AR and β-catenin as well as increased nuclear colocalization and interaction of these two proteins was observed in castrated xenograft mice but not in noncastrated controls [56]. These results further suggest a critical role of β-catenin in aberrant activation of the AR to promote prostate cancer progression and castration resistance.

Efforts have also been spent on *in vivo* studies to assess the role of β-catenin in prostate tumorigenesis. Early studies have shown that castration of rats and mice results in atrophy of the prostate, while administration of testosterone to castrated mice restores growth and prostate gland formation [57–59]. Interestingly, increased levels of nuclear β-catenin were observed in proliferating cells in the prostate, suggesting a potential role of β-catenin in androgen-induced prostate cell growth [43]. The biological role of β-catenin in the tumorigenesis of prostate cancer was further characterized using transgenic mouse models [60]. Specific expression of a mutant β-catenin, lacking exon 3, in prostate tissues results in the development of prostate intraepithelial neoplasia (PIN), a precursor to prostate cancer, in mice ranging between 10 and 21 weeks of age [60]. However, using similar approaches, a different group observed hyperplasia of the prostate and squamous metaplasia but no PIN lesion in a similar animal model [61]. Deletion of *Apc* in mouse prostate tissues elevated levels of β-catenin, resulting in hyperplasia around 4.5 weeks and adenocarcinoma by 7 months [62]. The above data implicated a promotional role of β-catenin in prostate cancer development. Future studies should focus on determining whether and how β-catenin-induced oncogenic transformation is mediated through androgen signaling in prostate cancer cells.

## 7.7 Effects of Other Signaling Pathways in Regulating Androgen Action, Wnt Signaling, and Prostate Tumorigenesis

### *7.7.1 IGF-1 Signaling Pathway in Androgen Signaling*

The insulin-like growth factor (IGF) signaling pathway plays an important role in prostate cancer progression and possibly in CRPC development [63]. Early studies showed that IGF-1 induces AR-mediated transcription in a ligand-independent

manner [64], but the mechanisms of this regulation are unclear. IGF-1 enhances tyrosine phosphorylation of β-catenin, resulting in dissociation of β-catenin from E-cadherin complexes and an increase in the cytoplasmic level of β-catenin [65]. In several prostate cancer cell lines, both the addition of IGF-1 and overexpression of a constitutively active mutant of the IGF-1 receptor increase endogenous AR-mediated transcription in the presence of a low level of androgens [66]. IGF-1 also enhances the stability of the β-catenin protein in prostate cancer cells [66]. A recent study has shown that expression of endogenous IGF-I was increased by androgens in prostate cancer cells, which further enhances the level of cytoplasmic β-catenin [67]. Functional depletion of IGF-1 or IGF-1 receptor diminishes PSA induction. These data elucidate a novel mechanism for IGF-1-induced AR activation.

### *7.7.2 PI3K/AKT and PTEN*

Multiple lines of evidence have shown that the androgen and phosphatidylinositol 3-kinase (PI3K)/Akt pathways cooperatively regulate prostate cancer development and progression [68–71]. The tumor suppressor PTEN is frequently mutated in prostate cancer and negatively regulates PI3K/Akt activity [72]. Previous studies have shown that PI3K/Akt activation regulates AR expression and AR transcriptional activity [73, 74]. In addition, androgens regulate the Akt pathway by both genomic and non-genomic effects. This explains why prostate tumors subjected to androgen ablation experience an increase in Akt phosphorylation and also suggests that tumors compensate for the loss of one pathway with another. Silencing of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and glycogen synthase kinase 3β (GSK3β) is frequently associated with advanced prostate cancer and likely serve a critical role in promoting AR and PI3K/Akt gain-of-function [75]. Specifically, PI3K/Akt can modulate the activity of β-catenin on AR-mediated transcription through GSK3β, one of the principle substrates of Akt [65, 70]. In a PTEN-null prostate cancer cell line, LNCaP, overexpression of PTEN and the addition of the PI3K inhibitor, LY294002, repressed the phosphorylation of Akt and GSK3β, resulting in an increase in β-catenin phosphorylation, which further induces the ubiquitylation and proteasome-mediated degradation of β-catenin [70]. These data suggest that GSK3β serves not only as a mediator of PI3K/Akt activation but also regulates the transcriptional activation that β-catenin confers upon AR. In addition, WIF-1, which is frequently downregulated in prostate cancer, could decrease the amount of phosphorylated GSK3β and the levels of soluble β-catenin in prostate cancer cells [76]. Overexpression of WIF-1 in the PTEN-null PC-3 cell line reduced the level of phosphorylated Akt. It has been reported that expression of exogenous GSK3β can represses AR-mediated transcription [77, 78]. This discrepancy may be generated by using different experimental conditions. More mechanistic studies are needed to fully characterize the regulation of AR-mediated transcription by GSK3β.

### 7.7.3 *Interaction Between AR and TCF/LEF Transcriptional Factors*

β-catenin is a promiscuous Wnt signaling member and participates in transcription through interaction with the members of the T-cell-specific transcription factor/lymphoid enhancer factor (TCF/LEF) families. TCF/LEFs bind directly to DNA through their high mobility group (HMG) domains but are incapable of independently activating transcription [18, 79]. Thus, the cross-regulation between β-catenin and TCF may facilitate androgen-induced AR-mediated transcriptional activation and repression in prostate cells. In addition, ligand-bound AR can repress Tcf-mediated transcription in prostate cancer cells [80], neuronal cells [45], and colon cancer cells [81]. These data suggest that the repression may result from a competition between the AR and TCF for binding to β-catenin and other transcriptional coactivators. The biological significance of this phenomenon is currently not well understood. Although both signaling pathways appear to promote cell growth, the presence of androgen appears to favor the interaction between β-catenin and AR to enhance AR-mediated transcription and cell growth in prostate epithelial cells [81]. In contrast, androgen ablation may increase the pool of β-catenin available for activation of TCF/LEF target genes, which may lead to tumor progression and relapse.

Our recent finding that ICAT enhances AR-mediated transcription and cell growth explore a new mechanism for cross-regulation between AR, β-catenin, and TCF factors [50]. It has been shown that ICAT directly binds to β-catenin and disrupts the formation of β-catenin and Tcf complexes, resulting in inhibition of β-catenin-mediated TCF transcription *in vitro* and *in vivo* [82, 83]. However, the ability of ICAT to augment AR-mediated transcription suggests a different role for the ICAT–β-catenin interaction. Since AR binds to the N terminus and arm repeats 1–6 of β-catenin, AR and ICAT can simultaneously bind to different regions of β-catenin to form a ternary complex. Thus, unlike β-catenin–TCF transcriptional complexes, ICAT binding to β-catenin allows AR recruitment. In addition, a direct interaction between AR and Tcf-4 has been reported [84]. Endogenous AR cooperatively bound a Tcf-4 response element with Tcf-4 on the c-myc promoter. The DNA-binding domain of AR was required and sufficient for binding to Tcf-4. The results suggest that AR may be directly involved in the transcriptional regulation of certain TCF target genes. However, the biological significance of this interaction needs to be further assessed in prostate tumorigenesis.

## 7.8 E-Cadherin, β-Catenin, AR, and Prostate Cancer

In normal epithelial tissues, E-cadherin forms complexes with the actin cytoskeleton via catenins to maintain the functional characteristics of epithelia [85, 86]. Disruption of this complex, primarily due to loss or decreased expression of

E-cadherin, is frequently observed in many advanced, poorly differentiated prostate cancer samples [87, 88]. A strong correlation between a lack of E-cadherin and the metastatic and/or invasive potential of prostate cancer was first identified in the Dunning rat model [89]. The observation was corroborated by later studies in prostate cancer patients, which showed reduced or absent expression of E-cadherin in about half of the tumor samples examined [90]. There was a strong association between aberrant expression of E-cadherin and an invasive and metastatic phenotype of human prostate cancers [90, 91]. It has been reported that β-catenin is accumulated in both the cytoplasm and the nucleus of prostate cancer cells in which there is a reduction or loss of E-cadherin expression [92, 93].

Since the cytoplasmic domain of type I cadherins binds to β-catenin, a question has been raised as to whether the cadherin-bound pool of β-catenin can be released and participate in signaling. A series of studies were carried out to elucidate the dynamic interaction of β-catenin with cadherins [94]. It has been shown that an increase in cytoplasmic and nuclear β-catenin resulted from E-cadherin loss in human tumor cells [95]. Reintroduction of E-cadherin to the E-cadherin negative cell line, TSU Pr-1, shifted the subcellular localization of β-catenin from the cytoplasm to the cell membrane. The characterization of different truncation mutants of E-cadherin revealed that the extracellular domain is important for the cell–cell contacts, while the cytoplasmic domain is necessary for growth suppression. It has been demonstrated that the loss or reduction of E-cadherin expression enhances AR-mediated transcription by increasing the level of the cytoplasmic and nuclear β-catenin in TSU Pr-1 cells. These above data suggest that during the process of prostate cancer progression, loss of E-cadherin expression or activation of the Wnt pathway can lead to an increase in the cytoplasmic levels of β-catenin. The excess free β-catenin proteins can translocate to the nucleus and interact with the AR to induce androgen-mediated cell growth or survival. As an AR coactivator, β-catenin can promote cell growth by compensating for decreased androgen levels in response to androgen ablation therapy. Interestingly, in the above experiments, no effect was observed on a TCF-induced promoter/reporter construct. A similar observation was also reported in breast cancer cell lines, in which the E-cadherin gene is transcriptionally silenced [96]. This raises the question as to whether the growth-promoting effect of β-catenin in prostate cancer and other tumor cells is mediated through partners other than the TCF/LEF transcription factors.

## 7.9 Conclusion

The Wnt signaling pathways and its key component β-catenin have recently emerged as important players in prostate tumorigenesis. Particularly, identifying β-catenin as an AR coactivator provided a direct link between androgen and Wnt signaling pathways. The current literature has shown that only a small percentage of prostate cancer samples possess mutations in the destruction complex and β-catenin itself,

suggesting that other possible mechanisms are involved in activating the Wnt signal and contribute to the pathogenesis of prostate cancer. Given the critical role of androgen action in prostate tumorigenesis, future studies should focus on the molecular mechanisms by which Wnt/β-catenin signaling regulate androgen action during the course of prostate cancer development and progression. The outcomes will help us to understand the pathogenesis of prostate cancer and to identify new therapeutic targets for the future treatment of prostate cancer.

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# Chapter 8
# Toward Revealing the Complexity of Androgen-Responsive Protein and Noncoding Transcripts in Prostate Cancer

**Melanie L. Lehman and Colleen C. Nelson**

**Abstract** The suppression of the androgen-AR signaling axis remains the primary avenue of treatment for men with advanced prostate cancer. Understanding the functions of androgens and the related treatment responses to androgen deprivation therapy and AR antagonists remains a large obstacle for the development of durable therapies. Advances in RNA profiling methods are rapidly expanding our understanding of the cellular role of RNAs and exposing some of our misconceptions around the human transcriptome and proteome. Microarray and RNA sequencing (RNAseq) technologies have revealed novel alternative protein-coding and noncoding transcripts not included in RNA references databases and therefore previously overlooked in the study of androgen-responsive genes in prostate cancer. Newly described unconventional roles for RNA that far exceed the established role of messenger between DNA and protein prompts a review of our understanding of the androgen-AR signaling axis.

**Keywords** Alternative transcripts • Alternative untranslated regions • Antisense transcripts • Chimeric transcripts • ChIPseq • Large intergenic ncRNA • Long ncRNA • Microarray • microRNA • Multitasking genomic loci • Noncoding RNA • RNAseq

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The prostate is dependent on androgens for its growth, differentiation, development, and homeostatic function and survival of prostate cancer (PCa) cells. Androgens directly regulate the expression of specific genes by binding to the androgen receptor (AR), a ligand-activated transcription factor. While organ confined PCa can be cured by surgery or radiation, the primary systemic treatment for advanced metastatic PCa is androgen deprivation therapy (ADT). ADT—often augmented with AR antagonists—exploits the inherent androgen dependency of PCa. Some PCa cells survive and adapt leading to tumor regrowth in the androgen-deprived environment, referred to as castrate-resistant prostate cancer (CRPC). CRPC is characterized by the paradoxical up- or downregulation of genes and biological processes normally regulated by androgens, such as PSA, despite castrate levels of circulating androgens [1]. Understanding the androgen-AR signaling axis and its role in the progression to CRPC is critical for the discovery of durable therapies for men with advanced disease.

A large amount of data has been generated in RNA transcript profiling experiments (i.e., microarray and RNA sequencing) in both PCa cells lines and animals in an effort to understand androgen transcript regulation (early efforts reviewed in Dehm et al. [2–9]). These experiments have mainly focused on conventional transcriptional regulation of protein-coding mRNAs where protein levels are inferred and pathway analyses have been generated (reviewed in Lamont et al. [10]). It has been estimated that thousands of RNA transcripts are regulated by androgens directly or indirectly and that the protein products generated from these transcripts have a role in many cellular functions including cell proliferation, survival, lipid metabolism, and cell differentiation [10].

Recent advances to high-throughput RNA profiling methods are rapidly expanding our understanding of the cellular role of RNAs and exposing some of our misconceptions around the human transcriptome and proteome. In this chapter, we will review these recent technological advances and the unconventional regulatory roles of RNA that these technologies have revealed. These unconventional roles of RNA, that far exceed the established role of messenger between DNA and protein, prompt a review of our understanding of androgen-responsive genes.

## 8.1 RNA Profiling Technologies

Advances in miniaturization and robotics and laser scanners have improved the density and quality of microarrays increasing the number of oligonucleotide sequences that can be attached to a single glass side to ~1 million features. High density microarrays are no longer limited to one microarray probe to measure the relative levels of a target protein-coding RNA. These microarrays can be used to tile across large genomic regions to detect novel RNA transcription or can be used to detect expression levels of alternative splicing in protein-coding transcripts. Advances in cRNA/cDNA generation and labeling protocols have increased strand specificity and reduced 3′ bias seen in earlier microarray protocols. Microarrays have a number of limitations:

they measure relative levels of RNA; they are limited to short (25–70mer) oligonucleotide probes; and they are limited by probe design. Microarray probe design requires a priori knowledge of target sequence as well as stringent oligonucleotide composition to optimize specific target–probe hybridization.

RNAseq is an application of next-generation sequencing technologies (e.g., Illumina HiSeq, Life Technologies Ion Proton) used to generate short sequence reads from cDNA libraries. Millions of short cDNA fragments are sequenced in parallel generating gigabytes of data in a single sequencing run. The short sequencing reads (current limit 200 bp) can be paired-end where both ends of a larger fragment of cDNA are sequenced. The sequencing reads can be aligned to a reference genome or can be used for de novo transcriptome assembly. RNAseq has a number of advantages over microarray technologies including an unbiased view of the transcriptome with the ability to detect novel transcripts and alternative splice variants and a larger dynamic range of measurement (depending on sequencing depth). Efforts are ongoing to reduce costs, increase read length, and increase efficiencies of high-throughput sequencing. Although analysis pipelines and storage methods exist to manage the data generated by RNAseq, we are still only at the edge of exploring the potential of what is possible with high-throughput sequencing. Targeted RNA sequencing technologies continue to unveil rare transcripts not yet seen in conventional RNAseq datasets [11].

## 8.2 RNA Reference Databases

Standardization of RNA sequences is critical for integration of genomic information with content relating to biological function and clinical significance. The two most widely used RNA reference databases are RefSeq from the National Center for Biotechnology Information (NCBI; Bethesda, USA) and Ensembl, a joint project between European Bioinformatics Institute (EMBL-EBI) and the Wellcome Trust Sanger Institute. These two databases provide a curated list of nonredundant RNA transcripts generated from primary mRNA and EST sequence repositories (e.g., NCBI GenBank). The reference transcripts are further consolidated and assigned a unique gene identifier (e.g., NCBI Entrez Gene ID) to facilitate integration with other resources including nomenclature (official gene names and symbols), links to citations, variation information, chromosomal location, expression, homologs, protein domains, and protein interaction information [12]. Gene IDs are used extensively to link to other databases (e.g., KEGG, GO ontology terms from the Gene Ontology Consortium) for pathway and functional analysis of high-throughput RNA profiling experiments. The majority of content in Entrez Gene is focused on protein-coding regions of the genome. RefSeq protein-coding transcripts, however, only cover 2.07% of the human genome with almost half of that being untranslated regions (UTRs) (Fig. 8.1).

A large consortia named ENCODE (Encyclopedia of DNA Elements) was launched in 2003 with the mandate to identify all functional elements in the human

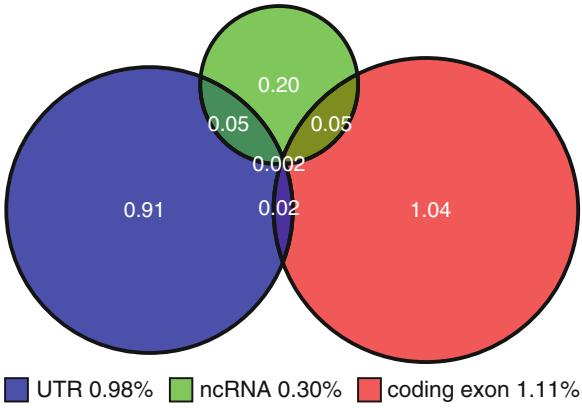


**Fig. 8.1** NCBI RefSeq transcript coverage on the human genome. The Venn diagram illustrates the percentage of coverage of exons and UTRs of protein-coding transcripts and exons of noncoding transcripts on the human genome (UCSC Genome Browser; hg19; June 2012)

genome sequence. An ENCODE pilot project examined 1% of the genome within a limited number of cell types [13]. The ENCODE project has expanded to a genome-wide survey of 294 cell types (June 2012). A subproject of ENCODE, called GENCODE, has the objective to annotate all genes in the human genome including protein-coding (with alternative transcripts), noncoding, and pseudogenes [14]. The most recent version of GENCODE (v12) has 53,934 genes with less than half being protein-coding.

Although the idealized concept of gene may simplify downstream analyses and is the foundation for many tools in systems biology, it is masking the detection of novel transcripts both protein-coding and noncoding. By ignoring nonreference transcripts, we may be misrepresenting the levels of reference transcripts and thereby, skew—and even invalidate—downstream work. Below are some examples of the recent discoveries that are shifting the mindset away from associating functions to genes.

## 8.3 Alternative Protein-Coding Transcripts

The expression of alternative protein-coding transcripts can be both tissue and condition specific and has been implicated in disease [15]. Most protein-coding regions encode for an average of five alternative transcripts [13, 16]. There are only 20,110 protein-coding genes found in GENCODE; whereas the number of distinct translations is 81,480 with 14,739 genes that have more than one translation (GENCODE v12; December 2011).

Alternative transcript splicing can alter protein function by altering the composition or conformation of a protein including protein domains and localization signals. Alternatively spliced transcripts of clusterin, for example, when translated to protein isoforms have opposing biological functions: one isoform inhibits while the other promotes apoptosis [17, 18]. AR can be alternatively spliced to generate

ligand-independent protein isoforms expanding our understanding of the continuing role of AR in a low androgen environment [19–23]. More than 20 additional AR transcript variants have been identified but little is known about their biological function or significance to disease [20]. The *KLK3* genomic region, encoding the androgen-responsive PSA biomarker, can generate at least 15 different transcripts [24]—many with unknown function and many that are not represented in reference RNA databases. High-throughput RNA profiling experiments continue to reveal cellular potential for alternative splicing [25, 26]. The biological significance for most of the alternative splice events, however, remains unknown.

Alternative transcripts exist that do not alter the protein-coding potential of the transcripts but instead alter UTRs which regulate protein translation efficiencies, RNA stability, or RNA localization. These alternative 5′ or 3′ UTRs are generated by alternative promoter usage and alternative polyadenylation sites, respectively, and are commonly seen to correlate with tissues and conditions [27]. Estimates suggest that over half of protein-coding mRNAs can have an alternative promoter [28, 29] and/or an alternative polyadenylation site [30]. Global analysis of 3′ UTR usage has shown that truncated 3′ UTRs can be associated with proliferating cells [31]. A coordinated shift to use truncated 3′ UTRs has also been identified in some types of cancer cells [32, 33]. The mechanism for these global changes in 3′ UTR length is unknown. mRNA with shorter 3′ UTR can be more stable by evading translation repression machinery (e.g., miRNA and the RISC complex) and therefore lead to high protein levels [32]. Longer 3′ UTRs extending well beyond previously annotated polyadenylation sites have also been identified [34, 35].

RNA sequencing projects are revealing chimeric transcripts that are encoded on distant DNA and sometimes different chromosomes. In the ENCODE pilot study, over half of the protein-coding genes studied utilized exons outside boundaries of an annotated gene [13]. Many transcriptional starts sites can be located at large distances upstream of the annotated start sites often skipping neighboring protein-coding regions of the DNA [36]. Some chimeric transcripts are formed by genomic rearrangements such as the fusion of the androgen-regulated promoter and first exons of *TMPRSS2* to protein-coding exons of *ERG* identified in ~50% of prostate tumors [37]. Many efforts have been made to identify these chimeric transcripts as they provide potential disease-specific biomarkers and therapeutic targets [38].

Chimeric transcripts of *SLC45A3-ELK4* are of particular interest as they are regulated by androgens and, though expressed in normal tissue, they are expressed at high levels in a subset of PCa samples (Fig. 8.2). Unlike the *TMPRSS2-ERG* fusion, the presence of *SLC45A3-ELK4* transcripts is not restricted to samples with genomic rearrangements [39]. *SLC45A3* and *ELK4* are located 25 kb apart on chromosome 1 but are fused by a *cis*-splicing or read-through mechanism of adjacent genes [40]. A recent RNAseq experiment identified additional read-through chimeric transcripts in prostate tumor samples; the function and clinical significance of these chimeric transcripts remains unknown [41]. The discovery of chimeric transcripts forces a shift in our perception of the role and complexity of RNA transcription. Through mechanisms such as *cis*- or *trans*-splicing, RNA may not be a simple linear copy of DNA but may use modular regions of the DNA [42].

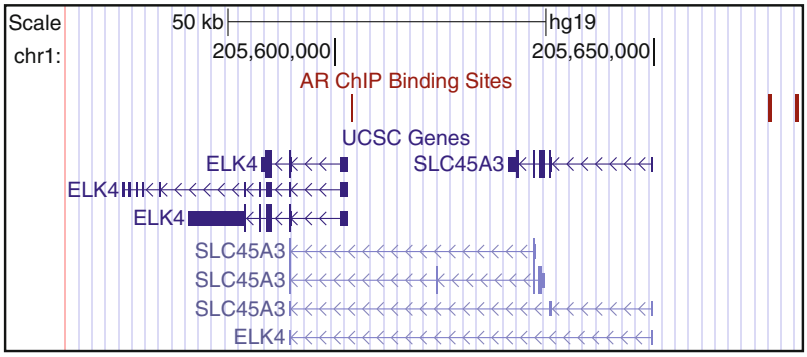


**Fig. 8.2** UCSC genome browser representation of chimeric *SLC453-ELK4* transcripts. The protein-coding transcripts for *SLC45A3* and *ELK4* encode on the negative strand on chromosome 1q32.1 are shown in *dark blue*. Chimeric transcripts of *SLC45A3-ELK4* are shown in *light blue*. Consensus AR binding sites from three genome-wide ChIP experiments [8, 9, 92] are shown in *red*

## 8.4 Noncoding RNA

Large scale high-throughput RNA profiling projects have revealed complex overlapping, interleaved, antisense, and intergenic transcription that cannot be attributed to protein-coding transcripts. Estimates from the ENCODE pilot project suggest that ~93% of the human genome is transcribed into RNA but less than 2% of that encodes for proteins [13]. Transcription from regions of "junk DNA" [43] that did not encode for a protein was previously viewed as transcriptional noise. Although the estimates for transcriptional potential may be liberal due to measurement sensitivity, it is becoming increasingly apparent that non-protein-coding RNA—termed noncoding RNA (ncRNA)—plays a significant regulatory role in cellular function. Many ncRNAs are spliced and polyadenylated and have comparable half-lives to protein-coding RNAs [44].

Although the sequences of many ncRNAs are highly conserved through evolution, it is difficult to rule out evolutionary conservation of the functional structure of an ncRNA [45]. Many functional ncRNAs have been shown to have rapid sequence evolution implying that a lack of sequence conservation does not equate to a lack of function [46]. Some of the examples of ncRNA described below will illustrate the potential for ncRNAs to provide a sequence specific signal for a generic protein complex [47]. Many therapeutics are designed to target the generic protein complex where it may be more effective to target a disease- or condition-specific RNA signal.

ncRNAs are crudely classified based on conventional RNA purification methods: small ncRNAs are shorter than 200 nt and long ncRNAs (lncRNA) are longer than 200 nt [48]. In the last decade, most of the research in the field of ncRNA has been focused on microRNA (miRNA). miRNA are ~22 nt single stranded RNAs that provide the sequence-specific component of the RISC protein complex that allows

for targeted translational repression of protein-coding transcripts. An miRNA can be processed from intronic transcription of a protein-coding RNA either independently (i.e., separate promoter) or as part of the protein-coding transcript or from an lncRNA. Small RNAs are difficult to detect using microarray technologies due to the short length of the RNA molecule and cross-hybridization potential. Early microarray experiments to detect miRNA expression gave inconsistent results especially in the context of miRNA profiling in PCa [49]. A recent small RNAseq experiment described 17 androgen-responsive miRNAs in LNCaP cells and 42 following castration in AR-positive xenografts [50].

Although early small RNA sequencing experiments were designed to detect miRNA, many other functional classes of small RNAs can be detected including small nucleolar RNA (snoRNA), involved in guiding chemical modifications of other RNAs; small nuclear RNA (snRNA), involved in RNA splicing; tiRNAs, associated with transcription initiation [51]; spliRNA, associated with RNA splice sites [52]; PIWI-interacting RNA (piRNA), involved in maintaining genome integrity and regulating the expression of transposable elements [53]. Small RNA sequencing experiments are revealing many new classes of small RNA whose mode of action is still unclear. It is intriguing that in vitro siRNA knockdown of *PIWIL1*, a protein that binds piRNAs to maintain genome integrity, increased the frequency of the *TMPRSS2-ERG* chromosomal translocation in normal prostate epithelial cells [54]. Evidence is mounting that small RNAs play an important role in cancer and cancer progression. The design of appropriate therapies to modulate small RNA function will open a whole new avenue for targeted therapeutics.

There has been less focus in the literature on the regulatory roles of lncRNA. Examples of lncRNAs have been shown to regulate numerous biological processes including chromatin-remodeling, transcription, and posttranscriptional processing [48]. Survey studies of RNA expression identified lncRNAs expressed in different tissues, cell types, subcellular locations, different stages of development, and during cell differentiation [55–58]. Functional classification of lncRNA has been mainly based on genomic context in relation to protein-coding transcripts: antisense (transcribed from the opposite strand of the chromosome), intergenic (transcribed outside of the annotated boundaries of a protein-coding gene), promoter or enhancer associated, intronic, and independent 3′ UTRs. Katayama et al. [59] estimated based on the FANTOM3 large scale cDNA sequencing project that ~72% of protein-coding transcripts in the mammalian genome have divergent, convergent, and fully overlapping antisense transcription [59]. The estimates for antisense transcription may however be liberal. The discovery of antisense artifacts generated by a reverse transcriptase enzyme during cDNA library construction has raised some concerns about current methods for detecting antisense transcription [60]. The possibility of artifact, however, should not detract from the significant potential of antisense transcription seen in such validated examples as X-chromosome inactivation regulated by the *XIST-TSIX* sense–antisense pair [61], *p15* tumor suppressor protein (also known as *CDKN2B*) inhibited by a *p15* antisense (*p15as*) transcript [62], and the *HOXD* genomic loci inhibited by *HOTAIR* (expressed antisense to the *HOXC* genomic loci) [63].

All three of the antisense transcripts described above interact with chromatin-remodeling machinery to regulate transcription by altering chromatin state. The interaction of these lncRNAs with chromatin-remodeling machinery suggests that lncRNAs could serve as scaffolds for the assembly of histone modification complexes at specific genomic loci [64]. In the *p15as* example, Yu et al. found an inverse expression between *p15as* and *p15* in tumor samples from patients with leukemia, a disease where epigenetic silencing of *p15* is common. Yu et al. showed that in vitro overexpression of *p15as* increased silencing of *p15*. The silencing of *p15* remained persistent after expression from the *p15as* construct was turned off. If continuing expression of an lncRNA was not required for persistent epigenetic silencing, low levels of the lncRNA would be sufficient to alter cellular state. The persistent epigenetic silencing of *p15* has interesting therapeutic implications from a global perspective suggesting that it may be possible to co-opt a cancer cell's epigenetic silencing mechanisms through RNA-based therapies to achieve persistent silencing of oncogenic pathways.

In addition to antisense transcription, many lncRNAs are transcribed in regions outside the annotated boundaries of known genes. These intergenic lncRNAs have recently been termed lincRNAs (large intergenic noncoding RNAs) [58, 65, 66]. Khalil et al. found that 24% of the lincRNAs that they tested physically associated with the chromatin-inactivating complex, PRC2—the complex responsible for the repressive H3K27me3 histone modification. One of the antibodies used in this experiment was against EZH2, a member of the PRC2 complex. This is particularly interesting in relation to PCa research as EZH2 has been reported to be increased in metastatic PCa [67, 68].

lncRNAs can be associated with transcriptional activation through interaction with chromatin-activating complexes [56] and through transcription at enhancers and promoters [69, 70]. Wang et al. [70] recently reported that transcription following FOXA1 binding at enhancer regions can regulate a subset of the AR transcriptional program in PCa cells. Promoter and enhancer associated transcripts may help to assemble transcriptional machinery at specific genomic loci.

lncRNAs can be associated with alternative splicing as in the case of *ZEB2*. *ZEB2* is a protein that plays a role in epithelial to mesenchymal transition (EMT) and migration of cancer cells. *ZEB2* antisense transcription masks a splice site in the 5′ UTR of *ZEB2* preventing the excision of the internal ribosomal entry site necessary for protein translation [71].

lncRNAs can act as decoys to compete with 3′ UTRs of protein-coding transcripts to alter the dynamic regulation by miRNA and RNA-binding proteins. Analysis of publically available cDNA and CAGE sequencing data (CAGE sequencing uses the 5′ cap of RNA to identify the first 20–25 nt in polyadenylated RNAs) detected expression of independent transcripts within 3′ UTRs of protein-coding transcripts [72]. Using in situ hybridization, Mercer et al. reported that some of these 3′ UTR transcripts had tissue- and condition-specific expression that was not correlated with the protein-coding region of the transcript. Independent 3′ UTR transcripts may bind miRNAs or RNA-binding proteins thereby altering their availability for regulation of protein-coding transcripts. A similar modulation of miRNA dynamics has been

identified through the transcription of pseudogenes [73]. Pseudogenes can be generated by genetic duplication or by retro-transposition of protein-coding transcripts. Although pseudogenes have lost protein-coding capacity, they retain regulatory regions such as UTRs and can still be transcribed. PTEN pseudogene 1 (*PTENP1*) transcripts can interact with *PTEN*-targeting miRNAs allowing *PTEN* transcripts to evade miRNA-mediated translation repression. Increased expression of the *PTENP1* transcript leads to increased *PTEN* protein levels [73]. The expression of decoy RNA sequences can alter the translation of many different proteins. The exogenous expression of decoy RNA sequences in vitro can inhibit the activity of a miRNA and may be an approach for therapeutic inhibition of miRNAs [74].

lncRNAs can interact with proteins to modulate their localization and function. An intriguing example is the interaction of a hairpin structure of an lncRNA, growth-arrest-specific 5 (*GAS5*), with the DNA-binding domain of the glucocorticoid receptor (GR). *GAS5* expression can decrease ligand-dependent transcriptional activity of GR. *GAS5* can also inhibit ligand-dependent transcriptional activity of other steroid hormone receptors that share similar DNA response elements such as androgen, progesterone, and mineralocorticoid receptors [75]. It is possible that other transcription factors may have similar unidentified decoy RNAs that mimic DNA binding to prevent DNA–protein interactions. In contrast to *GAS5*, the lncRNA, steroid receptor RNA activator (*SRA*), can interact with the N-terminal domain of steroid receptors to promote their transactivation [76].

## 8.5 Long Noncoding RNA Expressed in Prostate Cancer

A large focus of PCa research has been on androgen and the networks of proteins that it regulates with little effort spent on understanding the role of androgen regulated noncoding RNAs. Until recently three PCa specific lncRNAs have been described: *PCGEM1*, *PCA3*, and *PRNCR1*. *PCGEM1* (prostate-specific transcript 1) is overexpressed in primary prostate tumors compared to benign prostatic tissue in the majority of patients [77]. Overexpression of *PCGEM1* in an androgen-dependent cell line (LNCaP) promotes cell proliferation and an increase in colony formation, suggesting *PCGEM1* has a functional role in prostate tumorigenesis [78]. *PCA3* (prostate cancer antigen 3; also known as *DD3*) is a PCa-specific lncRNA which is being developed as a potential new diagnostic biomarker. In clinical trials, *PCA3* was able to predict the outcomes of prostate biopsies and in conjunction with PSA was shown to be more specific than PSA alone (reviewed in [79]). The biological function of *PCA3* has yet to be determined. *PRNCR1* (prostate cancer noncoding RNA 1) is encoded in a gene desert of the PCa susceptibility locus 8q24. Cell viability in LNCaP cells was decreased following siRNA knockdown of *PRNC1* [80]. Reis et al. performed a more systematic survey of lncRNA expression in PCa using a custom low density cDNA microarray to detect antisense transcription from intronic sequences. They detected many intronic transcripts in PCa cells with 39 being androgen responsive [81, 82].

Recent analysis of RNAseq experiments from 102 prostate tissues and cell lines detected 1,859 unannotated lincRNA. One hundred and twenty one of those lincRNA—called prostate cancer-associated transcripts or PCATs—were differentially expressed in PCa compared to benign tissue [83]. The lincRNAs were detected using ab initio reconstruction of the transcriptomes of 20 benign adjacent, 47 localized, 14 metastatic, and 21 PCa cell lines. In further functional characterization, *PCAT1* (encoded on 8q24) was found to interact with the PRC2 complex and with overexpression had a modest increase in cell proliferation. *PCAT1* was considerably increased in a subset of metastatic and high-grade localized cancers [83].

## 8.6 Multitasking Genomic Loci

Transcription is complex with many genomic loci encoding highly regulated sets of transcripts. The extent of biological complexity cannot be described by grouping transcripts encoded in a genomic locus into one gene with one associated function [84–86]. The term "gene" may function in the context of classical genetics where a gene is a unit of inheritance with an associated phenotype. The gene-centric view of biology does not fully describe the functional potential of a genomic locus. Gene-centric databases like NCBI Entrez Gene and GO Ontologies are invaluable for global analyses of large scale RNA profiling experiments and critical for systems biology but they may be masking the complexity of genomic loci. A genomic locus can have the potential to generate multiple protein isoforms with different and potentially opposing function through alternative RNA splicing and promoter usage. A miRNA with the potential to inhibit the translation of a large spectrum of protein-coding mRNAs can be processed itself from the intron of a protein-coding mRNA. A genomic locus can be transcribed in both directions and can have local (e.g., *p15* antisense) and distal (e.g., *HOTAIR*) regulatory roles. Figures 8.3 and 8.4 show examples of the types of transcripts encoded at multitasking genomic loci.

The lines defining a genomic locus as protein-coding or noncoding are further blurred by the *SRA* example. As described above, the lncRNA, *SRA*, can promote transactivation of steroid receptors. An isoform of *SRA* transcribed from the same genomic locus can encode for a protein called SRAP [87]. SRAP protein inhibits *SRA* RNA functional activity through direct protein–RNA interaction [88]. Although the functional significance of overlapping protein-coding and noncoding transcripts is rarely studied, 991 of the 20,286 protein-coding genes in the NCBI Entrez Gene database have 4,867 associated noncoding transcript variants (RefSeq release 52).

An individual protein-coding transcript may also have additional noncoding regulatory functions. An example is the *p53* mRNA that has both protein-coding and noncoding function. *p53* mRNA interacts directly with the MDM2 protein to prevent MDM2 from promoting the degradation of p53 protein. Cancer-derived silent point mutations in *p53* mRNA do not alter p53 protein composition but instead altered p53 protein stability. The point mutation prevents *p53* mRNA from binding to MDM2 permitting MDM2 to degrade p53 protein [89]. The UTRs of a protein-coding mRNA

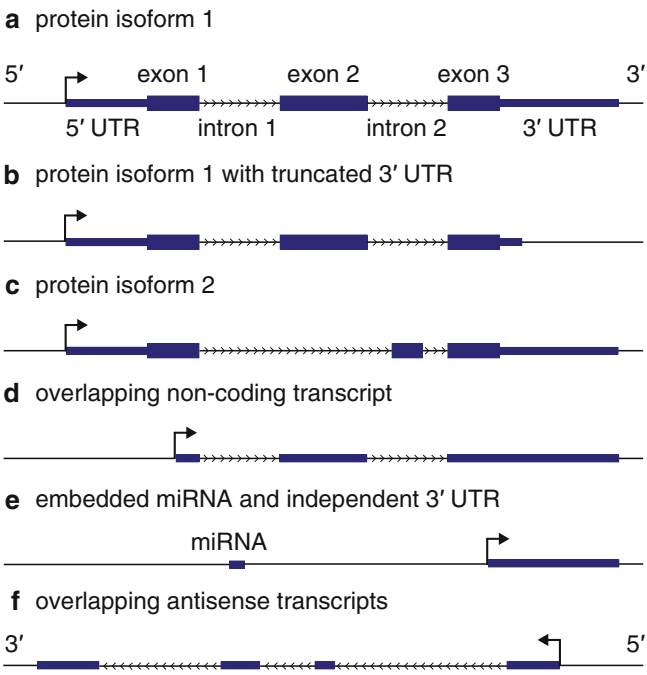


**Fig. 8.3** Examples of transcripts encoded at multitasking genomic loci. (**a**) the transcript encoding protein isoform 1 has a 5′ UTR (*short block on right*), three exons (*tall blocks*), and a 3′ UTR (*short block on the left*). Additional transcripts can be encoded at the same genomic loci including transcripts with alternative polyadenylation sites (**b**), alternative splicing (**c**), alternative transcriptional start sites (**d**, **e**), overlapping noncoding transcription (**d**, **e**), embedded miRNA (**d**), and overlapping antisense transcription (**f**)

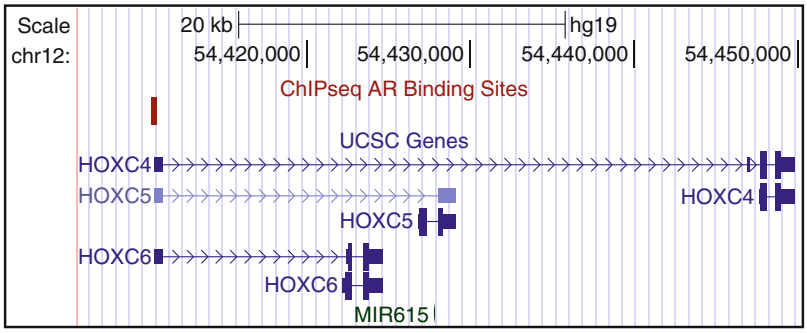


**Fig. 8.4** UCSC genome browser representation of a *HOXC* multitasking genomic locus A multitasking genomic locus on chromosome 12q13.13 encodes the protein-coding transcripts *HOXC4*, *HOXC5*, *HOXC6* (*dark blue*). The *HOXC4* protein may be translated from transcripts with a unique 5′UTRs or from a 5′UTRs shared with *HOXC5* and *HOXC6*. Usage of the common 5′UTR generates an overlapping noncoding RNA for *HOXC5* (*light blue*) and an alternative protein isoform for *HOXC6*. An AR-binding site is found upstream of the common 5′UTR (*red*) [8, 9, 92]. The miRNA, miR-615, is embedded in an intron of *HOXC4* and *HOXC5* (*green*)

may also have currently unknown regulatory functions. The example of the PTEN pseudogene (*PTENP1*) modulating miRNA dynamics brings into question the regulatory role of UTRs as part of a protein-coding mRNA. Many UTRs can be larger than their protein-coding counterparts and may themselves regulate miRNA dynamics or interact with RNA-binding proteins. Most functional experiments designed to elucidate the role of a protein-coding transcripts introduce a vector containing only the protein-coding sequence; overexpression of the complete mRNA sequence, including UTRs, may have a different influence on cellular state. Genome-wide experiments such as RNAseq and RNA coimmunoprecipitated with antibodies against RNA-binding proteins (RIPseq) [90, 91] will further elucidate state-specific RNA expression patterns and potential functional interactions with proteins.

## 8.7 Implications for the Detection of Androgen-Responsive Genes

The integration of genome-wide AR ChIP and gene expression profiling has advanced our understanding of androgen-AR signaling. AR-occupied enhancer regions can have different transcriptional capacity depending on histone acetylation and coregulator presence [5]. AR has a role in regulating central metabolism and biosynthesis in PCa [92]. AR interacts with ERG signaling in PCa with *TMPRSS2-ERG* fusions [9]. The expression of transcripts at AR enhancer sites (eRNA) by pioneering transcription factors such as FOXA1 can determine cell-type-specific AR signaling programs [70]. A distinct but overlapping androgen signaling program has been seen in androgen-sensitive and CRPC cell lines [8]. This difference in AR signaling in CRPC may, in part, be due to differences in transcriptional programs between full-length AR and ligand-independent AR splice variants [20, 23].

These genome-wide AR ChIP experiments show few AR bindings sites in classical upstream promoter regions (5 kb upstream of transcriptional start sites) of reference RNA. Many AR-binding sites are found in enhancer regions, introns, or distant intergenic regions [9, 92]. The apparent few numbers of AR-binding sites located at proximal promoters may be explained by chromatin looping. AR has been found to activate transcription by promoting chromatin looping which brings together AR binding at distant enhancer sites with AR binding at proximal promoter sites [93, 94]. Although genome wide AR ChIP experiments are starting to give an unbiased view of AR binding, it is difficult from a linear representation of the DNA to infer androgen regulation without a clear understanding of dynamic chromatin structure.

In addition to a limited understanding of chromatin structure, we have limited understanding of the extent of transcription in prostate cells and how it is dysregulated in cancer. AR may be regulating a subset of transcripts not previously profiled with microarray technologies. Most current computational approaches average expression across a reference RNA sequence or consolidate expression to a gene level. With lower density arrays, relying on expression values from probes in a 3′ UTR may be misleading as the 3′ UTR may be expressed independently of the coding

sequence or an alternative 3′ UTR may be used. With high density microarrays, averaging the expression of individual probes across a reference RNA sequence will mask alternative splice variants and overlapping noncoding RNA. These differences may explain discrepancies between the values obtained by microarray, qRT-PCR, and Western blots. The subtleties of multitasking genomic loci and alternative transcripts are often overlooked when integrating data across profiling platforms leading to inconsistent and sometimes invalid results.

Although RNAseq has many advantages over high density microarrays, incorrect assumptions made during analysis can be misleading. Most data currently generated by RNAseq is not strand specific; however, many genomic loci have overlapping antisense transcription. Incorrect assumptions about the direction of transcription can not only completely overlook antisense transcription but can also corrupt expression values for sense transcripts. RNAseq has the advantage of being a relatively unbiased technology; however, relying completely on RNA reference sequences for analysis reintroduces a gene-centric bias. The application of de novo transcriptome assembly algorithms to RNAseq data may identify novel transcripts as well as transcripts generated by read-through and *trans*-splicing mechanisms (algorithms reviewed in Garber et al. [95]). Integration of genome-wide experiments such as RIPseq, to examine RNA–protein interactions; ChIPseq, to examine DNA–protein interactions; CAGE, to identify transcript start sites; and strand-specific RNAseq, to identify splicing events, will provide additional information to further understand the androgen-AR signaling axis.

Expression profiling experiments have yielded invaluable information on the biological functions of androgens and AR and the related treatment responses to ADT and AR antagonists. The discovery of previously overlooked tissue- and disease-specific alternative transcripts, noncoding RNA, chimeric transcripts, and multitasking genomic loci should prompt a review of older profiling data as well as the design of new profiling experiments. The rapid advancement of profiling technologies will continue to push our understanding of biology with the goal of identifying potential targets to develop therapies for men with advanced PCa.

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# Chapter 9
# Androgen-Responsive Gene Expression in Prostate Cancer Progression

**Amy H. Tien and Marianne D. Sadar**

**Abstract** Benign and cancerous prostate tissue is dependent upon androgens. Androgen ablation causes prostate tissue to undergo apoptosis which thereby provides the rationale of castration as a systemic therapy for advanced prostate cancer. The full-length androgen receptor is a ligand-activated transcription factor that regulates the expression of genes required for growth, function, and survival of prostate cells in response to androgen. Androgen binds to the androgen receptor which then translocates to the nucleus to bind to androgen response elements on target genes termed "androgen-responsive genes" (ARGs) to regulate their transcription and levels of expression. Identification and characterization of ARGs may provide an understanding of androgen receptor signaling, resistance mechanisms to current hormonal therapies, and reveal biomarkers for patient selection and sequential application of current and new therapies targeting the androgen axis. This review addresses differential expression of ARGs following androgen ablation treatment during progression of advanced prostate cancer.

**Keywords** Androgen-responsive gene • Prostate cancer • Androgen receptor • Castration-resistant prostate cancer (CRPC) • Gene expression

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## 9.1 Introduction

### *9.1.1 Prostate and Androgen*

The prostate depends on androgen with androgen ablation therapy (castration) inducing programmed cell death and involution of the prostate. Androgen receptor mediates the effects of androgen and thereby regulates the development, function, and survival of prostate cells. Prostate cancer is also dependent upon androgens and this is why castration is a form of therapy for advanced disease. When androgen binds to the androgen receptor, the receptor becomes transactivated and translocates to the nucleus where it binds to androgen-response elements (AREs) in enhancer and promoter regions of target genes to regulate their transcription. The target gene is generally termed an "androgen-responsive gene" (ARG) and its levels of expression can be altered by addition or withdrawal of androgen. Expression of ARGs can be regulated at many different levels, such as transcription, RNA processing, RNA stability, protein translation, and protein stability [1]. ARGs are fundamental in normal prostate development, growth, survival, and function and many of these genes are speculated to be important in the progression of prostate cancer [2].

### *9.1.2 Androgen Receptor Mechanisms of Resistance*

Resistance of prostate cancer to androgen ablation therapy is inevitable at least by current approaches. Initially based upon the reexpression of ARGs such as prostate-specific antigen (PSA) in castration-resistant prostate cancer (CRPC), there have since been many proposed mechanisms that involve resumed androgen receptor signaling that include overexpression and/or gene amplification of androgen receptor; gain-of-function mutations in the ligand-binding domain of androgen receptor; androgen receptor splice variants lacking the ligand-binding domain; ligand-independent activation by alterative signaling pathways; and/or increased levels of intracellular androgen [3–8].

### *9.1.3 Identification of Androgen-Responsive Genes*

The quest to identify ARGs lies in their potential to reveal biomarkers for prognosis and patient selection for therapies as well as yield insight into novel approaches for the development of new treatments for prostate cancer. Identification of ARGs has encompassed numerous models including prostate cancer cell lines, rodents, and/or human xenografts. It is estimated that 1.5–4.3% of genes are responsive to androgen in LNCaP cells [9]. This percentage includes the genes directly or indirectly (as a

consequent event) regulated by androgen receptor. In 2009, 1,785 human genes, 583 rat genes, and 993 mouse genes were considered to be ARGs based on a literature search [10]. Most ARGs identified have functions in binding (72%) or catalytic activity (36%) according to gene ontology (GO) analysis from the Androgen-Responsive Gene Database website (http://argdb.fudan.edu.cn/index_info.php). There are many other cellular functions mediated by androgen receptor signaling. Understanding the changes in expression of ARGs between benign and cancerous prostate and ultimately in lethal CRPC may facilitate the development of therapeutic approaches to target androgen receptor signaling. Expression patterns of ARGs may provide indications of time, duration, and doses of therapies required to inhibit androgen receptor.

### *9.1.4 Prostate-Specific Antigen: The Prototype ARG*

The best characterized ARG is prostate-specific antigen (*PSA/KLK3*). The *PSA* gene contains several functional AREs in its enhancer and promoter regions and its transcription is highly inducible by androgen [11–14]. Although cellular levels of PSA may decrease in cancer compared to normal prostate tissue, especially in poorly differentiated tumors, levels of PSA in the serum enables the early detection of prostate cancer. It is important to note that serum levels of PSA do not correlate to tissue levels in any stages of prostate cancer [15–17]. Serum level of PSA is also a useful early indication of biochemical failure and recurrence of prostate cancer after primary therapy as well as after secondary and tertiary therapies. After primary therapy, 30% of patients will have biochemical failure and require secondary treatment, which involves androgen ablation by chemical or surgical castration. For those patients with metastatic disease, approximately 75–80% respond to androgen ablation therapies [18]. However, androgen ablation therapy is only palliative with ultimate failure and progression to lethal CRPC [19]. New approaches involving chemotherapy, immunotherapy, or new hormonal approaches recently approved by the FDA have improved overall survival by approximately 2–5 months for men with CRPC [7, 20–24]. Current approaches of androgen ablation and androgen receptor blockade are considered to fail by mechanisms that lead to resumed transcriptionally active androgen receptor and concomitant expression of ARGs, or at least a subset of ARGs.

## 9.2 Approaches of Gene Expression Analyses

### *9.2.1 Model Systems for Samples*

Gene expression profiles have been carried out in human prostate cancer cell lines treated with and without androgen such as dihydrotestosterone (DHT) or R1881,

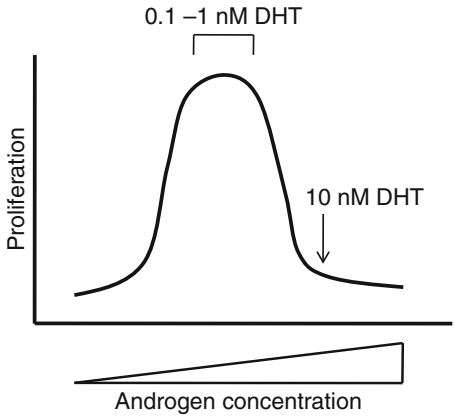


**Fig. 9.1** Effect of androgen concentration on LNCaP cell proliferation. The maximal proliferative response by androgen, DHT as an example in this diagram, is between 0.1 and 1 nM for in vitro LNCaP cell culture. The cells stop proliferating when DHT is higher than 10 nM

a synthetic androgen. Most of the cell lines used were originally derived from metastatic disease. LNCaP, derived from lymph node metastatic tumor, is the most frequently analyzed cell line for transcriptome changes in response to androgen. The frequent use of LNCaP cells is probably because it is a well-differentiated human prostate cancer cell line that expresses a functional androgen receptor and can be grown as a xenograft in murine hosts to mimic several aspects of human disease such as becoming castration resistant when the host is castrated [25]. LNCaP cells have a mutation in the ligand-binding domain at T877A making the receptor more promiscuous and activated by other steroids and some antiandrogens [26–30] which may impact gene expression signatures. It is important to note that androgen not only causes growth and survival but also causes differentiation of prostate cells. This is an important concept when studying ARGs as different subsets of genes may be expressed depending on whether cells are proliferating or become quiescent with differentiation. LNCaP cells display biphasic response on proliferation with increasing concentrations of androgen. The cells proliferate in response to DHT ranging between 0.1 and 1 nM. However, proliferation is inhibited and cells become more differentiated when DHT concentration is 10 nM or higher [31–33]. A simple diagram of the biphasic proliferative response is shown in Fig. 9.1. This biphasic proliferative effect may occur at slightly varying concentration ranges because of different cell line types, cell culture passage number, culture conditions prior to androgen treatment, and treatment duration [31, 34–36]. These variables may be responsible for the relatively few numbers of ARGs that are commonly detected amongst different gene expression profiling studies [37]. The spectrum of expression of ARGs over different concentrations of androgen still remains relatively uncharacterized with few studies that have examined ARGs using the relevant concentrations of androgens that would be encountered under castrate conditions.

Advantages of using cell lines include that they are relatively homogeneous, easily accessible, and stringent control of experimental conditions can be achieved thereby providing potentially highly reproducible data. However, cell lines growing as a monolayer on plastic culture dishes and passaged many times may not accurately

reflect the complexity of tumor environment nor represent different stages of disease. Therefore human xenografts and rodents have been explored as more complex models. Rodents with intact tissue structure including cell–cell interaction between different types of cells, such as epithelial cells, stromal cells, and blood cells make them suitable as an in vivo model for gene expression profiling analyses. However, these too may fall short and do not necessarily reflect the complexity of tumor progression in humans. Of note are the physiological differences between rodents and humans in the structure of the prostate. Unlike human prostate separating into zones, rodent prostate consists of distinct lobes. Rat ventral lobe is the most commonly studied because the epithelial cells in the ventral lobe undergo involution in response to androgen ablation [1]. Mouse dorsolateral lobe is the most commonly studied because the gene expression pattern in these two lobes is more similar to human prostate than other lobe [38]. Of note, the most characterized ARG in humans, *PSA*, is not expressed in rodents. This allows monitoring of serum PSA in mice carrying human xenografts to be a relatively accurate indication of tumor growth since only the human prostate tissue would be accountable for levels of PSA in the blood.

### *9.2.2 Clinical Samples*

Difficulties in obtaining clinical samples from patients before and after castration from both the prostate as well as distant sites that are suitable to measure levels of RNA have led to fewer gene expression studies with clinical tissues. Generally, clinical samples are categorized based on disease stages, treatment type, treatment duration, location of tissues (primary prostate or metastatic tumor), and androgen responsiveness (androgen sensitive or castration resistant). Several factors need to be considered when using clinical samples for transcriptome analyses. First, the sample quality and sample handling affect gene expression analyses. Freshly frozen tissues and paraffin-fixed tissues (and fixing duration) provide different RNA quality (stability and degradation) [39]. Second, heterogeneity of cell populations in a tumor sample may complicate the gene expression analyses. Therefore, enrichment of epithelial cells or tumor cells is usually achieved by laser capture microdissection. The importance of microdissection is emphasized for those clinical samples with a Gleason Score greater or equal to 7. This is because of the often potential lethality of a Gleason Grade of 4 or more. Combination of RNA from both fields, such as a Gleason 4 with a Gleason 3 from a Gleason Score 7 tumor may confound interpretation of data since Gleason Grade 3 is considered generally to be relatively benign [40–42].

### *9.2.3 Methods of Analysis*

Different platforms for genome-wide expression profiling have varying sensitivities to detect ARGs. Dehm and Tindall [9] summarized the following percentages of

genes that were detected to be regulated by androgen in LNCaP cells depending upon the approach: serial analysis of gene expression (SAGE) analyses yielded 1.5–2.1%; oligonucleotide array analyses, 2.8–3.7%; and cDNA array analyses, 4.3%. However, it is important to consider the total number of genes detected by each platform which may vary considerably.

Depending on the methods used to generate gene expression profiles, starting materials such as total RNA or mRNA (polyA$^+$ RNA) may result in different detection sensitivities due to RNA extraction manipulation and influence of non-transcripts. Reverse-transcribed cDNA or amplified RNA is used as the source for detection by different profiling methods. Other considerations include that tumor samples from individual patients are usually pooled to obtain enough material and to have an average expression value, but this approach may mask the variation between individuals. The choice of reference sample for comparison and to present data as fold change is also a critical factor that influences the data obtained, interpreting changes in expression, and for comparison amongst different studies. Ultimately data validation is carried out by quantitative polymerase chain reaction, tissue microarray, or immunohistochemistry due to the propensity of high-throughput approaches to generate false positives and less than optimal dynamic ranges of some approaches which may underestimate fold-change.

## 9.3 ARGs

### *9.3.1 ARGs Detected Using Human Cells Lines*

Human prostate cell lines have been used in a large number of ARG expression studies. Reviews from Dehm and Tindall [9] and Clegg and Nelson [1] comprehensively discuss the ARGs and their functions in prostate cancer and provide a list of the studies about gene expression profiling using human prostate cell lines or rodent models. As mentioned in Sect. 9.2.1, different cell lines may respond differently to varying concentrations of androgen, and even in the same cell lines, especially the extensively studied LNCaP cells. Such differences may be contributed from the strains of LNCaP cells or variations in culture and/or experimental conditions [37, 43]. For example, the presence or absence of serum in the control samples not treated with androgen will have profound effect on overall differences in the expression patterns obtained. This is because serum would provide cholesterol precursors for potential de novo androgen synthesis as well as contains a variety of growth factors and cytokines that may impact androgen receptor activity and gene expression.

Velasco et al. [37] cross-compared expression patterns of four in vitro studies and revealed that only 13 ARGs were common amongst four different studies: *BCHE*, *CDK8*, *CTBP1*, *DHCR24*, *FKBP5*, *FN1*, *HERC3*, *PSA/KLK3*, *LIFR*, *MMP16*, *NDRG1*, *PIK3R3*, and *RAB4A*. Interestingly, an oligonucleotide microarray and

LongSAGE analyses in LNCaP cells treated with R1881 also detected differential expression of 7 of these genes: *DHCR24*, *FKBP5*, *PSA/KLK3*, *MMP16*, *NDRG1*, *PIK3R3*, and *RAB4A* [44, 45]. One study that analyzed ARG identified in vitro for relevance in clinical samples revealed that transcripts of *CAMK2N1* and *GLO1* were significantly increased in the primary lesions from patients that later had biochemical failure [46].

Importantly LNCaP cells have been used to identify genes potentially associated with CRPC. The majority of CRPC involves osseous metastases and hence creating in vitro conditions that tried to mimic CRPC was attempted by culturing LNCaP cells with osteoblast-conditioned media [47]. In the absence of androgen, osteoblast-derived factors activate androgen receptor transcriptional activity and increase proliferation of LNCaP cells [47, 48]. The gene expression signature that was identified in LNCaP cells in response to osteoblast-conditioned media was enriched with ARGs such as *PSA/KLK3*, *ACPP*, and cell cycle-related genes that included *ASNS*, *AURKB*, *BUB1*, *BUB1B*, *CCNA2*, *CCNB1*, *CCNB2*, *CCNE2*, *CDC2*, *CDC6*, *CDC7*, *CDC20*, *CDC25C*, *CDKN3*, *CHEK1*, *DEPDC1*, *ESPL1*, *GTSE1*, *HCAP-G*, *MCM5*, *PLK1*, *SMC4L1*, and *STK6*. Blocking interleukin-6, which is abundant in osteoblast-conditioned media and also known to activate the androgen receptor in the absence of androgen [49], decreased expression of *AURKB*, *CCNA2*, *CCNB1*, *CCNB2*, *CDC2*, and *PSA/KLK3* in response to osteoblast-conditioned media [47]. Importantly, this gene expression signature could identify both clinical metastases and CRPC. All of the cyclins identified in response to osteoblast-conditioned media (*CCNA2*, *CCNB1*, *CCNB2*, and *CCNE2*) and most of the CDCs (*CDC2*, *CDC6*, *CDC20*, *CDKN3*, and *CDC25C*) were upregulated in clinical samples of metastases and the genes overlapping with CRPC clinical samples were *CCNB1*, *CDC20*, *CHEK1*, *ESPL1*, *HCAP-G*, *SMC4L1*, and *STK3* [47]. Later additional in vitro validation that androgen receptor regulates a distinct transcriptional program in androgen-insensitive cells was generated by comparing sublines of LNCaP cells [50]. Importantly, androgen receptor was shown to selectively upregulate M-phase cell-cycle genes in androgen-independent cells including *CCNA2*, *CCNE2*, *CDC2*, *CDC20*, *CDC25C*, *CDKN3*, and *UBE2C* [50]. Some of these genes known to be regulated by androgen receptor were associated with high expression of androgen receptor splice variant in bone metastases such as *CCNA2*, *CDC20*, *CDK1*, *HSP27*, *C-MYC*, *UBE2C*, and *UGT2B17*, while other known ARGs such as *PSA/KLK3*, *KLK2*, *NKX3.1*, *FKBP5*, and *TMPRSS2* were not associated with metastases containing high level of splice variant [51].

### *9.3.2 ARGs Discovered Using In Vivo Models*

More than a decade ago, dysregulation of ARGs was demonstrated in human xenografts including CWR22 and the CWR22-R1 xenograft derived from CWR22 cells. These studies revealed that some genes normally regulated by androgens (e.g., *FKBP5* and *S100P*), that had reduced levels of expression in response to

castration were reexpressed when the tumors began to grow again in the absence of testicular androgens [52, 53]. One of the largest in vivo study in terms of three million tags generated was from a LongSAGE analyses using the LNCaP Hollow Fiber model. This study uniquely used serial samples from the same mouse from before and after castration to reveal differential expressed genes in response to androgen ablation and with CRPC. The differential expressed genes identified in CRPC included genes known to be altered in response to androgen (*ABHD2*, *BM2*, *BTG1*, *C19orf48*, *CAMK2N1*, *CXCR7*, *EEF1A2*, *ELOVL5*, *ENDOD1*, *ENO2*, *HSD17B4*, *MAOA*, *MDK*, *NKX3.1*, *ODC1*, *P4HA1*, *PCGEM1*, *PGK1*, *PSA/KLK3*, *SELENBP1*, *TMEM66*, *TPD52*, and *TRPM8*) as well as those involved in androgen receptor signaling (*CCNH*, *CUEDC2*, *FLNA*, and *PSMA7*) and androgen biosynthesis and metabolism (*DHCR24*, *DHRS7*, *ELOVL5*, *HSD17B4*, and *OPRK1*) [54]. This library (GSE18402) is freely available at Gene Expression Omnibus and provides a resource where anyone can mine the data to determine if their gene of interest changes expression in vivo in the LNCaP Hollow Fiber model in response to castration and in CRPC.

It is important to note that the LNCaP hollow fiber model has genomic similarity to clinical samples in terms of differential gene expression during hormonal progression [55]. This model provided evidence for the reactivation of the androgen receptor signaling pathway in CRPC by hierarchical two-dimensional clustering algorithm based on similarity of expression patterns of 1,092 ARGs. Pathway-based characterization of gene expression revealed activation of Wnt/beta-catenin signaling pathway and interaction with androgen receptor in CRPC [55].

## 9.4 ARG Analyses Using Clinical Patient Tumor

### *9.4.1 Clinical Sample Analyses*

Gene expression profiling may be a useful tool to categorize tumor subtypes since the changes at molecular level are correlated with tumor progression [56–59]. ARGs may have important roles in the development of castrate resistance since androgen receptor protein is detected in most CRPC [37]. Profiling the expression of ARGs at different stages of prostate cancer will help elucidate the mechanism(s) leading to terminal CRPC as well as provide biomarkers for prognosis and patient selection for therapies. Several studies on the gene expression analyses have been carried out to compare androgen-responsive and castrate-resistant tumors [16, 60–63]. Studies by Holzbeierlein et al. [60] and Chandran et al. [16] analyzed tumor specimens without separating different types of cells to examine gene expression, while studies from Best et al. [61] and Tamura et al. [63] analyzed epithelial cells separated by laser capture microdissection. Heterogeneity of gene expression levels must be considered. For example, although the majority of lethal CRPC still express PSA, the levels are extremely variable [64]. Tissue levels of expression of *PSA/KLK3* and *AMACR* can vary considerably from high expression to non-detectable within tumors

from the same patient [16, 65]. Also there is no correlation between expression of PSA and androgen receptor [66]. Expression levels of a gene may also be high in the primary tumor and downregulated in the metastatic tumor such as seen with *FOS* and *JUNB* [16].

It is important to note whether change in levels of expression of an ARG is in direct response with change in occupancy of androgen receptor on an ARE on the ARG locus or if it is a downstream effect. The time from treatment to sample collection may affect gene expression. Studies from Holzbeierlein et al. [60] demonstrated that differential expression of ARGs was directly regulated by androgen after 3 months of androgen ablation therapy based upon in vitro validation that 22.8% of those genes had differential expression in LNCaP cultures after 36 h of androgen withdrawal. There are additional complexities that may be encountered with patient tissue as described by Mostaghel et al. [17] showing evidence that after short-term suppression of androgen, even though the serum testosterone levels are decreased, the intraprostatic levels may not be reduced. Hence, patients receiving androgen ablation therapy may not have complete suppression of androgen in the prostate microenvironment which would have profound effects on expression of ARGs. Therefore, duration of hormonal therapy and the residual levels of tissue androgen need to be considered for gene expression analyses on patient samples.

Finally the definition of CRPC needs to be consistent between analyses for correct interpretation of the data. Different criteria for clinical castrate resistance have been employed. For example, Best et al. [61] defined CRPC as patients with two rising PSA levels and at least one new lesion found in bone or progressive measurable disease, whereas Tamura et al. [63] defined CRPC as patients with three rising PSA levels.

### *9.4.2 ARGs and Androgen Ablation Therapy*

Despite the potential incomplete suppression of androgen within prostate tissues, the expression of ARGs is altered after short-term (1–9 months) androgen ablation therapy [17, 60]. Notably, there was a higher percentage of genes downregulated than those that were upregulated following androgen ablation therapy. These studies showed that 56% of the altered genes were downregulated while 44% of the altered genes were upregulated in response to androgen ablation treatment, even though the numbers of altered genes, profiling platform, analysis criteria, tissues sources, treatment agents, and treatment duration were different between the two studies [17, 60]. Comparison between these two studies revealed only nine ARGs that were in common and were differentially regulated after short-term castration in benign versus malignant prostate. One ARG, *TFF1*, was upregulated in response to castration, while six others (*ABCC4*, *CAMK1*, *DCXR*, *SORD*, *SPON2*, and *TMPRSS2*) were downregulated [17, 60]. *GSTM1* (involved in metabolism) was upregulated in the tumor [60], but downregulated in benign prostate [17] after castration, whereas *FOLH1* was downregulated in the tumor but upregulated in benign prostate after castration.

*TFF1*, *DCXR*, and *SORD* are involved in carbohydrate metabolism and these alterations consequently affect the cell proliferative capacity [16, 17]. CAMK1 has a role in calmodulin-dependent protein kinase cascade but its exact role in prostate cancer in response to androgen ablation requires investigation. ABCC4, a member of ABC-type multidrug transporters, transports a number of molecules. Its gene expression was highly upregulated in malignant compared to benign prostate and it is suggested to be involved in tumor progression [67]. Consistent with patient samples, expression of *SPON2* was reduced by castration and then highly expressed in CRPC in the LNCaP hollow fiber model [54]. SPON2 protein is a proposed serum biomarker for prostate cancer [68]. *TMPRSS2* encodes a serine protease and is one of the best characterized ARGs with bona fide AREs [69, 70]. Expression of *TMPRSS2* is decreased following short-term castration [17, 60]. The discovery of the *TMPRSS2-ERG* fusion gene has increased interest in this ARG with this fusion gene expressed in ~50% of primary prostate cancers [71, 72]. Comparison of gene expression profiles between *TMPRSS2-ERG* fusion-positive tumor cells to fusion-negative cells after androgen ablation treatment revealed that most of the differentially expressed genes are probably targets of ERG. Hence, these differentially expressed genes in fusion-positive tumor cells after short-term castration are indirect consequent of androgen receptor regulation. Moreover, these differentially expressed genes are associated with cell cycle and mitosis, indicating the *TMPRSS2-ERG* fusion has influence on proliferation-related genes that are potentially regulated by androgen receptor [73, 74].

The fact that only nine genes overlapped in the two gene expression studies (less than 5% of the genes detected), flags the importance of experimental design and/or highlights how different approaches of androgen ablation therapy may impact gene expression. Lehmusvaara et al. [74] reported that gonadotropin-releasing hormone (GnRH) agonist (goserelin) and anti-androgen (bicalutamide) treatments result in different gene expression profiles even though the clinical outcomes are similar. In their studies, only 16% of the differentially expressed genes were common in both treatments. *DCXR* and *TMPRSS2*, included in the list of the nine common genes found between Holzbeierlein et al. [60] and Mostaghel et al. [17] studies, were also found to be downregulated after bicalutamide treatment. Lack of high frequency of common genes amongst different treatments may be due to different targets at the molecular level. Moreover, some genes responding to androgen ablation were not detected in the above gene expression profiling analyses but were found in the prostate tumor after androgen ablation in studies from other researchers. Examples include beta1C (*b1C*) integrin and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death ligand [75, 76]. Expression of *b1C* integrin was upregulated after 1 month of androgen ablation therapy using goserelin and bicalutamide but was not differentially expressed after 3- or 6-month periods of androgen ablation therapy [75]. Expression of *TRAIL* and its receptor (*TRAIL-R*) was upregulated in patients with advanced prostate cancer after androgen ablation therapy, but expression was lower in CRPC. These results suggest that androgen ablation therapy may be effective only initially with tumors susceptible to apoptosis, and longer periods of androgen ablation enable the cells to escape apoptosis [76]. Such a scenario has been put

forth as an underlying hypothesis that intermittent androgen suppression may lead to increased apoptosis as compared to continuous androgen ablation [77, 78].

### *9.4.3 ARGs Change in CRPC*

Eight of the nine common genes differentially expressed after short-term castration (*TFF1*, *CAMK1*, *DCXR*, *SORD*, *SPON2*, *TMPRSS2*, *GSTM1*, and *FOLH1*) were not detected in CRPC. The one exception was *ABCC4* which decreased expression in CRPC [16]. However, comparing all the differentially expressed genes after androgen ablation therapy from Holzbeierlein et al. [60] and Mostaghel et al. [17] studies with differential gene expression analyses from CRPC samples in studies from other groups (list in Table 9.1), a few number of genes reversed their expression in CRPC. For example, *CYB561*, *NTN4*, and *RARRES1* had reversed expression in CRPC compared with tumor samples treated with androgen ablation [60]. *CYB561* was upregulated, while *NTN4* and *RARRES1* were downregulated in CRPC. *CYB561* (cytochrome b561) encodes a secretory vesicle-specific electron transport protein [79]. *NTN4* (Netrin 4) is a secreted protein and involved in anti-angiogenesis and has tumor suppressive activity in breast cancer [80]. *RARRES1* (retinoic acid receptor responder 1) also has tumor suppressive activity and plays an important role in tumorigenesis [81, 82]. The function of CYB561 is currently unclear in prostate cancer. The reversed expression of *NTN4* and *RARRES1* and their function as tumor suppressors suggest that they have important roles in prostate cancer during the transition from androgen sensitive to castration resistant.

Although the frequency of the same ARGs responding to androgen ablation and castration resistance is very low based on current gene expression profiling analyses, many studies have demonstrated that androgen receptor function is reactivated in CRPC as upregulation of androgen receptor was detected in CRPC, compared to androgen-sensitive tumors [16, 60, 62, 63]. This observation is consistent with studies that have examined xenograft models [83]. Although androgen receptor was not differentially expressed between androgen ablation treated prostate and untreated prostate [17, 60], upregulation of its expression and reactivation of its transcriptional activity in CRPC tumors indicate its critical role in CRPC [44, 52, 54]. The recent discovery of constitutively active splice variants of androgen receptor that lack ligand-binding domain, especially V567es that seems to be expressed exclusively in response to androgen ablation [51, 84], may provide an important marker of CRPC and indication of eminent failure to current therapies that target the ligand-binding domain of full-length androgen receptor. These variants appear to regulate a unique transcriptome that is different from full-length androgen receptor. A recent study demonstrated that androgen receptor splice variants regulate expression of cell cycle genes in the absence of full-length androgen receptor, while the full-length androgen receptor mainly regulates genes associated with macromolecular synthesis, metabolism, and differentiation in CRPC [85]. Importantly, their studies revealed that expression of splice variant of androgen receptor, but not the full-length

**Table 9.1** Profiling of androgen-responsive genes in clinical patient samples

| Study | Samples | Method |
|---|---|---|
| Holzbeierlein et al. [60] | (a) Benign prostate tissues (peripheral zone) of cancer patients<br>(b) Primary prostate cancer (radical prostatectomy; no other therapy)<br>(c) Primary prostate cancers [3-month androgen ablation therapy (goserelin + flutamide)]<br>(d) Metastatic prostate cancer (3 of 9 progressed after 5–10 year of androgen ablation therapy)<br>(e) LNCaP +/− R1881 (0.1 nM for 24 h and remove R1881 for 36 h) | Total RNA<br>Affymetrix U95 oligonucleotide array (~54,000 genes and EST) |
| Best et al. [61] | (a) Primary androgen-dependent tumor (untreated)<br>(b) Primary androgen-independent tumor (different hormonal treatments plus on clinical trial for docetaxel and thalidomide)<br>*Laser capture microdissection for epithelial cells | Total RNA<br>Affymetrix U133A oligonucleotide array (18,400 transcripts) |
| Varambally et al. [2] | (a) Benign prostate tissues<br>(b) Clinically localized, i.e., primary cancer<br>(c) Metastatic hormone-refractory cancer (androgen ablation, and/or chemotherapy and/or radiation therapy) [66] | Proteomics:<br>– High-throughput immunoblot (1,354 proteins; 521 at expected molecular weight)<br>– Tissue microarray |
| Stanbrough et al. [62] | (a) Primary androgen-dependent tumor<br>(b) Metastatic androgen-independent tumors from bone marrow<br>*Laser capture microdissection for tumor cells from primary tumor<br>*Select bone marrow metastatic samples with high tumor content and without erythroid and myeloid cells | Total RNA<br>Affymetrix U133A array (22,283 probes) |
| Mostaghel et al. [17] | (a) Prostate tissues from healthy men treated with or without 1-month Acyline (placebo, acyline or acyline + testosterone)<br>(b) Prostate tumor from localized prostate cancer (androgen deprivation therapy for 0, 3–6, or 6–9 months)<br>*Laser capture microdissection for epithelial cells | Total RNA<br>Custom cDNA array (~6,700 unique cDNA clones) |
| Chandran et al. [16] | (a) Normal prostate tissues<br>(b) Primary tumor tissues<br>(c) Metastatic androgen ablation-resistant samples (androgen ablation)<br>*Genes mainly expressed in stromal cells were removed | Total RNA<br>– Affymetrix HGU95av2, HGU95b, HGU95c oligonucleotide array<br>– CodeLink oligonucleotide array |

(continued)

**Table 9.1** (continued)

| Study | Samples | Method |
|---|---|---|
| Tamura et al. [63] | (a) Normal prostatic epithelial cells<br>(b) Primary hormone-sensitive prostate cancer (no treatment)<br>(c) Primary or metastatic hormone-refractory prostate cancer (androgen ablation)<br>*Laser capture microdissection for cancer or epithelial cells | Total RNA<br>Genome-wide cDNA array (36,864 cDNA) |
| Romanuik et al. [46] | (a) Primary androgen-dependent prostate tumor (no treatment)<br>(b) Primary tumor without recurrent within 5 years<br>(c) Primary tumor with recurrent disease within 5 years (biochemical failure)<br>(d) Lymph node metastatic androgen-independent tumors<br>*Laser capture microdissection for cancer or benign epithelial cells | Total RNA<br>QRT-PCR (27 genes) |

androgen receptor, was strongly correlated with expression of the cell cycle gene *UBE2C* in CRPC.

Interestingly, although downregulation of *TMPRSS2* was detected in prostate tissues (benign or malignant) following short-term castration, no differential expression was detected in clinical CRPC compared to primary tumors in gene expression profiling analyses. Instead, upregulation or restoration of expression of androgen receptor regulated *TMPRSS2-ERG* fusion, together with androgen receptor reactivation, were detected in other studies of CRPC [73]. Increased levels of *TMPRSS2-ERG* fusion may contribute to the progression to CRPC by altering genes associated with cell proliferation under regulation by androgen receptor. On the other hand, TMPRSS2-ERG fusion can inhibit androgen receptor signaling. Yu et al. [71] proposed a model showing that TMPRSS2-ERG interferes with the balance between androgen receptor-mediated differentiation and EZH2-mediated dedifferentiation of prostate cells as well as inhibits androgen receptor signaling, thus leading to a selective pressure for development of CRPC [71]. Of note, non-androgen receptor-mediated signaling pathway, such as phosphatidylinositol 3-kinase/Akt pathway involving cell survival and proliferation, may also contribute to CRPC development and the aggressive phenotype of tumor cells [60, 63, 86].

Similar to gene expression profiles of tumors after androgen ablation treatment, the expression profiles for CRPC showed very few common genes amongst different studies. However, several cellular functions including coordinated ARGs and response to androgen were altered in CRPC. These included altered expression of steroidogenic enzymes of androgen biosynthetic and metabolic pathways in CRPC as compared to androgen-sensitive tumors [16, 17, 60, 62, 87, 88]. Examples include *AKR1C3*, *HSD3B2*, and *SRD5A1* for biosynthesis and *AKR1C1*, *AKR1C2*, *UGT2B15*, and *UGT2B17* for metabolism. Increased expression of *CYP17A1* and *HSD17B3* was also detected in CRPC [89]. The enzyme CYP17A1 produces androgens from progestins and the enzyme HSD17B3 converts testosterone from androstenedione [89]. The altered expression of *SRD5A* genes (*SRD5A1* and *SRD5A2*), encoding 5-alpha-reductases, was detected in CRPC clinical samples as well as in the DuCaP cell line [89, 90]. Increased expression of these enzymes in CRPC provides the availability of androgen and conversion of low-level androgen to testosterone and DHT to allow reactivation of androgen receptor to ultimately overcome androgen ablation therapies. Identification of these genes enables development of therapeutic strategies to ultimately reduce levels of residual androgens in CRPC. One example of such a therapy is abiraterone, an inhibitor of CYP17A1 enzyme that blocks biosynthesis of androgen [91]. Mostaghel and Nelson [92] raised several questions regarding the targeting steroidogenic enzymes for CRPC treatment. The treatment outcome may be affected by the choice of a particular steroidogenic enzyme to inhibit duration of androgen ablation therapy in CRPC patients and the specific tissues/sites of targeting, e.g., adrenal gland or prostate tumor [92].

Other altered cellular functions of identified ARGs involve cell–cell interaction and cell adhesion. Upregulation of *SPP1*, *FN1*, or *CDH11*, all involved in cell adhesion, was detected in CRPC [16, 61, 62]. Osteopontin (*SPP1*) promotes metastasis [16] and cadherin11 (*CDH11*) contributes to metastasis [93]. The role of fibronectin-1 (*FN1*) in prostate cancer is unclear. However, induction of apoptosis by tumor necrosis factor-alpha can be blocked by fibronectin in LNCaP cells [94].

## 9.5 Summary

Comparison of gene expression profiling studies of cell lines, xenografts, and clinical tissues revealed that only a few of the differentially expressed ARGs were common amongst the different samples. These ARGs included *UBE2C*, *SPON2*, *FKBP5*, *TMPRSS2*, and *KLK3* (PSA). Although the majority of differentially expressed ARGs were different, three major cellular functions were commonly affected by androgen during progression of prostate cancer and were (1) androgen biosynthesis and metabolism; (2) cell cycle and proliferation; and (3) cell adhesion (Fig. 9.2). ARGs with differential expression included *CYP17A1* and *SRD5A* (steroidogenic enzymes), *UBE2C* (cell cycle), *TMPRSS2* (cell proliferation), and *SPP1* and *CDH11* (cell adhesion). These ARGs are potential candidates as diagnostic biomarkers or

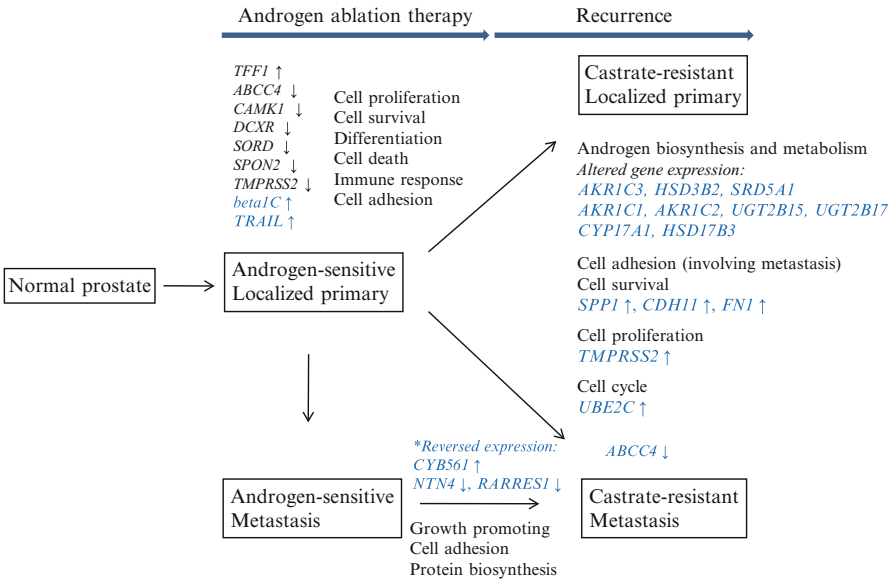


**Fig. 9.2** ARG change leading to disease stage change (under and following androgen ablation therapy) in clinical patients. Altered cellular functions and ARGs are listed. (*black*: common genes found between studies; *blue*: detected but may not be common between different studies). *Asterisk* indicates reversed expression detected in CRPC by comparing castrate-resistant metastatic samples with androgen-sensitive metastatic samples from Holzbeierlein et al. [60] studies

therapeutic targets. Evaluation of expression of these and other ARGs may provide insight of androgen receptor activity in prostate cancer and be utilized for personalized therapies by shedding light on efficacy of hormonal therapies, sequencing of therapies, selection of patients for clinical trials, and mechanisms of resistance.

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# Chapter 10
# Androgen Receptor-Regulated Genes in Prostate Cancer Initiation Versus Metastasis

**Soo Ok Lee, Chiung-Kuei Huang, Luo Jie, and Chawnshang Chang**

**Abstract** Despite extensive attempts to reveal downstream genes of androgen recepter (AR) signaling in each stage of prostate cancer (PCa), the critical switches that trigger PCa leading to castration resistant PCa (CRPC) and metastasis still remain unclear. Compared to the normal prostate, proliferation/cell cycle/apoptosis related genes are upregulated, but genes related to differentiation and secretory functions are downregulated by androgen/AR in PCa. After the androgen deprivation therapy (ADT), AR becomes less sensitive to androgens, but may be able to be activated via multiple ways, such as androgen-independent AR activation, non-genotropic activation of AR, chromosome rearrangement, and cofactor activation, all of these cross-talks may contribute to the progression at the castration resistant stage with metastasis.

Distinct sets of genes up- and downregulated by AR were demonstrated in the metastatic stage. More adhesion related molecules are upregulated in metastasis. Considering different functions of AR in PCa growth (promoter) versus metastasis (suppressor), understanding the exact downstream gene profiles of AR signaling in each stage of PCa is essential so that we can develop successful future therapies with better efficacy in each stage.

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## 10.1 Introduction

Prostate cancer (PCa) continues to be the most common cancer in the United States. The androgen deprivation therapy (ADT) remains the main systemic treatment for PCa, but in most cases PCa progresses into the castration-resistant PCa (CRPC) and becomes more advanced metastatic CRPC (mCRPC).

The androgen receptor (AR) is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors [1]. AR is present in virtually all epithelial cells of the prostate, including benign epithelium, high grade prostatic intraepithelial neoplasia (PIN), and cancer. AR signaling is necessary for the PCa development and, even after the ADT, the AR expression persists in most of the CRPC cells [2–5]. However, the AR expression in CRPC tumor tissues is heterogeneous and hard to correlate with the disease state [6, 7] and differential mechanisms might contribute to the genotropic and non-genotropic actions of AR.

Several mechanisms were suggested to be involved in the transactivation of AR in the CRPC cells. One of the mechanisms is the AR gene amplification, which is detected at a high frequency in recurrent tumors [8, 9]. These changes confer a growth advantage to the tumor cells due to the hypersensitivity of AR at the low castrated levels of androgens. The altered AR ligand specificity toward antiandrogens, adrenal androgens, and non-androgen steroids due to AR mutations could also lead to an increased AR transactivation in CRPC [10, 11]. Recently, frequent epigenetic aberrations such as DNA hypo- and hyper-methylation and altered histone acetylation have also been observed in PCa cells affecting the expression and function of a large array of genes, leading to tumorigenesis, tumor progression, and metastasis [12]. The overexpression of AR coactivators was reported to be involved in AR activation at low levels of androgens [13]. Another mechanism for mediating aberrant AR activation is through generation of splice variants of AR that are constitutively active, independent of androgen levels [14, 15]. Ligand-independent AR activation by cytokines and growth factors were also suggested [16–20] and recently intratumoral de novo steroid synthesis was reported to activate AR signal in CRPC [21].

AR's functions are mainly mediated through specific target genes. Recently, differential AR roles in tumor growth (as a promoter) and metastasis (as a suppressor) have been suggested. We believe that different profiles of AR downregulated genes are responsible for the ability of AR to play these opposite roles. In addition to the genotropic mediation, AR can activate signaling transduction pathways directly to trigger cell proliferation [22, 23], through the modulation of non-genotropic action.

In this chapter we will discuss differential roles of AR in prostate tumor development and in advanced stages including metastasis and then focus on differentially expressed gene profiles in the three different PCa stages, androgen dependent, castration resistant, and metastatic.

## 10.2 Differential AR Roles in PCa (Initial Stage Versus Metastatic Stage)

### *10.2.1 AR Role in Initial Stage of PCa*

Androgens and AR regulate normal prostate development and growth. AR signaling is essential in the development of prostatic diseases, including benign prostatic hyperplasia (BPH) and PCa. It was demonstrated that AR is also essential in PIN initiation [24]. It was found that the epithelial-specific loss of AR blocked PIN development in the prostate regeneration mouse model system, whereas adding back exogenous AR restored this response, suggesting a selective role for AR in the initiation of PIN [24]. The promoter effect of AR on PCa cell proliferation and survival was also demonstrated [25]. The AR in epithelial cells played a positive role to promote cell growth and crosstalk with several growth factors to enhance cell proliferation [26]. AR was also shown to promote G1-S cell transition (is discussed in Sect. 10.3.1) and to exert antiapoptotic ability through suppression of TGF-β signaling [27].

The effects of AR on PCa cell growth are different depending on the PCa cell lines. Even using the same LNCaP cell line, AR's function differs, functioning as a cell survival factor, stimulator, or suppressor depending on the culture conditions, cell environments, or passage number [25, 28, 29]. Moreover, the responsiveness of the LNCaP cells to androgen/AR signaling might be different among cells from the same xenografted tissue [30, 31].

The tumor mouse models using cell specific AR knockout (ARKO) transgenic mice were developed to study AR role in PCa. When the mice lacking AR in prostate luminal epithelial cells (pes-ARKO) were generated by mating the floxAR mice with the probasin-Cre (prostate epithelial specific) [32] mice and were then further crossed with the transgenic adenocarcinoma (TRAMP) mice to generate pes-ARKO-TRAMP mouse model [33], it was shown that the knocking out of epithelial AR led to decreased number of luminal epithelial cells. These results suggested that the luminal epithelial AR might function as a survival factor. Interestingly, in these pes-ARKO-TRAMP mice, an increase in CK5-positive (basal epithelial, stem/progenitor, and intermediate) cells was detected [34], implying that the depletion of AR acted in favor of proliferation of the CK5-positive cells. Similarly, Lee et al. [35] also reported the suppressor role of AR in basal epithelial cells in basal epithelium-specific ARKO (basal-ARKO) mice model studies. These results indicate that the AR roles in the CK8-positive luminal epithelial and CK5-positive cells could be different [36].

### *10.2.2 AR Role in PCa Metastasis*

While the classical concept of AR role in promoting cell growth and suppressing apoptosis driving the initial stage of PCa is well accepted, the role of AR signaling

in tumor metastasis has not been clearly elucidated yet. Although increased expressions of several metastasis-related markers were detected in clinical samples after the ADT [37], the correlation of AR expression/signaling status with the increased expressions of these genes remains unclear.

Niu et al. [33] demonstrated the increased number and larger foci of metastatic tumor developments in pes-ARKO-TRAMP mice than in the wild-type (WT) control mice. These data suggest that the epithelial AR acts as a suppressor of PCa metastasis. Consistently, the AR-negative lymph node metastatic tumors isolated from the pes-ARKO-TRAMP mice were shown to be more invasive than those isolated from the WT mice. Again, restoration of AR expression in pes-ARKO-TRAMP primary cells by AR cDNA transfection reduced the invasion to lymph nodes in the orthotopic xenograft mouse model [33]. Fleischmann et al. [38] examined AR levels in primary tumors and metastatic tissues and also found downregulation of AR in lymph nodal tissues. These data support the notion that the epithelial AR functions as a suppressor of PCa invasion and metastasis.

Nevertheless, some in vitro evidences reported the opposite observation by showing that AR promoted PCa cell invasiveness. Several groups suggested that continuous targeting of the AR signaling pathway will block metastasis since CRPC remains dependent on AR [39, 40]. Overall, more evidence are required to conclude whether AR promotes or inhibits cancer metastasis.

## 10.3 Alteration in AR Transcriptional Complex (Initial Stage Versus Metastatic Stage)

### *10.3.1 Epigenetic Alterations*

The CAG repeat (poly-glutamine) within exon 1 of AR has been associated with the PCa development. The shorter number of glutamine residues in the protein has been suggested to be associated with a higher transcriptional activity of AR and increased relative risk for PCa [41] although a report showing no association of AR CAG repeat length with PCa risk has also been published [42].

It was shown that the methylation/demethylation status of the CpG islands of the AR promoter region determines AR expression via analyzing the methylation profile of AR promoter in AR-expressing and AR-depleted PCa cells [43]. Interestingly, differences in methylation profiles in AR promoter were detected in AR-depleted PCa stem cells and AR-expressing non-stem cells [44].

One of the transcriptional regulations of AR-regulated genes is through histone modification. This modification was suggested to be a predictive factor of PCa recurrence [45]. Acetylation generally correlates with transcriptional activation of genes, while methylation can signal either activation or repression. Metzger et al. [46] showed that lysine-specific demethylase 1 (LSD1; also known as BHC110) colocalizes with the AR in normal human prostate and prostate tumors, interacts with

AR in vitro and in vivo, and stimulates AR-dependent transcription. Wissmann et al. [47] further found that cooperative demethylation by Jumonji C (JMJC) domain-containing protein, JMJD2C, and LSD1 promotes AR-dependent gene expression. Yamane et al. [48] also found that the JMJC-containing protein, JHDM2A, specifically demethylates mono- and dimethyl-H3K9 and facilitates hormone-dependent transcriptional activation of AR target genes. However, on the contrary, LSD1-mediated suppression of AR gene expression was also reported [49].

Ellinger et al. [50] analyzed global histone-3-lysine-27 (H3K27) methylation profiles in PCa tissues and found that the H3K27 mono-, di-, and tri-methylation patterns were different in nonmalignant prostate tissue, localized PCa, CRPC, and metastatic PCa. H3K4me1, H3K9me2, H3K9me3, H3Ac, and H4Ac were significantly reduced in PCa tissues compared to nonmalignant prostate tissues. H3K4me1, H3K4me2, and H3K4me3 levels were significantly increased in CRPC [51]. Welsbie et al. [52] has also suggested that histone deacetylases are required for AR function both in androgen-dependent PCa and CRPC.

However, little is known about the interplay of different epigenetic events [53]. Jia et al. investigated histone acetylation, coregulators, and transcriptional capacity in AR-occupied regions and found that AR mediates transcriptional control of target genes using the same transcription factor related to the chromatin structure, but by altering looping across varying genomic distances, suggesting that this may be assisted by various coregulators [54].

### *10.3.2 Chromosomal Rearrangements*

Chromosomal rearrangement of fusing the androgen/AR-regulated TMPRSS2 gene to the oncogenic E26 transformation-specific (ETS) transcription factors such as ERG or ETV1, occurs in PCa patients at a high frequency (40–50%) [55–57]. Using chromatin immunoprecipitation (ChIP) assays coupled with massively parallel sequencing, it was found that ERG disrupts AR signaling by inhibiting AR expression, binding to and inhibiting AR activity at gene-specific loci, and inducing repressive epigenetic programs via direct activation of the H3K27 methyltransferase EZH2, a Polycomb group protein [58]. These findings provided a working model in which TMPRSS2–ERG fusion plays a critical role in PCa progression by disrupting differentiation of the prostate tissues and potentiating the EZH2-mediated dedifferentiation program. Interestingly, it was shown that the androgen signaling promotes co-recruitment of AR and topoisomerase II beta (TOP2B) to sites of the TMPRSS2–ERG genomic breakpoints, triggering TOP2B-mediated DNA double strand breaks [59].

The switch/sucrose non-fermentable (Swi/Snf) family of nucleosome-remodeling complexes has been shown to play important roles in gene expression throughout eukaryotes and the requirement of this complex in AR function has also been suggested [60].

Taken together, these results suggest that chromosomal rearrangements are important in mediating AR functions.

### 10.3.3 Contribution of Co-regulatory Proteins in AR Transcriptional Complex

Co-regulatory proteins form a bridge between AR, the preinitiation complex, and RNA polymerase and possible alterations in these AR-co-regulatory proteins govern PCa growth and progression [61–64]. AR transactivation could be modulated by various co-regulators, including CREB-binding protein (CBP) [65, 66], p300 [67], steroid receptor coactivator 1 (SRC-1) [68], transcription intermediary factor 2 (TIF2) [69], glucocorticoid-interacting protein (GRIP1) [66], nuclear coactivator 3 (N-CoA) [70], SMRT [71], testicular zinc finger protein (TZF) [72], ARA 55 [73] and ARA70 [74, 75], and SNF2-related CBP activator protein (SRCAP) [76, 77], and all have been reported to influence AR transcription [78–81]. The interplay between AR and the AR co-regulators was also demonstrated [82], but generally they do not bind to androgen response elements (ARE) or the genomic sequence DNA. The detailed mechanisms of some AR co-regulator actions have not been clearly elucidated, but most of them could be considered to remodel or affect the chromatin structure [83, 84]. For example, the knockdown of SRCAP resulted in decreased histone variant H2A.Z binding at the chromosome and affected AR transactivity [77]. Cyclin D1 functions as a strong AR corepressor by directly interacting with and inhibiting receptor activity. However, the extent to which cyclin D1 functions to inhibit AR activity under conditions associated with cancer progression has not been determined [85]. Some growth factors, such as epidermal growth factor, were shown to increase TIF2/GRIP1 coactivation in CRPC [86].

### 10.3.4 The Mechanisms That Modulate AR Expression/Signal

As discussed earlier, AR expression persists in CRPC and many mechanisms are suggested to be involved in this process. AR amplification is one mechanism by which PCa cells survive at the low androgen concentrations present in CRPC.

Several mechanisms have been suggested to amplify AR expression and signals. Recently Cai et al. [49] identified that an enhancer in the AR second intron contributes to increased AR expression at the low androgen levels in CRPC. Plasma membrane-associated sialidase (NEU3), which is known as the key enzyme for ganglioside hydrolysis participating in transmembrane signaling, was reported to mediate AR expression/activation increase and be involved in the androgen-independent growth of PCa [87]. Ectopic expression of GATA binding protein 2 (GATA-2) induced AR transcript levels under androgen-depleted conditions [88]. Caveolin-1 (Cav-1), the main structural component of caveolae, has also been shown to promote the malignant growth [89] and invasion of prostate tumors [90] and was reported to act as an AR coactivator by enhancing its transcriptional activity. Cav-1 overexpression was shown to be associated with an increase in the phosphorylation of AR on serine 210

[89] and inhibition of the angiotensin II type I receptor (AT1R) has the potential to influence AR expression in PCa cells [91]. It was also shown that cyclin D1 negatively modulates AR-dependent expression of kallikrein-related peptidase 3/prostate-specific antigen (KLK3/PSA) [92]. Moreover, the neuroendocrine-derived peptide parathyroid hormone-related protein was shown to promote PCa cell growth by stabilizing the AR [93]. In addition, cooperative interactions between AR and heat-shock protein 27 (HSP27) were reported to facilitate AR transcriptional activity [94].

## 10.4 AR Downstream Target Genes in PCa Stages (Androgen-Dependent, CRPC, and Metastatic Stage)

### *10.4.1 AR Target Genes in Androgen-Dependent Stage of PCa*

AR regulates expression of the androgen-responsive genes by binding to the AREs in their promoter regions. Both dihydrotestosterone (DHT) and testosterone can bind to and activate AR, but DHT has a higher affinity to AR, thereby activates its target genes at lower concentrations than testosterone [95, 96]. The most commonly known and studied AR target genes in classical AR signaling in PCa include *KLK2*, *KLK3*, and *TMPRSS2* (transmembrane protease serine 2), which produce prostate gland enzymes including phosphatases and several serine proteases secreted by the epithelial cells into the seminal plasma. The PSA, since first revealed by Riegman et al. [97, 98], has been used as an indicator to monitor PCa disease progression and therapeutic outcome.

In the normal prostate, the AR's role is maintaining prostate homeostasis [99], secretory functions, and differentiation [100–102], and the AR pathway is still the driving force for PCa growth and proliferation. AR is suggested to be a master regulator of G1-S progression and possibly regulates DNA replication [103]. It was suggested that AR acts as a regulator of G1-S phase progression by promoting cyclin-dependent kinase (CDK) activity, which in turn, induces phosphorylation/inactivation of retinoblastoma (RB), thereby governs androgen-dependent proliferation [104]. The CDK-mediated RB phosphorylation/inactivation is suggested to be the key component of the proliferative response to AR. Loss of RB may play an important role in PCa development [104] and the conditional deletion of RB causes early stage PCa [105]. This mechanism is shown to be controlled by androgen; so androgen ablation blocks this pathway. Fang et al. [106] recently identified a pathway linking AR to the degradation of p27, the target of rapamycin complex 2 (TORC2) and AKT, which leads to cell cycle progression in PCa cells. Jin and Fondell [103] found a novel AR-binding element in the cdc6 gene, which is also important during cell cycle progression, suggesting AR regulated transcriptional control of cdc6. Mallik et al. [107] further found that androgen regulates cdc6 transcription through interactions between AR and the E2F transcription factor (See Fig. 10.1. summary).

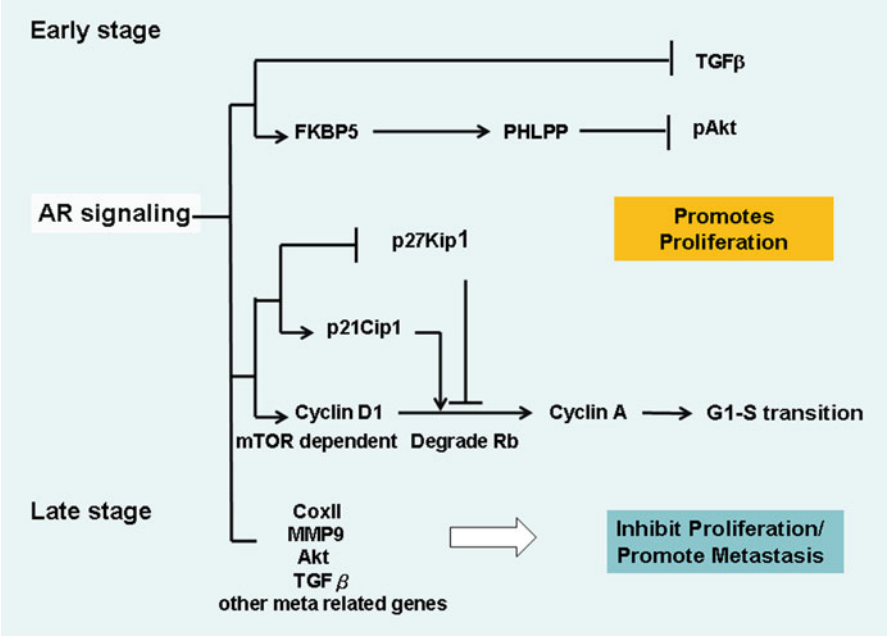


**Fig. 10.1** AR signaling in PCa proliferation and metastasis

The cell cycle inhibitor, p21, was also known as a target gene of AR [108] and the AR-dependent regulation of bcl-xL expression has been reported [109]. Transcriptional regulation of other growth factors, such as FGF8, by AR has also been suggested [110]. Amyloid precursor protein (APP) is a primary androgen/AR target gene that promotes androgen-dependent PCa [111].

Recently, Takayama et al. [112] performed high-throughput genome analyses of 5′-cap analysis of gene expression (CAGE) and ChIP-chip analyses and elucidated the AR-mediated transcriptional network and found that the novel target genes, such as *JAG1* (ligand for Notch1), *SOX4* [113], and *CAMKK2* (calcium-dependent protein kinase) [114] are associated with PCa cell proliferation.

Due to the dedifferentiated characteristic of PCa, transcriptional repression of differentiation-associated genes was also detected and these repressed genes were suggested to be dictated by the Polycomb group protein, EZH2, and repressive chromatin remodeling [115]. The silencing of transcription of these genes was suggested to result in lack of differentiation. It was also found that TMPRSS2–ERG gene fusions potentiate the EZH2-mediated dedifferentiation program [58]. Wang et al. [116] identified a noncanonical ARE as a *cis*-regulatory target of AR action in the TMPRSS2 gene.

In the earlier section, we discussed that shorter CAG repeat in AR gene may result in higher risks of PCa development [41]. Recently Li et al. [117] reported that

the resultant shorter poly-glutamine (polyQ) tract can enhance AR transcriptional activity. They found that an AR coactivator, ZMIZ1, can augment polyQ tracks with shorter length and thereby induced androgen-dependent transcription.

Some microRNAs (miRNAs) and novel RNA transcripts have also been reported to be associated with PCa development and progression. Researchers used a high-throughput transcriptome technology to analyze noncoding RNA expression as well as coding RNA profiles [118]. Only a few miRNAs have been suggested to be AR regulated. miRNA-101 negatively regulated EZH2 and its expression was shown to be modulated by AR and HIF-1alpha/HIF-1beta. miRNA-21 was also reported as an AR-regulated miRNA, which promoted hormone-dependent and hormone-independent PCa growth [119]. The contribution of miRNA-141 in PCa progression also has been suggested [120]. The high-throughput genome analyses mentioned above demonstrated that miRNA 125b-2 cluster was also regulated by androgen/AR [112].

Recently, a positive correlation between the expression of dynamin-related protein 1(Drp1), which is an important molecule in mitochondrial fission (fragmentation), and AR was suggested [121]. Since the mitochondrial fission is considered to be a part of cell mitosis and apoptosis, the AR regulation in this process seems significant.

### *10.4.2 AR Target Genes in CRPC*

AR signal is still believed to be the key factor in CRPC as well as in the androgen-dependent stage [122]. Disruption of AR inhibited the growth of androgen ablation-resistant PCa cells [123] and increased AR expression was shown necessary and sufficient to convert androgen-dependent PCa to CRPC [124]. In addition, increased expression of AR sensitized the PCa cells to low levels of androgens [125]. Therefore, it was generally believed that the AR signal remains responsible for the tumor growth after the ADT. However, the AR signature in CRPC may not the same as that in the androgen-dependent stage and selective up- and downregulations of AR target genes in two stages of PCa were suggested [126].

Expression of AR and AR-regulated genes in the androgen-deprived recurrent CWR22 cells were shown similar to the androgen-stimulated CWR22 cells suggesting that AR is transcriptionally active in the recurrent CWR22 cells [127]. Induction of these AR-regulated genes may enhance cellular proliferation in the relative androgen absence but through an AR-dependent mechanism. Chen et al. [128] investigated genome-wide analyses of AR-binding and AR-dependent transcription in two related castration-resistant cell lines derived from androgen-dependent CWR22-relapsed tumors, CWR22Rv1 and CWR22-R1, and found different transcripts in these two cell lines. These two cell lines are derived from the same ancestor but derived from different xenografted mice. They were reported to share many similarities but shown to have differences in AR function and characteristics [129].

Wang et al. [130] reported that AR selectively upregulates the M-phase cell cycle genes in CRPC including *UBE2C*, a gene that inactivates the M-phase checkpoint.

They found that silencing of *UBE2C* blocks CRPC but not androgen-dependent PCa cells growth. Thus, they suggested that the role of AR in CRPC is not to direct the androgen-dependent gene expression program without androgen but rather to execute a distinct program resulting in androgen-independent growth.

However, ligand-independent AR-mediated gene expression was also reported [131]. AR can mediate its action by peptide growth factors [17], cytokines [18, 19], and via other cellular pathways [16, 132–134]. For example, constitutive activation of the Src-MEK-1/2-ERK-1/2-CREB pathway was suggested [22]. Desai et al. [16] reported that the non-steroidal neuropeptides like bombesin can activate Src kinase that leads to the transactivation of AR and further identified that *c-myc* is a downstream target gene of src kinase. Crosstalk between AR and β-catenin in CRPC was also reported, with the suggestion that the interaction of β-catenin with AR leads to PCa progression into the CRPC stage [135]. Hedgehog/Gli interaction with AR was also suggested to be one of the mechanisms by which AR can be activated in the presence of the castrated level of androgen [136].

Alteration of the PTEN/PI3K pathway is suggested to be associated with CRPC [137]. PTEN genomic deletion was shown to be associated with the AR signaling in CRPC [137]. The crosstalk between AR and the Akt signaling pathway was first found by Lin et al. [138] and recently, Carver et al. [139] found that AR transactivation was decreased upon PTEN deletion in human and murine tumors, but on the contrary, the PI3K pathway inhibition activated AR signaling by relieving feedback inhibition of HER kinases and therefore suggesting reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient PCa. Similarly, it was shown that the inhibition of AR signal activates AKT signaling by reducing the levels of the PH domain leucine-rich repeat protein phosphatase (PHLPP). Thus, these two oncogenic pathways were suggested to crossregulate via reciprocal feedback. Mulholland et al. [140] further identified that the suppression of AR transactivation impairs the PHLPP function, which acts as an inhibitor for Akt signaling, thereby resulting in increased Akt phosphorylation. They also found that the AR target gene, *FKBP5*, is essential for PHLPP maturation.

The oligoarray analysis studies using androgen-dependent PC346C and their androgen deprivation-resistant derivative cell lines found that the AR target genes in androgen deprivation-resistant cell lines include the genes in signaling pathways, the metabolism of proteins, lipids, and carbohydrates, in addition to the genes involved in cell proliferation and differentiation (see review by Marques et al. [126]).

Using ChIP display, Jariwala et al. [131] discovered 19 novel loci occupied by the AR in castration-resistant C4-2B PCa cells. Only four of the 19 AR-occupied regions were within the 10-kb 5′-flanking regulatory sequences. Three were found located up to 4-kb 3′ of the nearest gene, eight were intragenic, and four were detected in gene deserts. Whereas the AR occupied the same loci in PCa C4-2B and LNCaP cells, differences between the two cell lines were observed in response to androgens. They found AR-dependent, DHT-independent regulation of two genes, *OAT* (ornithine aminotransferase) and *MRFAP1* (MORF4

family-associated protein 1, RB-associated protein), although their functions are not fully revealed yet.

Tamura et al. [141] performed a supervised analysis and permutation test using clinical specimens of different stages and identified 36 upregulated genes and 70 downregulated genes in CRPC tissues compared with the androgen-dependent stage tissues. They observed overexpression of AR, *ANLN* (anillin), and *SNRPE* (small nuclear ribonucleoprotein polypeptide E), and downregulation of *NR4A1* (nuclear receptor subfamily 4, group A, member 1), *CYP27A1* (cytochrome P450 oxidase), and *HLA-A* (a group of human leukocyte antigens), in CRPC progression. Armstrong et al. [142] also reported differential expression of 50 genes in androgen-dependent PCa and CRPC and specifically emphasized that upregulated FGFR1 expression is associated with the transition of androgen-dependent PCa to CRPC. Prescott et al. [143] reported the discovery of three genes, *KIAA1217* (sickle tail protein homolog), *CHRM1* (cholinergic receptor), and *WBSCR28* (Williams-Beuren syndrome chromosome region 28) in CRPC. These were newly identified in a screen for AR-occupied regions in C4-2B PCa cells and their transcription was shown repressed upon DHT treatment. AR knock-down resulted in increased mRNAs of those three genes, indicating that AR represses these genes even in the absence of added ligand. Unlike the other androgen-repressed genes studied thus far, AR occupancy at these genes was mapped outside their respective 5′-promoter regions [143]. However, functions and correlation to disease state are not known yet.

Chen et al. [144] reported negative regulation of AR in expressions of some genes in CRPC. They showed that androgen deprivation augments the receptor tyrosine kinase ErbB3 protein level and this contributes to the androgen-independent cell cycle progression and, in turn, increases AR transactivation. In the androgen-dependent stage, AR activation promoted ErbB3 degradation by upregulating transcription of neuregulin receptor degradation protein-1 (Nrdp1), an E3 ubiquitin ligase; however, in the androgen-depleted stage, Nrdp1 expression was decreased, which promoted ErbB3 protein accumulation, and led to AR-independent proliferation progression of PCa to CRPC. They explained this differential regulation of this pathway in CRPC in association with the inability of AR to transcriptionally regulate Nrdp1. In addition, *CRYM* (thyroid hormone T3-binding protein mu-crystallin) was recently reported as a novel AR downstream gene whose expression is elevated in the early stage of PCa but downregulated in CRPC [144].

Earlier we discussed increased TMPRSS2-ERG gene fusion in PCa compared to normal tissues, but Carver et al. [55] suggested that this genetic rearrangement may be more important in PCa progression than the earlier initiation stage. Similar observations were reported by Rajput et al. [145] by analyzing the frequency of the TMPRSS2:ERG gene fusion in moderate to poorly differentiated PCa specimens. Recently, it was also shown that the WNT and TGF-beta/BMP signaling pathways are significantly associated with genes upregulated in TMPRSS2–ERG fusion-positive tumors [146].

### *10.4.3 AR Target Genes in Metastatic PCa*

Whether androgen or AR influences PCa to progress into the metastatic stage is uncertain. It was reported that the prolonged exposure of the Nkx3.1/Pten mutant mice to low androgen levels accelerated PCa progression [147]. But a recent report using prostate-specific Pten null mice model showed that the loss of AR in prostate epithelium increased cell proliferation but has no significant effects on cancer invasion [148].

However, differential regulation of AR gene signature in tumor growth and metastasis was demonstrated by several groups. Marques et al. [126] found several genes that are differentially regulated in primary prostate tumors versus metastatic tumors. For example, *ENDOD1* (endonuclease domain containing 1), *ACSL3* (acyl-CoA synthetase long-chain family member 3), and *MCCC2* (3-methylcrotonoyl-CoA carboxylase) were upregulated in primary tumors but were repressed in metastatic tumors [126]. However, they did not detect much difference in gene profiles in CRPC and metastasis. Instead, they found a general trend in progression of primary tumors to CRPC and metastatic tumors: genes downregulated in CRPC tended to be downregulated in metastatic tumors. More importantly, they suggested that a considerable fraction of the AR pathway genes that were upregulated in primary PCa compared to normal prostate were downregulated in metastatic tumors. They further analyzed that the cluster of androgen-regulated genes overexpressed in metastatic tumors is enriched for genes involved in cell survival, proliferation, cytoskeletal remodeling, and adhesion, which are crucial functions in tumor progression and invasion [126].

Epithelial–mesenchymal transition (EMT) is the earlier process leading to metastasis. Many genes including N-cadherin [149], Cadherin-11 [150, 151], Mapsin [152, 153], or nestin [154] are involved in this process. N-cadherin [155] and cadherin-11 [156] levels were upregulated after androgen deprivation and suggested to be associated with metastasis in PCa. Interestingly, the expression levels of AR were shown to be inversely correlated with EMT in prostate tumor epithelial cells, pointing to a low AR content requirement for the EMT phenotype [26]. However, the AR effect in promoting EMT was shown to be different in the androgen-independent PCa DU145 cell line. When the stable transfected cell lines expressing functional AR were established and exposed to DHT, a significant reduction in cell–cell adhesion and aggregation accompanied by a decrease in E-cadherin expression was observed [157].

The involvement of androgen and AR in the EMT process has been studied by Zhu et al. [26], who found an inverse correlation between EMT and AR expression levels, which is consistent with the earlier notion that AR downregulation accelerates the migration/invasion potential of epithelial cells [33]. Considering that mesenchymal cells possess stem cell characteristics and high metastatic potential, the observed suppressive role of AR in the EMT process is not surprising.

EMT can be activated by soluble factors such as TGF-β [158]. TGF-β is also an AR target gene [34] and plays important roles in the EMT process and subsequent

metastasis. The AR repressed TGF-β signaling through interaction with Smad-related protein 3 (Smad3) [159] and the AR activation by DHT was shown to suppress the TGF-β-induced phosphorylation of Smad3 in LNCaP cells [160]. It was also shown that downregulation of AR increased TGF-β, and this regulation is suggested to be associated with the AR downregulation-mediated enhanced metastatic ability of PCa cells. The implication of TGFβ/BMP-SMAD4 signaling axis in metastatic PCa was recently reported in the study of the Pten null mice model [161].

A recent report also showed that in human PCa cells LuCaP35 xenografted mice, androgen deprivation can increase the EMT process in a zinc finger E-box binding protein 1 (ZEB1)-dependent manner [162], and this effect was also observed in the PC3AR9 cell line [163]. The TMPRSS2–ERG gene fusion was also reported to be associated with the EMT regulation by ZEB1 [164].

One of the most critical AR related signaling to play a pivotal role in transition of PCa into the metastatic stage is thought to be the Akt signaling [165, 166]. However, whether the Akt molecule is a suppressor or enhancer of PCa metastasis is uncertain. Recent studies have found that the two different Akt isoforms, Akt1 and Akt2, may function differently in cell migration, EMT [167], cancer initiation and progression [168]. It was shown that Akt1 accelerates mammary tumorigenesis but suppresses tumor invasion [169], while Akt 2 plays a critical role in colorectal cancer metastasis [170]. Moreover, the Akt1 was shown more dominantly located in the differentiated luminal-like cells, while the Akt2 was highly expressed in poorly differentiated basal-like or EMT-like cells [171].

The reciprocal regulation of AR and Akt activation has been also suggested by Chang et al. [138, 172] and recently, many other groups reported similar findings [24, 139, 140, 173]. The transcriptional activity of AR was shown increased when the PI3K pathway was inhibited [173] and the PTEN loss suppresses androgen-responsive gene expressions by modulating AR transcription factor activity [139]. These results might explain why the combination therapy of anti-AR and anti-Akt enhances the PCa metastasis undesirably.

Interestingly, the TGF-β/Smad3 signaling was activated by either anti-AR or anti-Akt therapy, which can mediate the cell growth suppression but enhance the cell invasion through activation of the MMP9 pathway. In addition, it was suggested that the AR signaling can upregulate the ETV1 gene expression and enhance the cell invasion [174].

Niu et al. [33] earlier observed the AR suppressor role in PCa metastasis. To investigate AR regulation of the metastasis related genes, they compared expression levels of MMP9, COX-2, IGF-2, TNF-α, and IL-6 in metastasized tumors from lymph nodes. It was found that the metastatic tumor tissues derived from the AR knocked out cells express higher levels of these genes compared to the tumor tissues derived from the AR expressing cells.

C/EBP beta expression was shown to be correlated with tumor invasiveness and increased expression of C/EBP beta was associated with increased COX-2 expression and AR downregulation [175], also indicating the importance of AR downregulation in PCa progression into metastasis. The TM4SF1 (transmembrane 4 L

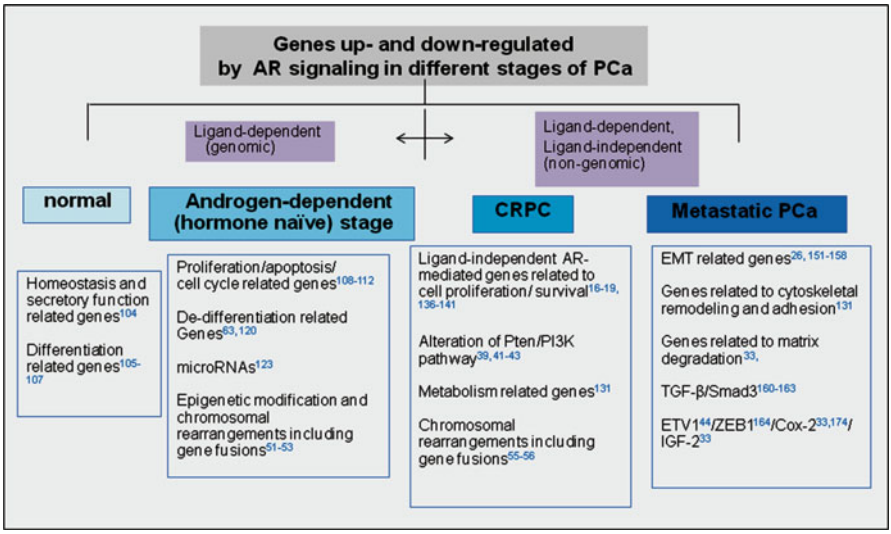


**Fig. 10.2** Genes up- and down-regulated by AR signaling in different stages of PCa

six family member 1) molecule, known to be involved in migration, was shown to be regulated by AR [176]. The studies of the role of cyclin A1 and AR in transcription of VEGF and MMP2 found that cyclin A1 interacts with AR and contributes to PCa metastasis by modulating the expression of these two molecules [177].

## 10.5 Conclusion

The AR signaling plays differential roles in different stages of PCa, accordingly, differentially regulated gene expression profiles in each stage were detected. The summary of these differentially regulated genes are described in Fig. 10.2. It should be noted that the universal targeting of AR signaling may not be the most effective therapy for all stages, so we need to develop different therapeutics for each stage for maximum outcomes to battle PCa in patients with different stages. Future researches should be focused on this aspect.

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# Chapter 11
# Androgen-Independent Induction of Androgen-Responsive Genes by Interleukin-6 Regulation

**Zoran Culig**

**Abstract** Androgen receptor (AR) is expressed in recurrent prostate cancer and its increased activation could be a consequence of protein stabilization, point mutations, and interaction with nonsteroidal compounds. One of the molecules which activate the androgen receptor in a ligand-independent and synergistic manner is the proinflammatory cytokine interleukin-6 (IL-6). IL-6 regulates prostate growth in an autocrine and paracrine manner. AR activation by IL-6 has different consequences for LNCaP and MDA PCa 2b cells. Whereas LNCaP cells are inhibited by IL-6, in vitro and in vivo growth of MDA PCa 2b cells is enhanced by the cytokine. AR activation by IL-6 is mediated by coactivators p300 and SRC-1 which are both expressed in prostate cancer tissues. Recent studies on suppressors of cytokine signaling and protein inhibitors of activated STAT revealed that there are several complex interactions between the pathways of IL-6 and androgen. Suppressor of cytokine signaling-3 is upregulated by IL-6 and response to androgenic hormones. The IL-6/AR axis is a target for novel therapies in prostate cancer. The monoclonal anti-IL-6 antibody siltuximab could delay progression of an androgen-sensitive xenograft. However, in clinical studies siltuximab was used mostly in the late stage disease in which it did not show clinical activity as a monotherapy.

**Keywords** Androgen receptor • Anti-interleukin-6 antibodies • Interleukin-6 • Prostate cancer • Suppressors of cytokine signaling

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## 11.1 Introduction

Progression of prostate cancer was in the past explained by inhibition of expression of the androgen receptor (AR), which is one of the main transcription factors in benign and malignant prostate. Although some of the cell lines used in research laboratories (PC3, DU-145) do not express the AR, it has been documented that primary tumors from patients with clinical recurrence and metastatic lesions are AR positive [1, 2]. In cellular models, it has been shown that AR expression is upregulated after steroid depletion. In vivo upregulation of the AR may be a consequence of amplification of the AR gene, which was detected in a subgroup of specimens from patients with relapsed prostate cancer [3]. That and other seminal publications were of major importance for better understanding of the mechanisms of prostate cancer progression and development of novel therapies which could be compared to classic antiandrogen treatment in prostate cancer. Nowadays, the inhibitor of intracrine androgen synthesis abiraterone acetate and the antiandrogen MDV3100 which prevents nuclear translocation of the AR are in clinical trials [4, 5]. AR mutations may additionally contribute to prostate cancer progression. It was shown that continuous treatment with antiandrogens in vitro causes the mutations which are exactly the same as those detected in patients treated with bicalutamide [6]. Mutant AR detected in LNCaP cells is activated by the antiandrogen hydroxyflutamide as well as by steroids estradiol and progesterone [7]. AR activity in prostate cancer is also enhanced by increased expression of coactivator proteins or interactions between the AR and coactivators. An important issue in prostate cancer development and progression is ligand-independent activation of the AR. This chapter will review our knowledge on regulation of cellular events in prostate cancer by interleukin-6 (IL-6), a multifunctional cytokine which activates different signaling pathways in cells which express the IL-6 receptor.

## 11.2 Interleukin-6 Signaling Pathways

IL-6 is one of the most frequently studied pro-inflammatory cytokines. In addition to its role in regulation of immune responses, IL-6 affects the proliferation and apoptosis in benign and malignant cells. IL-6 regulation of cellular events could be achieved in an autocrine or paracrine manner. The IL-6 receptor consists of two subunits, the ligand-binding subunit gp80 and the signal transduction subunit gp130. The ligand-binding subunit is specific for IL-6, whereas the gp130 subunit transmits the signals of other IL-6-related cytokines, such as IL-11 and leukemia inhibitory factor. Following binding of IL-6 to the receptor, distinct signaling pathways could be activated. Specifically, phosphorylation of Janus (JAK) kinases and signal transducer and activator of transcription (STAT)3 is induced. Enhanced STAT3 phosphorylation is observed in many cancers in which STAT3 contributes to tumorigenesis [8]. This signaling pathway is physiologically controlled by suppressors of cytokine

signaling (SOCS) and protein inhibitors of activated STAT (PIAS). Absence of inhibition of the JAK/STAT signaling pathway may cause a chronic inflammation. IL-6 also regulates in a cell type-specific manner the signaling pathway of mitogen-activated protein kinases p44 and p42 and Akt kinase. These kinases have been found to be upregulated in prostate cancer and phosphorylated Akt is a bad prognostic factor [9, 10].

IL-6 could also exert its effect through soluble IL-6 receptor and this process is known as trans-signaling. Soluble receptor for IL-6 is expressed in prostate cancer cell lines [11]. The soluble receptor is implicated in modulation of antiproliferative response of IL-6. However, on the other hand it enhances motility and migration and downregulates the tumor suppressor maspin in prostate cancer cells.

Chronic inflammation has been recognized as a risk factor for prostate cancer [12]. It could be caused by bacteria, urinary reflux, changes in testosterone to estradiol ratio, and increased consumption of food carcinogens. The evidence for its involvement in the disease pathogenesis is mostly indirect since it is difficult to establish and establish a reliable animal model for premalignant lesions such as proinflammatory atrophy and high grade prostate intraepithalial neoplasia. For this reason, little is known about specific signaling pathways regulated by IL-6 in prostate cancer precursor lesions. Most results available in the literature are obtained with commonly used cancer cell lines. Thus, there is a challenge to propose appropriate anti-cytokine prevention strategies on the basis of current experimental models which are more relevant to established prostate cancer.

## 11.3 Interleukin-6 and Androgen Receptor

Growth factors and growth factor-related receptors are implicated in AR activation. Ligand-independent and synergistic activation of AR by growth factors such as insulin-like growth factor and epidermal growth factor and peptide hormones which elevate intracellular levels of protein kinase A are of pathophysiological interest [13, 14]. Since androgen levels decrease during endocrine therapy for prostate cancer, synergistic activation by low doses of androgen and a growth factor may lead to maximal stimulation of the AR. Thus, stimulation of the LAPC-4 xenograft by the epidermal growth factor receptor-related oncogene ErbB2 leads to the enhancement of AR-mediated tumor progression [15]. ErbB2 causes activation of the mitogen-activated protein kinase pathway thus leading to AR phosphorylation in the N-terminal region [16]. So far there is a relatively little information about the in vivo significance of AR activation by nonsteroidal compounds. For example, there are a low number of genes which are regulated in the same way by androgen or protein kinase A, which causes an elevation of AR activity in reporter gene assays [17]. However, the neuropeptide bombesin induces AR activity and promotes in vitro growth of prostate cancer cells [18]. This mechanism may be relevant in vivo since paracrine stimulation by neuropeptides has been associated with unfavorable prognosis of prostate cancer.

The effects of IL-6 on AR activity were initially discovered in DU-145 cells which were transfected with an AR expression vector and androgen-responsive reporter gene [19]. Treatments with androgen and/or IL-6 led to a ligand-independent activation of the AR and also to potentiation of effects of low doses of androgen. The effects of IL-6 were blocked by bicalutamide thus confirming the activation of the AR. In breast cancer, some steroid receptor antagonists lack the efficiency under conditions of nonsteroidal activation of the progesterone receptor [20]. In addition to DU-145 cells, IL-6 activation of the AR was confirmed in LNCaP cells. In the absence of androgen, IL-6 causes elevation of the AR endogenous downstream target prostate-specific antigen (PSA). Regulation of AR activity by IL-6 may have a role in autocrine and paracrine loops in prostate cancer. Autocrine production of IL-6 was observed in androgen-insensitive PC3 and DU-145 cells, whereas LNCaP cells were found not to express IL-6 [21]. It is known that androgen negatively regulates the expression of the transcription factor nuclear factor kappa B and IL-6 [22]. Thus, in those cell lines the repression of IL-6 production by androgen may be abolished. Tumor cells express the IL-6 receptor which could be activated by IL-6 from epithelium and stroma. This conception is supported by data obtained with human specimens [23]. IL-6 and IL-6 receptor expression was found in most of the benign and malignant prostates investigated.

In addition to regulation of PSA by IL-6, it is of importance to evaluate the role of the IL-6/AR axis in proliferative and apoptotic responses in prostate cancer. In LNCaP cells, we have observed an inhibitory effect of IL-6 on cellular proliferation [19]. Prostate cancer cells developed after a prolonged treatment with IL-6 (LNCaP-IL-6+) show a downregulated AR expression and no inhibitory effect on proliferation [24]. It should be mentioned that phenylbutyrate, which also activates the AR, inhibits proliferation and was proposed for the differentiation therapy in cancer [25]. Mechanistically, two cytoplasmic kinases are involved in IL-6-induced AR activation [26]. Pim1 is the first kinase in the chain which is regulated by IL-6 and activates the Etk kinase. A kinase-deficient form of either Pim1 or Etk could block the effect of IL-6.

An effect of serum and/or other cell culture conditions on LNCaP cell proliferation cannot be excluded since some investigators demonstrated a positive growth effect of IL-6 [27]. In order to analyze whether the inhibitory effect of IL-6 on proliferation of AR-positive prostate cancer cells is a general phenomenon we have extended the studies to two other cell lines, LAPC-4 and MDA PCa 2b, which express either wild-type or mutant AR, respectively. Both cell lines are IL-6-negative. LAPC-4 cells similarly to LNCaP are inhibited by IL-6, whereas a modest stimulatory effect was seen in MDA PCa 2b cells, associated with increased mitogen-activated protein kinase phosphorylation [28]. Importantly, MDA PCa 2b xenografts were grown in mice after castration. The evidence showed that the growth of tumors in vivo is similar in noncastrated animals as well as in castrated ones which were treated with IL-6. The effect of IL-6 could be blocked by bicalutamide thus confirming that AR stimulation results in growth promotion. Thus, bicalutamide is as an antiandrogen efficient in inhibition of ligand-independent activation of the AR in vitro and in vivo. The reasons for differential effects of IL-6 in LNCaP versus MDA PCa 2b cells have not been clarified yet. There are different AR mutations in

**Table 11.1** Proliferative responses of prostate cancer cells induced by IL-6

| Cell line | Growth response | Signaling |
|---|---|---|
| LNCaP | Paracrine inhibition[a] | STAT3 phosphorylation |
| LNCaP-IL-6+ | Autocrine stimulation | Mitogen-activated protein kinase up-regulation |
| PC-3 | Autocrine stimulation | Phosphatidylinositol 3-kinase activation |
| DU-145 | Autocrine stimulation | [b] |
| LAPC-4 | Paracrine inhibition | [b] |
| MDAPCa 2b | Paracrine stimulation | Mitogen-activated protein kinase up-regulation |

[a]Some laboratories observed growth stimulation
[b]This issue has not been clarified

those cell lines. Differences in function of AR coactivators in these cell lines may be one explanation; however, such studies have not been performed so far. It is also possible to speculate that different subsets of genes are upregulated in the cell lines studied in response to IL-6. For example, it is known that the genes that regulate G1 to S cell cycle progression are downregulated by IL-6 in the LNCaP cell line [29]. A more comprehensive overview of proliferative responses of prostate cancer cells by IL-6 is shown in Table 11.1.

IL-6 activation of AR is influenced by two coactivators, p300 and SRC-1. P300 is a transcriptional integrator which regulates many different cellular events. Its expression in prostate cancer in vitro is upregulated following androgen ablation [30]. Interestingly, p300 may also regulate expression of AR downstream targets in conditions in which AR itself is expressed at a very low level [31, 32]. Thus, p300 is considered a target for novel therapies and consequences of its inhibition were investigated in experimental approaches in AR-positive and AR-negative cell lines. In addition to inhibition of AR activity in LNCaP and LAPC-4 cells, it was demonstrated that downregulation of p300 in AR-negative cells has an impact on the nuclear factor kappa B pathway, migration, and invasion [33]. SRC-1 is a coactivator whose expression correlates with prostate cancer progression [34]. Downregulation of p300 or SRC-1 leads to an abrogation of ligand-independent activation of AR by IL-6 [35, 36]. P300 histone acetyltransferase activity is required for ligand-independent activation of the AR by IL-6 [35]. SRC-1 phosphorylation by mitogen-activated protein kinases was demonstrated to be a requirement for AR activity regulation by IL-6 [36].

## 11.4 Suppressors of Cytokine Signaling and Protein Inhibitors of Activated STAT and Androgen Receptor-Mediated Events in Prostate Cancer

The role of SOCS-3 in prostate cancer has been investigated in AR-positive and -negative cell lines. In contrast to the results obtained in some other tumors, it was found that in AR-negative cells SOCS-3 inhibits apoptosis through both

extrinsic and intrinsic mechanisms [37]. Downregulation of SOCS-3 by siRNA also reduces the levels of the Bcl-2 oncogene. It was demonstrated that forced expression of Bcl-2 inhibits the effect of inhibition of SOCS-3 in prostate cancer cells. However, SOCS-3 expression was re-established in androgen-sensitive LNCaP cells by a doxycyclin-inducible system. Interestingly, SOCS-3 overexpression had a negative effect on androgen-induced proliferation and PSA secretion [38]. Thus, it could be postulated that there are several interactions between the androgen and IL-6 signaling pathways. Both androgen and IL-6 upregulate AR activity, although the implications may be cell type specific. In addition, IL-6 and androgen also elevate the expression of SOCS-3 which serves as a negative regulator of signaling of steroid hormone and cytokine. In contrast to SOCS-3, only tumor suppressive effects of SOCS-1 have been demonstrated in human prostate cancer [39].

In early studies, PIAS1 has been identified as a regulator of AR activity [40]. PIAS1 is expressed in the majority of prostate cancer cell lines and in malignant tissue its expression is higher than in the benign tissue [41]. PIAS1 has an effect on cell cycle progression through p21 downregulation. Interestingly, this effect could not be attributed to other PIAS proteins since it was shown that PIASy acts as a transcriptional corepressor in prostate cells [40].

## 11.5 Possibilities for Therapeutic Intervention Against IL-6 in Prostate Cancer

In several experimental studies, the anti-IL-6 monoclonal antibody siltuximab (CNTO328) has been used in prostate cancer [42, 43]. This antibody caused heterogenous effects in different prostate cancer models. For example, PC-3 xenografts have been inhibited by siltuximab and progression of an androgen-responsive xenograft LuCaP 35 to castration therapy resistance was delayed after treatment with this antibody. Importantly, siltuximab prevented upregulation of p300 and CBP coactivators following androgen ablation [43, 44]. Phase I and phase II clinical studies have been performed with the antibody. In a phase I study, it was demonstrated that a number of oncogenes are downregulated by siltuximab in tissue specimens treated with the antibody prior to radical prostatectomy [45]. Interestingly, genes involved in intracrine androgen synthesis were also downregulated. Thus, it seems that novel therapies may lead to inhibition of the IL-6/AR axis which could be beneficial in patients with locally advanced or metastatic prostate cancer.

The lack of clinical success in trials with siltuximab so far could be explained by the fact that the antibody was applied as a monotherapy at very late stages of prostate cancer [46]. It should be mentioned that docetaxel resistance represents heterogenous phenotypes which is difficult to target with a monotherapy. It is important to develop more personalized approaches in cancer medicine to develop anti-IL-6 therapies.

## 11.6 Outlook

There have been several improvements in prostate cancer therapy due to a better understanding of intracrine androgen synthesis and introduction of novel antiandrogens such as MDV3100. However, there is little progress in therapeutic inhibition of ligand-independent activation of the AR. This could be explained with insufficient knowledge about the specific genes regulated by nonsteroidal AR activators. From experimental models, there is a possibility to target IL-6/AR interaction. It is unlikely that this approach will be effective as a monotherapy in all patients with prostate cancer because of the heterogeneity of disease. The key question which has to be answered in the future is which pathway should be inhibited in addition to anti-IL-6 therapies in an appropriate patient group in order to improve the outcome of endocrine therapy for prostate cancer.

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# Chapter 12
# The Effect of AR Overexpression on Androgen Signaling in Prostate Cancer

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**Abstract** Androgen receptor (AR) signaling pathway is required for both development of normal prostate gland and prostate cancer (PC). Patient with advanced disease are usually treated with androgen deprivation therapy. However, this treatment is only palliative, since a castration-resistant PC (CRPC) usually arises within 2–3 years of treatment. The mechanism by which CRPC develops is yet to be fully understood. However, common alteration in CRPC is the overexpression of the AR. Several studies have addressed the molecular changes occurring in AR overexpressing

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PC cells. The overexpression of AR enhances the binding of the receptor to chromatin in the presence of low concentrations of androgens. Furthermore, under the same conditions, AR overexpression alters also the dynamics of chromatin binding of the receptor and the binding of basic components of the transcriptional machinery. These changes translate into global epigenetic changes, which deserve more attention. Many studies have found that AR activation in CRPC cell models stimulates a different transcriptional program, which may be influenced by cooperative functions of other transcription factors. Thus, not only a single target gene but also a network of genes could be responsible for the disease progression. In fact, functional studies have shown that androgen-regulated genes, which are overexpressed in CRPC, are also likely to be important in PC progression.

**Keywords** Amplification • AR • ChIP-seq • Overexpression • Prostate cancer

## 12.1 Introduction

In 1941, Huggins and Hodges reported the dependency of metastatic PC on androgens [1]. Already in the 1980s it was shown that castration reduced the levels of dihydrotestosterone (DHT) in prostate tissue of only about 50% [2]. Castration combined with ketoconazole treatment to block the production of adrenal androgen further reduced the levels of DHT [3]. Recent work by Titus et al. [4] showed that, even though the levels of DHT were reduced up to 90% in CRPCs tissues, the levels of testosterone were same as in non-malignant androgen-stimulated prostate. Moreover, the residual levels of DHT in the tissues were able to activate AR. These studies led to the idea that PC could progress to a CRPC stage as a result of androgens produced by the tumor itself [4, 5]. In fact, levels of enzymes required for intratumoral de novo androgen synthesis were recently found to be high in CRPCs [6–9]. Abiraterone was developed in order to avoid androgen synthesis in the cells, achieving an almost complete abrogation of androgens in the tissue. In a clinical trial, abiraterone extended lifespan by 4 months in patients with advanced disease and the use of this drug was approved by FDA last year [10].

Further evidence that CRPC is dependent on the androgen/AR signaling pathway was demonstrated in a recent clinical trial with next-generation antiandrogen, MDV3100. The drug improved survival of patients confirming that CRPCs are still androgen sensitive [11]. The emergence of CRPC has been associated with mutations in AR altering transactivation properties of the receptor, which also occur in about 10–20% of the CRPCs [12]. Moreover, expression of constitutively active AR splice variants and reexpression of androgen-regulated genes have also been found [13–15].

The AR gene is amplified in one-third of CRPCs [16], but not in untreated tumors suggesting that the androgen deprivation therapy selects for this genetic alteration. Furthermore, the patients with AR gene amplification respond better to a second line combined androgen blockade than patients without the amplification [17], suggesting that the tumors with the amplification are more androgen dependent than

tumors without the amplification. The finding of AR gene amplification led to the hypothesis that CRPCs are androgen hypersensitive, instead of androgen independent [16].

Because AR is overexpressed in approximately 80–90% of all CRPCs, other mechanisms than gene amplification leading to AR overexpression have also been hypothesized [18–23]. It has been suggested that such mechanisms could include loss of a transcriptional repressor complex found in CRPC specimens [24]. Also the upregulation of the lymphoid enhancer-binding factor 1 (LEF1) transcription factor has been shown to induce AR gene expression in model systems [25]. More recently, the loss of RB1 signaling, which is also very frequent in CRPC [23] was shown to correlate with AR overexpression. Sharma et al. [26] demonstrated that the loss of RB1 induces E2F1-mediated AR enhanced transcription. AR expression was also shown to be inversely correlated with the androgens concentration to which cells were exposed, through an AR-mediated feedback loop involving the chromatin modifier LSD1 [27].

Understanding the molecular mechanisms driven by the AR overexpression is critical and serves as the basis for identifying new drug targets and biomarkers for this disease. Several cell line models mimicking different stages of the disease and expressing different levels of wild-type and mutated AR are available today (Table 14.1). For instance, the cell line LNCaP carrying mutated AR, as well as wild-type AR expressing VCaP cells are widely used models for CRPC. The LNCaP was derived from a human lymph node metastasis [28], whereas VCaP from a metastatic lesion in a lumbar vertebral body [29]. VCaP caries both AR gene amplification and TMPRSS2:ERG gene fusion [30, 31]. It expresses AR >tenfold higher levels than LNCaP [32]. Long-term androgen starvation of the LNCaP led to the establishment of abl-LNCaP cell line, expressing fourfold higher AR protein levels compared to parental LNCaP cell line [33]. To study the consequences of AR overexpression in PC cells in a more controlled manner, we have developed a LNCaP-based cell line model overexpressing wild type AR 4–6-fold (LNCaP-ARhi) and 2–4-fold (LNCaP-ARmo) more that the control cell line (LNCaP-pcDNA3.1) [32]. In concordance with the results by Chen et al. [20], LNCaP-ARhi cells grow faster in the presence of low levels of androgens than the control cells and adapt better to long-term androgen starvation.

In this chapter we will describe how the overexpression of AR affects androgen signaling, leads to resistance to androgen ablation therapies, and how these mechanisms could be exploited to evaluate new approaches to treat CRPC.

## 12.2 AR Overexpression Is the Best Known Change in CRPCs

We showed already in 2001 [18] that almost all clinical CRPC samples express more AR than hormone-naïve PC specimens. In 2004, Chen et al. [20] showed that AR is consistently upregulated in hormone-refractory xenografts. To confirm that the AR overexpression is responsible for progression, they overexpressed AR in the hormone sensitive LNCaP cells and demonstrated their acquired capability to grow

**Table 14.1** Most commonly used AR-overexpression cell line models

| Cell line | Origin | AR level | AR aberration | ETS fusion | Reference(s) |
|---|---|---|---|---|---|
| LNCaP | Derived from a human lymph node metastasis of prostate cancer | + | T877A | SLC45A3:ETV1 | Horoszewicz et al. [28], Tomlins et al. [29], Veldscholte et al. [92] |
| abl-LNCaP | Long term androgen starvation of LNCaP cells | ++ (4 fold higher than LNCaP) | T877A | SLC45A3:ETV1 | Culig et al. [33] |
| LNCaP-ARmo | Transfection of wtAR vector and subsequent clonal expansion | ++ (3–4 fold higher than LNCaP) | wt (& T877A) | SLC45A3:ETV1 | Waltering et al. [32] |
| LNCaP-ARhi | Transfection of wtAR vector and subsequent clonal expansion | +++ (5–6 fold higher than LNCaP) | wt (& T877A) | SLC45A3:ETV1 | Waltering et al. [32] |
| LAPC4 | Derived from a human lymph node metastasis of prostate cancer | ++ (2-fold higher than LNCaP) | wt | none | Klein et al. [32], Waltering et al. [91] |
| VCaP | Derived from a metastatic lesion in a lumbar vertebral body of a CRPC patient | +++++ (10 fold higher than LNCaP) | Gene amplification and truncated splice variants | TMPRSS2:ERG | Korenchuk et al. [29] Lee et al. [93], Saramäki et al. [30] |
| DuCaP | Derived from a metastatic lesion in the dura mater of the same CRPC patient than VCaP | +++++ (10 fold higher than LNCaP) | Gene amplification and truncated splice variants | TMPRSS2:ERG | Korenchuk et al. [29], Lee et al. [93] |

in lower concentrations of androgens. Furthermore they showed that the same cells with forced expression of AR rapidly formed tumors in castrated mice, while the parental cells did not or developed tumors later. They found that the resistance was still due to activation of the AR by its ligand and that the AR overexpression was also able to induce resistance to the antiandrogen bicalutamide and to convert AR antagonist into agonists. More recently, we have demonstrated that the AR overexpression sensitizes AR signaling pathway to lower concentrations of ligand [32, 34]. Thus, the evidence that overexpression of AR is the main mechanism of castration resistance is strong and suggests that even a moderate sensitization of AR signaling may cause castration resistance. This is of fundamental importance, since it has been shown that the post-castration levels of androgens may vary significantly between individuals [5].

AR is a transcription factor that regulates the expression of a large number of genes [35–37]. Many AR target genes have been associated with the development of the disease. Therefore, the identification of genes downstream of AR signaling involved in the development and progression of PC remains an important area for future investigations. The identification of commonly altered downstream genes could potentially provide new drug targets and better biomarkers.

## 12.3 AR Overexpression Affects Gene Expression and AR Target Genes

The knowledge that the AR overexpression sensitizes the growth of PC cells to lower androgens concentration is not new. Already Kokontis et al. [38] showed that LNCaP cells, grown in hormone-deprived media, appeared to adapt to lowered androgen levels by increasing AR expression and transcriptional activity. The data suggested that AR is transcriptionally active in CRPC and can increase cell proliferation at low circulating levels of androgen reported in castrated men [39].

By comparing gene expression signatures of both LNCaP-ARhi and abl-LNCaP to parental LNCaP we [32] and Wang et al. [40] found independently that AR selectively upregulates M-phase cell-cycle genes in the LNCaP derivative cells, including the genes *UBE2C*, *CDK1*, and *AURKA*, involved in both inactivation of the M-phase checkpoint and driving cell cycle further. AR overexpression increases the number of androgen-regulated genes in the lower concentrations of androgens [32, 34]. Thus, it is conceivable that the role of the increased AR expression in CRPC cells is to sensitize the transcriptional program in order to achieve androgen-dependent cell growth in the presence of minimal androgen concentrations. The gained transcriptional program elicits expression of several cell cycle-associated genes in LNCaP-ARhi and VCaP cells, compared to control cells exposed to 10–100-fold less androgens [32]. AR regulation of CDKs and cyclins in CRPCs has previously been suggested [41]. We have demonstrated that also metabolism and mitosis-associated genes, such as ZWINT, SKP2 (S-phase kinase-associated protein 2 (p45)), and

FEN1 (flap structure-specific endonuclease 1) transcripts [32, 34] are upregulated in AR overexpressing cells, as well as overexpressed in CRPC specimens [34]. The importance of the androgen regulation of metabolic pathways by AR in CRPC was pinpointed also by the finding that aerobic glycolysis, biosynthesis, and anabolism in PC cells are crucial for the disease progression [42]. The work by Massie et al. identified Calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) as an androgen-regulated gene important in PC progression [42].

Androgen-repressed genes may also play important roles in PC cell growth and be major contributors in progression to CRPC [43]. Their reexpression during androgen deprivation is thought to contribute to disease regression, and they may become repressed once again in CRPC. Recently, the study by Zhao et al. [44] reported a systematic analysis of genomic data in order to establish the role of AR as transcriptional repressor. They presented evidences that AR directly inhibits a large number of target genes. Mechanistically, this repression is mediated by the polycomb group protein EZH2 and subsequently by repressive chromatin remodeling. These genes are developmental regulators functionally involved in cell differentiation and tumor suppression. Furthermore, forcing AR expression in LNCaP showed that increased AR binding in the AR-binding sites (ARBSs) in proximity of these genes further enhanced their repression. They also reported similar results using VCaP cells [44], thus confirming that AR overexpression alone may contribute, in CRPC to repression of this particular transcriptional program.

## 12.4 AR Overexpression Affects AR Coregulator's Expression

There is a large array of coregulators required for AR-dependent transcription and so far it has been very difficult to establish the rules for their assembly into transcriptional complexes and their hierarchy of action. Studies in knock-out mice of few coregulators including KDM1A (LSD1), NCOA1 (SRC1), NCOA2 (TIF2), and FKBP4 (FKBP52) revealed only mild phenotypes [45–49], suggesting that coregulators can supplement the function of the missing ones. This suggestion leads to the hypothesis that it is the stoichiometry of the coregulators that may be mostly important for the AR physiological functions.

We performed a systematic and comprehensive study to investigate the expression profile of the AR coregulators in PC [50]. More recently, Taylor et al. [23] have done the same. We found that the levels of AR coregulators do not change dramatically in PC, excluding their involvement in disease progression. Also, the study by Taylor et al. [23] seems to support this observation, because of the coregulators, only NCOA2 was found to be upregulated in a proportion of PCs. NCOA2 expression level has been shown to correlate with early biochemical recurrence in PC patients [51] and it has been recently reported to function as an oncogene in a subset of PCs. Chromosome 8q13.3, harboring the *NCOA2* gene, is the most common amplified locus in PC [23]. However, our data [50] do not support the overexpression of *NCOA2* in PC. There are studies that have found coactivators to be overexpressed

in PC [52–54]. For example, levels of CBP have been shown to be high in advanced PC and, in particular, in tissues from patients that failed endocrine therapy [55], although other reports do not support the finding (e.g., [23, 50]). Downregulation of AR corepressors has also been proposed to be involved in the development of CRPC. For example, the recently identified AR corepressor BTG2 [56], is frequently downregulated in PC and associated with PC aggressiveness [56–58].

We have investigated whether AR coregulators would be androgen-regulated, taking advantage of our LNCaP-AR overexpression model [32]. We studied the effect of AR overexpression on their expression and regulation. Of the over 25 coregulators studied with qRT-PCR, about half were androgen regulated. Coactivators such as *AIB1*, *CBP*, *MAK*, and *BRCA1* showed particularly enhanced upregulation in LNCaP-ARhi cells when compared to control cells [59]. It is difficult to attribute such effect to the presence of ARBSs in the proximity of the loci of these genes, since also non-androgen-regulated AR coregulators displayed ARBSs. However, all these coactivators displayed ARBSs in a putative enhancer region. More recently, Heemers et al. [60] profiled the expression and activity of 186 AR coregulators. Similarly with our results, 30% of them resulted to be androgen regulated [60]. CBP was one of the AR coactivators upregulated in the LNCaP cell line derivative LNCaP-Rf, which was established by long-term androgen ablation of LNCaP cells [55, 60].

These data suggest the existence of a potential positive feedback loop directed to enhance AR activity in CRPC in low concentrations of androgens. The AR coregulators deserve more attention, especially since, recently, evidences showing that targeting the activity of AR coactivator such as EP300 (p300) or CBP may be therapeutically advantageous [61].

## 12.5 AR Overexpression Affects AR Binding to Chromatin

The first attempt to use chromatin immunoprecipitation (ChIP) coupled with genomic array (chip) to identify genomic locations bound by AR was made by Wang et al. [62]. They not only reported the first AR-binding map in chromosome 20 and 21 but also opened the way to the extensive use of this method. In the same year, Massie et al. [63] published the first ARBSs map of the AR in the LNCaP cells using a promoter array. Genome-wide AR-binding profiles of all the available cell lines models have been generated over the past few years (Table 14.2) although a few technical and experimental variations characterize each single study. Recent studies have utilized ChIP coupled with next-generation deep sequencing (ChIP-seq).

The study by Wang et al. [40] and by Yu et al. [64] reported decreased genomic binding of AR in abl-LNCaP and in VCaP cells, respectively. In contrast, two studies by Sahu et al. [65] and by Massie et al. [42] reported dramatically increased AR binding in VCaP when compared with LNCaP [42] or LNCaP-derived cells [65]. Recently, we reported that a modest overexpression of the AR gene in the LNCaP

**Table 14.2** ARBSs studies made with different LNCaP-derived cell lines

| Study (technique used) | Cell lines used (ARBSs number found) →increasing AR levels→ | | | | Time point and androgen used |
|---|---|---|---|---|---|
| Wang et al. [40] (ChIP-chip) | LNCaP (8708) | abl-LNCaP (6353) | | | 4 h 100 nM DHT |
| Yu et al. [64] (ChIP-seq) | LNCaP (37193) | | | VCaP (12965) | 16 h 10 nM R1881 |
| Massie et al. [42] (ChIP-seq) | LNCaP (11053) | | | VCaP (51811) | 4 h 1 nM R1881 |
| Sahu et al. [65] (ChIP-seq) | LNCaP-1 F5 (6215) | | | VCaP (44879) | 2 h 100 nM DHT |
| Urbanucci et al. [34] (ChIP-seq) | LNCaP-pcDNA3.1 (1600) | LNCaP-ARmo (2296) | LNCaP-ARhi (2399) | | 2 h 1 nM DHT |
| | LNCaP-pcDNA3.1 (3265) | LNCaP-ARmo (1359) | LNCaP-ARhi (1207) | | 2 h 100 nM DHT |

Abbreviations: *ARBS* androgen receptor binding site, *ChIP-chip* chromatin immunoprecipitation followed by hybridization on array platform, *ChIP-seq* chromatin immunoprecipitation followed by new generation sequencing

cells led to enhanced binding of AR to the chromatin when cells were treated with low concentrations of androgens [34]. However, we also observed decreased AR binding when cells were treated with a 100-times higher concentration (Table 14.2). The reasons underlying the variation in these results are still not addressed experimentally. A possible explanation may be the concentration of androgens used and the experimental settings such as type of androgen used and timing of chromatin binding assessment. Yu et al. [64] used relatively high (10 nM) concentration of synthetic, stabile androgen (R1881) and a much longer treatment time window than in the other studies. This suggests that most significant correlation between AR levels and transcriptional response affects short timepoints and initial dynamics of AR recruitment to DNA. This hypothesis needs further investigation. ChIP-chip analysis by Takayama et al. [66] reported that the number of androgen-dependent ARBSs increased in LNCaP cells treated with same concentration of R1881 (10 nM) for 6 and 24 h, suggesting that the timing of assessing chromatin binding is an important issue.

The type of androgen used may also be a source of variation. For instance, it is known that R1881 (synthetic androgen methyltrienolone) binds AR with higher affinity and is able to stimulate its activation more potently than DHT [67]. Sahu et al. [65] used 100 nM DHT while Massie et al. [42] used 1 nM R1881, which may be assumed to achieve the same range of AR activation [67]. Both found increasing amount of ARBSs in VCaP than in LNCaP, while Yu et al. [64] stimulated the cells with 10 nM R1881, which may result in downregulation of AR levels and growth inhibitory on the long run [27, 68]. Thus, one can speculate that inhibitory effect on AR binding can be observed when cells overexpressing AR are treated with high concentrations of androgens. Moreover, the non-isogenicity of the cell lines used (LNCaP vs. VCaP) does not allow a true controlled comparison between the AR binding in the different cell lines. The experiments in isogenic cell models, supports the idea that AR binding is regulated by both the amount of androgens in the media and the levels of AR protein [34, 40]. Furthermore, the binding of AR to the target genes' regulatory regions is periodic, and it has also been demonstrated to be dependent on proteasome activity and on the activity of other cofactors [69, 70]. To address this issue, we treated our AR overexpressing cells with low and high concentrations of androgens and profiled AR binding to two well-known AR target genes: the PSA and the TMPRSS2 [71]. The binding to the regulatory regions of the genes reflected the periodicity reported previously [69, 72]. However, the overexpression of AR altered the binding to the loci studies in a manner, which seems to be gene and locus specific. We further proved that the AR binding is more rapid and potent at the enhancer and promoter region of these genes. We observed that the AR binding was affected differently in LNCaP-ARhi cells treated with higher concentrations of androgens [71], suggesting that higher concentration of ligand may mask the effect of the AR overexpression on the dynamic binding. We found also that the overexpression of AR alters also the binding dynamics of basal transcription factors, such as RNA Polymerase II as well as the chromatin structure as assessed by the enhanced histone 3 acetylation [71].

To confirm the association between the AR level and the AR-binding sites in an independent AR overexpression model, we used two CRPC tumors previously

xenografted into castrated mice. One of them, LuCaP69 harbors AR gene amplification, whereas the LuCaP73 does not [18]. Consequently, the expression of AR is about tenfold higher in LuCaP69 compared to LuCaP73 according to qRT-PCR [18]. AR ChIP-seq analysis revealed approximately 19,000 and 7,000 ARBSs in LuCaP69 and LuCaP73 respectively, confirming that there is an association between the AR level and the number of ARBSs in vivo [34]. We also compared AR-binding potency in the same ARBSs map in AR overexpressing cells compared to control cells [34]. At the same loci, the AR binding in AR overexpressing cells was more potent when cells were stimulated with low concentration of androgens and tended to decrease when cells were treated with higher ligand concentrations. This finding was validated for several loci also with ChIP-qPCR [34]. For example, 100 times less ligand was needed in order to achieve the same AR recruitment to the *PSA* enhancer in AR overexpressing cells as compared to control cells. Thus, the AR overexpression sensitized AR binding by 100-fold. ChIP-qPCR on the *PSA* enhancer in the xenografts also showed that the AR binding is stronger in LuCaP69 compared to LuCaP73 confirming that the strength of the AR binding is also associated with the AR level [34].

These data indicate that both the ligand concentration and the amount of receptor affect together the chromatin binding of AR. Moreover, these data are concordant with the results of a recent work by Makkonen et al. [73] who confirmed that the binding at single gene level's regulatory regions is enhanced in AR overexpressing cells, such as VCaP cells compared to LNCaP [73] and with the more recent report by Zhao et al. [44], associating AR binding and gene regulation.

## 12.6 AR Overexpression Affects the Chromatin Remodeling

It is known that the lineage-specific binding of transcription factors, such as AR, to chromatin is modulated also by other transcription factors such as FOXA1 translating epigenetic marks [65, 74, 75]. For example, the status of histone acetylation is critical for androgen receptor-mediated transcriptional activation of genes [76].

Chen et al. [20] found that a modest overexpression of AR can alter the abundance of AR coregulators recruited on the promoters of AR target genes, many of which have histone acetylation activity [77, 78]. There seems to be also a direct correlation between AR expression and chromatin modifiers. We found that many AR coactivators are targets of AR and the AR overexpression further enhances their expression [59]. Among those were *CREBBP* (*CBP*) and *NCOA3* (*AIB1*), which are known histone acetylases [79]. Sahu et al. [65] investigated the association between AR and FOXA1 protein expression in PC, finding a direct correlation between the two [65]. The finding is confirmed also in the data by Taylor et al. [23]. Thus, the AR overexpression seems to favor expression of chromatin remodeler which facilitates AR-mediated genes transcription. A proof of principle that chromatin remodelers may be involved in the emergence of the CRPC phenotype and that the AR overexpression selects for these types of mechanisms is the recent finding that curcumin, which is able to inhibit recruitment of the complex p300/CBP to the

regulatory regions of AR target genes, is able to slow growth of CRPC [61]. Indeed, to test whether the AR overexpression brings epigenetic changes to the chromatin, we investigated the chromatin structure in our AR overexpression model. LNCaP-ARhi cells showed enhanced acetylation of H3K9 and K14 [71], which are known markers of active transcription [80–82]. Furthermore we also showed preliminary results that the chromatin could be already open when cells are hormone deprived for few days [71]. These data are in agreement with the finding by Andreu-Vieyra et al. [83] showing nucleosome-depleted regions at AR enhancers in the absence of ligand. Moreover, we found that such chromatin opening was, once again, enhanced in AR overexpressing cells [71].

These data suggests that nucleosome disposal is an important mechanism to favor gene transcription and AR overexpression may affect also such mechanism. We anticipate that this mechanism is likely to favor ARBSs promiscuity for other transcription factors, which may concur to aberrantly modulate the AR transcriptional program observed in CRPC phenotype.

## 12.7 Summary

AR overexpression sensitizes cells to low androgen concentrations. A mechanistic explanation for such sensitization is that genome-wide chromatin binding of AR is enhanced in AR overexpressing cells. Chromatin binding of AR seems to be dependent on both the level of the receptor and the androgen concentrations to which the cells are exposed. Different concentrations are able to alter the dynamic of the AR recruitment to AR target genes regulatory regions depending on the level of the receptor. These changes translate into an enhanced AR target gene down- or upregulation, which may be different from gene to gene and due to intrinsic biological properties of such genes. The AR transcriptional program is sensitized 10–100-fold and enhances expression of AR coactivator, proliferation-associated genes, and chromatin remodelers, which may result in a positive feedback loop sustaining the AR activation in low androgen concentrations.

Altogether, these results indicate that the overexpression of AR in CRPC cells allows these cells to maintain and potentiate the AR signaling in lower androgen concentrations through several different mechanisms involving epigenetic, transcriptional, and stoichiometric changes.

## 12.8 Future Perspectives and Implications for Therapies

By analyzing the ARBS maps of the two xenografts, we realized that these maps overlapped poorly [34]. In order to investigate whether such variability may occur in other settings, we reanalyzed publicly available datasets and found that also in the independent study of Yu et al. [64] of a tumor specimen. Again, the ARBS maps overlapped poorly. Thus, it is difficult to attribute such variability to the different

AR levels in those cells. It seems rather that, at least in the cell lines, core AR binding is conserved and additional sites are gained in consequence of the AR overexpression. This is an observation which seems to be true across all the studies (Table 14.2). If so, the expression of pioneer transcription factors such as FOXA1 or GATA2 may play a role in redistribution of transcription factor binding in different cells [65, 84].

The LNCaP model and other in vitro cell models represent great tools in order to study the mechanisms associated with the upregulation of the AR in CRPC. However, they will never mimic completely the tumor environment. Thus, in the future, it will be essential to engage in experiments of coculturing the available cell lines models with macrophages and other cell types able to nourish these cells of cytokines and other signals. This setting probably will affect the AR signaling in vivo. Moreover, recreating a tumor environment will help to explore the insight of the AR binding variability observed in the studies described above. It will help also to explain the differences between cells growing in vitro and in vivo and elucidate whether there are differences in their transcriptional program, which may be explained via a different AR binding in the genome.

It is now evident that CRPC is not androgen independent; instead, it still rely on an enhanced AR signaling in order to face the shortage of androgens in the tumor environment. Thus, the future clinical strategies for treating men with CPRC still depends on finding active drugs that inhibit the androgen signaling pathway. However, targeting AR for therapy has turned to be not an easy task. Therefore, the identification and characterization of AR target genes that are relevant in the development and progression of PC remains an important area for future investigations, as these genes could provide alternative, and perhaps more efficacious, drug targets.

Rather targeting one gene at the time, it might be advantageous to target a network of genes or few targets in combination that master different deregulated networks. Moreover, the tight stoichiometry between the abundances of AR and ligand may be exploited in order to delay tumor growth via intermittent androgen deprivation therapy in CRPC patients [85]. Some evidences suggest that patients with CRPC may benefit from these treatments and side-effects of standard therapies may be diminished [86, 87]. Moreover, as it has been established that AR acts in concert with other transcription factors establishing a network of TF, which cooperate in order to maintain the PC phenotype, a critical point is to evaluate different drugs in combinations. For instance targeting AR in combination with PI3K pathway inhibitors [88, 89] or in combination with MYC inhibitors [90].

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# Chapter 13
# The Expression Signature of Androgen Receptor Splice Variants and Their Distinctive Transcriptional Activities in Castration-Resistant Prostate Cancer

**Stephen R. Plymate and Jun Luo**

**Abstract** In a normal male genome, the androgen receptor (AR) mRNA and protein is encoded by the single-copy *androgen receptor* (AR) gene located on the X chromosome. With the decoding of AR splice variants (AR-Vs) in prostate cancer, the diversity and complexity of transcribed AR sequences and the distinctive functional properties of their protein products began to be appreciated. The expression signature of AR-Vs has been characterized to some extent mainly in castration-resistant prostate cancer (CRPC), in which AR transcripts are almost always overexpressed, with or without involving genomic rearrangement of the *AR* gene locus. A recent tiling microarray study using CRPC specimens revealed an expression signature consisting of AR-Vs that are constitutive active, conditionally active, or inactive as transcription factors. Two constitutively active AR-Vs, AR-V7 and $AR^{V567ES}$, have been characterized in more details in the context of AR-directed CRPC therapies. Suppression of canonical ligand-dependent AR-FL signaling may lead to an adaptive increase of these two AR-Vs. However, endogenously induced AR-Vs may not be sufficient to "rescue" the suppressed AR-FL signaling. Instead, AR-Vs may direct distinct transcriptional programs featured by genes involved in cell cycle progression. Therefore, in CRPC tumors subjected to AR-directed therapies, an adaptive shift toward AR-V-directed transcriptional programs may occur, and this shift may signify the emergence of therapeutic resistance. Future investigations focusing on clinical validation of this concept are necessary and will facilitate clinical development of novel therapies to overcome this putative resistance mechanism.

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**Keywords** Androgen receptor • Androgen receptor splice variant • AR-splice variant transcriptome • ARV567ES • AR-V7 • Castration-resistant prostate cancer • Cell cycle

## 13.1 Introduction

In 1941, Huggins and Hodges [1] demonstrated the clinical efficacy of hormonal manipulation for the treatment of metastatic prostate cancer. Androgen deprivation therapies (ADT) have since become the mainstay of systemic treatment for advanced prostate cancer. In men with metastatic prostate cancer treated with ADT, progression to castration-resistant prostate cancer (CRPC) almost always occurs following a variable period of clinical response [2]. It is well established that prostate cancer progression upon ADT is not due to loss of dependence on hormonal signaling but instead is characterized by sustained AR signaling with the presence of tissue androgens sufficient to activate AR [3, 4]. As such, novel CRPC therapies, including abiraterone and MDV3100, have been developed to further suppress AR signaling [5]. These AR-directed CRPC therapies have improved patient outcome [6, 7]. However, the majority of patients progress with rising PSA, suggesting a return of AR signaling even with a near-complete deprivation of intra-tumor androgens [8].

Although resistance to AR-directed CRPC therapies may involve the inter-play of multiple mechanisms, recent studies on AR splice variants implicate a conceptually simple model with the potential to explain ligand-independent AR signaling in CRPC tumors progressing on abiraterone or DMV3100. Structurally, AR-Vs contain intact AR N-terminal domain (NTD) and DNA-binding domain (DBD) but lack the ability to bind to androgens due to truncation of the ligand-binding domain (LBD) [9, 10]. AR signaling mediated by AR-Vs is not targeted by and thus resistant to existing CRPC therapies designed to target the AR-LBD, calling for the development of novel agents that target all AR molecules [11–13]. Additionally, the ability to detect AR-V and AR-V signaling in clinical specimens may provide much-needed indicators of therapeutic response or resistance. Given the clinical relevance, this chapter will focus on AR-Vs that have been investigated in the context of CRPC.

## 13.2 Discovery of Truncated AR-Vs with Defined Coding Sequences

Since 2008, multiple groups have utilized a variety of approaches to clone mRNA for AR-Vs in the context of CRPC (Table 13.1). Dehm et al. [14] reported the identification and characterization of two AR-V transcripts, named AR1/2/2b and AR1/2/3/2b. Hu et al. [15] decoded seven AR variants that originated from splicing of 3 cryptic exons (CE1, CE2, CE3) within AR intron 3. The seven AR variants

**Table 13.1** Diverse characteristics of human AR-Vs in CRPC

| AR-Vs | Splicing junction | Length of variant-specific Peptide | Alternative names | Transcriptional activity | Detection with variant-specific antibody | References |
|---|---|---|---|---|---|---|
| AR-V1 | 3/CE1 | 19 | AR4 | Conditional | No | [15, 16] |
| AR-V2 | 3/3/CE1 | 19 | N/A | Unknown | No | [15] |
| AR-V3 | 2/CE4 | 53 | AR1/2/2b | Constitutive | Yes | |
| AR-V4 | 3/CE4 | 53 | AR1/2/3/2b, AR5 | Constitutive | Yes | [14–16] |
| AR-V5 | 3/CE2 | 1 | N/A | Unknown | No | [15] |
| AR-V6 | 3/CE2 | 6 | N/A | Unknown | No | [15] |
| AR-V7 | 3/CE3 | 16 | AR3 | Constitutive | Yes | [15, 16] |
| AR-V8 | 3/intron 3 | 10 | N/A | Unknown | No | [17] |
| AR-V9 | 3/CE5 | 23 | N/A | Conditional | No | [17, 19] |
| AR-V10 | 3/intron 3 | 39 | N/A | Unknown | No | [17] |
| AR-V11 | 3/intron 3 | 20 | N/A | Unknown | No | [17] |
| AR-V12 | 4/8/9 | 10 | $AR^{v567es}$ | Constitutive | No | [18, 19] |
| AR-V13 | 6/9 | 3 | N/A | Unknown | No | [19] |
| AR-V14 | 7/9 | 7 | N/A | Unknown | No | [19] |
| AR8 | 1/3/intron3 | 33 | N/A | N/A | Yes | [20] |

were named numerically, going from AR-V1 to AR-V7, with AR-V3 and AR-V4 identical to AR1/2/2b and AR1/2/3/2b, respectively. Guo et al. [16] cloned AR3, AR4, and AR5, containing coding sequences identical to those in AR-V7, AR-V1, and AR-V4, respectively. Subsequently, Watson et al. [17] identified four new AR-Vs, named AR-V8 through AR-V11. A constitutively active AR-V encoded by an AR transcript lacking exons 5–7, named $AR^{v567es}$, was reported by Sun et al. [18]. More recently, Hu et al. [19] employed a modified RNA amplification method, termed selective linear amplification of sense RNA (SLASR), for unbiased detection of transcribed AR sequences using arrayed 60-mer probes tiled across the human *AR* gene locus. This study provided a snapshot of the expression peaks along genomic sequences downstream of AR exon 3 and identified three new variants named AR-V12 to AR-V14. Finally, Yang et al. [20] reported functional properties of AR8. AR8 does not have AR-DBD and thus is not a putative transcription factor, yet mediates important functions in cell lines. Table 13.1 lists all these aforementioned AR-Vs that have been recently cloned from human cell lines and CRPC specimens. The 15 AR-Vs (Table 13.1) differ from each other in their relative abundance in clinical specimens, their functional properties (see below), and their suitability for antibody development. Common to all these AR-Vs is the presence of the canonical GT-AG sequence in all variant-specific splicing junctions, further confirming the transcript structure of these cloned AR-Vs. In addition to these cloned AR-Vs that constitute the AR expression signature in CPPC, other AR-Vs of unknown relevance to CRPC have been previously reported. Some of these

additional AR-Vs were included in a review article by Dehm et al. [9] and will not be discussed further in this Chapter.

## 13.3 Functional Diversity of AR-Vs

Among the AR-Vs listed in Table 13.1, AR-V7 (also named AR3, Table 13.1) and $AR^{v567es}$ have received more attention due to their relatively higher expression in clinical specimens and their unequivocal constitutively active nuclear functions [15, 16, 18]. Both AR-Vs activate transcription of canonical AR-FL target genes when overexpressed in cell lines with or without AR-FL. Other AR-Vs may be conditionally active, i.e., their transcriptional activities are cell type-specific [19]. Examples include AR-V1 and AR-V9, which do not have the basic amino acids characteristic of the bipartite nuclear localization sequence (NLS) [21]. Both AR-V1 and AR-V9 demonstrate transcriptional activity when introduced in AR-FL positive LNCaP cells but not in the AR-FL negative PC-3 cells. However, exogenous AR-FL does not affect these cell context-specific activities [19]. It is possible that the conditional activity of AR-V1 and AR-V9 may require nuclear localization independent of the canonical NLS [22], and they may still enter the nuclei, albeit inefficiently, by engaging the molecular motors and the nuclear import system that is intact in LNCaP cells but deficient in PC-3 cells. Yet another group of AR-Vs are predicted and validated to be inactive [19]. Transcriptionally inactive AR-Vs retain partially truncated AR-LBD, which were previously shown to inhibit AR activity [21, 23]. Examples include AR-V13 and AR-V14 [19].

Because knockdown or inhibition (by the anti-androgen MDV3100) of AR-FL abrogated AR-V7-mediated functions [17], it was proposed that constitutively active AR-V7 functions might require the presence of AR-FL. However, we show that the transcriptional activities of exogenous AR-V7 in LNCaP cells are not affected following AR-FL knockdown, in the presence or absence of ligand [19]. In addition, the conditionally active AR-V9 also remained strongly active in LNCaP cells following AR knockdown or in the absence of R1881 [19]. Moreover, we show recently that in the presence of AR-FL signaling, treatment with MDV3100 induces expression of AR-Vs [24]. Thus, the bulk of evidence supports that AR-V7 functions do not depend on AR-FL, though the two coexist in CRPC.

## 13.4 Detection of AR-Vs in Clinical Specimens

The majority of AR-Vs listed in Table 13.1 can be reliably detected in prostate cancer tissue specimens by RT-PCR [15, 16, 19]. A consistent finding is that AR-V expression levels are higher in CRPC specimens than in hormone-naïve prostate cancer tissues. In addition, although increased expression levels of AR-Vs in general parallel that of AR-FL, fold expression difference for AR-Vs between the

compared groups tend to be greater. Given that alternatively spliced transcripts containing premature stop codons may be degraded through the nonsense-mediated decay (NMD) mechanism [25], it is critical to detect the corresponding protein product to draw functional relevance. Thus far, variant-specific antibodies were reported for AR-V7 (AR3) [15, 16], AR8 [20], and AR1/2/2b [22], using predicted variant-specific c-terminal peptides (Table 13.1) as epitopes. Among these, AR-V7 remains the only AR-V with a proven protein product that can be detected using variant-specific antibodies in clinical specimens. For $AR^{v567es}$, there is evidence supporting the existence of the corresponding protein product. However, a variant-specific antibody has yet to be developed. Some AR variants are not suitable for antibody development due to short length of the c-terminal variant-specific sequences (Table 13.1). Using antibodies recognizing AR-NTD and AR-LBD, Zhang et al. [26] recently show a wide distribution of the AR-NTD/LBD ratio in clinical CRPC specimens, and that higher ratios are associated with more aggressive tumors. This finding is consistent with another report showing elevated AR-V mRNA in CRPC specimens (bone mets) with worse clinical outcome [27], suggesting excess AR-NTD detected in CRPC specimens may originate from the expression of AR-Vs.

## 13.5 Molecular Origin and Regulation of AR-V Expression

Genome-wide copy number analysis of CRPC specimens revealed complex genomic alterations involving the *AR* locus [28]. It remains to be determined whether genomic events contribute to the genesis of AR-Vs. This possibility has been recently investigated in the CWR22Rv1 cell line [29] as well as the LuCaP86.2 xenograft [30], each expressing high levels of the constitutively active AR-V7 and $AR^{v567es}$, respectively. Because *AR* amplification occurs frequently and specifically in CRPC, there may exist rare copies of AR DNA with intragenic rearrangements that may lead to AR-V transcripts, and cells with such alterations may be selected for during AR-directed therapies. However, this hypothesis will need to be tested in clinical specimens.

A second possible mechanism for generation of AR-Vs may be active cotranscriptional or posttranscriptional splicing, especially in response to androgen deprivation. We have reported that when intra-tumor androgen levels drop below those detectable by mass spectroscopy [18, 31], AR-Vs are rapidly induced. These expression changes occur rapidly well before doubling of the cell number. Thus there is not sufficient time for clones of cells with genomic rearrangements to dominate. Additionally, in vivo studies have shown that the expression of variants decreases within days in castrate mice when androgens are replaced [17]. Collectively, these data suggest that some xenografts and cell lines have survived the castration process by selecting for clones of cells in which there is a genomic rearrangement; however, we posit that cancer cells may respond to castration and survive by instituting an active splicing mechanism to generate AR-Vs. Of interest, one of the genes in the

AR-V transcriptional program is UGT2B17 [24, 27], a gene that encodes an enzyme for androgen glucuronidation, which would further lower cellular levels of androgens, possibly exerting further selective pressure on cells expressing AR-Vs.

Yet another explanation for AR-V overexpression is that increased AR-V levels may be coupled with enhanced transcription of the AR gene, possibly involving autoregulation by AR-FL [32]. AR-FL and AR-V are both overexpressed in clinical CRPC specimens [15] and induced concurrently in castrate conditions in some but not all CRPC xenografts [17]. We investigated the direction and extent of endogenous AR-V expression change upon suppression (or activation) of AR-FL signaling in cell lines. Because individual AR-V levels are typically lower than AR-FL [15, 17], we utilized two cell lines, LNCaP95 and VCaP, that recapitulate the relative expression levels of AR-FL and AR-Vs in clinical CRPC specimens, i.e., detectable but lower levels of AR-Vs than AR-FL [15, 24]. We employed three different strategies to suppress ligand-dependent AR-FL signaling, by siRNA targeting the AR-LBD (AR-LBD siRNA), by ligand depletion (R1881-), or by MDV3100, a potent anti-androgen that targets the AR-LBD [33] (Fig. 13.1a, reproduced from [24]). All three of these AR-LBD-targeting strategies lead to dramatically reduced AR-FL activity, as indicated by diminished expression of canonical AR-FL target genes KLK3 and TMPRSS2-ERG (in VCaP cells only, no TMPRSS2-ERG fusion in LNCaP95 cells). However, suppression of AR-FL is accompanied by an increase of the aggregate AR-V signal indicated by the ~80-kDa lower band. In both cell lines, expression levels of AR-Vs reach maximum levels when AR-FL target genes (e.g., PSA, TMPRSS2-ERG) are maximally suppressed (Fig. 13.1a, reproduced from [24]). Increased AR-V protein expression following suppression of AR-FL was confirmed by an increase in AR-V7, as shown by Western blot (Fig. 13.1a) and immunofluorescence staining ((Fig. 13.1b, reproduced from [24]), using a validated variant-specific monoclonal antibody. This adaptive shift to AR-V expression is regulated at the mRNA level, and the magnitude of AR-V mRNA change is greater than that of AR-FL (not shown). Therefore, in the setting of AR-directed therapies targeting AR-LBD, AR-V expression may not be strictly parallel that of AR-FL, and AR-V levels may be negatively regulated by AR-FL signaling. With all experimental findings considered, it is possible that multiple mechanisms may account for elevated expression of AR-Vs in CRPC.

## 13.6 Distinctive Transcriptional Programs Induced by AR-FL and AR-V

By definition, constitutively active AR-Vs are capable of activating canonical AR-FL-regulated genes (e.g., KLK3, TMPRSS2, NKX3.1) in the complete absence of AR-FL signaling [14–18, 24]. When focusing on genes regulated by canonical AR-FL signaling, it is clear that AR-Vs direct a similar transcriptional program as that directed by AR-FL [15, 18]. These findings suggest that elevated AR-V after suppression of AR-FL may compensate for the lack of AR-FL signaling. However,

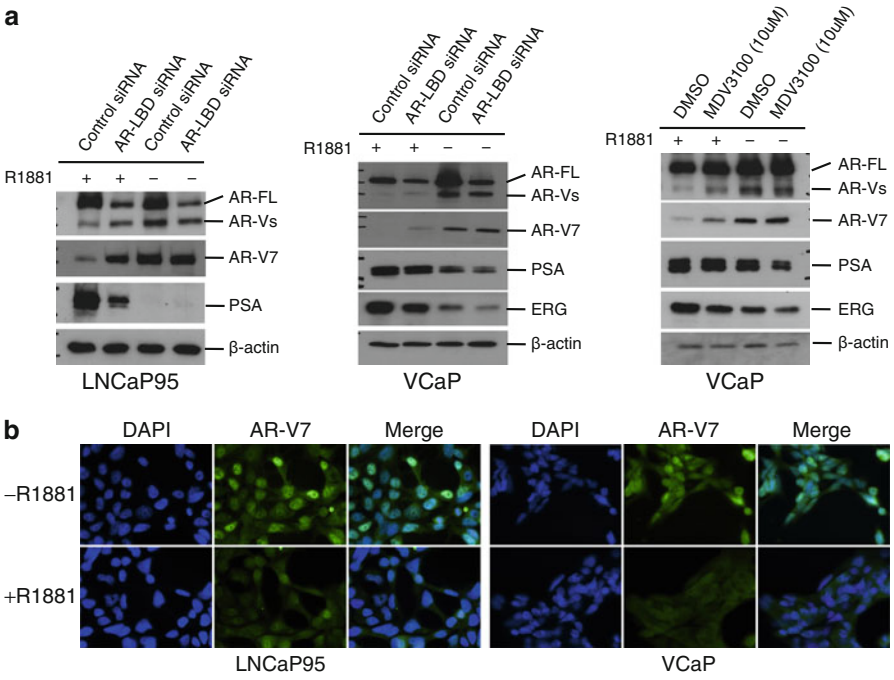



**Fig. 13.1** Regulation of AR-V expression by AR-FL signaling in LNCaP95 and VCaP cells. (**a**): Increased AR-Vs following suppression of AR-FL by ligand depletion (R1881-), siRNA targeting AR-LBD (AR-LBD siRNA), or MDV3100 (10 μM). Protein levels of AR (by the N20 antibody), AR-V7, PSA, ERG, and beta-actin were assessed by Western blot. Note that the N20 antibody detects both AR-FL and AR-Vs, while the variant-specific anti-AR-V7 antibody detects AR-V7 only. (**b**) Immunofluorescent images showing decreased or loss of AR-V7 nuclear staining following activation of AR-FL signaling in the presence of 1nM R1881. Figure is reproduced from [24]

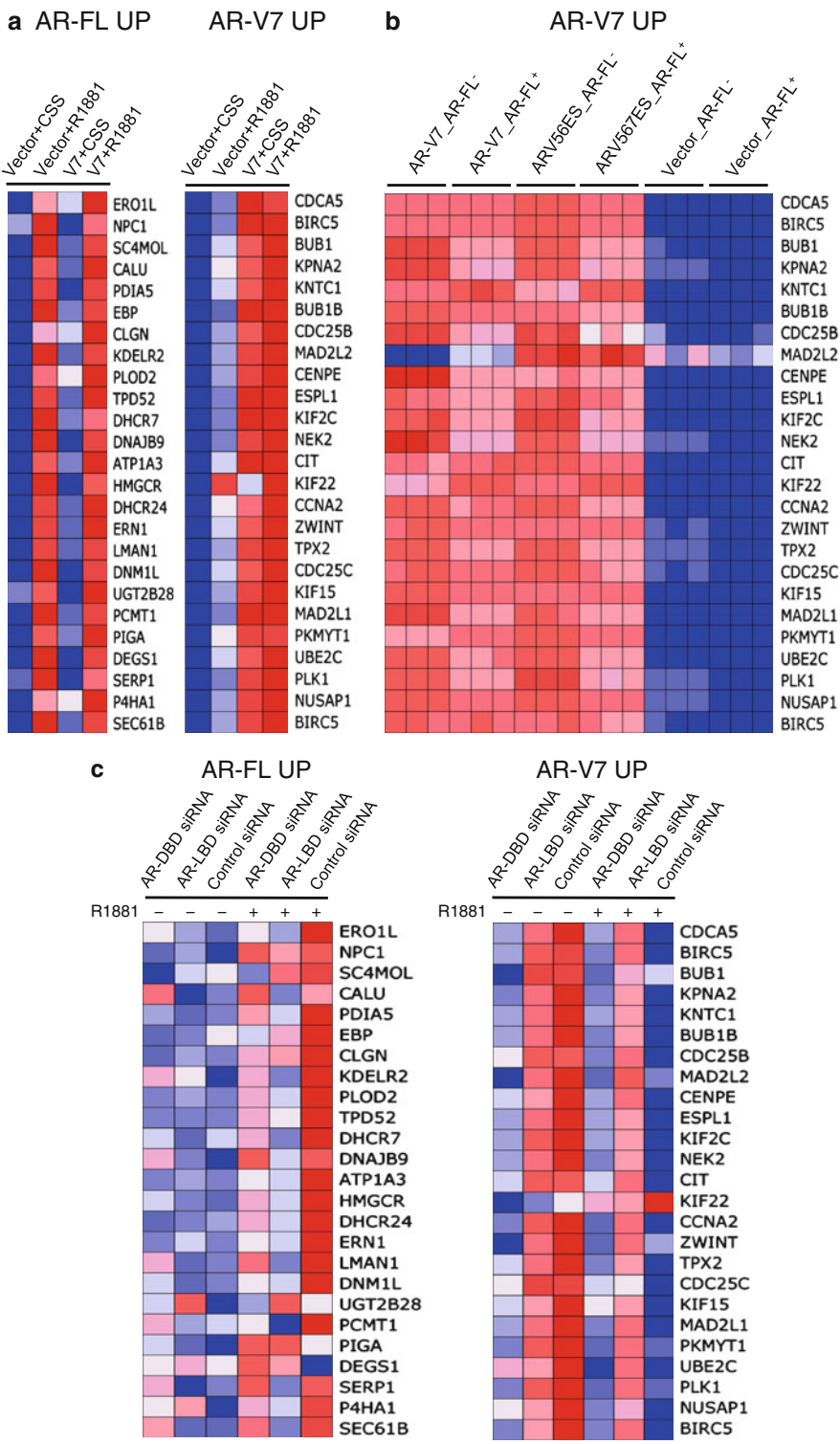
data presented in Fig. 13.1 suggest that AR-directed therapies can effectively block transcription of genes regulated by AR-FL despite the induction of AR-V expression. Lack of AR-V-mediated compensatory "rescue" of AR-FL signaling is evident in LNCaP95 cells in which PSA expression is not detectable although AR-V7 is abundantly expressed after AR-FL signaling is suppressed (Fig. 13.1, reproduced from [24]). In VCaP cells, sustained PSA gene expression may be explained by de novo synthesis of androgens [34]. Therefore, AR-V signaling may not be sufficient to "rescue" the suppressed AR-FL signaling.

To determine the role of ligand-independent AR signaling mediated by AR-Vs in the context of suppressed AR-FL signaling, initial efforts were made to dissect the genome-wide transcriptional programs induced by AR-V-mediated signaling in VCaP and LNCaP95 cells [24]. First transcriptional changes driven by forced expression of AR-Vs in the presence or absence of AR-FL signaling were examined by gene set enrichment analysis (GSEA) [24, 35]. This approach is different from those employed in previous studies in which expression differences are identified

solely based on fold expression change or by focusing on canonical AR-FL-regulated genes. Top-ranked gene sets enriched for upregulation by transient expression of exogenous AR-V7 in the parental LNCaP cells are predominantly cell cycle gene sets, and that these gene sets are not affected by the presence or absence of ligand-dependent AR-FL signaling [24]. On the other hand, top gene sets enriched for upregulation by ligand-dependent AR-FL signaling are dominated by those related to biosynthesis, metabolism, and secretion [24]. For illustration purposes, two new gene sets, termed "AR-V7 UP" and "AR-FL UP," were generated. Each gene set contains 25 probes (24 unique genes) that contributed to the core enrichment of top ranked gene sets driven by AR-V7 and AR-FL. As shown in Fig. 13.2a (reproduced from [24], the two representative gene sets demonstrate clearly independent expression patterns, with the "AR-V7 UP" genes upregulated only when AR-V7 is expressed, while the "AR-FL UP" genes are upregulated only in the presence of ligand-dependent AR-FL signaling. Other canonical AR-FL regulated genes (KLK3, TMPRSS2, NKX3.1) follow the same pattern as those in the "AR-FL UP" gene set (not shown). To further determine whether expression of the AR-V genes requires the presence of endogenous AR-FL, which is suppressed but still present in androgen-deprived conditions, stable LNCaP clones with or without endogenous AR-FL protein [24] were used. These cells were transiently transfected with AR-V7 or $AR^{V567ES}$ in androgen-deprived conditions, and subject to genome-wide expression analysis. Following mapping of the probes included in the "AR-V7 UP" and "AR-FL UP" gene sets to this independent dataset, we show that the "AR-V7 UP" gene set remains as the top ranked gene set induced by ARV567ES or AR-V7, and the absence of endogenous AR-FL did not attenuate induction of the "AR-V7 UP" signature (Fig. 13.2b, reproduced from [24]). Thus, neither AR-FL signaling nor the presence of AR-FL protein is required for induction of cell cycle genes by the two constitutively active AR-Vs (AR-V7, AR-V567ES).

As a second approach to further corroborate the functional distinctions between AR-FL and AR-Vs, gene expression correlates of endogenously induced AR-Vs were evaluated following suppression of AR-FL only or suppression of both AR-FL and AR-Vs (Fig. 13.2c, reproduced from [24]). The "AR-V7 UP" signature is again the top gene set enriched for upregulation following an increase of endogenous AR-V7 induced by AR-FL suppression. However, knockdown of both AR-FL and AR-Vs by targeting AR-DBD abrogated expression of the "AR-V7 UP" gene set (Fig. 13.2c, reproduced from [24]), confirming the essential role of induced AR-Vs

**Fig. 13.2** Distinctive expression patterns of gene sets representing the core transcriptional output of AR-V7 and AR-FL. (**a**) Expression of the "AR-V7 UP" and "AR-FL UP" gene sets in parental LNCaP cells transiently transfected with AR-V7 in the presence (R1881) or absence (CSS) of AR-FL signaling. (**b**) Expression of the "AR-V7 UP" gene set in stable clones of LNCaP cells with ($AR\text{-}FL^{+}$) or without ($AR\text{-}FL^{-}$) endogenous AR-FL following transient transfection with either AR-V7 or ARV567ES. Each experiment was repeated three times. (**c**) Expression profiles of the "AR-FL UP" and "AR-V7 UP" gene sets in LNCaP95 cells following suppression of AR-FL only (AR-LBD siRNA), or both AR-FL and AR-Vs (AR-DBD siRNA), in the presence or absence of 1 nM R1881. Figure is reproduced from [24]


a
AR-FL UP
AR-V7 UP
Vector+CSS
Vector+R1881
V7+CSS
V7+R1881
ERO1L
NPC1
SC4MOL
CALU
PDIA5
EBP
CLGN
KDELR2
PLOD2
TPD52
DHCR7
DNAJB9
ATP1A3
HMGCR
DHCR24
ERN1
LMAN1
DNM1L
UGT2B28
PCMT1
PIGA
DEGS1
SERP1
P4HA1
SEC61B
CDCA5
BIRC5
BUB1
KPNA2
KNTC1
BUB1B
CDC25B
MAD2L2
CENPE
ESPL1
KIF2C
NEK2
CIT
KIF22
CCNA2
ZWINT
TPX2
CDC25C
KIF15
MAD2L1
PKMYT1
UBE2C
PLK1
NUSAP1
BIRC5
b
AR-V7 UP
AR-V7 _AR-FL-
AR-V7 _AR-FL+
ARV56ES_AR-FL-
ARV567ES_AR-FL+
Vector_AR-FL-
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c
AR-FL UP
AR-V7 UP
AR-DBD siRNA
AR-LBD siRNA
Control siRNA
R1881
– – – + + +

in mediating expression of the "AR-V7 UP" signature. Conversely, the "AR-FL UP" genes demonstrated a change of direction opposite to those driven by AR-V. As shown in Fig. 13.2c (reproduced from [24]), the "AR-FL UP" genes are enriched for downregulation following suppression of AR-FL [24]. Thus, although AR-regulated genes are known to be cell-type and gene-specific [36], there is remarkable concordance in the distinct programs represented by the "AR-FL UP" and "AR-V7 UP" genes among VCaP and multiple LNCaP derivatives.

Thus, AR-Vs appear to play a broader function than simply another mechanism to bypass androgen depletion. Indeed, Hornberg et al. [27] suggests that tumors bearing high levels of AR-V7 or any $AR^{v567es}$ portend a rapid progression of the tumor measured by shorter disease-specific survival of the patients. These findings, combined with other correlative studies in clinical specimens as well as animal xenografts [24, 26, 27], suggest that AR-Vs drive a lethal mitotic phenotype. Targeting the AR-V and its transcriptome is an area of priority for discovery and development of novel approaches for CRPC.

## 13.7 Cell-Type-Specific Regulation of AR-Vs

Although AR-V expression and function is clearly upregulated following suppression of ligand-dependent AR-FL signaling in VCaP and LNCaP95 cells, two cell lines with detectable endogenous AR-V expression that is less abundant than that of AR-FL, the adaptive AR-FL to AR-V shift was not observed in the parental LNCaP and CWR22RV1 cells [24]. Unlike LNCaP95 cells, the parental LNCaP cells do not express detectable levels of AR-V protein and respond to androgen deprivation by increasing AR-FL mRNA but decreasing AR-V7 mRNA [24]. The CWR22Rv1 cells are known to express very high levels of AR-Vs [14–16]. CWR22Rv1 cells do not demonstrate apparent regulation of AR-V7 expression by AR-FL signaling [24]. Consistent with the role of AR-V in driving expression of cell cycle genes, in these two cell lines where AR-V expression cannot be induced, we did not observe increased cell cycle gene expression after suppression of AR-FL signaling (not shown). It is possible that regulation of endogenous AR-V expression by AR-FL signaling may be cell type-specific, raising the possibility that AR-Vs may play a role in supporting castration-resistant growth in some, but not all prostate tumors subject to AR-directed therapies.

## 13.8 Priorities in the Emerging Area of AR-V Research

Laboratory findings have revealed intriguing functional interplay and dichotomy between AR-FL and AR-Vs when AR-LBD is rendered inactive by AR-directed therapies. The combined in vitro and in vivo data predict an important shift toward AR-V-mediated signaling with effective CRPC therapies targeting AR-LBD including

abiraterone and MDV3100. If this shift is confirmed in a subset of CRPC patients receiving MDV3100 or abiraterone, a number of strategies to enhance the clinical efficacy of these therapies are rationalized. First, early indications of therapeutic efficacy may be detected as a consequence of this adaptive shift and utilized to guide treatment decisions. Second, novel agents for CRPC [12] may be designed to overcome adaptive activation of AR-V signaling. These clinical translational priorities call for development and validation of methods for reproducible detection and quantification of AR-V and AR-V signature in clinical specimens, as well as novel agents that target all AR molecules. The overall concept of an AR-FL to AR-V shift cannot be definitively established unless relevant studies are conducted in specimens obtained before, during, and after AR-directed therapies, which will allow concurrent clinical validation of a number of competing mechanisms of drug response and resistance. Key to this effort is the continued development and validation of variant-specific probes and antibodies, as well as preclinical and clinical testing of novel agents that target all AR molecules.

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# Chapter 14
# Androgen Regulation of the Cell Cycle in Prostate Cancer

**Haojie Huang**

**Abstract** Androgens control both growth and differentiation of the normal prostate gland and are the major factors on which prostate cancer cells depend for growth and survival. However, the mechanisms by which androgens act upon the cell cycle machinery to regulate both growth and differentiation are not fully understood. Research over the past decades reveals that expression of several key cell cycle regulators such as SKP2, $p27^{KIP1}$, E2F1, and EZH2 is regulated by androgens in a biphasic manner, that is, stimulated by low dose of androgens but repressed by higher doses of androgens. Because age is one of the key risk factors for prostate cancer, it is possible that with the decline in the levels of serum testosterone during aging, androgenic regulation of cell cycle genes may shift from the pro-differentiation to pro-proliferative mode. It is also likely that residual levels of androgens produced via intracrine mechanism in castration-resistant prostate cancer may be sufficient to activate androgen-mediated pro-proliferative gene program, but insufficient to initiate anti-proliferative program, thereby favoring castration-resistant progression. Finally, the possibility that the androgen-regulated anti-proliferative gene program may be outlawed by frequently deregulated oncogenic pathways in human prostate cancers is discussed.

**Keywords** Androgen • Androgen receptor • SKP2 • $p27^{KIP1}$ • E2F1 • EZH2 • p300 • Prostate cancer • Cell cycle • Aging

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## 14.1 Introduction

Testosterone (T) and dihydrotestosterone (DHT) are two major forms of androgens in adult males. T is mainly synthesized and secreted by Leydig cells in the testes and further converted by the 5α-reductases into DHT, the more active form of androgens in target tissues such as the prostate [1]. Males deficient in type II 5α-reductases have an underdeveloped prostate [2, 3] indicating that androgens are important for the development and function of the prostate. Animal studies show that androgens induce biphasic growth response in the prostate. Low dose of androgen administrated to castrated rats promotes proliferation of prostatic epithelial cells whereas higher doses of androgen cause growth arrest by inducing prostatic epithelium differentiation [4]. The biphasic or the so-called "bell-shaped" response to androgens is also well documented in the LNCaP prostate cancer cell line. Androgens at concentrations between 0.1 and 1 nM stimulates LNCaP cell proliferation; however, cell growth is either not stimulated or inhibited by androgens at concentrations below 0.1 nM or above 1 nM [5–8].

Similar to normal prostatic epithelial cells, prostate adenocarcinoma cells also largely depend on androgens for their development and maintenance. Prostate cancer incidence is rare or none in males castrated at young ages [9, 10] indicating that androgens are key risk factors of prostate cancer. Moreover, androgen deprivation induces programmed cell death or the so-called apoptosis in both normal and cancerous prostate epithelium [11]. Thus, in addition to promoting proliferation and differentiation, androgens are also important for the survival of normal and cancerous prostatic epithelial cells. The androgen action is realized by the androgen receptor (AR). AR belongs to the nuclear receptor superfamily of transcription factors that regulates expression of a myriad of genes that are involved in cell differentiation, proliferation, and survival. In this chapter, I focus on the findings regarding androgen regulation of expression of cell cycle-related genes in prostatic cells.

For each cell division cycle, there are two major phases, one for DNA synthesis/replication, so-called "S" phase, and the other for mitosis or so-called "M" phase. There are two "gap" phases between S and M phases, which are gap 1 (G1) and 2 (G2) phases [12]. Cells prepare for themselves at G1 for DNA synthesis and at G2 for mitosis. Upon the withdrawal of mitogenic factors such as growth factors or hormones, cells exit the cell cycle and enter into a resting stage deemed "G0" phase. For example, majority of epithelial cells in the adult prostate gland are at the G0 state and are terminally differentiated cells. Transition into and within the mitotic cell cycle are fulfilled by the coordinated activation of cyclin-dependent kinases (CDK)/cyclin complexes. Upon the mitogenic stimuli, expression levels of D-type cyclins including cyclins D1, D2, and D3 increase. Cyclin D proteins bind to and activate the early G1 kinases CDK4 and CDK6, thereby resulting in phosphorylation and inactivation of the tumor suppressor protein retinoblastoma (RB) and its related pocket proteins p107 and p130. Prior to phosphorylation, these pocket proteins bind to and inhibit the E2F/DP1 transcription complex. CDK4/6 phosphorylation results in the partial release of RB inhibition of the E2F/DP1 complex, leading

to the transcription of cylin E and cyclin A, which bind to and activate CDK2. Activation of CDK2 by cyclin E and cyclin A results in hyperphosphorylation and complete inhibition of RB and other substrates, thereby promoting DNA replication, centrosome duplication, and histone protein expression. Transition of the cell cycle through the G2 and M phases are driven by coordinated activation of CDK1-cyclin A and CDK1-cyclin B complexes. Cyclin B protein levels increase in G2. CDK1-cyclin B activity continues to rise, thereby ensuing mitosis, throughout early mitosis until anaphase, wherein activation of the anaphase-promoting complex/cyclosome (APC/C) promotes rapid degradation of cyclin B, triggering loss of CDK1 activity, mitotic exit, and completion of the cell cycle [13].

In contrast to cyclins and CDKs, another group of proteins called cyclin-dependent kinase inhibitors (CDKi) mediate cell cycle arrest by binding to and inhibiting the activities of the CDK/cyclin complexes following growth inhibitory stimuli [14]. There are two families of CDKi, the inhibitor of CDK4 (INK4) family including $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$, and the KIP family including $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. The INK4 family members bind specifically to CDK4 and CDK6 and inhibit D-type cyclin binding and the KIP family members bind to and inhibit targeted CDKs acting in G1, S, and G2/M phases. As discussed above, androgens function as both differentiation and proliferation factors in prostatic epithelial cells depending on the developing stage and physiological conditions. Androgens execute such biological effects by activating the AR, which in turn functions as a transcription factor that activates or represses an array of genes that either promote or inhibit cell cycle transition.

## 14.2 Biphasic Regulation of Cell Cycle-Regulating Genes by Androgens

### *14.2.1 S-Phase Kinase-Associated Protein 2*

S-Phase Kinase-Associated Protein 2 (SKP2) is a gene that was originally cloned due to its association with S-phase kinases and overexpression in many types of cancer cell lines [15]. Further studies show that it primarily acts as an ubiquitin E3 ligase by forming a SKP1/CULIN 1/F-box protein SKP2 ($SCF^{SKP2}$) complex [16]. SKP2 binds to and targets a number of cell cycle negative regulators/tumor suppressor proteins, including $p27^{KIP1}$, $p57^{KIP2}$, p130, and FOXO1 for polyubiquitination and proteasome degradation [17–20]. These observations suggest that SKP2 may function as an oncogenic protein. Expression of SKP2 protein is elevated in human prostate cancers and correlated with disease progression [21, 22]. A recent study with integrative genomic profiling of human prostate cancers shows that the genomic region containing the SKP2 gene at chromosome 5p13.3-p13.1 is amplified in a subset of patients [23]. In support of this clinical data, prostate-specific expression of SKP2 in transgenic mice induces hyperplasia, dysplasia, and high-grade prostatic

intraepithelial neoplasia (PIN) lesions in the prostate [24]. Gene knockout studies show that SKP2 plays an essential role in the progression of PTEN-knockout prostate tumors in mice [25]. These findings indicated that SKP2 is a bona fide oncogenic protein in the prostate.

Expression of SKP2 is regulated by androgens in both normal and cancerous prostatic cells. SKP2 protein is overexpressed in androgen-refractory metastatic prostate cancers [26]. An animal study shows that continuous testosterone propionate (TP) administration to castrated rats triggers epithelial cell proliferation, which peaked at 72 h, and then declined despite further treatment. The expression of Skp2 was seen to increase with androgen administration, preceding maximal proliferation [27]. Using the LNCaP cell line as a working model, Lu and colleagues demonstrated that G1 cell cycle arrest of LNCaP cells induced by mibolerone, a synthetic androgen at 1 nM or higher is associated with decreased expression of SKP2 [28]. Androgenic regulation of SKP2 requires a functional AR [29]. Further analysis demonstrates that androgens regulate SKP2 expression in a biphasic manner [6]. Treatment of LNCaP cells with R1881, another synthetic androgen at concentrations from 0.01 to 0.1 increased SKP2 expression [6]. Consistent with the other reports, treatment of LNCaP with 1 nM or higher concentrations of R1881 completely repressed SKP2 expression [6]. This regulation of SKP2 expression by androgens is mediated through p107-dependent and -independent pathways [6]. Thus, biphasic regulation of this cell cycle driving gene by androgens may provide a potential mechanism underlying the biphasic roles of androgens in control of both growth and differentiation of the normal prostate gland.

### *14.2.2 p27$^{KIP1}$*

The CDK inhibitor p27$^{KIP1}$ acts during G0 and the early G1 phase of the cell cycle to inhibit G1 cyclin/CDK complexes. p27$^{KIP1}$ knockout mice develop multiple organ hyperplasia, including prostatic hyperplasia [30], suggesting that p27$^{KIP1}$ is an important inhibitor of prostate cell proliferation. Increasing evidence obtained from studies of p27$^{KIP1}$ in human prostate cancers further suggests a role of this KIP protein in regulation of prostate epithelial cell growth. Low or undetectable levels of p27$^{KIP1}$ protein in primary prostate cancers are correlated with increased proliferation index, increasing tumor grade, shorter disease-free survival and decreased overall survival [30–33]. p27$^{KIP1}$ increases during differentiation in many cell types, including differentiation induced by vitamin D3 and androgens in the prostatic epithelium [4, 27]. Specially, it has been shown that castration-induced atrophy of the ventral prostate (VP) was associated with a significant increase in p27$^{KIP1}$ expression as compared with the VP of intact animals. Twelve hours after the initiation of androgen treatment, total p27$^{KIP1}$ levels significantly dropped in the VP of castrated rats. During the period of the regenerative process, whereas both proliferating basal and secretory epithelial cells did not express p27$^{KIP1}$, the protein was

selectively upregulated in the nonproliferating secretory epithelial compartment. This upregulation of $p27^{KIP1}$ expression was coincident with an increase in its association with, and presumably inhibition of, CDK2 [27]. In agreement with the findings in animals, low doses of R1881 decreased but higher doses increased $p27^{KIP1}$ protein levels in cultured LNCaP cells [6, 33]. The biphasic expression pattern of $p27^{KIP1}$ is inversely corrected with the levels of SKP2 [6]. Indeed, it has been shown that SKP2 is responsible for androgen control of $p27^{KIP1}$ stability in LNCaP cells [34]. Moreover, a study showed that a subset of tumors, treated with preoperative androgen deprivation therapy prior to radical prostatectomy, exhibits higher expression of $p27^{KIP1}$ protein than that in untreated cases [33]. Together, changes in cellular pathways that regulate $p27^{KIP1}$ levels may contribute to growth and differentiation of both normal and cancerous prostatic cells in response to stimuli by different doses of androgens.

### *14.2.3 E2F1*

The transcription regulator E2F1 partners with DP1 protein to form a transcriptional activator complex. The E2F1/DP1 complex regulates a number of genes that control the transition of the cell cycle from G1 to S phase. Animal studies using CWR22 prostate cancer xenografts show that expression of E2F1 protein is increased in castration-resistant tumors [35], suggesting that expression of E2F1 could be androgen regulated. Further studies show that expression of E2F1 mRNA and protein is repressed by high doses of androgens in LNCaP cells [29, 36] but increased by stimulation of LNCaP cells with low doses of androgens [36]. Thus, biphasic regulation of this cell cycle promoting gene by androgens is consistent with the biphasic role of androgens in regulation of prostatic cell growth and differentiation.

### *14.2.4 Enhancer of Zeste 2*

Enhancer of Zeste 2 (EZH2) is a Polycomb protein that primarily functions as an epigenetic gene silencer and plays a role in oncogenesis by promoting cell proliferation and invasion. Expression of EZH2 is elevated in prostate cancer compared to normal prostate tissues [37]. Importantly, increased expression of EZH2 is associated with hormone-refractory progression of human prostate cancers [26, 37] suggesting that EZH2 expression can be regulated by androgens. It has been shown recently that expression of EZH2 mRNA and protein can be slightly induced by R1881 at concentrations from 0.01 to 0.1 nM, but repressed by R1881 at concentrations of 1 nM or higher [38]. Small interference RNA studies show that androgenic regulation of EZH2 requires a functional AR, but is likely mediated by p130-dependent and -independent pathways [38]. Further studies reveal that

androgenic repression of EZH2 could be mediated by androgen-induced upregulation of microRNA 101 [39], expression of which represses EZH2 [40]. It is warranted to further investigate whether or not biphasic regulation of EZH2 by androgens plays a role in differentiated effects of androgens on proliferation and differentiation.

## 14.3 Upregulation of Cell Cycle-Regulating Genes by Androgens

### *14.3.1 Proliferating Cell Nuclear Antigen*

As aforementioned, androgen-regulated cell cycle progression is a complex process, meaning that androgens at low concentrations stimulate cell proliferation and at higher concentrations inhibits proliferation under both in vitro and in vivo conditions. Proliferating cell nuclear antigen (PCNA) plays an essential role in cell proliferation by binding to DNA polymerase-γ and regulating DNA replication. Treatment of LNCaP cells with 1 nM of mibolerone results in an increase in the protein level of PCNA [41]. A non-consensus androgen response element (ARE) was identified in the PCNA gene promoter. However, this ARE appears to be nonfunctional because no change in PCNA message RNA (mRNA) was detected after androgen treatment in LNCaP cells. In agreement with this observation, androgen stimulation-induced increase in PCNA protein levels results partially from increased half-life of the protein and partially from an increase in translational efficiency of PCNA [41]. These data suggest that androgen induction of prostate cell proliferation may be mediated, at least in part, through PCNA at the posttranscriptional level.

### *14.3.2 UBE2C*

UBE2C is an M-phase cell cycle gene. It plays a critical role in inactivating the M-phase checkpoint [42] and silencing of UBE2C causes a G2/M arrest in prostate cancer cell lines [43]. It has been shown that expression of UBE2C is regulated specifically by the AR under androgen-depleted conditions in a castration-resistant prostate cancer cell line [43]. Although overexpression of UBE2C in androgen-dependent LNCaP cells is not sufficient to promote cell growth in the absence of androgens, silencing of UBE2C selectively decreases proliferation of castration-resistant LNCaP-abl but not LNCaP cells [43]. These findings highlight that castration-resistant cells develop unique means to foster AR-dependent cell cycle control in the absence of androgens.

### *14.3.3 p21$^{CIP1}$*

p21$^{CIP1}$ is a universal inhibitor of CDKs [44] and involved in development and differentiation [45]. Using LNCaP cells as a working model, it has been shown that treatment of LNCaP cells with 10 nM of R1881 upregulates p21$^{CIP1}$ expression [28]. A putative ARE has been identified and electrophoretic mobility gel shift assay (EMSA) shows that it can be bound by the AR [28]. The finding that expression of p21$^{CIP1}$ is upregulated by high doses of androgen is consistent with the differentiation-promoting function of the AR in the normal prostate epithelial cells. It is worth noting that p21$^{CIP1}$ can be directly phosphorylated by AKT and this phosphorylation results in cytosolic localization and inactivation of p21$^{CIP1}$ [46]. Moreover, AKT is highly activated in LNCaP cells due to the mutated PTEN tumor suppressor gene [47]. Because of the well-known function of p21$^{CIP1}$ in development and differentiation, it can be speculated that androgenic regulation of p21$^{CIP1}$ may play an important role in development and differentiation of the prostate gland and that this effect of androgens can be abrogated due to deregulation of oncogenic pathways.

## 14.4 Downregulation of Cell Cycle-Regulating Genes by Androgens

### *14.4.1 p300*

This protein primarily functions as an acetyltransferase that promotes histone and non-histone protein acetylation. It has been demonstrated that p300 expression correlates with proliferation of human prostate cancers [48]. Moreover, high levels of p300 in biopsies predicted larger tumor volumes and prostate cancer progression after surgery. The disruption of p300 transcripts through small interfering RNA inhibited prostate cancer cell proliferation [48, 49]. Further studies show that expression of p300 protein, but not mRNA, is repressed in LNCaP prostate cancer cells treated with R1881 at concentrations of 1 nM or higher [50]. Consistent with this result, expression of p300 protein is increased in androgen-refractory prostate cancer cells. At present, however, how p300 protein is regulated by androgens in prostate cancer cells is not fully understood.

## 14.5 Perspectives

Evidence from both in vitro and in vivo studies indicates that androgens at physiological or higher levels increase expression of CDK inhibitors p27$^{KIP1}$, p21$^{CIP1}$, and p15$^{INK4B}$ [6, 28, 29, 33, 34] and decrease expression of cell cycle promoting genes such as

SKP2, E2F1, EZH2, and p300 [29, 34, 36, 38, 50]. In consistent with these observations, androgens at these levels decrease the activity of CDKs [33, 34]. In contrast, sub-physiological concentrations of androgens (e.g., 0.1 nM of DHT) reduce expression of cell cycle inhibitory proteins such as $p27^{KIP1}$ [6, 27], increase expression of cell cycle promoting proteins including SKP2, E2F1, and EZH2 [27, 36, 38], and enhance CDK activity [51]. In agreement with these molecular studies, androgens and the AR are well known as differentiation-promoting factors in the developing prostate gland. Moreover, it has been shown that knockout of the AR in the prostate epithelial cells results in an increase in prostate cell proliferation and loss of cell differentiation [52, 53] further supporting the notion that epithelial AR controls prostate growth by suppressing epithelial proliferation in the mature gland.

The differential effects of high versus low doses of androgens on expression of cell cycle-regulated gene may also be related to the role of androgens and the AR in prostate cancer development and progression. Epidemiological studies show that age is one of the key risk factors for prostate cancer. Clinical prostate cancer is extremely rare in men younger than 45 years of age, with less than 1 in 10,000 occurrences. The incidence increases dramatically over the ensuing decades, with a 1 in 6 chance of cancer detection between the ages of 60 and 80 [54, 55]. This relationship between prostate cancer incidence and aging is consistent across ethnic and racial groups [55]. Intriguingly, serum levels of testosterone decline with age [56]. Thus it can be speculated that with the decline of serum testosterone levels during aging, androgenic regulation of cell cycle genes may shift from the anti-proliferative to the pro-proliferative mode, thereby increasing age-related incidence of prostate cancer.

Because numerous signaling pathways are deregulated in cancerous cells, androgen regulation of the expression of cell cycle genes and the biological consequence of these genes are often affected by deregulated oncogenic pathways during prostate cancer development and progression. For example, the PTEN tumor suppressor is frequently mutated in primary and metastatic prostate cancers [57]. Loss of PTEN leads to activation of AKT in prostate cancer cells [58]. It has been shown that AKT directly phosphorylates $p21^{CIP1}$ and promotes cellular location of $p21^{CIP1}$, thereby blocking the cell growth-inhibitory activity of $p21^{CIP1}$ [46]. Thus, it is likely that while androgens still can induce expression of $p21^{CIP1}$, its inhibitory effect on growth may be outlawed by AKT phosphorylation in PTEN-null prostate cancer cells. Moreover, it has been shown that repression of cell cycle-regulating genes such as E2F1, SKP2, and EZH2 by high concentrations of androgens can be abolished by wild-type or mutated viral protein E1A, which binds to and inhibits the pocket proteins including RB, p107, and p130 [6, 29, 38]. Similar effects were observed in cells treated with gene-specific siRNAs for RB and its related proteins [6, 29, 38]. Given that the RB signaling pathways are deregulated in approximately 34 % of primary and 74 % of metastatic prostate cancers [23], it is conceivable that androgen-mediated repression of expression of these cell cycle-driving genes is likely disrupted in those cases with the deregulated RB pathway.

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# Chapter 15
# Androgen Receptor Signaling Interactions Control Epithelial–Mesenchymal Transition (EMT) in Prostate Cancer Progression

**Sarah K. Martin, Michael V. Fiandalo, and Natasha Kyprianou**

**Abstract** The role of the androgen receptor (AR) signaling axis in the progression of prostate cancer is a cornerstone to our understanding of the molecular mechanisms behind this important disease. Understanding the innate signaling axis of the AR and the aberrations of this axis in progression of prostate cancer has facilitated the development of emerging therapeutic interventions. Furthermore, the crosstalk of AR with other critical signaling pathways may explain the advancement of prostate cancer to metastatic castration-resistant prostate cancer (CRPC). Of particular interest to such crosstalk are the pathways associated with epithelial to mesenchymal transition (EMT). The reactivation of EMT is a hallmark of metastatic cancer spread, and recent evidence suggests the involvement of AR in the signaling pathways regulating EMT. Cadherin switching, EMT inducing transcription factors, Wnt, TGF-β, and Notch signaling can all be modulated by crosstalk with the AR. Overexpression and localization of the AR to the nucleus has been associated with reactivation of the androgenic signaling axis and progression to metastatic CRPC in patients. In this chapter we consider the current understanding of the functional exchanges between the androgen signaling championed by AR activity and key growth factor

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signaling pathways that impact EMT towards prostate cancer progression to metastatic CRPC and we discuss the clinical relevance of these insights in the effective targeting of advanced disease.

**Keywords** Androgen receptor • Epithelial to mesenchymal transition • Metastasis • TGF-β • Cadherin switching

**Abbreviations**

| | |
|---|---|
| CRPC | Castration-resistant prostate cancer |
| ADT | Androgen deprivation therapy |
| EMT | Epithelial–mesenchymal transition |
| TGF-β | Transforming growth factor-β |
| DHT | Dihydrotestosterone |
| ARE | Androgen-responsive elements |
| PSA | Prostate-specific antigen |

## 15.1 Introduction

The pioneering work of Huggins and Hodges [1], first established the significance of male steroid hormones in prostate cancer cell proliferation and that their withdrawal diminished prostate tumor growth [1]. Seventy years later, this observation is still a cornerstone of the clinical treatment paradigm in the management of patients with metastatic prostate cancer. Androgen deprivation therapy (ADT) by a variety of surgical and/or pharmacological methods ultimately, fails to effectively cure patients with prostate cancer; they relapse and progress to the more aggressive disease state "castration-resistant prostate cancer" (CRPC) or "hormone-refractory prostate cancer." Therapeutic failure ADT is assessed by biochemical recurrence, monitored in patients by sequential evaluation of serum prostate-specific antigen (PSA). It is indeed the alarmingly high number of 70,000 American men who develop disease recurrence each year that represents the treatment challenge for urologists, oncologists, and radiation oncologists, as well as basic scientists [2, 3]. Despite the shortcomings of PSA screening, it facilitates the identification of at risk patients experiencing biochemical recurrence to metastatic CRPC progression. The androgen signaling axis still remains the focal point of the first line clinically viable therapeutic approach to impeding prostate cancer progression [1, 3]. Progression to CRPC is characterized by increased androgen receptor (AR) expression, elevated intraprostatic androgens, and perpetually activated AR signaling despite physiologically castrate levels of androgens [4, 5]. Mechanisms via which androgenic/AR signaling is maintained in androgen-depleted environments and effects on target gene expression include the potential AR mutations, amplification, alternative splicing,

overexpression, and altered sensitivity yielding altered AR expression and/or aberrantly activated function in diverse cellular processes involved in tumorigenesis [6]. Emerging evidence suggests that reactivation of epithelial–mesenchymal transition (EMT) processes may facilitate the development of prostate cancer [7], with increasing number of studies focusing on the direct involvement of androgen/AR signaling in EMT/MET transitions. The clinicopathological significance of EMT in human cancers continues to be a topic of debate. As the cellular landscape EMT is being interrogated by proteomic analysis toward defining its role in prostate cancer progression to metastasis. Investigating the regulatory mechanisms by which EMT programs are controlled by the androgen/AR signaling, is fundamentally important for understanding the functional contribution of EMT processes to various stages of prostate tumor progression to metastasis and emergence of CRPC disease.

### *15.1.1 The Androgen Receptor in Control of Prostate Growth*

The AR is a member of the steroid–thyroid–retinoid nuclear receptor superfamily found on the X chromosome (Xq11-12) spanning approximately 180 kb of DNA with 8 exons [8]. In the normal AR signaling axis, testosterone synthesized in the testis or by the adrenal gland is sequestered by sex hormone-binding protein circulating in the blood sera. Dissociation from SHBP and diffusion across the plasma membrane brings testosterone into proximity with the cytochrome p450 enzyme 5 α- reductase (SRD5A1, SRD5A2) producing the cognate ligand of AR (dihydrotestosterone; DHT) [9–11]. Binding of DHT to the AR facilitates the rearrangement of AR domains and within the heat shock protein 90 super complex and subsequent transcriptional activation. AR bound to DHT homo-dimerizes and becomes activated via phosphorylation by the Protein Kinase A signaling pathway [12, 13]. The homo-dimer translocates into the nucleus and is able to bind androgen-responsive genes (ARG) at specific palindromic DNA sequences known as androgen- responsive elements (ARE) (Fig. 15.2a) [14]. This binding to ARE DNA allows the homodimeric AR to act as a scaffold and recruit coregulators and modulate transcription via actions as transcription factor [14–16]. The binding of the AR to the ARE forms a stable pre-initiation complex near the transcriptional start site facilitating the recruitment and initiation of RNA polymerase II (Fig. 15.2a) [17].

### *15.1.2 The Structure of AR*

The AR is composed of an amino-terminal-activating domain (NTD), a carboxy-terminal ligand-binding domain (LBD), a DNA-binding domain in the mid-region that contains two zinc finger motifs to facilitate the interaction of the protein with the DNA double helix (DBD), and a hinge region to facilitate the change in protein folding upon binding to the ligand and dimerization (Fig. 15.1). These four domains

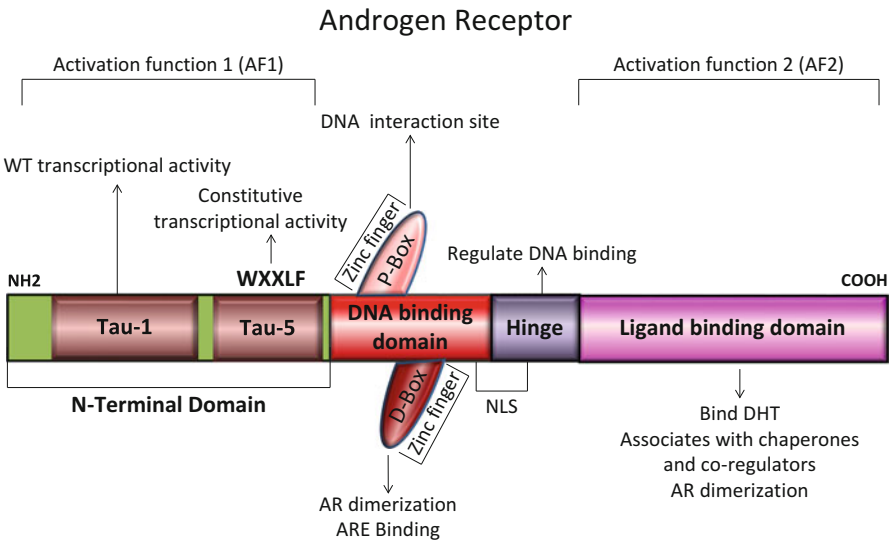


**Fig. 15.1** Androgen receptor structure: schematic diagram of protein domains and functions

comprise the 919 amino acid protein with a mass of 110 kDa. N-terminal Domain: The NTD (exon 1, amino acids 1–537) has been shown to possess multiple transcriptional activating units (TAU): TAU-1 and TAU-5 (Fig. 15.1) [17]. TAU-1 is associated with wild type AR transcriptional activation and is characterized by a high number of acidic amino acids, three glutamine repeats, and a phosphorylation site. Conversely, the TAU-5 sequence is characterized by stretches of proline, alanine, and glycine [17]. Having been attributed with 50 % of aberrant AR activity in CRPC, TAU-5 is responsible for the constitutive transcriptional activity of the NTD and is mediated by a core sequence of $^{435}$WHTLF$^{439}$ in between the aforementioned alanine and glycine stretches (Fig. 15.1) [9, 18]. DNA-binding domain: The cysteine-rich DBD (exons 2 and 3, amino acid: 68) contains two important motifs [19]. The P-box motif found in the first of two zinc fingers facilitates the interaction of the AR with gene-specific nucleotide sequences inside the major groove of the DNA double helix (Fig. 15.1) [20]. The D-box motif mediates the DBD/LBD interaction that allows for inter-domain interaction and AR homo-dimerization after activation and facilitates the spacing of the AR over the half sites and binding on the ARE (Fig. 15.1) [9, 20–22]. The DBD contains one of the nuclear localization signals (NLS) as discussed below.

*Hinge Region*: With only approximately 50 amino acids, the hinge region packs a big punch in a small space. The nonconserved and flexible hinge region separates the LBD and the DBD while containing part of the bipartite nuclear localization signal (Fig. 15.1) [23, 24]. The hinge region (as well as DBD and LBD) also contains a site for interaction with Filamin A, an actin interacting protein and signaling

scaffold required for nuclear translocation of the AR [9, 25]. The hinge region plays a role in nuclear localization, DNA-binding inhibition, coactivator recruitment, and the N-terminal/C-terminal interaction of the AR [23]. Interestingly, a span of highly basic residues between 629 and 636 (629-RKLKKLGN-636) is conserved in all AR sequences known and decreases the affinity of AR for DNA binding as demonstrated by deletion constructs [23]. Ligand-Binding Domain: The LBD (exons 4–8, ~250 amino acids) mediates the binding of the AR ligand (testosterone or DHT) to the AR protein and initiates the downstream cascade of the androgen signaling axis [9]. In addition to ligand binding, the LBD associates with the heat shock protein super-complex, interacts with numerous coregulators, and participates in receptor dimerization (Fig. 15.1 and 15.2a) [26–29]. The AR protein is composed of two activation domains (AF-1 and AF-2). The AF-1 domain is localized to the NTD and is composed of TAU-1 and TAU-5 domains contributing to the transcriptional activation program (Fig. 15.1). The AF-2 domain is localized to the LBD and interacts with LxxLL-containing coregulators such as the steroid receptor coactivators (SRC) and TAU domains in the NTD [9, 30].

### *15.1.3 The AR Localization: Translocation Matters*

The AR is sequestered in the cytosol by the Hsp90 super-complex awaiting its cognate ligand DHT for initiation of the nuclear translocation protocol. The bipartite nuclear localization signal (NLS1) spans the DBD and hinge regions with exons 3 and 4 represented in [22]. This NLS is composed of the sequence, **RK**CYEAGMTLGA**RKLKK**, and two basic motifs represent important sequence components which facilitate AR nuclear translocation (indicated in bold) [22]. The bipartite nature of the NLS ensures that there is cooperation between the different protein domains to allow nuclear shuttling. The AR NLS is regulated by the binding of the LBD to the cognate ligand, facilitating a conformation change in the protein that places the NLS in a functional orientation for translocation [22]. After AR has bound DHT, homo-dimerized, activated by phosphorylation, and translocated to the nucleus, the exposure of the NLS allows binding to the importin-α adaptor protein and importin-β carrier protein [31]. This allows movement through the nuclear pore complex and Ran-dependent release into the nucleus [32–37]. There is however a second NLS sequence (NLS2) in the LBD, that allows the AR to enter the nucleus in an importin-α-independent mechanism [27, 38, 39]. In addition to the importance of the bipartite NLS of the AR itself, the binding of AR to the Hsp90 super-complex aids to prevent aberrant signaling without cognate ligand activation. The hinge region of the AR is able to mediate an interaction with Filamin-A (FLNA) protein in the cytosol [25, 40]. The 280 kDa cytoskeletal protein, Filamin-A is a critical regulator of the solation–gelation equilibrium at the cell membrane by cross-linking F-actin fibers into orthogonal arrays and interacting with the AR and Hsp90 complex [25, 41]. Filamin-A is essential to mediating the

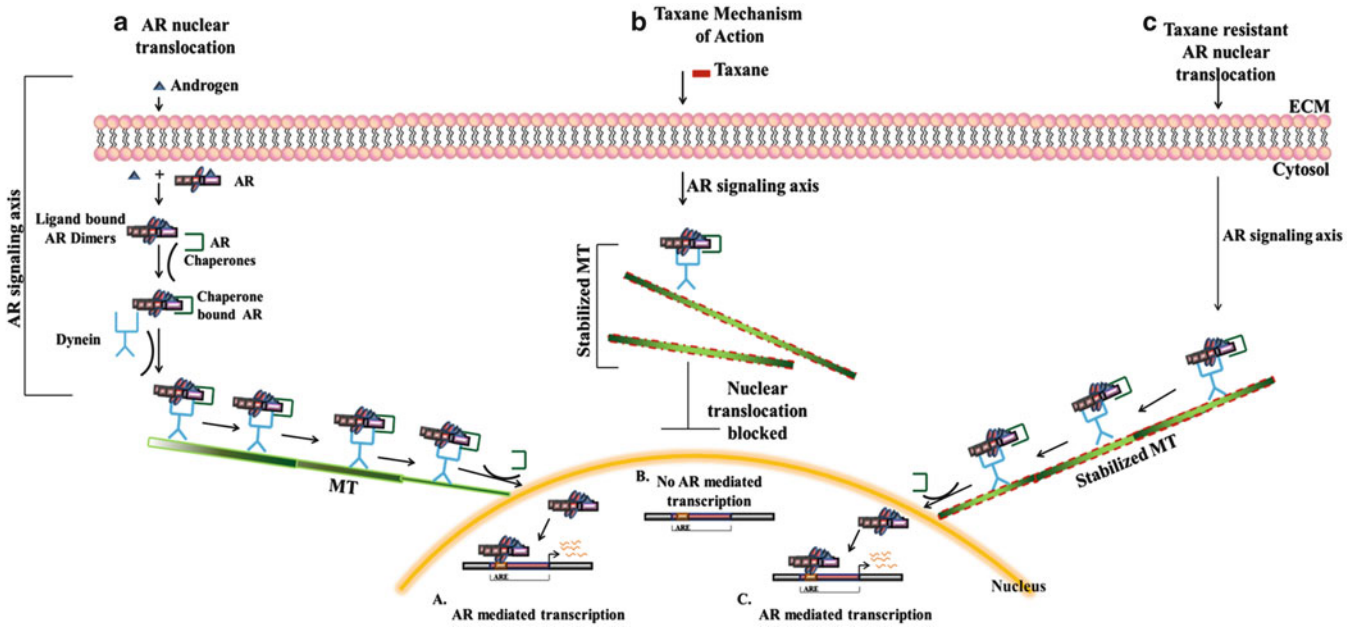


**Fig. 15.2** Targeting AR translocation to the nucleus in prostate cancer. (**a**) After binding of cognate ligand, molecular handling by super-chaperone complexes, dimerization, phosphorylation, and association with dynein motor proteins onto microtubules (MT), AR translocates to the nucleus to mediate transcription of Androgen-responsive elements (ARE). (**b**) Taxane chemotherapeutics stabilize the interaction of β-tubulin subunits within the proto-filaments of the microtubule preventing the de-polymerization of the structure resulting in G2M arrest, apoptosis, and increased accumulation of AR in the cytoplasm. (**c**) With regard to Docetaxel (Taxane) resistant—CRPC, largely unknown mechanisms are employed which facilitate translocation of AR to the nucleus despite microtubule stabilization

translocation of the AR to the nucleus upon activation as well as the microtubule-associated motor protein, dynein [25, 40, 42]. Many of these important protein interactions have been mechanistically involved with the AR translocation to the nucleus; the molecular chain of events, however, responsible for this movement is not completely defined. The AR undergoes numerous interactions with coregulatory proteins that have been extensively reviewed [43]. The intramolecular interactions within the AR and intermolecular protein:protein interactions between AR subunits in a homo-dimeric complex are important to the activation and nuclear translocation [21]. Upon ligand binding, the D-box of the DBD interacts with the TAU-1 domain of the NTD, an N-terminal to C-terminal protein domain interaction that is initiated in the cytoplasm [21, 44]. This interaction between the D-box and the NTD of the AR is essential for the transition from intramolecular domain associations to intermolecular homo-dimerization, a process that occurs independent of DNA binding [44]. In fact, upon DNA binding the AR N/C interaction is lost, facilitating coregulator interaction with the AR and AR interaction with the major groove of DNA double helix [21, 45].

### *15.1.4 Pathways Bypassing AR*

During the last decade there has been a plethora of mechanisms pursued as potentially engaged by prostate tumors to bypass or perpetuate AR signaling toward CRPC [5, 6, 12]. These mechanisms, comprehensively considered, lend credence to the need for personalized medicine and continued research in the landscape of alternate pathways to CRPC [5, 6, 12]. Alterations to the regulation, structure, and posttranslational modifications of the AR itself can perpetuate continued androgen signaling. The mRNA and protein expression of the AR is commonly overexpressed in CRPC [4]. However, the structure of the AR can be altered as well. Point mutations increasing the affinity of the AR for ligand have been identified causing the pathway to become hypersensitive [46]. Promiscuous mutations cause binding flexibility in the LBD allowing the AR to become activated by adrenal androgens, androgenic metabolites, and even some anti-androgen therapeutics such as flutamide and bicalutamide have been described [5, 47–49]. Moreover, over twenty splicing variants of AR, some lacking LBD, and therefore constitutively active have been identified and associated with progression of CRPC and metastasis [17, 50–54]. AR can be activated independent of ligand interactions by aberrant signaling pathways causing the activation of the protein and homo-dimerization by growth factors, receptor tyrosine kinases, and the Akt pathway via loss of PTEN [5, 55–57]. Recent evidence has elucidated the potential for prostate cancer cells to synthesize their own androgens “hijacking” adrenal synthesis enzymes [6, 58–60]. The entire AR signaling axis can even be bypassed by overexpression of the apoptosis blocking protein Bcl2, frequently found overexpressed in prostatic intraepithelial neoplasia (PIN) [61, 62].

## 15.2 The Therapeutic Impact of Impairing Androgen Signaling in Prostate Cancer

Taxanes were first identified in the bark of yew trees, and their cytotoxic effects against cancer cells were pursued with zest [63]. The underlying mechanism of action behind such drugs as Taxotere (Docetaxel) and Paclitaxel has been historically considered the binding to microtubules, leading to stabilization or destabilization of microtubules and ultimately mitotic catastrophy [63]. Specifically, taxanes bind two subunits of β tubulin, stabilizing the interaction and preventing depolymerization of the protofilament within microtubule complex [64]. This stabilizing interaction ultimately results in G2M arrest and apoptosis [63, 64]. Taxanes are able to counteract the effects to some extent of Bcl-2 protein overexpression. Bcl-2 is a pro-survival protein and important effector of apoptosis frequently overexpressed in prostate cancer [65, 66]. Taxane treatment counteracts the anti-apoptotic effect of Bcl-2, one of significant modes of overcoming androgen dependence and progression to CRPC [67, 68]. The clinical evidence delivered much therapeutic promise. In 2004 the findings of two landmark clinical trials, TAX327 and SWOG (Southwest Oncology Group) 9916, demonstrated a benefit of Docetaxel-based treatment regimen in patients experiencing CRPC [69]. Docetaxel treatment produced benefits in palliative relief and overall survival and these results have persisted with extended follow ups [70, 71]. Since the approval of Docetaxel from the US Food and Drug Administration, this clinical use as chemotherapeutic drug has generated an additional, but sometimes modest, survival benefit to patients progressed to CRPC. Work from our lab revealed that in addition to these aforementioned effects in stabilizing microtubules and inducing G2M arrest, taxanes are particularly poignant in prostate cancer, because they possess the ability to block translocation of the AR to the nucleus and inhibit AR-driven gene transcription (Fig. 15.2b) [72]. Using clinical specimens from patients treated with Docetaxel versus untreated, immunohistochemical analysis of tissue microarrays strikingly revealed significantly diminished AR nuclear localization in the Docetaxel-treated patients [72]. Significantly enough these translational studies revealed that while AR protein expression levels were not affected by the taxane treatment, the nuclear transport and localization of AR was markedly reduced in response to Docetaxel (38% decrease), evidence that provided a intriguing new insight into the mechanisms of action of microtubule-targeting agents, as well as resistance in CRPC. Further investigation into the domain of the AR responsible for mediating the interaction with the taxane target tubulin revealed that the NTD negotiated this association [72]. These important mechanistic insights serve as a roadmap to understanding why Taxane chemotherapeutic served as our only clinically relevant treatment for CRPC for nearly a decade and guide our pursuit of future therapeutics (Fig. 15.2a). Taxane treatment ultimately fails, however, as the majority of patients develop resistance. The mechanisms driving prostate cancer progression after Docetaxel treatment are far from completely understood and this has been the focus of pursuit by investigative efforts from our group and others (Fig. 15.2c). A potential mechanism of resistance can be attributed to the adenosine triphosphate-dependent drug efflux

pump P-glycoprotein-1. More recent evidence supports that Docetaxel has a high affinity for this pump and that an increase in expression of the efflux pump itself is observed over the course of prostate cancer progression [73, 74]. Exacerbating the insult of progression to CRPC, biochemical recurrence is associated with other clinical manifestations. Bone, brain, and lymph node metastasis as well as increasing amounts of pain secondary to the metastatic lesions are common in CRPC patients [73]. Emergence of new therapeutic interventions such as Cabazitaxel, Abiraterone acetate, and MDV 3100 have demonstrated additional survival benefits to the Docetaxel-resistant metastatic CRPC patients (DR-CRPC) [75], with emerging strategic combinations of microtubule-targeting taxane-based drugs with the androgen signaling agents for effective therapeutic outcomes in DR-CRPC patients.

### *15.2.1 Cabazitaxel*

Cabazitaxel is a novel, next-generation Taxane chemotherapeutic drug that has been shown to be effective in the DR-CRPC landscape [75, 76]. It has been shown to be highly cytotoxic and have a low affinity for the adenosine triphosphate-dependent drug efflux pump: P-glycoprotein 1, known to confer chemotherapeutic resistance [77]. Cabazitaxel was shown in a multicenter, randomized, phase 3 clinical trial (Treatment of Hormone-Refractory Metastatic Prostate Cancer (TROPIC)) to result in a significant increase in overall survival [76, 78]. Tumor response, biochemical recurrence, and tumor progression were all favored by Cabazitaxel treatment and consequently the drug was approved by the US Food and Drug Administration for use in DR-CRPC patients [75, 76, 78]. In addition to imparting overall survival benefits to chemotherapy naïve patients, the findings associated with Cabazitaxel are its ability to confer additional overall survival benefits in patients with biochemical recurrence on ADT, Docetaxel chemotherapy, or both [73, 75].

### *15.2.2 Abiraterone*

Abiraterone Acetate (AA) is a novel anti-androgen therapy designed to target the adrenal androgen-mediated signaling axis by blocking the synthesis of adrenal products which serve as precursors for testosterone and DHT synthesis [75, 77, 79]. AA acts as a pregnenolone analog, inhibiting the rate limiting enzyme, cytochrome P450 (CYP17A1), further inhibiting androgen biosynthesis [75, 77]. AA inhibits both the 17α-hydroxylase and 17,20 functions of CYP17A1 [77]. The efficacy of AA was demonstrated in the COU-AA-301 trial, confirming that AA imparted additional survival benefit compared to DR-CRPC men treated with placebo and prednisone. In addition to overall survival increase, benefits were seen with regard to time to disease progression, biochemical recurrence, and tumor burden [75, 77, 80]. These results highlight the importance of targeting the AR signaling axis in conferring survival benefits in DR-CRPC patients.

### *15.2.3 MDV3100*

The translational significance of AR targeting in DR-CRPC gains further support by the development of the direct AR antagonist, MDV3100 [77, 81]. This androgen-targeting agent is a diarylthiohydantoin member of the family of AR antagonists rationally designed from the crystal structure of the AR bound to its ligand [81]. MDV3100 is effective in the context of AR overexpression, in addition to inhibiting AR nuclear translocation, preventing binding of the AR to DNA, blocking recruitment of co-activators to AR target genes, and inducing apoptosis [81–83]. MDV3100 has been shown to be efficacious in improving survival in prostate cancer patients previously treated with ADT (CRPC), in Docetaxel-resistant patients, as well as in DR-CRPC patients [77, 84, 85]. Recent reports indicate that MDV3100 inhibits translocation of constitutively active AR splice variants lacking portions or all of the LBD, implicating MDV3100 in circumventing progression to CRPC [86].

## 15.3 AR Navigates Emergence of CRPC

With the outlook of the therapeutic horizon evolving and improving rapidly, prostate cancer is still treated as a single disease [87]. Other major human malignancies (breast, non-small cell lung cancer, colon cancer) are classified based on molecular features, for example, breast cancer is subclassified based on the presence of estrogen receptor, progesterone receptor, Her2/neu, and BRCA-1 [87, 88]. This provided effective therapeutic targets for successful drug development for specific molecular subtypes [87, 88]. Considering that the therapeutic repertoire in androgen-dependent prostate cancer is being hijacked by the extensive tumor heterogeneity of the disease and the stromal–epithelial interactions, it is of paramount importance to identify subpopulations in which the AR axis that can be actively targeted via optimized therapeutic strategies and carefully designed treatment sequencing. One must recognize the functional promiscuity of AR in targeting repressors and activators during prostate cancer progression. Until recently, investigation into the identity and functional contribution of AR-targeted genes was conducted in a hypothesis-driven, meticulous, and linear manner, building pillars to base future pursuits of drug and biomarker discovery in prostate cancer. In 2012, the challenge of wading through the tremendous output of data from bioinformatics lies towards development of molecular signatures and target identification in advanced disease patterns remains. Application of combination techniques of proteomic analyses coupled with microassay analysis of gene expression facilitated identification of proteins in prostate cancer signaling landscape, which are significantly upregulated on both the protein and gene level by AR signaling [89–91]. Such “topologically significant” nodes allow interpretation of the pathways which are affected most by androgen signaling in prostate cancer [91]. Functional validation of critical signaling pathways regulated by the androgen axis and AR activity provides valuable opportunities for exploitation

at the mechanistic and translational level in the management of prostate cancer [91]. The main pathways that have been interrogated in recent years include AR nuclear signaling, AR crosstalk with growth factor signaling, androgenic regulation of epithelial to mesenchymal transition (EMT), extracellular matrix (ECM) adhesion and integrin priming, and regulation of angiogenesis by androgens. Our efforts focus on dissecting the role of AR signaling and its crosstalk with critical signaling effectors of apoptosis in controlling the process epithelial to mesenchymal transition (EMT) towards progression to metastatic CRPC.

### *15.3.1 Epithelial to Mesenchymal Transition : "Moving and Shaping" the Metastatic Journey*

The biological process of EMT was first described in the context of normal organ development [92]. Reactivation of EMT quickly became a hallmark of metastatic tumors. EMT is observed extensively in nonpathological conditions such as mechanisms of development including gastrulation and neural crest development in which epithelial cells must de-differentiate to a mesenchymal form, migrate, and redifferentiate into a new structure or organization [93]. EMT can be classified into three distinct subtypes based on the biological setting hosting its manifestation [94]. Type 1 EMTs are associated with embryonic implantation and gastrulation facilitating the stratification of the germinal layers [94]. Unlike Type 1, Type 2 EMTs are associated with wound healing, tissue regeneration, and organ fibrosis [94]. Type 2 EMTs are characteristically induced by inflammatory signaling, either as a response to injury-induced inflammation as seen in wound healing or ongoing inflammation of certain organs resulting in fibrosis [94]. Type 3 EMTs occur in neoplastic cells that undergo a manifold of genetic or epigenetic changes resulting in localized tumor cell proliferation [94]. The Type 3 EMT is responsible for changes that facilitate tumor cell invasion and metastasis [94].

The significance of EMT in cancer emerges as tumor cells must physically detach from their immediate primary tumor, invade into the surrounding microenvironment, intravasate into the vasculature, endure the turbulence of circulation in the blood stream or lymphatics, and extravasate from the circulatory system at a secondary site [93, 94]. Each step required for execution of EMT requires a vast number of molecular events [7, 93, 94]. Epithelial cells must begin their transition to a mesenchymal phenotype by disrupting their intercellular adhesive contacts [95], a phenomenon manifested by formation of apical constrictions and disorganization of the basal cytoskeleton resulting in detachment and loss of apical-basal organization [95–98]. The phenotype of detached cells becomes spindle-like and exhibits a front-rear polarity conferring enhanced motility and invasive shape [7, 99–101]. Further breakdown of the basal membrane and extracellular matrix (ECM) must occur for migration to ensue and this is accomplished via secretion of proteases and acquisition of migratory/invasive properties [95, 102]. Key mechanisms activating

EMT include TGF-β and receptor tyrosine kinase (RTK)/Ras signaling in addition to the well-known canonical Wnt-/B-catenin, Notch, Hedgehog, and NFκB-dependent pathways [103]. Cadherin switching is an important milestone and regulatory step in EMT development regulated by transcriptional regulators including Snail and Twist [103].

Recent investigations from this laboratory strongly implicate the androgen signaling axis as an active participant in the progression of the mechanistic sequelae of EMT [7, 104]. In what is seemingly becoming a controversial twist, androgens can induce EMT-associated changes in prostate cancer cells, regardless of their androgen sensitivity and AR status, conferring enhanced invasive and motile capacity therein as well as modulating known EMT transcriptional regulator, Snail [104]. Moreover, an inverse relationship between AR expression level and extent of androgen-induced EMT induction was established suggesting that very low level AR expression such as that seen immediately after beginning ADT may be contributing to metastatic spread of prostate cancer tumor cells [104]. Others have recently shown that prostate cancer cells expressing AR in androgen-deprived conditions undergo an EMT, indicated by decreased E-cadherin and increased N-cadherin and vimentin [105]. Confirmed previously, increased N-cadherin expression and metastasis was seen in LNCaP xenografts and human clinical specimens [106]. The β-catenin/Wnt-dependent signaling pathway is already a well-known accomplice in progression to EMT and metastasis, but the implication of this pathway under androgenic drive is essential to understanding prostate-specific EMT. Recent exciting insights into EMT regulation in prostate cancer implicates β-catenin in the androgen-modulated EMT effect [104].

### *15.3.2 Cadherin Switching and the Master Regulators of EMT*

The physiological phenomenon known as “cadherin switching” has been accepted as a hallmark of EMT. E-cadherin or epithelial cadherin is an important cell adhesion protein mediating intercellular contacts and facilitating maintenance of tissue architecture. This is a protein essential to formation of adherens junctions which in combination with tight junctions mediate intercellular adhesion [93]. E-cadherin is structurally characterized as a single pass transmembrane glycoprotein which forms calcium-dependent homotypic interactions with E-cadherin on cell neighbors [93]. These essential interactions are anchored to the cytoskeleton by interactions with microfilaments composed of actin and mediated by β-catenin and α-catenin [93]. E-cadherin expression can be lost, nonpolar, or cytoplasmic expressed or alternatively transcriptional repression of E-cadherin can occur by diverse mechanisms engaging AR and its transcriptional coregulators [107]. Loss of E-cadherin expression results in loss of normal cell–cell interactions and facilitates progression of EMT and leads to metastasis [103, 108, 109]. Upon E-cadherin loss, N-cadherin expression is enhanced to promote the mesenchymal cell phenotype. N-cadherin or Neural-cadherin is a mesenchymal cell association protein that allows transient

cell–cell contacts typically expressed in cell types including smooth muscle, myofibroblasts, endothelial cells, neurons, and neoplastic cells [7, 93, 110]. The cell types usually expressing N-cadherin are also typical components of the reactive stroma composing the microenvironment of the prostate cancer tumor cell [93]. The interactive mode employed by N-cadherin is not unlike that used by E-cadherin; this single span transmembrane protein engages in homotypic interactions with N-cadherin on neighboring cells [93]. Loss of E-cadherin has been associated with increasing Gleason grade in prostate cancer and the concept of cadherin switching is traditionally considered as predictive of metastatic development [111, 112].

E-cadherin expression is repressed by the zinc finger transcription factor Snail (SNAI1) [95]. Snail not only gained notoriety as a master regulator of EMT induction but also plays an essential role in embryonic development and cell survival [95]. Snail employs a mechanism of action whereby the transcription factor binds to the E-box of the E-cadherin promoter and silences gene expression promoting a mesenchymal phenotype (Fig. 15.3a) [7]. Interestingly enough, Snail is capable of modulating expression of proteins involved in tight junctions, including claudins, occludins, mucin-1, and cytokeratin 18 [113]. Further fulfilling its infamy of "master regulator," Snail increases expression of mesenchymal phenotype-associated markers and proteins associated with invasive capacity: vimentin, fibronectin, metalloproteinase-2, -9, ZEB1, and LEF-1 [113]. To dissect the functional contribution of Snail to prostate EMT, one must focus on its crosstalk with the AR signaling axis. Indeed, AR may function in an analogous manner to Snail, thereby repressing the expression of E-cadherin and promoting EMT by itself (Fig. 15.3a) [114]. Work from this laboratory has demonstrated that in androgen responsive, TGF-β responsive, prostate cancer cell line, expression of Snail is significantly increased by exposure to DHT alone or in combination with TGF-β [104]. These observations support a functional involvement of the AR signaling navigated by Snail in acquisition of EMT characteristics of prostate tumor cells towards metastatic progression. Recent high throughput DNA analyses have furthered this investigation at the molecular level by identifying an ARE/ARG in the promoter region of Snail2 (slug), suggesting the direct modulation of Snail2 by AR [89].

A plethora of transcription factors impact expression and transcriptional activation of genes controlling the EMT phenomenon. Identification of those which specifically interact with the AR signaling axis provides a unique molecular platform begging exploration in prostate cancer (Fig. 15.3, panel A). Zeb1 (ZFHX1a gene) and Zeb2 (ZFHX1b) are closely related transcription factors whose activity has been strongly implicated in EMT [115]. These transcription factors are characterized by separated clusters of Zinc finger domain (7 total) which recognize the CAGGTA/G E-box promoter element [116]. ZEB1 modulates diverse-function genes, it significantly contributes to EMT by repression of E-cadherin expression, genes encoding basement membrane components, and regulators of cell polarity; other affects run the spectrum from tumor suppression to anti-adipose accumulation in vivo [7, 115–118]. Progression to metastasis is an event mediated by ZEB1, in addition to its important involvement in facilitating transendothelial migration [119, 120]. Clearly, ZEB1 plays an important role in orchestrating complex physiological

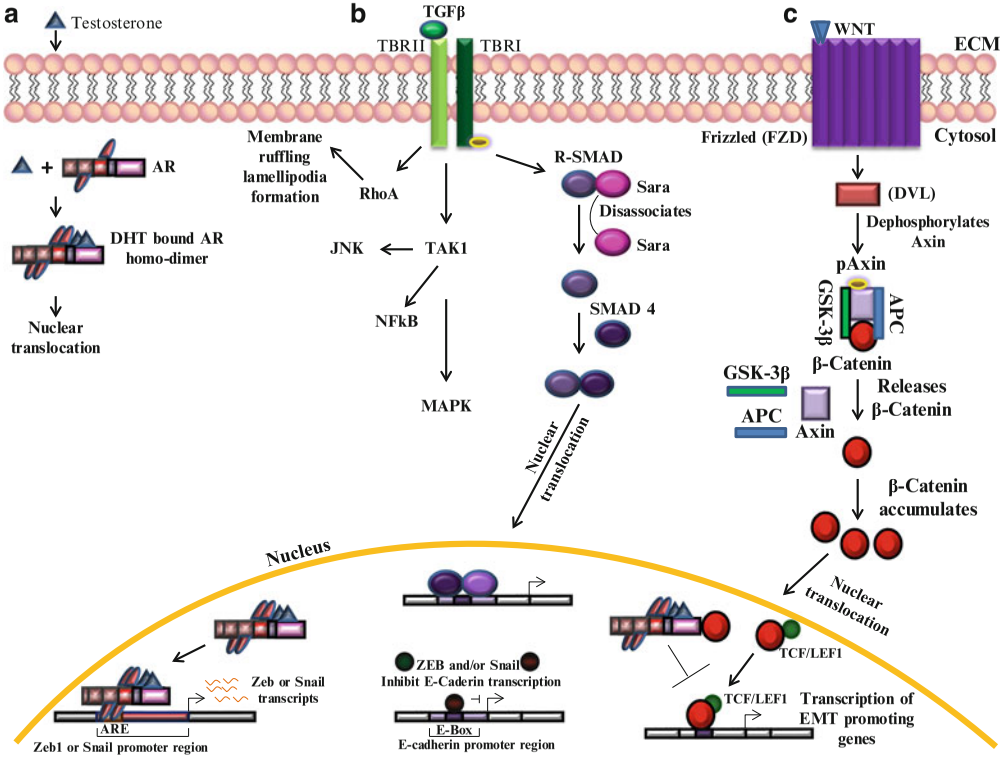


**Fig. 15.3** Role of AR in prostate cancer and EMT (**a**) Cadherin switching, Zeb1 feedback loop, and expression of Snail2 are modulated by the crosstalk with Androgen Receptor (AR). (**b**) TGFβ signaling can induce EMT-associated changes in Prostate cancer via Smad-dependent and -independent pathways and their crosstalk with the AR. (**c**) Wnt/β-catenin signaling engages in direct crosstalk with AR. AR and TCF-LEF1 transcription factors compete for β-catenin acting as a coactivator of transcription promoting EMT

processes such as, but certainly not limited to EMT. Recent work has revealed a bidirectional negative feedback loop between AR and Zeb1 that has implicated ADT in inducing EMT signatures in prostate cancer cells and human tissues [105]. Without androgenic stimulation, AR expression is diminished during early ADT, but in the absence of AR, Zeb1 expression cannot be inhibited and thereby becomes increased. With increased Zeb1 transcription factor expression, EMT promotion becomes transiently facilitated as a result of ADT leading to metastasis [105]. ZEB2 (SIP1) was originally described within the context of TGF-β signaling [116]. ZEB2 interacts with SMADs and promotes tumorigenic invasion and downregulates E-cadherin expression [121]. Expression of ZEB transcription factor has been correlated with progression to malignant carcinoma in various cancer types (including prostate), and that expression could be induced by both estrogen and progesterone [115, 122, 123]. Moreover, activated AR signaling induces ZEB1 in human prostate cancer cells and in triple negative breast cancer [115, 124]. Identification of AREs in the promoter of the ZEB1 gene confirms that expression of this dynamic regulator is controlled by AR signaling [115].

Identification of Enhancer of Zeste Homolog 2 (EZH2) from Chinnaiyan's research group has been significant in understanding the role of epigenetic modifications in prostate cancer progression to EMT as well as in renal and breast cancer [125]. EZH2 expression is associated with cancer metastases and is markedly localized to tumors with poor prognosis in combination with depressed E-cadherin, both markers associated with poor disease-free survival [126, 127]. EZH2 functions as a histone lysine methyltransferase and its overexpression has been detected in mCRPC [126, 128]. Both EZH2 mRNA and protein levels are significantly elevated in prostate cancer compared to benign prostate hyperplasia (BPH) or human high grade PIN (HGPIN) [129]; however, in 2012 the precise role of EZH2 in prostate cancer progression is not fully understood. EZH2 targets NKX3.1 inducing repression of the homeobox gene, phenomenon observed in up to 85% of HGPIN lesions and prostatic adenocarcinomas [128]. Furthermore, EZH2 targets other genes undeniably linked to EMT, including E-cadherin and DAB2IP [130, 131]. The fusions of TMPRSS2, an androgen-regulated gene, and the oncogenic ETS transcription factor ERG place ERG under androgenic drive [132–137]. ERG activates EZH2 transcription allowing the methyltransferase to induce its repressive epigenetic agenda [138]. The neuronal chemorepellant and tumor supressor gene SLIT2 has also been linked to EZH2 [139]. EZH2 targets SLIT2 and inhibits its expression under the drive of AR-dependent TMPRSS2-ERG fusion [139]. SLIT2 is downregulated in a majority of prostate cancers and low levels of SLIT2 are associated with agressive disease [139]. ERG overexpression interferes with AR binding to ARE/ARGs, thus providing an additional layer of selection pressure to AR overexpression and mutation, driving progression to CRPC [138].

### *15.3.3 The Wnt Signaling and AR: Up, Close, and Intimate Interactions in Metastasis*

The Wnt signaling pathway plays an important role in embryonic development and differentiation and is a highly conserved pathway among organisms. The deregulation of Wnt signaling is associated with tumorigenesis and EMT [140]. In prostate cancer cells, this pathway can engage in direct crosstalk with AR, with the central protagonist being β-catenin [140]. This molecule is located in distinct cellular locations: sequestered at the adherens junctions in concert with E-cadherin, in the cytoplasm, or in the nucleus [140]. Wnt ligand binds with the seven pass transmembrane receptors: FZD (Frizzled) at the plasma membrane interface with the extracellular environment (Fig. 15.3c). FZD receptors transduce a signal to Disheveled (Dvl) and Dvl subsequently dephosphorylates an associated protein Axin. Axin functions as a signaling scaffold protein coordinating the interactions of Adenomatous Polyposis Coli (APC), glycogen synthase kinase 3β (GSK3β), β-catenin, and Conductin (Fig. 15.3c). The coordination of these proteins by Axin facilitates the phosphorylation of β-catenin and APC by GSK3β. The dephosphorylation of Axin diminishes its capacity to coordinate β-catenin in complex with GSK3β causing decreased phosphorylation of β-catenin (Fig. 15.3c). The phosphorylation of β-catenin mediates subsequent ubiquitylation and degradation, but without phosphorylation by GSK3β, β-catenin accumulates in the cytoplasm. Accumulation of β-catenin results in nuclear translocation of the protein and interaction with lymphoid enhancer binding factor 1/T-cell factor (LEF1/TCF) transcription factors and transcription of β-catenin target genes, such as c-MYC, c-Jun and fra-1, in addition to EMT important urokinase type plasminogen activator receptor (uPAR), matrix metalloproteinases and cyclin D1 [140–144]. AR and β-catenin interact directly with one another (Fig. 15.3c), impacting the EMT outcome [145]. In vitro androgen-stimulated transcriptional responses are enhanced by functional involvement of Wnt signaling, consequently opposing the effects of antagonistic anti-androgenic treatment (bicalutamide) [146]. Together, these data reveal that β-catenin acts as a coactivator of AR gene target transcription and thereby associated with progression to CRPC under conditions of overexpression [140]. Furthermore, cognate ligand-induced AR signaling possesses the capacity to attenuate Wnt signaling and TCF/LEF1-dependent gene transcription.

The pioneering work of Arul Chinnaiyan's discovery of TMPRSS2: ERG gene fusion has been paramount in advancing our molecular understanding of prostate cancer. These gene fusions result in androgen-driven expression of the transcription factor ERG. The consequences of these fusions on cell fate are diverse and intriguing, but an important observation that ERG fusion-positive tumors and Frizzled4 (Fzd4: 7 pass transmembrane receptor of Wnt signaling pathway) cooverexpression were consistently identified in clinical prostate cancer [147]. Moreover, overexpression of ERG induced the EMT phenomenon in androgen-responsive cell lines (VCaP), including repression of E-cadherin and induction of N-cadherin [147]. The effects of

ERG overexpression could be abrogated by the modulation of FZD4, demonstrating that FZD4 was both necessary and sufficient to mediate the oncogenic effects of ERG overexpression and defining the impact of direct crosstalk of AR-driven ERG overexpression with the Wnt signaling on prostate cancer EMT [147].

### *15.3.4 The Star Power of AR Partner: Transforming Growth Factor-β*

EMT induction is characteristically associated with Transforming Growth Factor-β (TGF-β) signaling and its cooperation with oncogenic Ras or receptor tyrosine kinases (RTKs) is commonly associated with growth factor receptor to induce EMTs and subsequent metastasis [103, 148]. TGF-β is a ubiquitously expressed growth inhibitory cytokine [149–152]. TGF-β contributes to tissue and organ homeostasis by inducing a system of proliferative versus apoptotic balances [149, 150]. TGF-β signaling is critical in diverse cell types by impacting important features of cellular behavior including migration, adhesion, alterations to the extracellular environment, apoptosis, and promoting formation of osteoblastic metastatic lesions [149, 151, 153]. The TGF-β pathway traditionally engages signaling involving the SMAD proteins (Fig. 15.3b) [149, 151, 153–155]. TGF-β signaling is mediated by the Serine/Threonine Kinase domains of the TGFβRI and TGFβRII receptors and the formation of hetero-tetrameric complexes (Fig. 15.3b) [155]. Binding to TGF-β causes TβRII receptor to phosphorylate the regulatory GS domain of TβRI, initiating a downstream signaling cascade mediated by SMAD proteins [153, 155]. TβRI selectively phosphorylates regulatory SMADs (R-SMADs) at the SSXS motif on the carboxyl terminus of the SMAD [149, 153]. The R-SMADs, SMAD2, and SMAD3, activated by the TβRI [149], are sequestered in the cytoplasm via their interactions with SMAD anchor for receptor activation (SARA) [149]. Once activated by TβRI, R-SMADs lose affinity for SARA and become free to interact with SMAD4 [149]. SMAD4 is essential for formation of SMAD-mediated transcriptional complexes, components of which are continuously shuttled between the cytoplasm and nucleus via nuclear pores [149, 150, 153]. The SMAD complex dictates transcriptional activation, via recruitment of coactivators such as p300, CBP, or SMIF. Conversely, for transcriptional repression, the SMAD complex recruits p107, SKI, SNON, TGIF, EVI1, and ZEB2 (SIP1) [149, 156]. Expression of these coregulators is dependent on cell type, developmental stage, and microenvironment-hosted crosstalk facilitating a broad cellular response repertoire [149]. Defective/lost TGF-β receptors and SMAD mutations are not directly responsible for the effects of EMT in cancer progression [151]; rather, loss of apoptotic response occurs in cancer cells despite production of TGF-β ligand [157].

Smad-independent signaling proceeds via MAPK pathways, involving activation of Erk, JNK, and p38 MAPK signaling pathways by TGF-β. Oncogenic Ras contributes to the activation of Erk/MAPK signaling, in a context-dependent manner.

TGF-β activates TGF-β-activated Kinase 1 (TAK1), a MAPK kinase kinase family member (MAPKKK), leading to activation of JNK and p38 MAPK. TAK1 can also phosphorylate IκB, thereby activating NFκB signaling [153]. Direct mechanistic link of EMT to cancer progression is mediated by the effect TGF-β signaling on activation of Rho A (Fig. 15.3b). Rho A and $p160^{ROCK}$ (effector kinase) activation in conjunction with activation of Cdc24, p38, MAPK, and Smad signaling, correlate with stress fiber formation, membrane ruffling, lamellipodia formation and the physical mechanisms of EMT [153]. Rho A is upregulated in prostate cancer cells as compared to the benign prostate and this elevated expression is linked to aggressive disease and diminished disease-free survival in patients after radical prostatectomy [158]. In fact, Rho A activation by TGF-β is similarly activated by action of AR on Serum Response Factor target genes further corroborating the crosstalk between TGF-β and AR [158], in the context of EMT cellular "landscaping." Elevated TGF-β correlates with increasing tumor grade in numerous human malignancies, including prostate cancer [159–162]. And furthermore, overexpression of TGF-β ligand is detected in advanced prostate cancer [150, 153, 154]. TGF-β ligand binds to and induces phosphorylation of TβRI by TβRII resulting in SMAD signaling in prostate cancer cells. SMADs 3 and 4 serve as transcriptional coregulators of AR target genes and conversely, ligand-bound AR transcriptionally modulates SMAD3 in prostate cancer [163, 164]. SMAD4 (alone or in conjunction with SMAD3) can coregulate AR transactivation via binding to the DBD and LBD domains of the steroid receptor thereby modulating its DHT-induced activity [151, 152]. SMAD3 can bind AR as well, but this interaction is mediated by the NTD [165]. In a mechanistic twist, AR overexpression enables prostate cancer cells to overcome the growth inhibitory effects of TGF-β under DHT deprived conditions [164]. Moreover, expression of SMAD3 enhances AR-mediated transactivation, whilst co-overexpression of SMAD3 and 4 repressed AR transactivation [166]. Our group has pursued the impact of dysfunctional TGF-β signaling in functional interaction with AR signaling in vitro and in vivo models of prostate cancer. The TGF-β/Smad signaling pathway elicits a downstream activation in Snail thereby repressing E-cadherin expression in a number of cancer cell types [167, 168]. In LNCaP TβRII human prostate cancer cells, DHT (alone or in combination with TGF-β) significantly induced Snail expression [152], pointing to a dynamic crosstalk between the AR and TGF-β pathways in control of EMT. Recent studies identified a role for Hexim-1 in mediating such a crosstalk between AR and TGF-β in prostate cancer progression. Hexim-1 is an inhibitor of cyclin-dependent kinase 9 (Cdk9) of transcription elongation factor (pTEFb) complex, which is upregulated and translocated to the cytoplasm during tumor progression [169]. Cdk9 interacts with AR and phosphorylates the AR at serine 81 [170] and transcriptionally programs Smads 1 and 3 via phosphorylation of linker region [171, 172]. Such refined mechanistic control of Hexim-1 expression supports its role as a converging modifier of activity for AR and TGF-β signaling crosstalk towards EMT [169].

### *15.3.5 Notch (Hi)-Jagged AR in Metastatic CRPC*

Notch signaling is fundamentally significant in development and tissue homeostasis. Notch signaling facilitates an important mode of cell–cell communication. Notch proteins (1–4) are type I, single pass transmembrane receptors [173]. The extracellular domain of the Notch protein participates in ligand binding and is composed of a variable number of epidermal growth factor (EGF)-like domains (essential for ligand binding) and three cysteine-rich LIN12/Notch repeats (LNR) (ensure signaling only transduced in the presence of ligand) [173, 174]. The intracellular domains of the Notch receptor include RAM23 domain, six ankyrin/cdc10 repeats, two nuclear localization signals, transcriptional activation domain, and a PEST sequence [173]. The ligands recognized by the Notch receptor are Delta 1,3, and 4 as well as Jagged 1 and 2; these ligands are membrane bound and composed of an amino-terminal domain known as DSL and variable number of EGF-like repeats [175–179]. The Jagged ligands possess a cysteine-rich (CR) domain [173] and ligand receptor-initiated signaling cascade results in cleavage of the Notch receptor and ultimately translocation of the Notch intracellular domain (NICD) to the nucleus. There is growing evidence identifying Notch signaling as characteristic component of EMT. As discussed above, Snail1 is a transcription factor responsible for repressing E-cadherin transcription, with Notch1 activation upstream of Snail1 [173]. This observation has been further validated in the immortalized porcine aortic endothelial cell line, whereby overexpression of NICD induced an EMT via activation of Snail1 and subsequent repression of E-cadherin [180]. The correlation between expression of Notch ligand, Jagged1, and high grade and metastatic prostate cancer compared to localized prostate cancer [181] is of major translational value as Jagged1 may serve as an independent prognostic indicator of prostate cancer recurrence and progression [181], potentially driven by a link with androgenic signaling [181, 182]. Notch1 signaling is associated with osteoblast differentiation and Notch1 expression is markedly elevated in osteoblast skeletal-derived prostate cancer cells [182], validating the role of this EMT promoting pathway in prostate cancer metastasis to the bone.

## 15.4 Conclusions

The AR acts as a cornerstone of the aberrant signaling mechanisms associated with prostate cancer. Intense pursuit of the anomalous pathways via which androgen signaling is perpetuated in CRPC has identified “diverting” mechanisms that still impact tumor progression and therapeutic response in patients. The androgenic signaling axis can become altered in a number of ways: point mutations, truncations, variant expression of the AR itself, posttranslational modifications deviating from the normal signaling by RTKs and downstream of growth factor signaling pathways, and the ability of prostate cancer cells to commandeer androgen synthesis in the face of

ADT. In close exchanges directed by AR, EMT can be reactivated in prostate cancer epithelial cells by the key signaling controllers of prostate growth and their functional interactions (TGF-β and androgen axis/AR), towards metastatic behavior. Thus unfolding the key players in EMT-activating signaling pathways engaged in crosstalk with AR signaling is paramount to recognizing potential therapeutic targets for CRPC. The landscape becomes progressively defined: Loss of E-cadherin expression and induction of N-cadherin are regulated by key transcription factors, Snail and Slug, which transcriptionally repress E-cadherin via the androgenic signaling axis. In a more prominent role, Zeb1 directly recruited by the AR signaling, engages in a bidirectional negative feedback loop, highlighted in ADT. In the absence/repression of AR (as in early ADT), Zeb1 is overexpressed facilitating the mechanistic events leading to EMT. Also impacted by the androgenic status, β-catenin, accumulates in the cytoplasm and translocates to the nucleus, to induce transcription of LEF1/TCF genes and others which mediate EMT processes. Interestingly, β- catenin can also interact with AR directly and act as a transcriptional coactivator of AR driving not only EMT but also progression to CRPC.

In a less direct crosstalk event, AR drives overexpression of TMPRSS2: ERG genes fusion products resulting in highly overexpressed transcription factor ERG (ETS family of transcription factors). At the clinical setting this overexpression of ERG and FZD is associated with prostate tumor progression. At the cellular level, elevated ERG induces EMT, an effect that can be abrogated with silencing of FZD, thus implicating the Wnt signaling pathway in driving the effects of ERG gene fusions. In view of the documented significant association between elevated TGF-β ligand and increasing prostate tumor grade, a dynamic crosstalk of TGF-β signaling with AR, in controlling EMT during progression to metastasis, becomes central to the cellular landscape of CRPC development. Moreover, Smads3 and 4 interact directly with AR to reciprocally modulate both target gene transcriptional activation and expression. Androgen treatment of human prostate cancer cells significantly upregulates Snail and promotes the TGF-β and AR interaction in controlling EMT. Notch signaling is essential to intercellular communication, and expression of Jagged1 (main effector) emerges as a potential independent prognostic indicator of prostate cancer recurrence and progression, since expression of Jagged1 correlates with high grade and metastatic prostate cancer compared to benign disease or localized tumors. Moreover Jagged1 bypasses AR in prostate cancer metastasis, and Notch1 signaling is functionally involved with osteoblast differentiation in skeletal-derived prostate cancer cells. As our understanding of the role of AR signaling in navigating EMT towards prostate cancer metastasis and CRPC expands, so do the opportunities to exploit the interactions of AR with lead partners, in pursuit of novel therapeutic targets and prognostic indicators of disease progression.

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# Chapter 16
# Androgen Receptor Regulation of Serum Response Factor Signaling in Prostate Cancer

**Alissa R. Verone and Hannelore V. Heemers**

**Abstract** The importance of androgens and the androgen receptor (AR) for the progression of PCa has been recognized for more than a century. Androgen deprivation therapy (ADT), which interferes with the interaction between AR and androgens, has been the standard of care for patients with non-organ confined PCa for seven decades. After initial remission, PCa almost invariably recurs as castration-recurrent (CR) disease during ADT, and despite low levels of circulating androgens, AR signaling remains essential for CR PCa growth. Insights in the molecular mechanisms by which AR affects transcription of target genes that drive disease progression may lead to more effective and PCa-specific forms of ADT. Here, we describe the isolation of a novel Serum Response Factor (SRF)-dependent mechanism of AR action that separates malignant from benign disease, is enriched in PCa compared to benign prostate, and is associated with aggressive disease and poor outcome. The molecular mechanism(s) by which AR control SRF action and the implications for therapeutic intervention are discussed.

**Keywords** Androgen • Androgen receptor • SRF • RhoA • Coregulator • Transcription factor • GTPase • Outcome

## 16.1 Introduction: The AR in PCa Progression

The role for androgen signaling in the progression of PCa was first recognized in the late 1800s and has served as the rationale to develop ADT as the first systemic PCa treatment in the 1940s [1]. The recurrence of disease during first-line ADT was

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interpreted originally to indicate that AR had lost its significance as a target for therapy in this stage of the disease. The appreciation that a reawakening of AR signaling axis underlies, at least in part, PCa recurrence in the presence of castrate levels of serum androgens, dates back only a decade [2–5]. Insights in two of the key molecular mechanisms that underlie resistance to first-line ADT, AR overexpression and intracrine androgen synthesis, have led to the development of second-generation ADT, which results in a survival benefit for CR PCa patients that failed first-line ADT and subsequent chemotherapy [6, 7]. AR-dependent mechanisms similar to those that underlie reemergence of PCa during initial ADT appear responsible also for disease recurrence during second-line ADT [8, 9], and the resulting disease has been suggested to be sensitive to further manipulation of the AR signaling axis [10].

Thus, the AR signaling axis remains a valid therapeutic target throughout the clinical progression of PCa. Nonetheless, AR-dependent genes and transcriptional programs that are critical for PCa cell proliferation and metastasis have remained largely elusive. Due to extra-prostatic actions, ADT is associated with significant side effects that affect negatively a patient's quality of life [11, 12]. The long-term goal of our laboratory is to identify and characterize the mechanisms of AR action that drive PCa progression and can lead to more effective and PCa-specific forms of ADT. Insights in the basic mechanisms by which AR affects transcription of target genes may lead to isolation of clinically relevant androgen action that is actionable for therapeutic intervention. Here, we describe, to our knowledge, the identification of the first mechanism of androgen action that is associated with PCa aggressiveness and progression.

## 16.2 AR Signaling, the Basics

A general description of AR structure, function, and mechanism of action is included to provide the information necessary and critical for the account of the SRF-dependent mechanism of androgen action. For a more detailed explanation of other aspects of AR-dependent transcription, the reader is referred to several excellent contributions to this volume.

### *16.2.1 AR Structure and Function*

AR is a 110-kDa ligand-activated transcription factor (TF) that belongs to the nuclear receptor family. AR is composed of an N-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) (reviewed in [13]). The NTD harbors the major transactivation function of AR

(AF1) that is constitutively active when isolated from LBD. The DBD controls AR dimerization and the specificity and selectivity of AR binding to genomic recognition sites, known as Androgen Response Elements (AREs). Located next to the DBD is the hinge region that contains the nuclear localization signal, which controls AR entry into the nucleus. The C-terminal LBD contains the ligand-dependent transactivation function (AF2) and forms the ligand-binding pocket. AR ligand-induced transcriptional activity depends on intramolecular and intermolecular interactions that involve NTD and LBD. During progression to CRPC, AR undergoes structural rearrangements and mutations that affect predominantly the LBD and allow AR to adapt to the selective pressure of the androgenic milieu and to develop resistance to ADT [14, 15].

### *16.2.2 Mechanism of AR Action*

The classical mechanism of AR action starts with testosterone, the principal circulating male sex steroid, entering its target cell, where it is converted to the more active metabolite dihydrotestosterone (DHT) by 5-alpha-reductases (reviewed in [13]). DHT binds to the AR with greater affinity than testosterone. A growing body of evidence indicates that alternative routes of steroid synthesis pathways can lead also to intracrine production of DHT in PCa cells [16, 17]. Ligand binding promotes a conformational change that causes alterations in the heat shock protein complex that is associated with AR. Ligand-activated AR translocates to the nucleus and binds to AREs, where it recruits other components that are necessary to form a productive AR transcriptional complex [13].

### *16.2.3 AR-Interacting Proteins*

An ever increasing number of proteins have been identified that are needed to interact with DNA-bound AR to ensure efficient transcription of AR target genes (reviewed in [13]). To date, more than 200 such AR-associated transcriptional regulators have been described. These proteins can be assigned to two major groups: secondary TFs and coregulators. The latter group of proteins is recruited to AREs in the regulatory regions of AR target genes by AR without necessarily binding DNA themselves. Coregulators that induce and promote AR transactivation are known as coactivators; coregulators that repress AR transcriptional activity are termed corepressors. A wide variety of cellular functions has been ascribed to coregulators. AR-associated coregulatory proteins can function in cell processes and pathways that are associated readily with transcriptional control, such as chromatin remodeling and histone modification. Other coregulator-associated functions, which are not

as easily reconciled with control over target gene expression, include but are not limited to DNA repair, ubiquitination, cytoskeletal functions, and endocytosis. Recently, in addition to specificity for select AR target genes [18–20], context dependency has been reported that allows a given coregulator to act as a corepressor or coactivator for different target genes [21]. Further complicating an already complex situation, feedback and feed-forward loops have been described in which AR imparts androgen regulation over AR-associated coregulator expression [18, 22–24]. Insights in the manner by which the more than 190 AR-associated coregulators that have been isolated to date contribute to expression of the AR-dependent transcriptome may identify distinct modes of androgen action in PCa cells.

Similar variability in the mechanism(s) by which they regulate AR transcriptional activity has been observed for TFs that interact, either physically or functionally, with AR to regulate transcription of AR target genes. In addition to TFs that are part of the general transcription machinery [13], AR action relies on action of specific secondary TFs that bind their respective consensus binding sites within the genome. These TFs can affect AR action in multiple ways (for summary, see Fig. 16.1). Some TFs bind to their consensus binding sites in the immediate vicinity of AREs (e.g., FoxA1) (Fig. 16.1a); others are recruited as a cofactor to ARE-bound AR without binding DNA (e.g., Runx2) (Fig. 16.1b). Conversely, AR can act as a cofactor for DNA-bound TFs (e.g., HoxB13) (Fig. 16.1c) which may underlie observations of AR recruitment to genomic regions that do not harbor sequences that resemble consensus AREs. The scenarios described above all involve recruitment, directly or indirectly, of AR to regulatory DNA elements in or near androgen-responsive genes. Other, somewhat underappreciated, so-called indirect mechanisms of androgen action exist in which AR affects the activity of DNA-bound TFs (e.g., SRF) (Fig. 16.1d) or induces activation and/or recruitment of secondary TFs to ARE-less TF binding sites (e.g., SREBP1) (Fig. 16.1e). The latter mechanisms not only lead to amplification of the original androgenic signal, but they also harbor a mediator downstream of AR that governs a fraction of AR action on target cells and may represent a novel target for therapy.

### *16.2.4 AR as Target for PCa Therapy*

The AR is the main target for therapy for PCa patients whose cancer recurs after initial surgery or radiation with curative intent and for patients who present with non-organ-confined PCa. First generation ADT consists of castration, which can be achieved surgically or medically, the administration of anti-androgens that compete with natural ligands for binding to AR, or a combination of both approaches [25]. Second-line ADT exploits insights into the mechanisms by which AR bypasses first-line ADT to impede intracrine androgen biosynthesis or involves dispensing of novel, more potent anti-androgens that bind AR with higher affinity [26, 27]. Ongoing investigations indicate that AR remains relevant in a significant fraction of

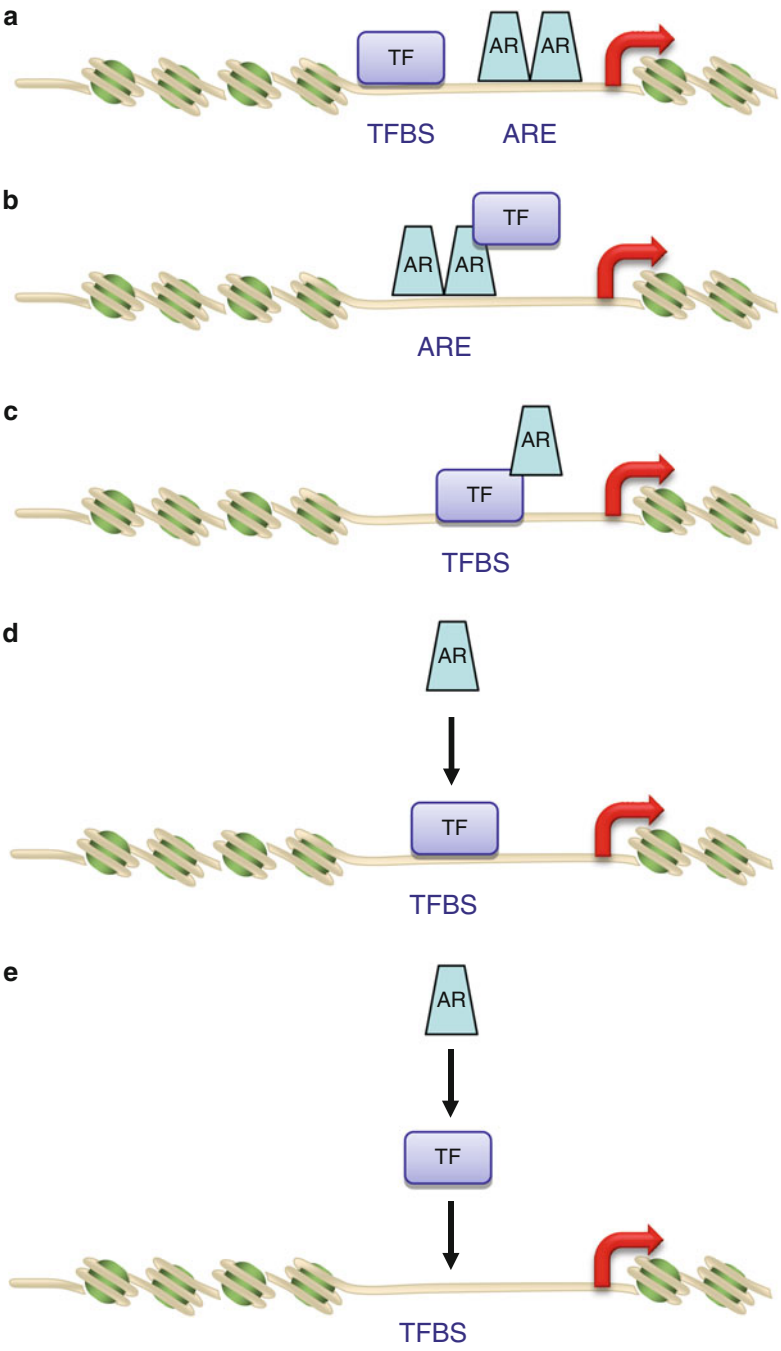


**Fig. 16.1** Overview of the different mechanisms by which TFs affect AR action. *ARE* androgen response element, *TFBS* transcription factor binding site

PCas that fails second-line ADT and suggest a role for tertiary forms of ADT at this stage of the disease [8–10]. A common theme among all currently available forms of ADT is that they target the AR LBD and by their nature block all androgen action in PCa cells, irrespective of relevance to disease progression. In view of the emergent role for NTD in CR PCa, efforts have been directed towards identifying small molecule inhibitors that block AF1 function. EPI-001 is a lead compound that interferes with AR NTD action. In preclinical studies, EPI-001 was found to block androgen-induced proliferation and cause cytoreduction of CR PCa in xenografts dependent on AR for growth and survival without causing toxicity [28]. Its safety and efficacy in humans remains to be determined. As an alternative to approaches to target directly AR, novel therapeutic avenues that are directed selectively against the action of secondary TF that act downstream of AR to drive disease progression may be of clinical benefit.

## 16.3 A Clinically Relevant SRF-Dependent Mechanism of Androgen Action

Investigation into the molecular mechanism that underlies the feed-forward mechanism by which AR induces expression of its associated coactivator four-and-a-half LIM domain protein 2 (FHL2), led to the identification of a novel-indirect mechanism of androgen action. Androgen stimulation of FHL2, an AR coactivator that is overexpressed in PCa where its expression is associated with a poor prognosis [29, 30], depended on active transcription that is mediated by action of Serum Response Factor (SRF) on FHL2 proximal promoter [23].

### *16.3.1 SRF Signaling, the Basics*

SRF is a founding member of the MADS (MCM1, Agamous, Deficiens and SRF) box-containing family of TFs [31]. SRF, which is expressed ubiquitously, was identified originally based on its ability to regulate the immediate early response and has since been shown to play a critical role in the organization of the actin cytoskeleton. Several other cellular and physiological functions including roles in cell proliferation, cell cycle control, mesoderm formation, angiogenesis, vascular integrity, and neurogenesis have been attributed to SRF (for review see for instance [31–34]) . SRF is organized in a modular structure that is common among MADS-box proteins: a regulatory NTD that is subject to posttranslational modifications which modulate its DNA binding ability and transcriptional activity, the DBD that mediates homodimerization and DNA recognition, and a C-terminal transcriptional activation domain. SRF regulates expression of target genes through a regulatory

genomic domain that is known as the serum response element (SRE). Within the SRE, specific DNA sites promote the recognition of SRF. These sites are referred to as CArG boxes and consist of a ten base pair sequence with a series of six A/T repeats flanked by CC and GG or $CC(A/T)_6GG$ sequences. Prior to DNA binding, SRF homodimerizes. SRF occupies CArG boxes constitutively and, a weak TF by itself, derives its transcriptional activity from interaction with a multitude of cofactors. More than 60 such factors have been described and selective interaction with these regulatory proteins allows SRF to toggle between disparate transcriptional programs [35]. Recruitment and/or activation of specific cofactors rely on activating signaling cascades. The 2 major signaling pathways that regulate SRF activity are MAP kinase, which induces phosphorylation and activation of ternary complex factors (TCFs) that are recruited to the SRE adjacent to CArG boxes, and the RhoA signaling axis that controls nuclear translocation of the critical SRF cofactor MAL [34].

### *16.3.2 Indirect Mechanism of Androgen Action Via SRF*

Our laboratory's research program aims to make use of insights in the basic regulation of the AR transcriptional complex to isolate mechanisms of androgen action that are relevant to PCa progression. Overexpression of several AR-associated coactivators has been shown to be essential for AR activation during PCa progression [13, 36]. The stimuli and signaling pathways leading to overexpression of these coregulators, however, remain largely elusive. Our previous work that explores the regulation of the expression of FHL2 demonstrated that expression of this key AR coactivator is induced strongly by androgens [23]. Expression of FHL2 is enriched in prostate epithelial cells, where FHL2 colocalizes with the AR. FHL2 expression is increased in PCa cells compared to benign epithelial cells. Moreover, the nuclear content of FHL2 increases with prostate tumor dedifferentiation where it is predictive of postoperative PCa recurrence [29, 30]. Thus, understanding the mechanism by which AR governs FHL2 expression may unravel mechanisms of androgen action that contribute to disease progression.

Characterization of the mechanism by which androgens induce FHL2 expression pointed towards the involvement of active transcription that relies on AR expression and activity. The increase in FHL2 expression required 8–16 h of androgen stimulation; these kinetics are slow compared to androgen induction of ARE-driven genes such as PSA which is obvious already at 4 h. These results indicated that androgen stimulation of FHL2 may not follow the canonical, direct AR signaling cascade, but rather involves an indirect, ARE-independent mechanism of androgen action. In line with this hypothesis, the regulatory regions of the FHL2 gene did not harbor an ARE-like sequence, nor could AR recruitment be observed to these sites. Instead, androgen regulation of FHL2 was mediated by a 145 bp proximal promoter

fragment that harbors a motif that matches the consensus sequence for a CArG box. Site-directed mutation of this site, to which SRF binding was observed under both androgen-deprived and -supplemented conditions, prevented androgen regulation of FHL2 expression, as did siRNA-mediated loss of SRF expression. These studies were the first to indicate a role for SRF in androgen action in PCa cells [23].

### *16.3.3 SRF-Dependent Androgen-Responsive Gene Signature*

In view of the critical roles of SRF in cell proliferation and regulation of the actin cytoskeleton, it was tempting to speculate that androgen control over its activity could have implications for PCa cell proliferation and migration. To explore this possibility, a microarray analysis was performed using RNA from LNCaP cells in which androgen stimulation was combined with siRNA-mediated SRF silencing. From this approach, a set of 158 genes that depend on SRF to achieve full androgen responsiveness was isolated. Genes that belong to this signature showed at least 2-fold androgen-dependent changes in expression, relied entirely on the presence of SRF for androgen dependence, and their basal expression was not affected by loss of SRF. The signature represented less than 6 % of all androgen-regulated genes and 11 % of SRF target genes in the LNCaP cell line. Using real-time RT-PCR, AR- and SRF-dependency of these genes was confirmed. Moreover, the kinetics and ligand-dependency of androgen regulation, which were validated in the independent AR-positive cell line VCaP, was consistent with regulation via an indirect mechanism of androgen action. The majority of these genes was not known previously to be androgen responsive or to be relevant to PCa. Pathway analysis indicated that genes belonging to the 158 gene expression profile function in cellular processes such as cell division, morphology, cell cycle control, cell assembly and organization, and cellular development, all critical cellular processes that often become deregulated during cancer development and progression. The gene signature also correlated with various diseases and disorders, such as cancer, reproductive system disease, genetic disorders, respiratory disease, and cardiovascular disease [37].

### *16.3.4 SRF Mediates Clinically Relevant AR Action in PCa*

In view of the PCa cell line-derived observations, the relevance of the SRF-dependent androgen-responsive gene signature for the clinical situation was assessed. The expression of the 158 gene set was analyzed against mRNA expression datasets that were derived from 99 laser-capture microdissected human prostate specimens that had been generated before [38]. Prostate tissues included in this profiling study consist of benign (normal epithelium, benign prostatic hyperplasia) and malignant (prostatic intraepithelial neoplasia (PIN), PCas of Gleason Pattern (GP) 3, GP4,

GP5 and lymph node (LN) metastases) samples. Unsupervised clustering analyses indicated that the 158 gene signature is able to separate benign from malignant prostate specimens. Of the original 178 probe sets that cover the 158 genes, 20 were found to be consistently deregulated in PCa samples versus normal prostate epithelium. The expression patterns of these 20 probe sets were examined in prostate tissue mRNA profiles available through the Oncomine database for validation purposes. The 20 core probe sets were expressed consistently differentially between normal and malignant prostate samples in 12 independent profiling studies, which had used different tissue procurement methods, RNA extraction procedures, and microarray platforms. Similar analyses for random sets of 20 direct AR target genes failed to show consistent alterations in gene expression between malignant and benign prostate samples. These findings confirmed the significance of the SRF- and AR-dependent (core) gene signature in PCa and corroborated the validity of the microarray experiment. The relevance of the SRF-dependent androgen-responsive gene signature to PCa progression was assessed by exploring its correlation with GP number and metastatic status. Linear regression model analyses demonstrated that expression is associated with aggressive disease, suggesting that this gene signature may also be indicative of clinical outcome after initial surgical treatment. Consequently, the correlation between expression of the probe sets and PSA failure, defined as detectable levels of the PCa serum marker PSA (>0.4 ng/dl) after radical prostatectomy (RP), was explored. Expression of 15 probe sets was significantly associated with PSA failure. Similar analyses for 10 sets of 158 randomly selected AR-target genes, demonstrated that the fraction of SRF- and AR-dependent genes associated with PSA failure is larger than the fraction of direct AR-target genes [37]. These results were validated using independent gene expression profiling data available from the Memorial Sloan Kettering Cancer Center data portal. For the generation of this dataset, tissues had been macrodissected, and mRNA profiling was done using procedures and a microarray platform that differed from those used in the original study [39]. Survival analysis was performed using Cox proportional hazard model where PSA recurrence was the end point. Eighteen genes were identified that were associated significantly with biochemical failure. Gene set analysis was conducted for the gene set as a whole, which showed that it is significantly associated with biochemical failure. Gene set analysis was performed for both the SRF- and AR-dependent genes and 10 randomly selected similarly sized sets of AR-dependent genes. These results indicated that the SRF- and AR-dependent gene set is associated more significantly with PSA recurrence than similarly sized sets of AR-target genes. Evaluation of gene set enrichment between PCa and normal prostate tissues indicated that all 10 random AR-target gene sets are significantly enriched in normal tissues, whereas the SRF- and AR- dependent gene set is enriched in cancer tissues.

This work isolated SRF as mediator of clinically relevant androgen action in PCa. Since then, other groups have independently implicated SRF in biochemical recurrence after RP [40] and proposed that SRF is a candidate driver of PCa development [41].

## 16.4 Molecular Mechanism by Which AR Conveys Androgen Responsiveness to SRF

Understanding the mechanism(s) by which SRF imparts androgen regulation to its target genes may provide novel opportunities to target clinically relevant androgen signaling. Initial exploration of the molecular mechanism(s) by which androgens regulate SRF activity failed to show a direct interaction between SRF and AR, a reciprocal effect on SRF and AR protein expression, androgen-induced changes in SRF phosphorylation status or cellular localization. In addition, overexpression of SRF, which has been described in clinical specimens, did not affect androgen regulation of SRF target genes. Recruitment of AR to SRF genomic binding sites did not occur under either androgen-deprived or androgen-supplemented conditions. Instead, these data supported the established model of SRF action in which SRF is bound constitutively to CArG boxes in regulatory regions of target genes and is activated by the recruitment of cofactors or activation of upstream signaling pathways.

### *16.4.1 RhoA Conveys Androgen Responsiveness to SRF*

The RhoA signaling axis is a well-known activating pathway upstream of SRF [34]. Like other members of the small GTPase family, RhoA functions as a molecular switch that is active in GTP-bound state and inactive when GDP-bound. Extracellular stimuli induce RhoA GTPase activity which leads RhoA to engage RhoA effectors. Activated RhoA effectors such as Rho associated kinase (ROCK1) facilitate actin polymerization, which results in the release and nuclear translocation of the SRF coactivator megakaryocytic acute leukemia (MAL) from monomeric actin. In the nucleus, liberated MAL regulates SRF-mediated transcription.

Interference at every step in the RhoA/actin/MAL signaling axis prevented full androgen induction of the SRF target gene FHL2. Androgen regulation of FHL2 was attenuated severely or completely following siRNA-silencing of RhoA, the use of the ROCK inhibitors Y-27632, hydroxyfasudil and Ro-318220, and treatment of PCa cells with latrunculin B, which hampers the actin polymerization that is needed to allow release and nuclear translocation of the SRF coactivator MAL. In addition, androgen exposure of LNCaP cells led to increased nuclear levels of MAL, and nuclear accumulation of MAL coincided with kinetics of androgen induction of FHL2. ChIP experiments confirmed androgen-stimulated recruitment of MAL to the CArG box in the FHL2 promoter, and loss of MAL prevented androgen induction of FHL2. These data suggested that androgen exposure of PCa cells may affect the expression and/or activity of RhoA. Whereas RhoA expression levels remained unaltered following androgen treatment, rhotekin pull-down assays demonstrated

androgen induction of RhoA activity. Androgen stimulation of RhoA activity preceded nuclear translocation of MAL and androgen regulation of FHL2.

To verify to what extent the role of RhoA in androgen-dependency of FHL2 can be generalized to the 158 AR- and SRF-gene expression profiles, the impact of siRNA-mediated silencing of RhoA on other SRF target genes was assessed using real-time RT-PCR. Androgen regulation of a quarter, half, and a quarter of SRF target genes was found to be either lost completely, partially, or not affected, respectively, following silencing of RhoA. Noteworthy, a siRNA-induced decrease in MAL expression affected the androgen modulation of these same genes in a similar manner. These findings indicate that RhoA-mediated androgen modulation affects a substantial segment of SRF-dependent gene expression [42].

### *16.4.2 RhoA Overexpression in PCa Specimens Is Associated with Disease Progression*

As the androgen- and SRF-dependent gene signature is associated with aggressive disease and poor outcome after RP, the relevance of RhoA to clinical PCa progression was explored. Expression of RhoA was examined using immunohistochemical analysis of prostate tissues from 91 patients with biopsy-proven diagnosis of PCa who were treated with RP without neoadjuvant hormonal therapy. In prostate tissue samples RhoA expression presented as a membraneous cytoplasmic staining pattern, which is consistent with the cellular distribution of activated RhoA. In 80 specimens that contain both cancer and benign cells, RhoA intensity was higher in cancer cells. Increasing levels of cancer RhoA intensity were associated with larger tumors, higher GPSM scores (scoring algorithm that takes into account Gleason score, PSA levels, seminal vesicle involvement and margin status to predict biochemical recurrence following RP), LN involvement, and extraprostatic extension at RP. When the association of RhoA expression with PCa progression after RP was assessed, patients whose specimens contained marked levels of cancer RhoA intensity are over twice as likely to progress compared with patients whose specimens contained absent, focal, or moderate levels. Fifty of the eighty specimens that contained both benign and malignant cells had a higher percentage of cancer cells staining positive for RhoA intensity. Intensity and percentage were multiplied to summarize overall expression of RhoA. RhoA expression was higher in cancer cells. Moreover, increased RhoA expression was associated with extraprostatic extension at RP and a significant increase in risk of progression after RP [42].

In cell culture experiments, increased expression of RhoA led to a marked amplification in the androgen-responsiveness of SRF target genes that rely on RhoA to achieve full androgen regulation, but not for genes for which androgen-dependency is not affected by RhoA silencing. Moreover, loss of SRF attenuated the effect of

RhoA overexpression on androgen-induction of SRF target genes. Introduction of site-specific mutations in RhoA expression constructs revealed that the observed changes on androgen-regulation of select SRF target genes were not merely the result from overexpression of RhoA but rely also on its GTPase activity [42].

### *16.4.3 Activity of the AR-RhoA-SRF Signaling Axis Is Maintained in CR PCa*

In view of the observation that higher RhoA levels in RP samples are associated with poorer disease outcome, the possibility that RhoA expression correlates with progression to CR disease was examined. RhoA protein levels were compared in parental LNCaP cells and its CR sublines C4-2 and LN-Rf and in the CWR22 xenograft PCa progression model. C4-2 and LN-Rf cell lines were established following androgen deprivation of LNCaP cells in vivo or in vitro, respectively. The CWR22 xenograft recurred as CR disease 4–5 months after castration. CWR22 tissues were harvested pre-castration, at several time points post-castration, and at recurrence. C4-2 and LN-Rf cells showed levels of RhoA expression similar to those observed for LNCaP. Similarly, RhoA immunohistochemistry signals did not alter during the progression of CWR22 tumors to CR PCa. These data provided evidence that RhoA expression does not change significantly during disease progression. Moreover, they suggested that the level of RhoA that is present at the time of RP determines the aggressiveness of PCa. In addition, expression levels of FHL2 and other SRF target genes in C4-2 and LN-Rf cells were maintained at levels that are similar to those observed for the parental cell line LNCaP indicating that the AR-SRF signaling axis is active in the CR LNCaP sublines. These observations were validated in the CWR22 model of PCa progression. Exposure to androgens reinstated androgen regulation of a significant fraction of SRF target genes in CR LNCaP cell lines and in androgen-deprived CWR22 tumors. Further confirming the activity of SRF dependent mechanism of androgen action in CR PCa, CArG-box-driven reporter gene activity in CR PCa cells was androgen-responsive in a RhoA-, MAL-, and SRF-dependent manner [42].

## 16.5 Conclusions

Here, a rational approach is described that starts from knowledge about the basic mechanisms by which AR affects expression of its target gene expression and validates insights in molecular mechanisms that are derived from PCa model systems in well-annotated clinical PCa specimens to identify novel actionable targets for therapeutic intervention downstream of AR. Specifically, systematic investigation of the molecular mechanism by which AR imparts androgen regulation to its coactivator FHL2 identified SRF as a TF that mediates clinically relevant AR action and isolated

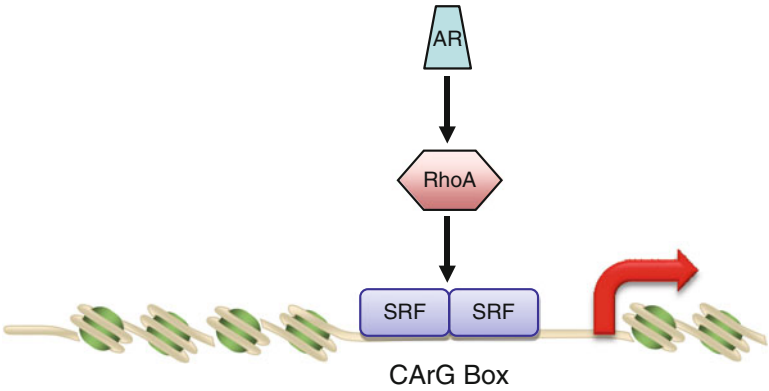


**Fig. 16.2** Schematic representation of the AR-RhoA-SRF signaling cascade

the RhoA signaling axis, which harbors drugable targets that have shown promise for anticancer therapy in preclinical studies [43], as a novel therapeutic target in PCa (Fig. 16.2). In the long term, this type of approach may lead to more effective forms of ADT that target selectively and specifically androgen action that drives progression of PCa to the lethal phenotype.

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# Chapter 17
# Regulation of Angiogenesis by Androgen-Responsive Gene EAF2

**Laura E. Pascal and Zhou Wang**

**Abstract** Androgens regulate vascular regeneration through the induction of angiogenic factors such as VEGF in both normal prostate epithelial cells as well as hormone-responsive cancer cells. Androgen deprivation induces the apoptotic death of androgen-sensitive luminal epithelial or prostate cancer cells. Androgen deprivation also has an impact on the other cell types in the prostate tissue microenvironment including vascular endothelial cells. In animal models, castration results in not only a rapid involution of the prostate but also a reduction in prostatic endothelial cell proliferation, blood flow, and the induction of a hypoxic environment. Hypoxia is a well-known inducer of angiogenesis and neovascularization. Under hypoxic conditions, VHL protein levels decrease and the transcription factor HIF1α is stabilized and transactivates angiogenic factor VEGF. Previous studies have suggested that the tumor suppressor p53 is also induced by hypoxia. Increased p53 expression usually induces cell cycle arrest or apoptosis. However, p53 can also play a role in limiting angiogenesis through the upregulation of anti-angiogenic TSP-1, inhibition of HIF1α, and the transcriptional repression of VEGF. ELL-associated factor 2 (EAF2) was identified as an androgen-responsive tumor suppressor gene that is decreased in prostate cancer and has been shown to regulate p53 target gene TSP-1,

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to bind and stabilize VHL and to upregulate HIF1α. Interaction of EAF2 and its binding partners p53 and VHL in the regulation of pro-angiogenic HIF1α and anti-angiogenic TSP-1 in the prostate may be critical for maintaining normal tissue homeostasis. Dysregulation of these interacting pathways could lead to increased vascularization and prostate tumorigenesis.

**Keywords** ELL-associated factor 2 • Von-Hippel Lindau • p53 • Thrombospondin 1 • Vascular endothelial growth factor • Hypoxia inducible factor 1 alpha • Prostate cancer • Angiogenesis • Neovascularization

## 17.1 Angiogenesis in Prostate Carcinogenesis and Progression

Angiogenesis, or the formation of new blood vessels, is critical for tumor progression and has been associated with prostate cancer metastasis [1]. Tumors require new vasculature in order to grow beyond a volume of 1 $mm^3$ and can induce the formation of new blood vessels through the secretion of specific angiogenic factors [2]. As tumors grow beyond this critical volume, diffusion of oxygen and nutrients to the cells in the center of the tumor is reduced. This induces a state of cellular hypoxia that triggers the onset of tumoral angiogenesis. The so-called "angiogenic switch" is an alteration in the balance between pro- and anti-angiogenic factors that results in increased tumor angiogenesis and subsequent tumor growth. Tumor cells under hypoxia secrete angiogenic molecules that attract and promote the proliferation of inflammatory and endothelial cells. Inflammatory cells also secrete molecules that further intensify the angiogenic response. Endothelial cells lining existing blood vessels respond by differentiating and by secreting matrix metalloproteases (MMP), which digest the blood vessel walls to enable the escape and migration of endothelial cells toward the site of the angiogenic stimuli. Several protein fragments produced by the digestion of the blood vessel walls intensify the proliferative and migratory activity of endothelial cells, which then form a capillary tube by altering the arrangement of their adherence-membrane proteins. Finally, through the process of anastomosis, the capillaries emanating from the arterioles and the venules will join, thus resulting in a continuous blood flow. Inhibition of this critical rate-limiting step in tumor development has proven successful in delaying progression (reviewed in [3]).

Prostate tumors are characterized by increased microvessel density and a more chaotic vascular network compared to benign prostate tissue [4, 5]. A number of autocrine and paracrine factors in the tumor microenvironment have been identified that contribute to tumor vascularization including VEGF, basic FGF (FGF-2), acidic FGF (FGF-1), matrix metalloproteinases, IGF-1, and angiopoietin-1 [6]. In prostate cancer treatment, the most studied angiogenic factor is vascular endothelial growth factor (VEGF) and antiangiogenic therapies have largely focused on the inhibition of VEGF [7, 8]. VEGF is an angiogenic factor transcriptionally regulated by hypoxia inducible factor (HIF1α) particularly in response to hypoxia. Hypoxia in prostate tumors has been associated with tumor progression [9], as well as a shorter time to

biochemical failure and progression-free survival [10]. VEGF is secreted by tumor cells including prostate cancer cells and promotes the formation of new capillaries by stimulating endothelial cell division and migration and by increasing capillary permeability [11–13]. In prostate cancer xenografts, VEGF expression was positively correlated with MVD [14].

Although antiangiogenic therapies can result in decreased tumor growth and longer periods of progression-free survival, recent clinical studies have found that patients given anti-VEGF therapies eventually become resistant [15, 16]. Furthermore, anti-VEGF therapies in preclinical models have recently been associated with increased tumor invasiveness and metastasis [17, 18]. Several mechanisms have been identified by which tumors become resistant to antiangiogenic therapy. One such mechanism, adaptive evasion, is the up-regulation of alternative angiogenic pathways to circumvent the blocked angiogenic pathway [19–21]. Intrinsic nonresponsiveness is another mechanism by which the tumor is characterized by an innate resistance to antiangiogenic therapy. Intrinsic resistance is likely to involve similar molecular and cellular mechanisms to those that mediate adaptive evasion. Clinical observations suggest that certain tumors may have upregulated redundant angiogenic pathways [22].

One alternative mechanism to prostate tumor angiogenesis promoted by VEGF upregulation is the downregulation of TSP-1. TSP-1 is a potent antiangiogenic factor upregulated by p53 that inhibits the proliferation and migration of endothelial cells and increases apoptosis [23]. Studies in murine melanoma and human lung and breast cancer cell lines have shown an inverse correlation between TSP-1 expression and malignant progression [24]. In androgen-dependent prostate tumors, TSP-1 expression is inversely correlated with MVD [25–27]. Conversely, transfection of TSP-1 in tumor cells inhibits tumor progression and metastases [28, 29]. Studies suggest that TSP-1 could inhibit tumor progression through the inhibition of tumor angiogenesis. The role of p53 and TSP-1 in prostate cancer remains unresolved. Some studies have shown that in prostate cancer loss of TSP-1 expression is correlated with loss of p53 [26], yet others have demonstrated increased TSP-1 and p53 in prostate tumors [30]. Recently, TSP1 expression was shown to increase with prostate tumor hypoxia and was associated with prostate tumor invasiveness and recurrence [31]. Furthermore, TSP1 was shown to be a potent stimulator of prostate tumor migration [31]. Increased TSP-1 expression has been associated with vascular regression in androgen-sensitive prostate tumors but not in castration-resistant tumors, suggesting the development of tumor resistance to TSP-1 inhibition of angiogenesis in advanced disease [25]. A complete understanding of how angiogenesis in prostate tumors is enhanced by VEGF or inhibited by TSP-1 and how the mechanisms regulating these factors can contribute to tumor resistance will be critical for optimizing the use and timing of powerful antiangiogenic agents in prostate cancer treatment. Furthermore, understanding the mechanisms involved in regulating neovascularization will aid in designing combination strategies (i.e., anti-VEGF combined with anti-TSP-1) to overcome the development of resistance to antiangiogenic inhibitors in the clinic.

Androgens regulate vascular regeneration in a sex-dependent manner [32], and testosterone has been shown to induce the secretion of angiogenic factors such as

VEGF in both normal prostate epithelial cells [33] as well as hormone-responsive cancer cells [34]. Androgen deprivation therapy (ADT) reduces clinical symptoms in the majority of patients with advanced prostate cancer, but most tumors recur within 2 years as castration resistant prostate cancer. The contribution of angiogenesis to prostate cancer progression to castration resistance has not been fully elucidated; however, increased microvessel density (MVD) is associated with metastatic potential [35]. ADT induces the apoptotic death of androgen-sensitive luminal epithelial or prostate cancer cells. However, androgen deprivation also has an impact on the other cell types in the prostate tissue microenvironment, including vascular endothelial cells. In animal models, castration results in not only a rapid involution of the prostate but also a reduction in prostatic endothelial cell proliferation, blood flow, and the induction of a hypoxic environment [36–40]. In human prostate xenografts, androgen deprivation induced acute vascular damage, characterized by leaky vessels and an increase in endothelial cell apoptosis; however, tumor vasculature eventually recovered completely [41].

Angiogenic inhibitors such as aflibercept and tasquinimod are currently under clinical investigation for treatment of castration-resistant prostate cancer. The genetic instability of tumor cells can promote the development of acquired drug resistance. Because the microvascular endothelial cells present a relatively stable genetic target [42], antiangiogenic therapies hold significant promise. Aflibercept is a soluble form of human VEGF receptor that binds to VEGF and inhibits tumor progression in preclinical castration-resistant prostate cancer models [43]. In an ongoing Phase III clinical trial, aflibercept is being tested in conjunction with docetaxel and prednisone versus docetaxel and prednisone alone in metastatic castration-resistant prostate cancer patients (NCT00519285). Tasquinimod is thought to prevent the downregulation of TSP-1 via a mechanism involving the downregulation of hypoxia-inducible factor 1α and VEGF in prostate cancer cells [44]. In a Phase II clinical trial of metastatic castration-resistant prostate cancer patients, tasquinimod monotherapy increased progression-free survival at 6 months versus placebo (69% vs. 34%) as well as median progression-free survival (8.4 months vs. 3.8 months) and Phase III clinical trials have been initiated (reviewed in [45]). Fully characterizing the mechanisms by which neovascularization is triggered in tumors will provide critical insight into establishing the best sequence and combination of therapies such as these in order to prevent or reduce the development of prostate tumor resistance.

## 17.2 EAF2 in Prostate Disease

ELL-associated factor 2 (EAF2) and other eleven-nineteen lysine-rich leukemia gene (ELL) family proteins function to positively regulate transcription elongation of RNA polymerase II. EAF2 and its functional homologue EAF1 contain a transcriptional activation domain and are capable of immortalizing hematopoietic cells when fused to myeloid/lymphoid or mixed-lineage leukemia (MLL) [46].

Mutagenesis and transfection studies showed that ELL-EAF2 binding plays an important role in EAF2 nuclear localization, nuclear speckle formation, stabilization, and transactivation activity suggesting that ELL is required for EAF2 function [47]. The C terminus domain of EAF2 is a serine-rich region that can activate transcription [47] and mediates nucleolar targeting potentially involved in the sensing and repairing of DNA damage or in triggering apoptosis [48]. Additionally, cotransfection of ELL and EAF2 induced a decrease in cell survival compared to that of either gene alone suggesting a significant role for their interaction in growth suppression [49]. In the mouse, EAF2 expression is spatially and temporally regulated during embryonic development [50]. EAF2 transcripts were detected in murine branching structures of organs including the tooth, mammary placodes, vibrissae follicles, submandibular glands, lung, pancreas and kidney suggesting EAF2 may be actively involved in organ development involving epithelial and mesenchymal interaction [50]. Human EAF2 mRNA is widely expressed, with the most abundant expression in the prostate, kidney, bone marrow, and lymph nodes [51].

EAF2 is upregulated by androgens and was identified as a tumor suppressor gene [51–53]. EAF2 downregulation, allelic loss, promoter hypermethylation, and possibly homozygous deletion was identified in ~80% of advanced prostate cancer specimens examined (Gleason ≥8) [51], whereas EAF2 was upregulated in benign prostatic hyperplasia [54]. More recently, data from gene array analyses have indicated that loss of EAF2 may also occur in early stage prostate cancer (Gleason ≤8) [53, 55]. EAF2 transfection in prostate cancer cell lines induced massive apoptosis and inhibited the growth of xenograft prostate tumor models [51], and EAF2 knockout mice developed high-grade murine prostatic intraepithelial neoplasia (mPIN) [53, 56]. Although EAF2 overexpression induced dramatic apoptosis in cultured prostate cancer cells and in xenograft tumors, it is unlikely to play a role in castration-induced apoptosis in the normal prostate because EAF2 expression is downregulated in the absence of androgens [51]. EAF2 may function differently in normal epithelial cells than in cancer cells, or androgens could be prohibitive of EAF2-induced apoptosis. This protective role could be via the blockage of caspase activation [51]. EAF and ELL family proteins were recently associated with the regulation of collagen expression during cuticle morphogenesis in *C. elegans* [57]. Collagens are major components of the extracellular matrix and play a critical role in organogenesis, tissue homeostasis, and carcinogenesis.

## 17.3 EAF2 in Prostate Angiogenesis

EAF2 has multiple binding partners which include the tumor suppressors pVHL and p53 [58, 59]. Von Hippel-Lindau disease is characterized by highly vascular tumors and results from the inactivation of the VHL gene. One major function of VHL is the degradation of the angiogenic hypoxia inducible factor (HIF). HIF1 is a basic helix-loop-helix transcription factor that transactivates genes encoding proteins involved in homeostatic responses to hypoxia. HIF1 induces expression of

proteins controlling glucose metabolism, cell proliferation, and vascularization. Under hypoxic conditions, HIF1α accumulates and controls the upregulation of a number of factors important for solid tumor expansion including VEGF. Increased HIF1α expression has been observed in prostate cancer [60, 61] and has been associated with aggressiveness [62]. pVHL stabilizes and enhances p53 transcriptional activity inducing p53-mediated cell cycle arrest and apoptosis, particularly in response to DNA damage [63].

EAF2 deficiency in mice was found to reduce Von Hippel-Lindau protein (pVHL) levels and increase expression of HIF1α [58]. EAF2 and pVHL were shown to co-immunoprecipitate in transfected cells under hypoxic conditions. EAF2 and pVHL binding increased the stability of both proteins. EAF2 loss and VHL heterozygosity in a murine model induced an increased incidence in hepatic vascular lesions as well as increased liver and prostate vascularity compared to wild-type, see Fig. 17.1 [56]. Increased vasculature in the liver was characterized by an increase in HIF1α and VEGF immunoreactivity. Prostates displayed an increased incidence of mPIN, stromal inflammation, fibrosis, and smooth muscle proliferation. PIN and hepatic vascular lesions displayed decreased expression of pVHL suggesting that combined loss of EAF2 and VHL could contribute to the development of neoplasia in the liver and prostate [56]. It has been postulated that upregulation of HIF-1α is likely to be an early event in the development of prostate cancer, given that increased levels were observed in high-grade intraepithelial neoplasia, which is considered the precursor of prostatic adenocarcinoma [64]. EAF2 loss could trigger increased angiogenesis through the upregulation of HIF1α contributing to prostate tumorigenesis.

In addition to activation of the VHL pathway, EAF2 was shown to colocalize and co-immunoprecipitate with p53 suggesting that EAF2 may functionally interact with p53 [59]. The p53 pathway is activated in response to DNA damage, hypoxia, oxidative stress, or other cellular stress. Loss of p53 function in tumor cells enhances HIF1α levels and augments HIF-1-dependent transcriptional activation of the VEGF gene in response to hypoxia and contributes to increased neovascularization [65]. p53 has also been shown to inhibit angiogenesis through the upregulation of TSP-1 [66]. Recently, expression of VEGF and p53 status and an inverse association between TSP-1 and VEGF expression was demonstrated in human prostate cancer tissue specimens suggesting that VEGF and TSP-1 may interact in the control of prostate tumor angiogenesis [26].

In EAF2 knockout mice, expression of TSP-1 was reduced in the prostates and livers, while VEGF expression was unaltered [59]. The livers of knockout mice had a 3-fold increase in the number of CD31 positive blood vessels, indicating an increase in vasculature. In a luciferase assay, transfection of exogenous EAF2 alone in multiple cell lines had no significant effect on TSP-1 promoter activity, whereas p53 transfection significantly repressed TSP-1 promoter activity. Cotransfection of EAF2 and p53 showed that EAF2 overexpression alleviated p53 repression of TSP-1 promoter in all assayed cell lines. Interestingly, in LNCaP and H1299 cells, cotransfection of EAF2 with p53 not only alleviated p53 repression but also increased TSP-1 promoter activity suggesting EAF2 and p53 could functionally interact in the

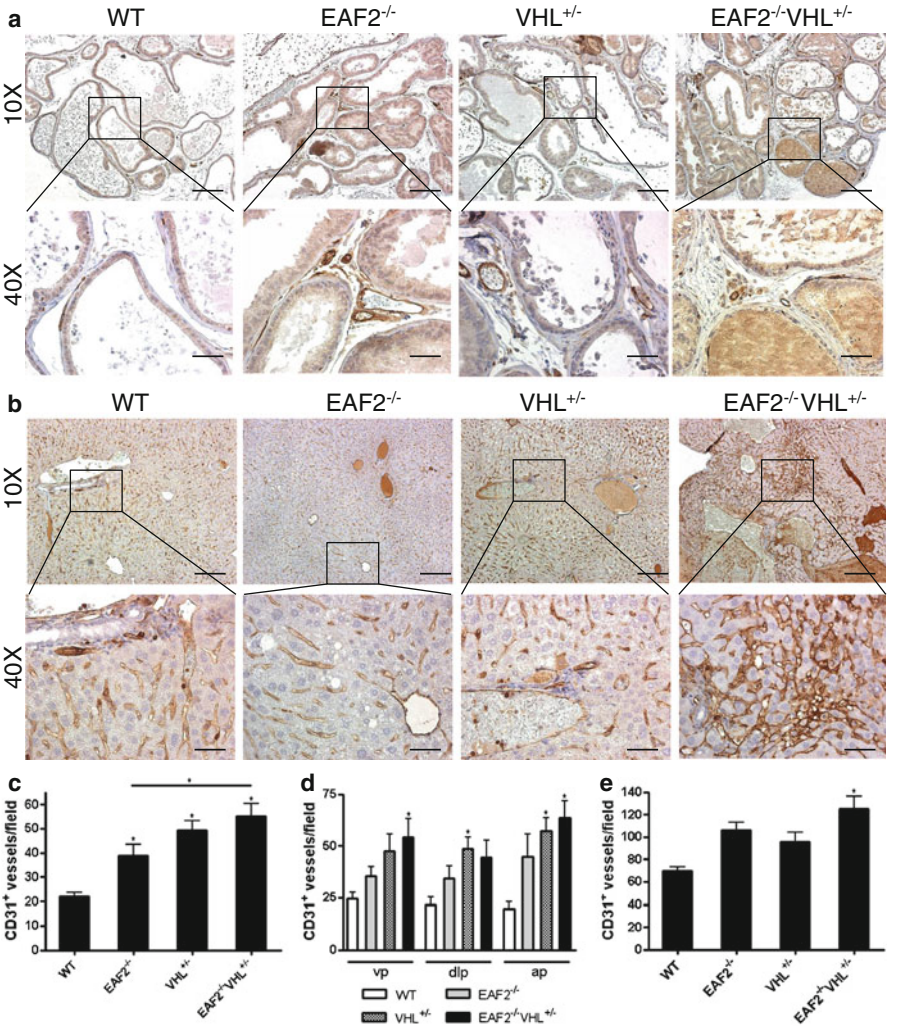


**Fig. 17.1** Cooperative effects of EAF2- and VHL-deficiency on CD31+ blood vessel formation in the prostate and liver. (**a**) CD31 immunostaining of vessels in transverse sections of prostate ventral lobes from wild-type control (WT), EAF2−/−, VHL+/−, and EAF2−/−VHL+/− mice at age 20–24 months. (**b**) CD31 immunostaining in transverse sections of liver from WT, EAF2−/−, VHL+/−, and EAF2−/−VHL+/− mice at age 20–24 months. Original magnification ×10, inset ×40. *Scale bars* indicate 200 mm in ×10, 50 mm in ×40. (**c**) Quantification of CD31+ vessels in prostate. (**d**) Quantification of CD31+ vessels in the ventral (vp), dorsal-lateral (dlp), and anterior (ap) prostate. (**e**) Quantification of CD31+ vessels in the liver. Data represent average of 5–8 mice per group (*, $p<0.05$). (Adapted from Angiogenesis. 2011 Sept 14(3):331–43)

repression of angiogenesis through up-regulation of TSP-1. Loss of EAF2 function in the prostate might represent an important pathway by which prostate tumors could escape from angiogenic inhibition via the downregulation of TSP-1.

The role of EAF2 in prostate cancer has yet to be fully elucidated. EAF2 may suppress prostate tumorigenesis and neovascularization through multiple pathways. EAF2 could inhibit prostatic cell proliferation directly, and loss of EAF2 expression could enhance prostate epithelial expansion. Downregulation of the EAF2/ELL collagen axis could disrupt and compromise the extracellular matrix and the basement membrane, thereby promoting tumor invasion and vascular sprouting. EAF2 loss could also promote tumor angiogenesis via upregulation of pro-angiogenic HIF1α through the VHL or p53 pathway, and/or downregulation of antiangiogenic TSP-1 through interaction with p53. AR can also regulate TSP-1 expression in the prostate [67]. EAF2 expression is regulated by AR as well, and androgens could influence HIF1α and TSP-1 expression in the prostate directly through AR or indirectly through EAF2. Further studies will be required to fully elucidate the role of EAF2 in prostate neovascularization and tumorigenesis.

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# Chapter 18
# The Role of miR-21, an Androgen-Regulated MicroRNA, in Prostate Cancer

**Judit Ribas and Shawn E. Lupold**

**Abstract** MicroRNAs (miRNAs) are an established family of small non-protein coding RNAs which broadly regulate gene expression through posttranscriptional mechanisms. Differential miRNA gene expression is common in cancer and other disease states, and there is significant evidence that many miRNAs play a functional role in disease processes. miR-21, a miRNA which imparts cellular growth and survival properties, is commonly overexpressed in malignant and inflamed tissues, including prostate cancer. Its expression is directly induced by the activated androgen receptor (AR), and elevated miR-21 alone is sufficient to impart castration-resistant tumor growth. Therefore, the miR-21 gene is of particular interest in prostate cancer biology. This chapter focuses on the miR-21 gene, its expression and processing, and miR-21 regulated pathways in the prostate and other tissues. The association of miR-21 with clinical prostate cancer is also reviewed.

**Keywords** MicroRNAs • miR-21 • VMP1 • Androgen receptor • Prostate cancer

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## 18.1 Introduction

MicroRNAs (miRNAs) are a widely studied class of small non-protein coding RNAs (ncRNAs) that posttranscriptionally inhibit gene expression through complementary binding to messenger RNAs. The origins of mammalian miRNAs are lost in the dawn of metazoan evolution and accordingly have been subjected to high evolutionary pressures to reach the current repertoire in animal phyla [1]. Despite the initial association of miRNAs with the development of the nematode *Caenorhabditis elegans*, current knowledge suggests that miRNAs rarely contribute to essential functions of mammalian development, such as the establishment of the body plan and the specification of multiple cell lineages. On the contrary, mammalian miRNAs appear to more regularly modulate the response of mature tissues facing stressors [2]. This chapter focuses on a single androgen receptor (AR)-regulated microRNA, miR-21, and its role in the prostate and prostate cancer (PCa).

### *18.1.1 A Brief History of miRNAs*

In 1993 Victor Ambros and Gary Ruvkun laboratories identified the first microRNA in the model organism, *Caenorhabditis elegans* [3, 4]. Their discovery came from the observation of the LIN-14 protein, which decreased in quantity during larval development preceding the appearance of multiple cell lineages of the nematode. The loss of LIN-14 protein was known to correlate with the overexpression of a ncRNA, lin-4. It was determined that lin-4 posttranscriptionally regulated the translation of LIN-14 by binding to the 3'untranslated region (UTR) of its mRNA. Ergo for the first time a ncRNA was determining the switch between developmental cell proliferation and differentiation. Seven years later a new miRNA named let-7 was observed in the development of nematodes [5, 6]. This event marked the classification of a new family of genes and their homologs across many species [7–9]. Presently the 18th release of miRBase, the primary online repository for miRNA sequences and annotations, contains a total of 1921 mature human miRNAs.

### *18.1.2 miRNA Biogenesis and Function*

Mature miRNAs are derived from pre-miRNA hairpin sequences within larger initiating transcripts known as primary miRNAs (pri-miRNAs). The biogenesis of pri-miRNAs is initiated with the recruitment of transcriptional complexes to gene promoter and enhancer elements. RNA polymerase II accounts for most pri-miRNA transcription [10], albeit the transcription of a minor set of pri-miRNAs is RNA polymerase III dependent [11]. The resulting pri-miRNA products may be ncRNAs or coding "host" mRNAs which include a passenger miRNA. Pri-miRNA processing

is initiated in the nucleus with the enzymatic cleavage of the approximately 70 bp pre-miRNA hairpin by a complex known as the Microprocessor. In its basic form, the Microprocessor is comprised of the RNase III enzyme Drosha and a double stranded RNA binding protein (dsRBP) called DGCR8 (DiGeorge syndrome critical region gene 8) [12]. Evidence suggests that transcription and Microprocessor processing of pri-miRNAs occurs concomitantly [13]. Nuclear pre-miRNAs are then shuttled to the cytoplasm through a RanGTPase complex including Exportin 5 [14, 15] and subsequently processed in the cytoplasm by another RNase III enzyme, Dicer1 [16]. Like the Microprocessor complex, Dicer1 utilizes a partnering dsRBP known in vertebrates as the Trans-activator RNA (TAR)-binding protein (TRBP). The specific signature of Dicer1/RNA III cleavage generates two-nucleotide overhangs at each of the 3' ends of the newly formed RNA duplex. The 19–24 nucleotide RNA duplex is then loaded onto the Argonaute (Ago) protein within the RNA-induced silencing complex (RISC) [17]. To acknowledge the facilitating role of Dicer1/TRBP in this process, the term RISC loading complex (RLC) was coined. Once the miRNA duplexes are loaded within RISC, a critical phenomenon takes place: duplex unwinding and strand selection. Specifically, one of the strands remains associated with RISC (guide strand) whereas the other one will be removed (passenger strand). Conventionally the strands are termed miRNA and miRNA*, assigning the (*) to the minor product of the stem [18, 19]. However, this nomenclature will soon be replaced with -5'p or -3'p to refer to the original arm of the hairpin which encompasses the miRNA. The RISC-associated mature miRNA is then available to bind target mRNAs through sequence complementarity. In most cases, miRNA binding does not involve full sequence complementarity and instead is driven by nucleotides 2–7 of the 5'-end, known as the miRNA "seed region." Because of the frequency of such small complementary regions in the genome, each miRNA is thought to regulate between 20–200 mRNAs [20–22]. This interaction generally takes place within the 3'UTR of the target mRNA and leads to diminished levels of protein. The exact mechanism remains controversial but appears to involve RISC-mediated inhibition of translation and/or induction of mRNA decay (reviewed by [23]).

### *18.1.3 miRNAs and Human Disease*

The first insight into miRNA function in human disease came from cancer research. Genetic mapping of chromosomal deletions and mutations at the 13q13.4 loci in patients with chronic lymphocytic leukemia (CLL) identified the miRNA gene cluster miR-15a and miR-16-1 [24]. Over the last decade, since this discovery, thousands of reports have interrogated the role of miRNAs in cancer and cancer-related pathways. In addition to cancer, miRNAs have been shown to directly play a role in cardiovascular disease (reviewed in [25]) and in some Mendelian and complex genetic diseases (reviewed in [26]). This chapter focuses on the putative oncogene miR-21 [27], one of the most commonly overexpressed miRNAs in cancer [28] and its role and regulation in prostatic cells.

## 18.2 The Role of AR in Prostate Cancer and miRNA Gene Expression

### *18.2.1 The AR and Prostate Cancer*

AR is a central component in the biology of the normal prostate and in PCa. As a nuclear receptor, hormone binding leads to AR conformational changes and nuclear translocation where it then transcriptionally regulates the expression of numerous genes. Under normal physiological conditions, stromal AR induces the production of andromedins, which act as paracrine growth factors by inducing the proliferation and survival of the prostatic epithelia. On the other hand, in the epithelia, AR inhibits the andromedin-mediated proliferation and promotes transcription of prostate-specific differentiation markers. This balance is lost in PCa, where AR activity shifts towards an autocrine loop and promotes direct survival and proliferation of the epithelia (reviewed by [29]). In view of the central role of AR in PCa, endocrine therapeutics aiming to diminish physiological levels of androgens or inhibit AR itself, have become the first line therapy for advanced and metastatic PCa. Nonetheless, clinical data shows that after a short period of remission, a fatal castration-resistant form of the disorder will develop. Curiously, this disease form still appears to rely on AR activity. In cellular and animal models, Cheng and colleagues found that a subtle increase of AR mRNA and protein were sufficient to convert tumors from androgen sensitive to castration resistant [30]. This is supported by the finding that castration-resistant PCa (CRPC) often presents an amplification of the AR gene locus [31, 32]. In addition to gene amplification, it has been shown that alternative splicing can generate a constitutively active AR or ligand-independent variants [33, 34]. Thus, the transcriptional role of AR and its downstream pathways remain valid targets for therapeutic intervention in early PCa and CRPC.

### *18.2.2 miRNAs and the Androgen Receptor*

In light of the elemental and broad role of AR in PCa, we and others speculated that the AR may directly or indirectly regulate miRNA gene expression and that AR-regulated miRNAs may then contribute to PCa growth and castration resistance. Our approach consisted of evaluating the common changes in miRNA gene expression following the treatment of two androgen-dependent PCa cell lines, LNCaP and LAPC-4, with increasing concentrations of androgens. The resulting list of candidates contained 16 androgen-induced miRNAs, including miR-21 [35]. Androgen-induced miR-21 expression was confirmed in additional AR-positive PCa cell lines by northern blotting. Chromatin Immunoprecipitation (ChIP) found that ligand-activated AR was directly bound to the miR-21 promoter, miPPR-21

[35, 36], indicating direct AR regulation of miR-21 transcription. Walterling and colleagues similarly screened for androgen-responsive miRNAs by microarray analysis in LNCaP sublines and VCaP cells [37]. In support of our data, they confirmed that miR-21, miR-29a, and miR-29b were induced in an androgen-dependent fashion. They also found androgen induction of miR-141, miR-22, and miR-494 expression. In a third study, Jalava and colleagues performed a screen for miRNAs that were differentially expressed in CRPC cases and which could also be regulated by androgens in PCa models. This study found miR-21, miR-32, miR-590-5p, and miR-148a to be upregulated under these conditions [38]. Other androgen-induced miRNAs include miR-125b [39] and miR-338 [40]. It is important to emphasize that expression of these androgen-induced miRNAs are enhanced by, but are not dependent upon, the activated AR. For example, these miRNAs are known to be expressed in non-prostatic tissues which lack the AR. To date there have been no reports of miRNAs which are exclusively expressed in the prostate or which are fully dependent upon the AR for expression. It is notable that the AR can act as both a transcriptional activator and repressor. We did not identify any androgen-suppressed miRNAs in our study [35]; however, Walterling and colleagues found miR-1225-5p, miR-10a, miR-194, miR-221, miR-630, miR-638, and miR-940 to be suppressed by androgens [37]. Androgen suppression of miR-221 was also found in two other high throughput studies [38, 40]. Thus, androgen appears to significantly regulate a small handful of miRNAs.

While the AR is clearly regulating miRNA gene expression, it has also been reported that the AR itself is the target of miRNA regulation. In a protein lysate microarray study, Östling and coworkers screened 1,129 miRNAs to reveal 52 miRNAs which reduced AR protein expression and 19 miRNAs which caused increased AR protein levels [41]. This work also identified a broadly expressed form of AR mRNA with an extended 3'UTR of approximately 7 kilobases (kb). Thirteen miRNAs were shown to functionally target and repress this extended 3'UTR region. An inverse correlation between AR and two of these miRNAs, miR-34a and miR-34c, was also observed in samples from malignant PCa epithelia. These findings are suggestive of loss of AR regulation by miR-34 family members during PCa progression.

## 18.3 miR-21

Of the androgen-regulated genes identified thus far, miR-21 is clearly the best characterized. It was one of the first miRNAs identified in human cells [7] and was recognized early as the most commonly overexpressed miRNA in cancer [28]. The MIR21 gene is located on the long arm of chromosome 17 in a region which is commonly amplified in cancer [42, 43] and its expression is induced by multiple cancer-associated pathways [35, 36, 44–46]. Moreover, miR-21 is known to target broad cellular survival, proliferative, and metabolic pathways.

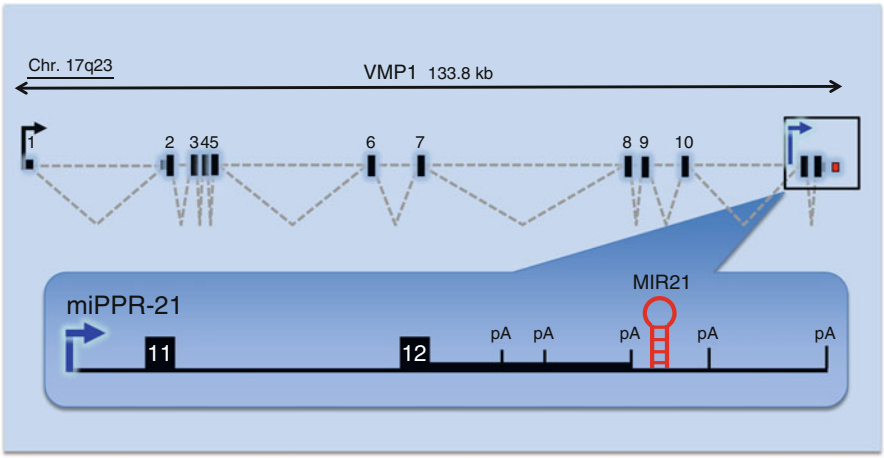


**Fig. 18.1** Genetic organization of VMP1 and MIR21 loci at chromosome 17q23. VMP1 coding exons are depicted as *black boxes*. The VMP1 3'UTR is differentiated by means of a *narrower black box*. MIR21 gene is represented as a *red narrow box*. *Gray dashed* lines are located at intronic sequences. Promoters are indicated through *black* (VMP1) and *blue* (miPPR-21) *arrows*. The inset magnifies the pri-miR-21 region with exons numbered in *white* and polyadenylation sites represented as (pA). The miR-21 hairpin is represented in *red*. The non-spliced pri-miR-21 transcript primarily utilized the 4th pA signal from the left, whereas the VMP1-miR-21 transcript preferentially utilizes the rightmost pA signal

### *18.3.1 The MIR21 Gene*

Approximately half of all miRNAs are located in intergenic regions and some exist within policistronic transcripts. The remaining miRNAs map to known intronic regions of protein coding genes, with a few lying within exons. Finally, a very limited set of miRNAs have been mapped to the 3'UTR of protein coding genes. For instance, miR-198 is located within the 3'UTR of the FRP gene [47] and miR-BHRF1-1 and miR-BHRF1-2 are both found within the 3'UTR of the Epstein-Barr Virus BHRF1 gene [48]. Interestingly, miR-21 is similar to these unique miRNAs as it is located immediately downstream of the 3' terminus of the VMP1 coding gene (also known as TMEM49) [49] (Fig. 18.1). While separate promoters have been identified for VMP1 and pri-miR-21 (Fig. 18.1, blue arrows), these two genes overlap for approximately 4 kb of genomic sequence and the resulting transcripts share over 1 kb of common RNA sequence. The VMP1 transcript, which initiates over 130 kb upstream of the miR-21 hairpin, is spliced to produce an approximately 2.5 kb mRNA that can be polyadenylated as close as 194 bp upstream of the miR-21 hairpin [50] (Fig. 18.1, inset). On the other hand, primary miR-21 transcripts (pri-miR-21) initiate within introns 10 or 11 of VMP1, are not spliced, and extend through the VMP1 polyadenylation signals to include the miR-21 hairpin [36, 44, 49] (Fig. 18.1, inset). Recently we reported that VMP1 can also be commonly alter-

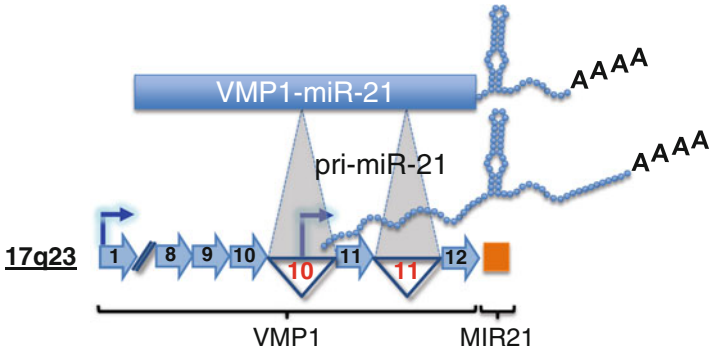


**Fig. 18.2** Two primary miR-21 transcripts: VMP1-miR-21 and pri-miR-21. Schematic of two well-characterized primary transcripts encompassing miR-21: pri-miR-21 as an unspliced, non-coding transcript and VMP1-miR-21, an alternatively polyadenylated form of the VMP1 coding gene. Terminal poly(A) tails are labeled as "A." Below the transcripts is a schematic representation of the VMP1 and MIR21 gene region. Coding exons are depicted as *light blue arrows* whereas the two last intronic regions of VMP1 are highlighted as *inverted triangles*. Promoters and transcription start sites are indicated as *dark blue arrows*. The *orange square* represents the genetic location of the miR-21 hairpin

natively polyadenylated to produce extended transcripts which utilize the polyadenylation signals downstream of the miR-21 hairpin, thus producing an alternative pri-miR-21 transcript termed VMP1-miR-21 [50] (Fig. 18.2). Therefore, there are at least two independent mechanisms for the expression of miR-21. Remarkably, the overlapping pri-miR-21 and VMP1-miR-21 utilize different polyadenylation signals. The non-spliced pri-miR-21 transcript is preferentially polyadenylated by the most distal poly(A) signal, with respect to the miR-21 hairpin, while the spliced VMP1-miR-21 utilizes the proximal signal. This likely reflects known effects of local introns and splicing on polyadenylation [51, 52].

VMP1 was initially identified as a protein of 406 amino acids induced by acinar cells in response to acute pancreatitis [53]. Similarly VMP1 mRNA was increased in kidney after transient ischemic injury and was therefore surmised to be a stress-induced protein [53]. As for its role in cellular biology, expression of VMP1 promotes the formation of vacuoles and precedes cell death [53]. Several lines of evidence support its role in the poorly understood phenomenon of autophagy [54–59]. This fact notwithstanding, significant levels of VMP1 are present in several tissues under physiologic conditions [53]. In a recent report, VMP1 was shown to mediate the nutrient-replete autophagy triggered by oncogenic K-RAS. In the first attempt to decipher the mechanism of VMP1 transcriptional induction, the new pathway AKT1-GLI3/p300-VMP1 was identified [60]. This study found the histone acetyltransferase p300 to be part of the circuit that regulates VMP1. Supporting their data, we also observed that acetylation is an important regulator of the VMP1 transcript as well of the mature miR-21 [50]. In summary, the MIR21 gene locus is unique and complex, with the capability of generating multiple different primary

miR-21 transcripts which can be regulated by at least two different gene promoters and by alternative polyadenylation.

### *18.3.2 miR-21 Transcription*

Transcriptional initiation of miR-21 expression has largely been characterized for the non-spliced pri-miR-21 transcripts, which originate within the terminal VMP1 exons (Fig. 18.1). Three separate putative promoters have been identified in this region. The most characterized promoter, miPPR-21, is located within intron 10 of VMP1 and produces a 4.3 kb gene product [36]. The functional activity of this promoter region has been validated [61], and northern blotting indicates that the non-spliced 4.3 kb product is the most abundant form of pri-miR-21 [50]. Two additional overlapping but functionally independent promoters have also been identified within intron 11 [44, 49]. Multiple upstream pathways have been found to directly induce miR-21 expression. Transcriptional induction by AP-1 transcription factors likely accounts for the high levels of miR-21 expression in cancerous tissues [36, 61]. AP-1 activating agents can also stimulate VMP1 and VMP1-miR-21 [50]. In addition, the IL-6/STAT3 pathway contributes to pri-miR-21 expression and leads to elevated miR-21 levels in cancer and inflamed tissues [44]. Similar stresses associated with pancreatitis and ischemic injury also induce VMP1 [53]. As described above, the activated AR directly induces miR-21 expression and can also induce elevated VMP1 expression [35, 37, 38, 50, 62]. We therefore believe that aberrant AR activity contributes to the elevated miR-21 levels reported in PCa [28, 35, 63, 64]. Epigenetic forces also contribute to miR-21 gene expression [45, 65]. Two CpG islands are present in the VMP1/miR-21 gene region, one near the miPPR-21 promoter and a second upstream of the VMP1 transcription start site. However, only the miPPR-21 associated CpG island was found to be methylated by bisulfite sequencing [50]. Nonetheless, both VMP1 and pri-miR-21 transcripts were enhanced upon treatment with epigenetic modifying agents [45, 50].

### *18.3.3 miR-21 Processing*

In addition to transcription initiation and alternative polyadenylation, miR-21 levels are directly influenced by miRNA processing and maturation pathways. Several lines of evidence suggest that the Microprocessor is a highly regulated complex that can exist in the small dibasic form, consisting of Drosha and DGCR8, or alternatively as part of a larger complex with multiple accessory proteins [66]. One group of proteins found to interact with the Microprocessor complex are the two highly conserved DEAD-box RNA helicase subunits, p68 (DDX5) and p72 (DDX17). The characteristic DEAD-box motif is thought to confer ATP binding/hydrolysis and RNA binding/unwinding activities to the proteins containing it. Both members of

the family share approximately 90% homology across the conserved core of the protein albeit the N- and C-termini are significantly different (reviewed by [67]). RNA extracts from p68 or p72-deficient mice displayed diminished levels of a subset of 94 miRNAs including miR-21. Restoring levels of p72 with the wild type helicase, but not a mutant lacking the ATPase activity, reestablished the impaired levels of miRNAs [68]. These experiments support that processing of a specific subset of pri-miRNAs, including pri-miR-21, is influenced by p72 RNA unwinding activity.

In an independent system, 29 miRNAs (including miR-21) were commonly diminished either by activated estrogen receptor alpha (ERα) or p72 deficiency, suggesting that ERα may communicate with the p68/p72-Microprocessor complex. Using MCF-7 cellular extracts, Yamagata and coworkers found that ERα interacted with the RNase domain of Drosha, resulting in an inhibition of its processing activity. In this model, p68/p72 was potentiating the ligand-dependent association of ERα with the C-terminal domain of Drosha. The biological significance of this interaction was illustrated by the stabilization of hVEGF mRNA after treatment with estrogens. The results support that VEGF-targeting miRNAs (for example miR-125 and miR-195) were reduced by estrogen-mediated inhibition of pri-miRNA processing. Altogether, this pathway provides a putative indirect mechanism for observed hormone-mediated RNA stabilization and provides evidence for a new nontranscriptional level of miRNA regulation imparted by a steroid receptor [69]. It is logical to surmise that the AR could be involved in a similar pathway. It is known that p68 interacts with AR and that androgens enhance this interaction [70]. However, only a role for p68 as a transcriptional coactivator of the AR complex has been reported, whereas the same observation failed to be reproduced with p72. This finding underscores the possibility that, albeit both proteins present overlapping functions, they could also have distinct and nonredundant roles. Interestingly, p68 has also been shown to enhance pri-miR-21 processing following induction by transforming growth factor β (TGF-β) or bone morphogenetic protein (BMP) signaling. By binding p68, TGF-β and BMP ligand-activated SMAD transducers could be recruited to the Drosha Microprocessor complex resulting in a fast increase of mature miR-21 [71]. Thus, there is some evidence that AR may directly or indirectly influence miRNA processing.

## 18.4 miR-21 Targets and Regulated Pathways

miRNAs, like other signaling components, can have direct or indirect effects on cellular pathways. A direct effect would involve the specific interaction of a miRNA with a target mRNA, causing significant reduction in the levels of the protein. In some cases, such with as lin-4 and lin-14, a single primary miRNA–target interaction produces a given phenotype. In these rare cases, the miRNA-induced phenotype can be replicated by knocking-out or knocking down the target gene alone. However, such focused gene regulation by miRNAs appears to be uncommon in mammalian

systems. Instead, most miRNAs regulate multiple targets in an apparent concerted effort to produce a phenotype. Because miRNA binding is dependent upon the available mRNA milieu, miRNA targets and phenotypic effects may vary depending on the tissue, or tissue state, and the genes which are expressed under those conditions. Therefore, a miRNA may have different target mRNAs in different tissues, cell lines, or conditions.

There are a number of experimental means to interrogate direct miRNA gene regulation. A direct mRNA target would be expected to contain at least one 6–8 bp region of perfect complementarity to the miRNA seed sequence within its 3' UTR. Both direct and indirect miRNA gene regulation can be evaluated by western blot quantification of target protein levels following transfection of a miRNA mimetic or of a miRNA expression vector. This should result in decreased levels of the target protein when compared to a control miRNA. Likewise, one can inhibit miRNA function through transfection of a stabilized miRNA inhibitor or sponge, which should increase target protein levels. However, the presence of a seed sequence and corresponding changes in protein levels following miRNA transfection does not validate direct miRNA regulation. Direct miRNA targeting can be further supported by a 3'UTR reporter assay, where a portion of the 3'UTR region from the target gene is placed downstream of the coding region of a reporter gene, such as luciferase. Using this assay, inhibition or enhancement of reporter gene activity should be observed following the transfection of a miRNA mimetic or inhibitor which specifically targets the 3'UTR, respectfully. Specificity can then be validated by mutation of the seed sequence binding region. While these assays are widely applied and accepted, it is notable that miRNA–target interaction may be influenced by structural differences between the native mRNA and reporter transcripts. Also, the physiologic relevancy of these studies should be carefully weighed as transient transfection can often lead to an extreme excess of miRNA. Other methods, such as RNA pull-down or cross-linking and precipitation can be applied to support direct interactions between a miRNA and mRNA transcript under more physiologically relevant conditions. Below we review a series of genes and pathways which appear to be directly regulated by miR-21 and summarize them, and others, in Fig. 18.3.

### *18.4.1 Direct miR-21 Targets in Cancer-Related Pathways*

To date, over fifty miR-21 targets have been reported in the literature using various cells, tissues, and animal models. However, only a fraction of these are direct targets which have been validated by multiple assays. The most broadly observed and validated miR-21 target today is the Programmed cell death protein 4 (PDCD4). Direct miR-21 regulation of PDCD4 has been supported by western blotting and 3'UTR reporter assays by several laboratories in numerous human and mouse cells, tissues, and disease models [72–82]. PDCD4 is a ubiquitously expressed gene which is commonly down-regulated in many cancer types (reviewed by [83]). It was originally found as a gene which was induced during apoptosis [84], but it has since been

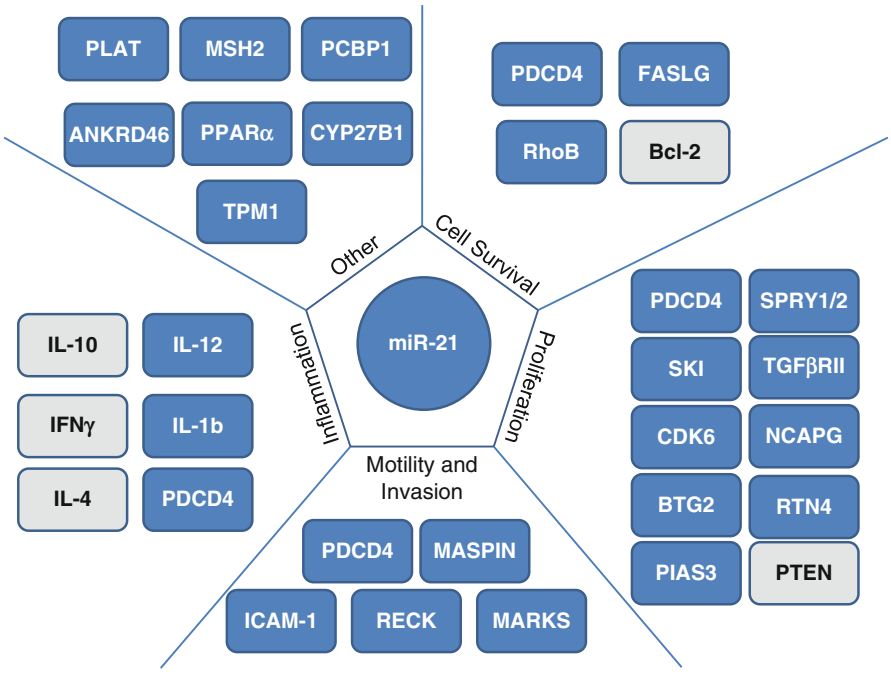


**Fig. 18.3** miR-21 targets and pathways. Validated direct miR-21 targets are summarized in *blue rectangles* and grouped into functional groups. A number of the targets are multifunctional in nature and are therefore present in more than one category. Finally, those targets colored in *light gray rectangles* represent genes which may be indirectly regulated by miR-21

functionally linked to neoplastic transformation [85], invasion [86], tumor progression, the cell cycle, and differentiation [87]. The multifunctional nature of PDCD4 may be explained by its role as a translational inhibitor through interactions with eIF4Aa and eIF4G [88, 89]. In addition to PDCD4, many validated miR-21 targets play a role in cell cycle and proliferation. Using proteomics, Yang and colleagues identified NCAPG, a protein involved in mitotic chromosomal condensation, as a miR-21 target [90]. The cell cycle progression genes BTG2 and CDK6 are also direct miR-21 targets [73, 91]. Two factors involved in TGFβ signaling and cellular growth regulation, TGFβRII and SKI, have also been validated as miR-21 targets [91, 92]. Two related antagonists of the fibroblast growth factor (FGF) pathways, Protein Sprouty homologues SPRY1 and SPRY2, have also been reported by multiple groups as miR-21 targets [91, 93, 94]. The neurite outgrowth factor RTN4 is also a candidate target for direct miR-21 regulation [90]. In addition to cell cycle regulation and proliferation, miR-21 also appears to play an important role in cell survival and apoptosis. This is particularly illustrated by PDCD4, as described above, as well as by RhoB and FASLG [91, 95–97]. miR-21 expression can also modulate cell motility and invasion. Elevated miR-21 reduces cellular adhesion

through downregulation of MARKS and ICAM-1 [98, 99] and can also activate matrix metalloproteases (MMP) by suppressing the MMP-inhibitors TMP3 and RECK [80, 91, 97, 100]. Other cancer-associated pathways have also been reported as direct miR-21 targets including the DNA repair factor MSH2 [101, 102], the metabolic enzymes CYP27B1 and PPARα [103, 104], Tropomyosin alpha-1 chain (TPM1) [99, 105], Poly(rC)-binding protein 1 (PCBP1) [106], and Tissue Plasminogen Activator (PLAT) [98].

The role of miR-21 in proliferation and survival is best reflected in recent transgenic mouse models of cancer. Hatley and colleagues evaluated a K-RAS driven model of non-small cell lung cancer in mice that overexpress the miR-21 gene and in mice with deletion of the miR-21 gene [91]. The results clearly demonstrated that miR-21 expression influenced the number of tumors, the tumor size, and the tumor incidence. Moreover, immortalized mouse embryo fibroblast cells (MEFs) derived from these miR-21 knockout (KO) mice were more sensitive to doxorubicin, when compared to wild type cells, and therefore confirmed previous studies indicating a role for miR-21 in drug resistance [107]. In a separate miR-21 KO mouse model it was found that miR-21 expression also affected chemically induced skin cancer [93]. Specifically, fewer miR-21 KO mice developed papillomas when compared to wild-type littermates, indicating a role for miR-21 in carcinogenesis. The miR-21 KO mice also had fewer papillomas. Further, the keratinocytes of the KO mice were less proliferative and had increased rates of apoptosis following treatment with phorbol esters. Finally, in a third transgenic mouse model, Medina and colleagues studied whether elevated expression of miR-21 alone was sufficient to cause cancer [27]. Using a Nestin-driven Cre-recombination cassette to activate gene expression, they selectively overexpressed miR-21 in a limited series of tissues, including hematopoietic cells. Within two months the transgenic mice began to develop a pre-B malignant lymphoid-like phenotype with large transplantable tumors. Moreover, the tumors regressed when miR-21 was inactivated. These results indicate that miR-21 is a true oncogene. In summary, there is evidence from numerous animal models that miR-21 can contribute to cancer initiation, growth, and therapeutic resistance.

### *18.4.2 Direct miR-21 Targets in Inflammatory Pathways*

In addition to cancer, there is significant evidence that miR-21 plays a role in inflammation and inflammatory disease. miR-21 expression is stimulated by a number of inflammatory pathways, allergenic stimuli, and injury [36, 44, 79, 108–112]. In a model of leprosy, miR-21 was found to be highly upregulated and to directly diminish CYP27B and IL-1β [104]. In separate mouse models of asthma, miR-21 expression was highly induced by several allergens [110]. These studies led to the identification IL-12p35 as a direct target of miR-21. Subsequent studies using miR-21 KO mice validated its role in IL-12 expression and revealed that it caused an imbalance in Th1 and Th2 response to allergen challenge [113]. The loss of miR-21 and subsequent overexpression of IL-12 correlated with an enhanced Th1 response with

increases in IFNγ and CXCL9 in addition to decreases in IL-4 and CCL17 levels. PDCD4, a direct miR-21 target, also influences expression of both IL-4 and IL-10 [114]. These pathways, particularly IL-12, may shed light onto the reduced interstitial fibrosis observed in miR-21 KO mice in response to kidney injury [109].

### *18.4.3 miR-21 Targets in the Prostate and PCa*

To date, the role of miR-21 in AR signaling and prostate tumorigenesis has been limited to human PCa cell lines and xenografts. We have previously reported that elevated miR-21 expression was sufficient to impart castration-resistant growth to LNCaP cells and xenograft tumors [35]. Using these prostate cancer cell line models, we also studied the role of miR-21 in androgen-induced cell growth. Our results estimate that miR-21 may contribute to approximately 20% of androgen-induced growth. However, the role of miR-21 in prostatic development and PCa initiation has not yet been determined. Moreover, the pathways which miR-21 regulates in AR positive tissues, such as the prostate, are just starting to be studied. Li and coworkers confirmed MARCKS, PDCD4, and TPM1 are regulated by miR-21 in a DU145 xenograft model [115]. We anticipate that miR-21 targets may vary under different conditions, such as prostatic development, inflammation, and cancer. This is supported by some surprising results from the miR-21 KO mouse models of kidney fibrosis [109]. Expression profiles from normal healthy kidneys of miR-21 KO and wild type mice did not show significant differences in the level of mRNAs containing miR-21-binding sites. However, following kidney injury, substantial changes in miR-21 target mRNAs were observed between these two groups. This indicates that miR-21, as well as other miRNAs, may play a larger role in injury or stressor response than in the normal growth and development of tissues.

## 18.5 miR-21 in the Prostate and PCa

One of the biggest improvements in the early diagnosis of PCa was realized with systematic screening through a combination of serum prostate-specific antigen (PSA) quantification and digital rectal exam, which is credited with the early identification of cancer at a curable state [116, 117]. This fact has translated into enhanced therapeutic success and survival for many patients [118–120]. However these and other studies [121] also reflect the overdiagnosis and overtreatment of PCa today. Thus the true value of PSA screening and early treatment have been called into question and a practice of careful observation or “watchful waiting” is being widely evaluated (reviewed by [122]). Nevertheless, PCa remains as the second leading cause of cancer death in men the United States [123]. Therefore, the PCa field is still in dire need of innovative treatments, yet-to-be identified targets, biomarkers, and novel prognostic factors that could ideally predict the tumor

malignancy, stratify risk, monitor therapeutic response, and ultimately cure metastatic disease. There is some preliminary evidence that miRNAs may have value in some of these categories. Below we focus on studies which have found miR-21 expression within PCa specimens.

### *18.5.1 miR-21 in PCa Tissues*

Some of the earliest studies of miRNA expression revealed elevated miR-21 levels in PCa [28]. We initially quantified its expression in 10 tumor-normal matched specimens from early grade radical prostatectomies (T1 to T2c). Despite the small size of our collection, we reported an average increase of 3.52-fold in the evaluated samples [35]. However, in our small study, miR-21 expression did not correlate with common clinical parameters such stage, grade, or PSA levels. By using an independent data set with information on miR-21 levels from 3 benign hyperplastic prostates (BPH), 5 hormone naïve PCa, and 4 CRPC, we also found that miR-21 expression was increased in CRPC [61]. Other studies have also found elevated miR-21 levels in patients with PCa and CRPC when compared with BPH [38]. Li and colleagues evaluated the potential of this miRNA as a predictor for the risk of biochemical recurrence. Using locked nucleic acid probes and in situ hybridization against a bank of formalin-fixed tissues from 168 radical prostatectomy patients, they observed that elevated miR-21 staining was associated with adverse histological features and PSA recurrence following surgery [115]. However, the impact of miR-21 as an independent prognostic marker appears to be modest (reviewed in [124]). Additional studies with larger patient populations and additional quantitative techniques may shed more light on the role of miR-21 and other miRNAs in stratifying patient risk for recurrence following surgery.

### *18.5.2 miR-21 in Other Biological Fluids*

miRNAs can also be routinely profiled from other bodily fluids, for instance urine and blood. This practice presents obvious advantages, for example, by reducing invasive techniques such as biopsy. Circulating miRNAs have been found as soluble-free miRNA or within encapsulated in lipid structures, such as exosomes [125], as well as with high-density lipoproteins (HDL) [126], as part of protein complexes [127], and within circulating tumor cells [128]. Thus, cancer-associated miRNAs are present and stable in the serum and may represent a valuable new class of PCa biomarkers. Here we focus specifically on studies which evaluate miR-21 in clinical specimens from PCa patients. Zhang and coworkers specifically quantified serum miR-21 in a small set of patients with BPH, localized PCa, androgen-dependent prostate cancer (ADPC), and CRPC. The highest levels of miR-21 expression were observed in

patients with CRPC and in those with ADPC with a serum PSA > 4 ng/ml. A subgroup of the CRPC patients which had been unsuccessfully treated with docetaxel also had elevated miR-21 serum levels when compared to chemotherapy-sensitive patients [62]. In an independent study, Shen and colleagues measured the absolute copy number of four miRNAs in the plasma of PCa patients with defined risks of progression. Elevated miR-21 was observed in patients with high risk "Cancer of the Prostate Risk Assessment" (CAPRA) scores when compared to those with intermediate or low scores. Similarly, increased miR-21 was found in patients with intermediate or high-risk D'Amico scores when compared to those with low risk. Finally, it was the combination of four miRNAs (miR-20a, miR-21, miR-145, and miR-221) that significantly distinguished patients with high versus low risk D'Amico scores [129]. Consistently, elevated miR-21 levels were found in the plasma of patients with localized, locally advanced, and metastatic PCa when compared to healthy donors [64]. The highest miR-21 levels were associated with the metastatic group. Nonetheless, none of the miRNAs analyzed in this study reached the power of PSA to distinguish metastatic from localized PCa. In summary, these preliminary studies support that miR-21 is elevated in PCa and that serum levels of miR-21 and other miRNAs may provide a means to identify patients with higher risk disease. We anticipate that future research studies will soon determine the clinical value of miRNAs as biomarkers in the management of PCa.

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# Chapter 19
# Androgen-Dependent Oncogenic Activation of ETS Transcription Factors by Recurrent Gene Fusions in Prostate Cancer: Biological and Clinical Implications

**Albert Dobi, Taduru Sreenath, and Shiv Srivastava**

**Abstract** Half of all prostate cancers in the Western countries harbor gene fusions that involve regulatory sequences of the androgen receptor (AR)-responsive genes (predominantly *TMPRSS2*) and protein coding sequences of nuclear transcription factors of the ETS gene family (predominantly *ERG*). This leads to unscheduled androgen-dependent expression of ETS-related transcription factors in tumor cell-specific manner. Extensive evaluations of *ERG* alterations at genome, transcript, and protein levels demonstrate unprecedented specificity of ERG for detecting prostate tumor cells. Assessment of *ERG* alterations in combination with other common prostate cancer gene alterations (*AMACR*, *PCA3*, *p63*) has potential in improving CaP diagnosis. Utility of ERG in assessing the clinical behavior of prostate cancer is uncertain. Strong correlation of *ERG* expression with known androgen-responsive genes in prostate tumors has potential in developing gene panels inclusive of ERG for monitoring androgen receptor functional status in the disease continuum. Studies focusing on oncogenic functions of *ERG* point to its involvement in: abrogating differentiation; facilitating cell invasion and epithelial to mesenchymal transition; and disrupting epigenetic, inflammatory, and DNA damage control mechanisms. Therapeutic targeting of *ERG* or ERG interacting proteins, such as PARP hold promise in developing new strategies for the treatment of prostate cancer. In summary multipronged evaluations of the *ERG* in CaP continue to reflect the critical role of this prevalent oncogenic activation in a CaP.

**Keywords** Prostate cancer • Hormonal regulation • Androgen receptor • Gene fusion • ETS transcription factors • ERG oncogene • ERG oncoprotein

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## 19.1 Introduction

Prostate cancer (CaP) is the most common non-skin cancer of men in the USA. Despite recent advances in early detection and continued refinements in treatment strategies, CaP is still the second leading cause of cancer mortality in American men [1]. Intense investigations of the CaP-specific gene expression and/or mutational alterations are beginning to make a significant impact on improving the understanding of this complex and heterogeneous disease. Continued discovery and evaluation of causal molecular alterations are leading to the development of more precise strategies in diagnosing, prognosing, and treating CaP [2–10].

Common CaP gene alterations leading to loss of function include *GSTP1* [11], *NKX3.1*, *PTEN*, *p53*, and *SPOP* [12, 13], whereas those genes exhibiting gain of function include *C-MYC* [14], *PI3K*, *AKT* [13, 15], *AR* [16, 17], and the ETS-related oncogenes (*ERG*, *ETV1*, and *ETV4*) [5, 9, 18, 19]. Functions of the *AMACR* and *PCA3* genes that are overexpressed in most prostate cancers remain to be understood [20]. Dysfunctions (both gain and loss) of the male hormone receptor, the androgen receptor (AR)-mediated signaling, play important roles in CaP development or progression [3, 16]. Androgen-dependent oncogenic activations, as a result of the gene fusions of the AR-responsive promoters (primarily *TMPRSS2*) and the ETS gene family members (predominantly *ERG*), represent highly prevalent causal alterations in CaP. This review focuses on biological and clinical aspects of the *ERG* oncogenic activation in prostate cancer.

## 19.2 Prevalence of the *ERG* Oncogene Activation Due to Recurrent Gene Fusions in Prostate Cancer

Evaluations of CaP epithelium transcriptomes suggested the elevated expression of *ERG* proto-oncogene in CaP [21–23]. Quantitative analyses of *ERG* mRNA in a large patient cohort of matched benign and prostate tumor cells highlighted the frequent tumor cell-specific overexpression of *ERG* in 60–70% of CaP patients [23]. The landmark report of recurrent gene fusions between the androgen-regulated *TMPRSS2* gene promoter and coding regions of *ERG* unraveled the underlying mechanism of *ERG* overexpression highly prevalent in CaP [24]. Further assessment of gene fusions revealed a variety of gene fusions between AR-regulated gene promoters (*TMPRSS2*, *SLC45A3*, *NDRG1*, *Herv-K22q11.23*, *CANT1*, and *KLK2*) and coding sequences of ETS gene family (*ERG*, *ETV1*, *ETV4*, and *ETV5*) [25, 26]. Promoter analysis of the *TMPRSS2* gene (loc:21q22.3) revealed the presence of binding sites for AR. Evaluation of a further upstream AR enhancer suggested the recruitment of AR in CaP cells [27]. While ETS-related transcription factors are predominantly oncogenic, activation described in CaP, androgen-dependent activation of other oncogenes, *RAF* and *K-RAS*, has also been described in smaller subsets of CaP. Of note, CaP harboring *RAF* fusions exhibited highly aggressive phenotype [28].

A comprehensive description of gene fusions in CaP has been covered in earlier excellent reviews [5, 19, 25].

Gene fusion types have been classified on the basis of the 5′ sequences and predicted 3′ coding sequences of fusion partners confirming the highest frequency of *TMPRSS2–ERG* fusion type [29–34]). However, the heterogeneity or homogeneity of fusion transcripts in the multifocal context of CaP remains to be better understood. Evaluation of the presence or absence of *TMPRSS2–ERG* fusion positive tumor foci transcripts suggested homogeneity within the same tumor focus and heterogeneity between various foci [26, 35–40].

The most common *TMPRSS2–ERG* fusion junction in CaP described to date results in the deletion of the first 32 N terminus amino acid coding sequences of *ERG* [24, 31, 41]. Specific *TMPRSS2–ERG* exon fusion transcripts have been shown to associate with poor prognostic features of CaP (PSA recurrence and seminal vesicle invasion) [29]. Cloning and sequencing of the commonly expressed full-length transcripts from a cDNA library of pooled RNA from six *TMPRSS2–ERG* positive patients revealed two major forms of *TMPRSS2–ERG* cDNA sequences [31]. The near full-length *TMPRSS2–ERG* transcripts (*Type I*) coded for ERG 1, 2, and 3 protein products containing deletion of first 32 amino acids, intact protein–protein interaction (pointed/SAM) and DNA-binding (ETS) domains, and nuclear localization signal [31, 41]. Surprisingly, shorter *TMPRSS2–ERG* transcripts (*Type II*) were also commonly expressed in the *TMPRSS2–ERG* positive CaP cells. The *Type II* transcripts (*ERG8* and *TEPC1*) lacked the ETS DNA-binding domain and nuclear localization signal but contained *Type I* amino terminal configuration with 32 amino terminal deletion and an intact SAM domain. The *Type II* transcripts contained unique carboxy terminus (Fig. 19.1). Assessment of *Type I* (*ERG1*, *2* and *3*) and *Type II* transcripts levels (*ERG8* and *TEPC1*) in RNA specimens from laser capture microdissected paired normal and normal cells revealed that higher *Type I* over *Type II* ratio correlated with poor prognosis (poorly differentiated tumor cell morphology, elevated Gleason sum, positive margin, and biochemical recurrence) [31]. Evaluations of *TMPRSS2–ERG Type I* and *Type II* encoded ERG protein products for primary structure, subcellular localization, and transcription factor activity suggested that *Type I* ERG proteins are functionally active. Since the *Type II* encoded ERG proteins are cytoplasmic and inactive as transcription factor, a dominant negative function has been suggested for the *Type II* ERG. It has also been reported that endogenous ERG mRNA expression is induced in CaP cells that harbor *TMPRSS2–ERG* fusions leading to overexpression of endogenous *ERG* transcripts in addition to *TMPRSS2–ERG* transcripts [42]. Therefore, a better understanding of the *ERG* transcripts, as well as function of the ERG protein products is warranted in CaP.

Towards understanding of mechanisms leading to recurrent gene fusions in CaP, cell culture-based experiments have shown that male hormone in combination with gamma radiation exposure can induce *TMPRSS2–ERG* fusions [43–45]. It has been proposed that AR-mediated chromatin looping may bring the DNA sequences of fusion partners into close proximity. Under genotoxic stress conditions [43, 44] compromised recombination events lead to genomic rearrangements [45]. In summary, the proposed mechanisms highlight the role of androgen receptor-mediated

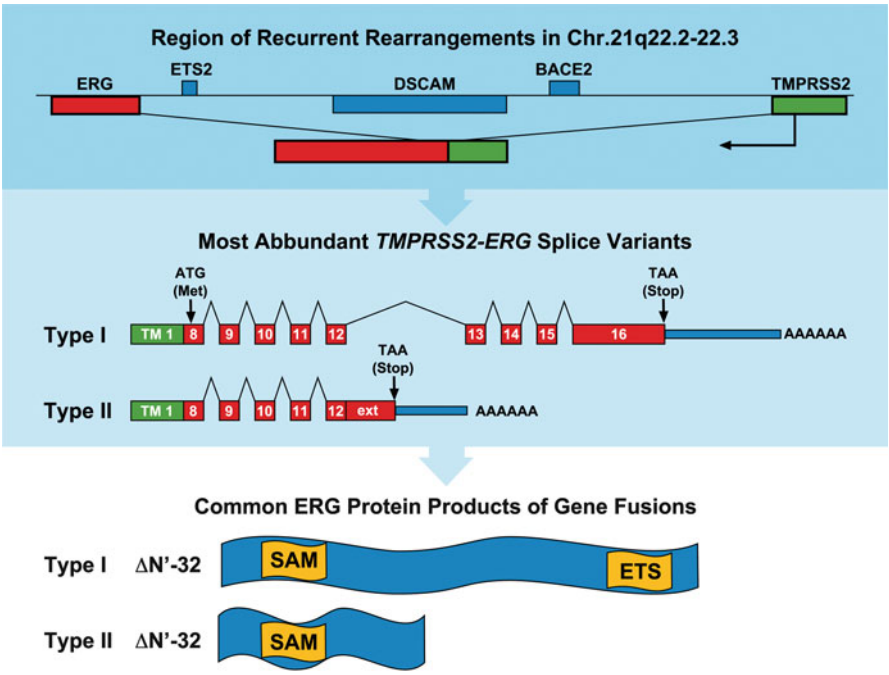


**Fig. 19.1** Recurrent rearrangements between the *ERG* and *TMPRSS2* gene loci on chromosome 21q give rise to *TMPRSS2–ERG* fusion transcripts in prostate cancer cells. The most abundant splice variants of fusion transcripts depicted on the figure represent longer (*Type I*) and shorter (*Type II*) splice variants. Common ERG protein products of fusion transcripts lack a 32 amino acid N-terminal segment (ΔN′-32). Type I protein products include the SAM (pointed) domain as well as the ETS DNA-binding domain of ERG. In contrast, Type II protein products lack the ETS DNA-binding domain

early genetic defects that lead to the activation of common oncogenic alteration, *TMPRSS2–ERG* fusion, in prostate cancer.

As noted above *ERG* oncogenic activation is a common genomic alteration in CaP in Western countries. However, differences of *ERG* alteration frequencies between African American and Caucasian American patients have been observed in previous studies [23, 31, 46, 47]. Further, much lower frequency of *ERG* alterations has been described in CaP from patients of Asian origin [47]. A recent comprehensive study has examined ERG frequencies in the multifocal tumor context of African American and Caucasian patients matched for age, pathologic grade, and stage [48]. Evaluations of all tumor foci in whole-mount prostate sections showed a significantly higher frequency of ERG positive prostate tumors in Caucasians American patients. Intriguingly higher frequency of the ERG oncoprotein expression was noted between the index tumors of Caucasian Americans (63.3%) than African Americans (28.6%). This study further noted that in African American patients the higher grade index tumors were predominantly ERG negative. Thus, ERG negative

index tumors may indicate less favorable outcome in African American patients. ERG typing of prostate tumors may indeed help to unfold the biological differences between ethnic groups.

## 19.3 Causal Role of the *ERG* Oncogenic Activation in Prostate Cancer

Gene fusions involving *ERG* (*EWS–ERG*) or its close homolog *FLI* (*EWS–FLI*) were initially reported in 5% or 95% Ewing's sarcoma (EWS), respectively [49]. *ERG* as a fusion partner has also been reported in certain acute myeloid leukemias (AML) [50, 51]. Fusion of *ERG* to other genes in these malignancies results in a chimeric protein [52]. In these chimeric oncoproteins, transactivation and DNA binding domains of fusion partners result in abnormal regulation of downstream genes. *ERG* oncogenic activation without rearrangement has also been noted in cytogenetically normal AML with poor prognosis [53].

The involvement of *ERG* in epigenetic reprogramming by activating histone deacetylase 1 (*HDAC1*) and enhancing WNT signaling is the earliest recognized oncogenic function of *ERG* in prostate cancer [54]. The results of further investigations suggested direct involvement of *ERG* in disrupting the normal epigenetic regulatory function of *EZH2* gene that is a key component of the polycomb group II (PcG II) [6, 55, 56]. Disrupted function of PcG II, including elevated expression of *EZH2* results in epigenetic reprogramming, inactivation of protective genes, and the activation of oncogenes. Among epigenetic targets of ERG direct suppression of histone acetyl transferases (*HAT*s) and activation of histone deacetylases (*HDAC*s) have been highlighted recently [57–59].

*ERG* mediated induction of cellular invasion and invasion-related genes such as matrix metalloproteinases (*MMP1*, *MMP3*, *MMP9*, and *ADAM19*), the urokinase plasminogen activator (*PLAU*), chemokine receptor type 4 (*CXCR4*), and the plasminogen activator inhibitor type1(*PAI-1*) genes have been widely reported in CaP cell models with ectopic expression of *ERG* [60–64]. Direct activation of osteopontin (*OPN*) by *ERG* has been described recently [65]. The *OPN* gene is an established regulator of metastasis and elevated expression of *OPN* has been shown to correlate with metastasis in human cancers including prostate cancer [65, 66].

One of the *ERG* downstream targets in the *TMPRSS2–ERG* context of CaP cells is a widely studied oncogene, *C-MYC* [67]. ERG binds to the *C-MYC* P2 promoter downstream ETS element resulting in the activation of *C-MYC* expression. Quantitative evaluation of *ERG* and *C-MYC* expression levels in LCM selected human prostate cancer cells with well-differentiated cellular phenotype revealed a strong positive correlation. Moreover, inhibition of *ERG* or *C-MYC* in *TMPRSS2–ERG* positive CaP cell culture model, resulted in an elevated expression of prostate differentiation-associated genes such as *KLK3*(PSA) and *SLC45A3*(Prostein). Knockdown of *ERG* and *C-MYC* also affected cellular morphologies consistent with abrogation the prostate epithelial differentiation program [41, 67, 68]. These

observations together suggested that activation of *C-MYC* by ERG may be one of the important mechanisms of prostate tumorigenesis.

Investigation of the *ERG* activated WNT signaling pathways revealed a role for *ERG* in facilitating the epithelial-to-mesenchymal transition (EMT), a phenotypic and functional transition of epithelial cells described in oncogenic transformation and metastasis [58, 69, 70]. Follow-up studies on whole genome scales has revealed enrichment of canonical and non-canonical WNT signaling genes in association with *ERG*-mediated tumorigenesis [71]. These studies highlighted coexpression of *WNT11* and *FZD4* in high-grade prostate tumors with *ERG* rearrangement [58, 72]. *ERG* may also facilitate EMT by downregulating the *E-cadherin* gene through the activation of its negative regulators such as *ZEB* [70] and *SNAIL* pathways in prostate cancer [73]. Disruption of cell adhesion and polarity may contribute to increased invasive characteristics of *ERG* expressing prostate cancer cells.

*ERG* may also contribute to the activation of inflammatory pathways in prostate cancer [74]. A recent report has described the activation of *NF-κB* by *TLR4* in response to *ERG* overexpression resulting in compromised immune response to Gram negative bacterial infection models [75]. Another study has shown that *ERG* negatively regulates the 15-hydroxy prostaglandin dehydrogenase gene (*HPGD*), encoding a key enzyme in antagonizing COX-2 function [76]. Oncogenic activation of *ERG* may indeed augment inflammatory signaling pathways. Within the initial phase of prostate tumorigenesis, genotoxic signals including inflammatory signals may disrupt DNA damage-sensing repair pathways resulting in genomic instability including the generation of *TMPRSS2–ERG* fusions [43–45]. A recent report suggests direct interaction between ERG and the DNA damage repair enzyme Poly (ADP-ribose) polymerase (PARP) revealing the potential role of ERG in genomic instability [77].

In majority of prostate tumors, *ERG* is expressed under the control of the androgen-regulated *TMPRSS2* promoter due to genomic fusion events. To understand the in vivo function of *ERG* in prostate cancer, *ERG* transgenic mice models have been developed targeting *ERG* expression to the prostate under the control of the androgen inducible rat probasin promoter [41, 61–63]. In these in vivo models preinvasive lesions of prostate epithelial cells, resembling prostatic intraepithelial neoplasia (PIN), were observed. However, adenocarcinoma was not found in these models [61, 62]. Although, in other reported *ERG* transgenic mice models PIN phenotype was not observed, transgenic expression of *ERG* in combination with *AKT* overexpression or loss of *Pten* have been shown to accelerate the development of adenocarcinoma [41, 63]. Transformation of adult prostate epithelial cells combining *ERG* and pAKT or *AR* has been shown to recapitulate prostatic adenocarcinoma in prostate tissue regeneration models [68, 78]. Genome-wide assessment of genomic alterations in human prostate cancers revealed the association of *TMPRSS2–ERG* fusion events with chromosomal deletions of the *PTEN* (10q23.31) and the *p53* (17p13.1) loci and focal deletions within the 3p14.1–3p13 region known to harbor various tumor suppressor genes [79]. A recent comprehensive evaluation of prostate cancer-associated genomic alterations has also revealed presence of *PTEN* and *p53* alterations in the *TMPRSS2–ERG* positive prostate tumors [80]. In contrast, a

recently described *SPOP* mutations was exclusively present in a subset of *TMPRSS2–ERG* negative tumors [13].

While continuing efforts are being made to clarify the oncogenic function of *ERG*, accumulating data point to the involvement of *ERG* in abrogating differentiation; facilitating cell invasion and epithelial to mesenchymal transition; and disrupting epigenetic, inflammatory, and DNA damage control mechanisms. Moreover, studies also highlight direct involvement of ERG in aberrant function of AR in prostate tumorigenesis [6, 34, 67, 81, 82].

## 19.4 Mechanisms of Hormonal Regulation of ETS Gene Fusions in Prostate Cancer

Due to the therapeutic potential of emerging ERG networks, the androgenic regulation of *TMPRSS2-–ERG* fusions has been in the focus in recent years (Fig. 19.2). Evaluation of regulatory elements in promoter regions of *KLK3*(PSA) and *SLC45A3*(prostein) genes indicated overlap between ERG and AR binding sites. This was shown by increased expression of the *KLK3* or *SLC45A3* mRNA by ERG knock-down along with increased binding of the AR to these regulatory sequences [67]. Mapping of the AR recruitment to regulatory regions of chromosomes 21 and 22 by chromatin immunoprecipitation (ChIP) and gene expression profiling assays highlighted AR binding to noncanonical sequences including in the *TMPRSS2* gene along with the association of cooperating factors such as GATA2 and OCT1 in both *TMPRSS2–ERG* fusion and non-fusion contexts [27]. Thus, these findings suggest a hierarchic role for pioneering transcription factors facilitating AR recruitment to canonical [83] as well as noncanonical AR binding sites within the prostate cancer context [27]. Further assessment of the genome-wide recruitment of AR and ERG in CaP cells and CaP tissues revealed a global pattern of overlapping ERG and AR binding sites [6]. This study explained these intriguing observations by suggesting that *ERG* may negatively regulate its own expression by disrupting AR signaling through interference of AR recruitment to cognate elements.

In contrast to *ERG*, a less frequent ETS-related fusion partner, *ETV1* may positively cooperate with AR transcription factor function. *ETV1* has been shown to bind to the *KLK3* (PSA) upstream AR-responsive enhancer facilitating the transcriptional activation of the *KLK3* (PSA) [84]. Thus, *ETV1* facilitates the superinduction of transcription through the overlapping AR-responsive enhancer of the *KLK3*(PSA) gene in response to androgen stimulation. These findings suggest deleterious functions for *ETV1* oncogene by positive interactions with AR. Although functional evaluations of ETS family of transcription factors have been in the focus of extensive research, the role of ETS family of transcription factors in prostate tumorigenesis has just begun to unfold [85–87]. An intriguing finding of these studies is the tight functional clustering of prostate cancer-linked ETS transcription factors, including ERG, ETV1, ETV4, and FLI1, based on DNA-binding specificity and protein-binding characteristics.

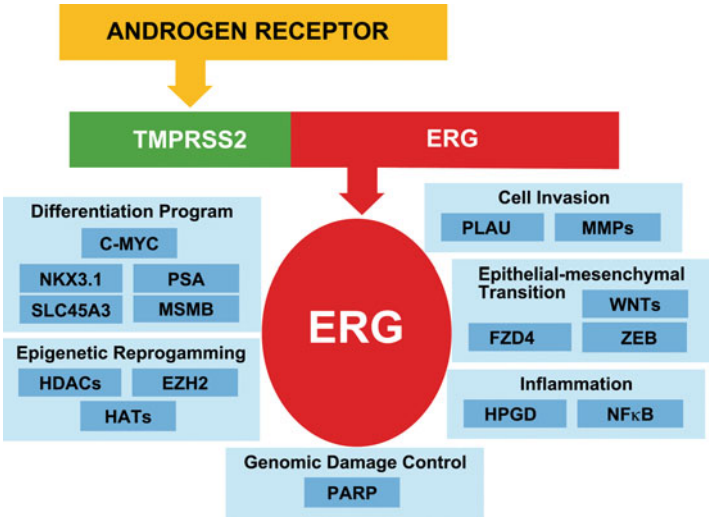


**Fig. 19.2** Androgen receptor activates the expression of *ERG* oncogene in prostate cancer due to genomic fusion events between androgen-responsive regulatory sequences of the *TMPRSS2* gene and coding sequences of *ERG*. Affected pathways in ERG expressing prostate cancers include disrupted differentiation program, activation of cell invasion-associated pathways, epigenetic reprogramming, activation of epithelial to mesenchymal transition, inflammation-associated pathways, and interference with genomic damage control throughout cancer initiation and progression

## 19.5 ETS Factors as Surrogate of Functional Androgen Axis in Fusion Positive Prostate Cancer Cells

The remarkable discovery of gene fusions between androgen-regulated gene promoters and oncogenes of the ETS transcription factor family has provided renewed impetus for searching potent inhibitors of the androgen signaling axis including new generations of androgen biosynthesis or androgen receptor inhibitors [88, 89]. Although most prostate cancers are initially responsive to androgen ablation therapy, they become treatment resistant in two plus years as tumor cells develop mechanisms to evade the treatment. Multiple mechanisms invoking gain of AR signaling (increased intra-tumoral androgen biosynthesis, elevated AR function) or loss of AR signaling (activation of AR independent survival pathways) can lead to androgen ablation refractory or Castration-Resistant Prostate Cancer (CRPC).

Initially, CaP development is driven by the androgen receptor (AR) pathway [90]. Frequent alterations of AR structure and function are well recognized during CaP progression especially with metastatic disease. Other genetic pathways may cooperate with altered function of the AR signaling axis in castration-resistant CaP and these include mutation or reduced expression of *PTEN* or *p53* and *BCL2* overexpression [15]. Indeed, cancer-associated defects of p53 and PTEN pathways may

also affect AR functions [91–93]. However in the clinical practice, identification of patients with functional defects of AR poses a great challenge. Moreover, recent demonstration of alternative pathways supplying androgen in CRPC adds to the complexity of monitoring functional defects of AR [94]. Although AR expression can be detected throughout prostate tumorigenesis, the diagnostic or prognostic utility of monitoring AR levels has been challenging. Studies evaluating the association of AR protein levels with poorly differentiated tumors, higher Gleason score or with decreased PSA recurrence-free survival are inconclusive [95–99]. During the past decade, significant efforts from several prostate cancer research laboratories, including ours, have provided novel insights into the androgen-regulated transcriptome. These endeavors identified direct transcriptional regulatory targets of AR which have promise in defining the role of AR dysfunctions, as well as, in providing novel, functionally relevant biomarkers, and potential therapeutic targets [3, 27, 100–107]. Recent data obtained from prostate cancer cell culture models highlighted a distinct AR-regulated transcription program in androgen blockade-resistant derivatives of LNCaP cells [106]. Although AR can be altered by numerous mechanisms, the net effect of these changes is reflected in defective transcription factor functions of the AR. An intriguing association of androgen-regulated genes that includes ETS transcription factors, such as ERG and ETVs in the context of prostate cancer has been reported noting a signature of attenuated AR function in late stage prostate cancers in human specimens [108–112]. Decreased expression of androgen-regulated genes in association with attenuated response to 5α-reductase inhibition in benign prostate tissues has also been recently reported [107]. This strategy has a great potential to provide early warning signs of androgen independence [113] and to predict failure of treatment response [107].

Enrichment of poor prognostic features of the CaP (Gleason score: ≥8; increased PSA recurrence or metastasis) has also been linked to low *ERG* or *ERG* negative CaP cells [114]. Attenuation or complete loss of AR-regulated genes, including *ERG* in *TMPRSS2–ERG* fusion context, were noted in this subset of predominantly poorly differentiated tumors suggesting that AR status may indeed explain the reported association between decreased *ERG* expression and more advanced prostate cancers [109, 110]. However, studies evaluating *ERG* at the genomic rearrangement level have indicated that the *TMPRSS2–ERG* fusions associate with a poorer prognosis [32, 115–118]. These seemingly paradoxical findings may actually reflect the functionality of the AR axis in the tumor cell microenvironment which may also be reflected in the status of ETS factors such as ERG levels. Therefore, a panel of defined androgen receptor-regulated genes including *ERG* may serve as surrogate for AR function in CaP progression [114].

Detection of gene fusions in hematologic cancers has led to a remarkable success in the diagnosis, classification, and treatment options [119–121]. The detection of *ERG* rearrangement by FISH in prostate biopsies and prostatectomy has led to extensive evaluations of these common genomic alterations in CaP in diagnostic and prognostic settings [39, 115, 122, 123]. Since CaP-associated gene fusions including the prevalent *TMPRSS2–ERG* fusions are exclusively present in prostate tumor cells and are virtually absent in normal cells, detection of *ERG* fusions have

promise in improving diagnosis as well as molecular classification of prostate tumors [25, 124]. A recent report suggests the need for close follow-up of patients with *TMPRSS2–ERG* positive high grade PIN in needle biopsy specimens [125].

Conflicting results have been reported regarding associations between *ERG* gene rearrangements and cancer aggressiveness [5, 126]. For example, presence of *TMPRSS2–ERG* fusion predicted cancer recurrence after surgery [32] or lethal outcome in a watchful waiting cohort [117]. However, association of the fusion or *ERG* expression with favorable outcome was also reported [23, 127, 128]. As noted above, *ERG* expression in CaP is androgen dependent due to *TMPRSS2–ERG* fusion, alterations of AR transcription factor activity may result in altered *ERG* mRNA expression as noted in poorly differentiated tumors [114]. These data also suggest that *ERG* in combination with a panel of androgen receptor-regulated genes (*PSA*, *PMEPA1*, *NKX3.1*, *ODC1*, *AMD1*) may serve as a biomarker panel for Androgen Receptor Function Index (ARFI) in CaP. Thus, ARFI may provide new opportunities in AR function-based stratification of CaP, where ERG expression evaluation could play important role in over half of CaP [114]. Consistent with this concept, a study of large patient cohort showed strong association between ERG and AR protein levels [129]. Along these lines a recent study showed that in a subset of *TMPRSS2–ERG* fusion positive CRPC, there was no detectable of the ERG oncoprotein [130]. This observation suggested upstream AR alterations leading to lack of *TMPRSS2*-dependent ERG oncoprotein expression. These findings also suggest AR alterations as the potential mechanism for initial intriguing observations on association of decreased or no *ERG* mRNA expression with poor prognosis of CaP [23]. Subsequent studies confirmed a significantly reduced AR levels in *TMPRSS2–ERG* fusion positive and ERG protein negative CRPC specimens. *TMPRSS2–ERG* fusion isoforms have variable tumor promoting biological activities and certain isoforms are correlated well with more aggressive disease [29] and others with favorable prognosis [131]. Similarly, the ratios of full-length splice forms *Type I* and *Type II* also shown to have prognostic association [31]. However, some studies have reported no significant association of *TMPRSS2–ERG* fusion or *ERG* expression with disease progression after prostatectomy [33, 129, 132]. Therefore, multicenter studies are needed for further clarification. The observations of combination of *TMPRSS2–ERG* fusion and *PTEN* deletions associating with poorer prognosis have been supported with functional studies showing cooperation of these genes in mouse models of CaP [41, 63, 68, 133]. Further assessment of the utility of combinatorial prognostic markers is warranted.

Utility of detection of *TMPRSS2–ERG* fusion or *ERG* transcripts in post-digital rectal examination (post-DRE) urine are also being evaluated for improving CaP diagnosis using minimally invasive assays [46, 134, 135]. Promising results from evaluations of highly CaP specific noncoding RNA, *PCA3*, in post-DRE urine specimens, have led the way for evaluation of additional CaP specific expression markers [136–138]. A CaP gene panel (*PCA3*, *ERG*, and *AMACR*) with diagnostic potential in which overexpression of at least one of three genes associated with virtually all of the LCM-derived prostate tumor specimens suggested for careful evaluation of such panels in post-DRE urine [23]. Evaluation of *ERG* [46] or

*TMPRSS2–ERG* [134] transcripts in post-DRE urine have provided promising data on diagnostic potential of *ERG* in this minimally invasive bio-specimen. A recent multicenter study of 1312 men showed promising data with respect to association of *TMPRSS2–ERG* in post-DRE urine with clinically significant CaP [135]. This study further showed a superior performance of the combination of *TMPRSS2–ERG* and *PCA3* in post-DRE urine in comparison to serum PSA for detecting clinically significant CaP in biopsy [135].

## 19.6 Emerging Observations from Evaluations of the ERG Oncoprotein in Prostate Cancer

Evaluations of ERG oncoprotein in CaP as the product of *ERG* fusions has been a challenge as ETS family of proteins share high homology. Recent development and evaluation anti-ERG monoclonal antibodies have paved the way for evaluation of ERG protein in routine pathologic specimens. Through exhaustive analysis of 132 whole-mount prostates sections (261 tumor foci and over 200,000 benign glands) for the ERG oncoprotein nuclear expression by an anti-ERG mouse monoclonal antibody (clone 9FY), this study demonstrated 99.9% specificity for detecting tumor cells in prostate. The ERG oncoprotein expression correlated well with fusion transcript or gene fusion in selected specimens. Strong concordance of ERG positive prostatic intraepithelial neoplasia (PIN) lesions with ERG positive carcinoma (96.5%) affirmed the biological role of ERG in clonal selection of prostate tumors in 65% (86 out of 132) of patients [132] (Fig. 19.3). These observations lend a support to the functional role of ERG in initiation of preneoplastic lesions [61, 62]. Evaluations of anti-ERG rabbit monoclonal antibody (EPR 3864) in CaP tissue microarrays from 207 cases established correlation between detection of ERG protein expression by IHC and *ERG* rearrangement by using fluorescence in situ hybridization (FISH) [139]. Detection of the ERG protein expression in CaP exhibited 95.7% sensitivity and 96.5% for the presence of *ERG* rearrangement. Further, presence of ERG protein in CaP also correlated with less common *ERG* rearrangements. Since ERG expression is almost exclusive to prostate tumor cells and IHC is easier to perform in comparison to FISH, it is expected that ERG protein evaluations in pathologic specimens will greatly facilitate the evaluations of biological and clinical utility of ERG in CaP. Among the currently known CaP biomarkers, detection ERG oncoprotein offers unprecedented opportunities in the diagnostic setting. With the availability of highly specific ERG monoclonal antibodies, better and more effective monitoring, treatment, and therapies may also be available in the near future [140, 141].

A recent review has summarized emerging studies of the ERG oncoprotein in CaP [10] and reports positive and negative predictive values of 82–100% and 88–100%, respectively, for ERG oncoprotein-based detection of CaP. Moreover, studies have reported significant improvement in diagnosing prostate cancer by combining ERG detection with previously established biomarkers, such as p63 and

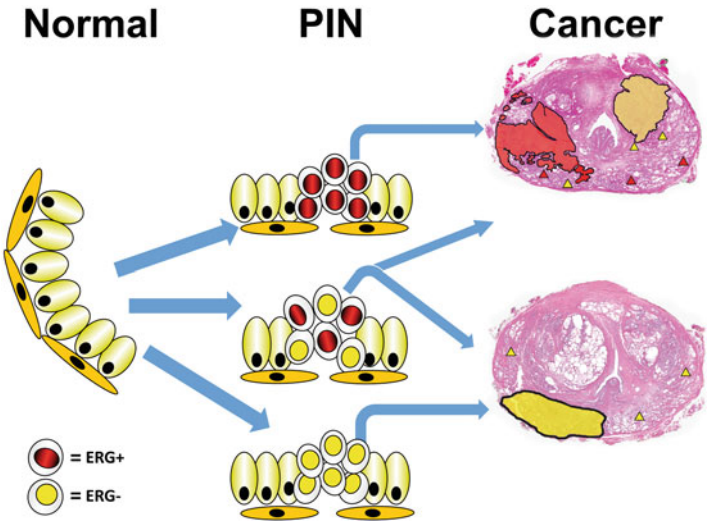


**Fig. 19.3** Normal prostate epithelial (*light green*) and basal (*orange*) cells form highly organized glandular structures (*Normal*). Schematic figure of clonal selection of prostate cancer cells from prostatic intraepithelial neoplasia (PIN) to prostatic adenocarcinoma (Cancer) indicate the clonal relation of ERG positive (*red*) and ERG negative (*yellow*) cells (Figure modified from [9, 132])

AMACR [123, 142, 143]. Although, in rare cases of cancer suspicious atypical glands (ATYP), the utility of ERG has not been seen [144], more studies are needed to assess if detection of ERG oncoprotein may assist pathologists in stratifying patients for a closer follow-up including repeat biopsy in ERG positive PIN cases [10, 123, 125]. Importantly, the major strength of incorporating ERG detection in prostate cancer diagnostic panels is the remarkable specificity due to the oncogenic function of ERG in prostate cancer. Since ERG-MAb 9FY is highly ERG specific as illustrated by lack of recognition of its closest homolog, FLI [132, 145], the presence of ERG protein in hemangiomas, lymphangiomas, angiosarcomas, epithelioid hemangio-endotheliomas, and Kaposi sarcomas [146] serve as an excellent new marker for vascular tumors [145, 146]. Similar studies are also warranted in Acute Myeloid leukemia where ERG has been suggested as prognostic marker based on evaluations of *ERG* mRNA levels.

## 19.7 New Therapeutic Opportunities Targeting *ERG* in Prostate Cancer

Unprecedented focus on evaluations of *ERG* biologic functions in CaP has led to the studies assessing the *ERG*-targeted therapeutic strategies. Transgenic mice studies have shown cooperative effects of the *ERG* overexpression with PTEN/PI3K axis

alterations, leading to progressive features of CaP [41, 63]. Studies have shown inhibitory effects of the ERG siRNA in *TMPRSS2–ERG* positive VCaP cells and VCaP-derived tumors in SCID mice suggesting for therapeutic potential of *ERG* inhibition in CaP [29, 67]. Further, it was shown that *ERG* siRNA effects were mediated through inhibition of *C-MYC* and induction of prostate epithelial cell differentiation program [67]. Thus targeting the inhibition of ERG pathway may provide a promising therapeutic strategy. YK-4-279, a derivative of the lead compound from the small molecule screen, has proven to effectively bind to ERG and subsequently downregulate its transcriptional activity as well as tumor cell invasion in cell culture model [147, 148]. Inhibitors of HDACs are currently being considered as one of the potent anticancer agents. HDAC inhibitors, such as SAHA, MS-275, TSA, and VPA have been evaluated both in vitro and in vivo prostate cancer models [57] and in a number of clinical trials [149]. HDAC inhibitor also downregulate *TMPRSS2–ERG* and alter the acetylating status of p53 [59].

Targeting nuclear transcription factors is often difficult in designing therapeutic strategies; hence, targeting component of the "ERG Network" may serve as an effective alternative strategy to combat the CaP. Recent findings showed physical interaction of ERG protein with PARP in inducing DNA damage and inhibition of PARP-impaired ERG-mediated cell invasion and tumorigenesis [77]. These findings suggest a promising therapeutic potential for PARP inhibitors for a large subset of CaP harboring oncogenic activation of the *ERG* or *ETV1*. In recent years, PARP inhibitors have been increasingly considered as a viable option in exploiting the DNA-repair defects of *BRCA1/2*-deficient tumors to induce cell death [150–152]. As CaP is heterogeneous and potentially involves multiple molecular pathways leading to complex phenotypes, development of small molecule inhibitors targeting multiple targets (AR, ERG, PARP, PTEN, PI3K, AKT, and mTOR) may incorporate new therapeutic strategies for CaP [153, 154]. Importantly, ERG network-targeted therapy may be an effective strategy for more than half of CaP in early stages when cancer cells may be more responsive to treatment.

## 19.8 Concluding Remarks

Evaluations of *ERG* oncogenic alterations represent one of the most studied and validated genomic alterations in CaP. Androgen-dependent expression of *ERG* transcription factor as a result of *TMPRSS2–ERG* fusion is detected in 50–70% of CaP patients in Western countries. Other ETS-related gene fusions (*ETV1* and *ETV4*) are lower frequency (~10%) events in CaP and need to be better understood. Significantly lower frequencies of the ERG alterations are noted in (20–40%) CaP from African American patients and this is even lower (10–25%) in CaP patients from Asia. Biological basis of the ethnic differences of ERG frequencies need to be better understood. Since *ERG* fusions described in CaP are highly specific to this cancer type, numerous studies have evaluated clinical utility of *ERG* as a diagnostic or prognostic biomarker in CaP. Detection of *ERG* rearrangement by FISH or ERG

protein by immunostaining has streamlined in pathologic specimens and results from these studies suggest the role of *ERG* in clonal expansion of ERG positive PIN (preinvasive lesion) to carcinoma. While *ERG* alteration is homogenous within a tumor focus, heterogeneity of ERG alteration is apparent in multifocal tumor context by simultaneous presence of ERG positive and negative tumor foci in the malignant prostate of a patient. Detection of ERG alterations in tissue- or urine-based assays with other CaP biomarkers (AMACR, PCA3, p63) have promise in improving prostate cancer diagnosis and continued investigations are anticipated along these lines. Prognostic value of *TMPRSS2–ERG* fusion or ERG protein expression is uncertain, however, combination of ERG alteration with other CaP gene alterations such as *PTEN* may define prognostic marker panels for progressive disease. Additional studies are also warranted to further assess the prognostic properties of specific *ERG* fusion types or relative abundance of *Type I* and *Type II* splice variants of *ERG* in CaP. *ERG* mRNA or ERG protein expression may serve as a surrogate of AR functional status in prostate tumors and therefore evaluations of *ERG* along with other androgen-responsive genes has potential in companion diagnostic setting for therapeutics targeting androgen/AR axis.

Functional evaluations of *ERG* in experimental models support causal role of *ERG* oncogenic activation in prostate tumorigenesis. In engineered mouse models, *ERG* induces preinvasive lesions and *ERG* in combination with loss of *Pten* function or gain of *AKT* or *AR* function cooperate in neoplastic transformation. *ERG* knock-down by *ERG* siRNA inhibits in vitro and in vivo growth of *TMPRSS2–ERG* positive prostate cancer cell. Studies focusing on ERG transcriptional targets in prostate cancer cells suggest the role of *ERG* in deregulating genes involved in differentiation, cell invasion, DNA damage, epigenetic control, inflammation, and epithelial–mesenchyme transition. The emerging "ERG network" defines new facets of *ERG* functions in CaP and underscores the functional interface of *ERG* with genes (*AR*, *C-MYC*, *NKX3.1*, and PI3K/PTEN axis) known to have critical functions in CaP. Studies focusing on therapeutic targeting of *ERG* or its network are promising as shown by therapeutic potential of PARP inhibitors for *ERG* and *ETV1* positive tumors in preclinical models. Taken together, strategies developing ERG-based biological classification of prostate tumors and therapeutic targeting of the ERG network in prostate cancer represent new paradigm in prostate cancer stratification and treatment.

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# Chapter 20
# Clusterin as a Target for Treatment of Castration-Resistant Prostate Cancer

**Amina Zoubeidi and Martin Gleave**

**Abstract** Secretory clusterin (sCLU) is a stress-induced cytoprotective molecular chaperone upregulated in an adaptive survival manner by various triggers to confer acquired treatment resistance. sCLU inhibits stress-induced apoptosis and induces epithelial mesenchymal transition by modulating pro-survival signaling and transcriptional networks. sCLU is associated with poor prognosis and treatment resistance including radiation, hormone, or chemotherapy. sCLU acts through an ATP-independent mechanism making this target less amenable to inhibition by small molecules, and so strategies to inhibit CLU at the gene expression level are required. In this chapter, we will review the rationale for targeting sCLU in castration-resistant prostate cancer (CRPC) and discuss the current status of preclinical and clinical studies using sCLU antisense (OGX-011) as a therapeutic target in CRPC.

**Keywords** Castrate resistance • Clusterin • Molecular chaperones • Protein homeostasis • Stress response • Apoptosis • OGX-011

## 20.1 Introduction

Many strategies used to kill cancer cells induce stress responses that promote the emergence of a treatment-resistant phenotype. In PCa, androgen ablation not only induces remission in most patients but also precipitates progression to CRPC. Intriguingly, over 80% of CRPC specimens continue to express androgen receptor

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(AR) and androgen-responsive genes [1], indicating that the AR axis remains paradoxically activated despite castration. Many mechanisms have been postulated to account for AR activation in CRPC tumors: (1) activation of AR by nonsteroids such as growth factors and cytokines via deregulated multiple signaling pathways, (2) genetic mutation(s) or amplification(s) of AR that render the receptor hyperactive which sensitizes cells towards low levels of androgen, (3) altered expression of activity of AR coactivators or chaperone proteins, (4) expression of AR splice variants that lack the ligand-binding domain (LBD) and are constitutively active in a ligand-independent manner, (5) intratumoral steroidogenesis, and (6) increase of molecular chaperones network.

Molecular chaperone bind misfolded proteins to facilitate substrate refolding or degradation. They protect cells against protein aggregation, but they must release their aggregate-prone clients to other downstream chaperones that facilitate refolding. The mechanisms of molecular chaperones function are poorly defined. Molecular chaperones expression is induced after many varied insults including hypoxia, heat shock, anoxia, UV, glucose deprivation, toxic radicals, carcinogens, hormone therapy, and chemotherapy [2–5]. In this chapter we will discuss the role of CLU in stress response and acquired treatment resistance, as well as its correlation with poor cancer prognosis and current status as therapeutic target in CRPC.

## 20.2 CLU Structure

Secretory CLU (sCLU) is a ubiquitously expressed, multifunctional, stress-induced, ATP-independent molecular chaperone, previously known as apolipoprotein J, testosterone-repressed prostate message-2, ionizing radiation-induced protein-8, complement lysis inhibitor, gp80, glycoprotein III, or sulfate glycoprotein-2. *CLU* is a single copy gene organized into 9 exons located on chromosome 8p21-p12 extending over 16 kb [6]. In humans, *CLU* gene codes for two secretory isoforms (sCLU-1, sCLU-2) originating from transcriptional start sites in exons 1 and 2, respectively; only sCLU-2 is expressed in subprimates. sCLU is an ER-targeted, 449-aa polypeptide that represents the predominant translation product of the human gene. Proteolytic removal of the ER-targeting signal peptide produces a 60 kDa ER-associated, high mannose, cytoplasmic form (sCLUc) [7]. sCLUc is further glycosylated in the Golgi and cleaved at $Arg^{227}$–$Ser^{228}$ into two 40 kDa α- and β-subunits. These subunits are assembled in an antiparallel manner into a ~80 kDa mature, secreted, heterodimeric form (sCLUs), in which its cysteine-rich centers are linked by five disulfide bridges and flanked by two coiled coil α-helices and three predicted amphipathic α-helices. The coiled-coil domain is a highly versatile protein folding and oligomerization motif, facilitating its interaction with client proteins involved in many protein signal-transducing events [8–10]. These properties of sCLU resemble survival chaperones associated with tissue injury and pathology like acute phase protein haptoglobin [11] and small Hsps [12]. Indeed, sCLU is involved in many biological processes ranging from mammary and prostate gland

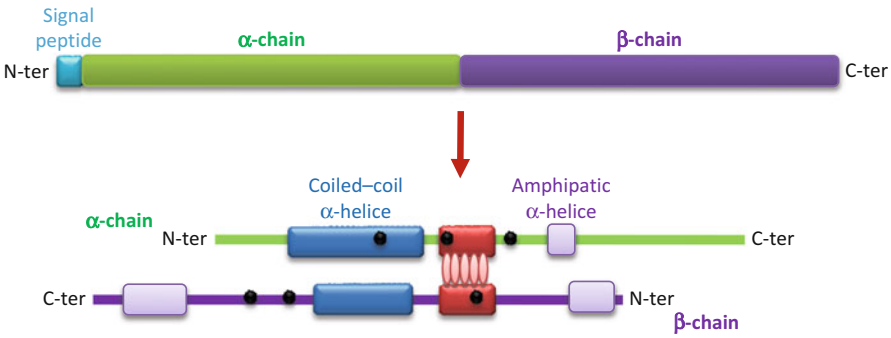


**Fig. 20.1** CLU structure: the cytoplasmic precursor peptide (sCLUc) is cleaved proteolytically between amino acids 22/23 to remove the signal peptide and between residues 227/228 to generate the α and β chains. The α and β chains are assembled in antiparallel to form a mature heterodimer (sCLUs). The cysteine-rich centers are linked by five disulfide bridges. These are flanked by two predicted coiled-coil α-helices and three predicted amphipathic α-helices. 6 N-glycosylation sites are indicated as *black dots*

involution to amyloidosis and neurodegenerative disease, as well as cancer progression and treatment resistance (Fig. 20.1) [13].

Promoter sequences of *CLU* gene are conserved during evolution and include stress-associated sites like activator-protein-1 (AP-1), AP-2, SP-1 (stimulatory element), HSE (heat shock element), CRE (cAMP response element), a "CLU-specific element" (CLE) recognized by HSF-1/HSF-2 heterocomplexes [14], Y-box binding protein (YB-1) [15], and 8 E-box known to interact with Twist [16] (Fig. 20.2). Steroid response elements include GRE (glucocorticoid response element) [17–19] and androgen response element (ARE) sites [20]. In prostate, sCLU was originally cloned as "testosterone-repressed prostate message 2" (TRPM-2) [21] from regressing rat prostate, but was later defined as a stress-activated and apoptosis-associated, rather than an androgen-repressed, gene [22, 23]. CLU promoter regions contain CpG-rich methylation domains [18, 24] indicating CLU may be regulated by DNA methylation and histone acetylation [25]; indeed, 5-aza-2′-deoxycytidine significantly increases sCLU expression in prostate cancer cells [26]. sCLU expression increases downstream of survival signaling pathways and in response to ER-stress.

CLU regulates a stress-activated feed-forward regulation loop involving HSF-1 and the heat shock response [27]. CLU silencing abrogates, while CLU overexpression enhances, Hsp90 inhibitor-induced HSF-1 transcription activity, identifying a role for CLU in the regulation of HSF-1 and the heat shock response itself. Indeed, CLU knockdown blocks the translocation of HSF-1 to the nucleus following treatment with Hsp90 inhibitors and enhances activity of Hsp90 inhibitors. CLU is upregulated downstream IGF-1 via Src-Mek-Erk-EGR-1 [28], TGF-b via twist [16], and cytokines via Jak/STAT1 [29] and downstream of ER stress inducer including paclitaxel via Y-box binding protein-1 (YB-1). Y-box binding protein-1 (YB-1)

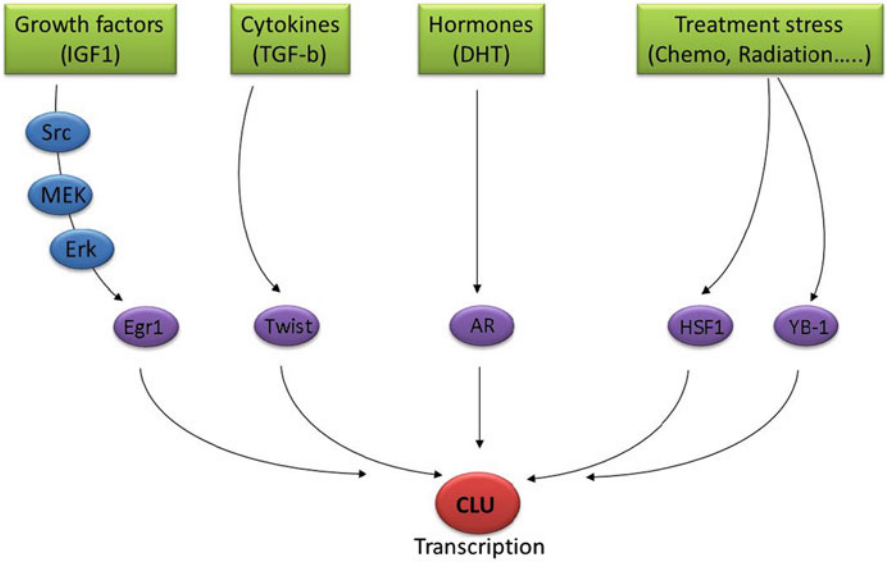


**Fig. 20.2** Regulation of CLU expression. CLU is transcriptionally activated by a variety of stimuli including growth factors such as IGF-1 via MAPK-EGR-1, cytokines such as TGF-b via Twist, downstream of chemotherapeutic stress via HSF-1 and YB-1, and downstream of androgen via androgen receptor

directly binds to CLU promoter regions to transcriptionally regulate clusterin expression. TGF-β induced CLU expression and that Twist1 regulates basal and TGF-β induced *CLU* transcription by binding to the distal promoter region of *CLU* gene between −1,998 bp and −1,116 bp from transcription start site [16] to regulate epithelial mesenchymal transition (Fig. 20.2).

## 20.3 CLU Regulates Protein Homeostasis Via Unfolded Protein and ER Stress Responses

sCLU functions to protect cancer cells from many varied therapeutic stressors that induce apoptosis including androgen withdrawal, radiation, cytotoxic chemotherapy, and biologic agents [30]. Since sCLU binds to a wide variety of biological ligands and is regulated by HSF1 [14], an emerging view suggests that sCLU functions like small HSPs to chaperone and stabilize conformations of proteins at times of cell stress. Indeed, sCLU is more potent than other Hsps at inhibiting stress-induced protein precipitation. sCLU inhibits stress-induced protein aggregation by binding to exposed regions of hydrophobicity on non-native proteins to form soluble, high molecular mass complexes [31, 32]. During amorphous aggregation of proteins, sCLU interacts with slowly aggregating species on the off-folding pathway.

Cytoplasmic sCLU (sCLUc) inhibits endoplasmic reticulum (ER) stress, retro-translocating from the ER to the cytosol to inhibit aggregation of intracellular proteins and prevent apoptosis [33] (Li et al. 2012). Interestingly, and likely related to its role in inhibiting protein aggregation, sCLU is the most abundant protein associated with β-amyloid deposits in Alzheimers [32]. In response to ER stress inducers, including paclitaxel, YB-1 is translocated to the nucleus to transactivate clusterin [15]. Furthermore, higher levels of activated YB-1 and CLU are seen in taxane resistant, compared with parental, prostate cancer cells. Knockdown of either YB-1 or CLU sensitized prostate cancer cells to paclitaxel, whereas their overexpression increased taxane resistance. CLU overexpression rescued cells from increased paclitaxel-induced apoptosis following YB-1 knockdown; however, YB-1 overexpression did not rescue cells from increased paclitaxel-induced apoptosis following clusterin knockdown. Collectively, these data indicate that YB-1 transactivation of CLU in response to stress is a critical mediator of paclitaxel resistance in prostate cancer [15]. Under ER stress, GRP78 (Bip) interacts with CLU to facilitate its retro-translocation and redistribution to the mitochondria (Li et al. 2012). ER stress increased association between GRP78 and CLU, which led to increased cytoplasmic CLU levels while reducing sCLU levels secreted into the culture media. GRP78 stabilized CLU protein and its hypo-glycosylated forms, in particular after paclitaxel treatment. Moreover, subcellular fractionation and confocal microscopy with $CLU_{GFP}$ indicated that GRP78 increased stress-induced CLU retro-translocation from the ER with colocalized redistribution to the mitochondria, thereby reducing stress-induced apoptosis by cooperatively stabilizing mitochondrial membrane integrity. These findings reveal novel dynamic interactions between GRP78 and CLU under ER stress conditions that govern CLU trafficking and redistribution to the mitochondria, elucidating how GRP78 and CLU cooperatively promote survival during treatment stress in prostate cancer (Li et al. 2012). Collectively, the preceding indicates sCLU plays an important role in protein homeostasis (proteostasis) via unfolded protein and ER stress responses.

## 20.4 CLU Inhibits Apoptosis

sCLUc inhibits mitochondrial apoptosis by suppressing p53-activating stress signals and stabilizes cytosolic Ku70-Bax protein complex to inhibit Bax activation [34]. sCLUc specifically interacts with conformationally altered Bax to inhibit apoptosis in response to chemotherapeutic drugs [35]. sCLU knockdown alters the ratios of anti-apoptotic Bcl-2 family members, disrupting Ku70/Bax complexes and Bax activation [34, 35]. In addition, sCLU increases Akt phosphorylation levels and cell survival rates [36]. sCLU promotes prostate cancer cell survival by increasing NF-κB nuclear transactivation, by scaffolding heterocomplexes between E3 ligase family members that ubiquitinate COMMD1 and I-κB and increase their proteasomal degradation [8, 9]. sCLU knockdown stabilized COMMD1 and I-κB, suppressing NF-κB translocation to the nucleus, and suppressing NF-κB-regulated gene signatures [8, 9].

## 20.5 CLU Regulates Invasion and Metastasis

High levels of sCLU expression are associated with migration, invasion, and metastasis [37]. sCLU induces a spindle-shaped morphology adopting an epithelial mesenchymal transition phenotype via ERK-Slug pathway [37]. sCLU induces cell mobility, increase Smad2/3 stability, and enhances TGF-β-mediated cell invasion [38] and Smad transcriptional activity [39]. Targeting sCLU using siRNA, antisense OGX-011, or antibody induces a mesenchymal-to-epithelial transition (MET) evidenced by the spindle-to-cuboidal morphological change, increased E-cadherin expression, and decreased fibronectin expression. CLU knockdown reduces TGF-β induction of mesenchymal markers N-cadherin and fibronectin and, in turn, inhibited migratory and invasive properties induced by TGF-β [16]. Interestingly, both anti-CLU monoclonal antibodies and OGX-011 significantly reduce in vivo lung metastasis in breast cancer [38] and prostate cancer model [16], respectively. Taken together, these data reveal a role for sCLU as an important promoter of EMT and suggest that targeting CLU may inhibit tumor metastasis.

## 20.6 CLU Correlates with Adverse Prognosis

CLU is expressed in many human cancers, including breast, lung, bladder, kidney, colon–rectum, and prostate [40–44]. sCLU expression correlates with loss of the tumor suppressor gene Nkx3.1 during the initial stages of prostate tumorigenesis in Nkx3.1 knockout mice [45]. CLU levels are low in low grade untreated hormone-naive tissues but increase with higher gleason score [44]. Levels of sCLU increase several fold after androgen ablation, suggesting that CLU expression is a stress-activated, cytoprotective response induced by the anticancer treatment [46]. Biochemical recurrence-free survival in patients with strong CLU expression in radical prostatectomy specimens was lower than those with weak CLU expression [47, 48]. Plasma levels of sCLU were significantly higher in patients with high grade prostate cancer with extracapsular extension compared to organ-confined tumors [49]. These data illustrate that CLU correlates with higher grade, post-treatment stress, and/or poor outcome in many cancers.

## 20.7 CLU as Therapeutic Target

Experimental and clinical studies associate sCLU with development treatment resistance, where sCLU suppresses treatment-induced cell death in response to androgen withdrawal, chemotherapy, or radiation [22, 23, 46]. CLU is upregulated after different stress including hormone and chemotherapy; CLU levels are high in CRPC [22, 23] or after estrogen withdrawal in breast cancer tumors cells [50]. Indeed,

CLU is stress induced by many anticancer therapies including docetaxel or irradiation in breast and prostate cancers [50–53], cisplatin [54, 55], doxorubicin [56, 57], Herceptin [58], Hsp90 inhibitors [27], and HDAC inhibitors [59].

In keeping with its cytoprotective function, recent reports support the concept that silencing CLU can enhance the cytotoxicity of hormone- radiation-, and chemotherapies [8, 9, 53, 60]. An antisense oligonucleotide (ASO) inhibitor targeting the translation initiation site of human exon II CLU (OGX-011) was developed at the University of British Columbia and out-licensed to OncoGeneX Pharmaceuticals Inc. OGX-011 (custirsen), is a second-generation antisense oligonucleotide with a long tissue half-life of ~7 days that potently suppresses CLU levels in vitro and in vivo. OGX-011 improves the efficacy of many varied anticancer therapies by suppression treatment-induced CLU expression and the stress response [27] with increased apoptotic rates in preclinical xenograft models of prostate, lung, renal cell, breast, and other cancers [8, 9, 47, 48, 61, 62]. For example, OGX-011 enhanced the growth-inhibitory effect of trastuzumab [58], an HER-2-targeted monoclonal antibody, in breast cancer cells. CLU ASO sensitizes bladder cancer to cisplatin [55], breast, prostate, and lung to paclitaxel [22, 23, 50, 63], and prostate to hormone therapy [22, 23] where it delays CRPC progression [22, 23, 27, 55, 63]. OGX-011 suppresses Hsp90 inhibitor-induced increases in HSF-1 transcriptional activity and CLU and synergistically enhances Hsp90 inhibitor activity in vivo in PC-3 and LNCaP models. Collectively, these results highlight, for the first time, a biologically relevant feed-forward regulation loop of CLU on HSF-1 and the heat shock response [27]. These preclinical studies demonstrate that cotargeting CLU and the stress response antisense with chemo, hormonal, or other biologic therapies enhances anticancer activity in many cancer types.

## 20.8 Clinical Trials of CLU Inhibitor, OGX-011

To date, over 300 patients have been treated with OGX-011 in 6 phase I and II clinical trials. The first-in-human phase I study with OGX-011 used a novel neoadjuvant design to identify the effective biologic dose of OGX-011 to inhibit sCLU expression in human cancer [64]. In this dose-escalation study, cohorts of 3–6 patients with localized prostate carcinoma and high risk features were treated with OGX-011 in doses of up to 640 mg given as a 2-h intravenous infusion on Days 1, 3, 5, 8, 15, 22, and 29 with prostatectomy performed within 7 days of the last OGX-011 dose. Neoadjuvant androgen deprivation was administered concurrently to induce sCLU expression. The presurgery design was used to correlate changes in expression of sCLU to drug dose received and drug levels within the prostate tissue itself. In this study, treatment was well tolerated and at doses of 320 mg and higher, concentrations of full-length OGX-011 or >600 nM were achieved that were associated with preclinical activity. OGX-011 produced statistically significant, dose-dependent >90% knockdown of sCLU in normal and tumor tissue. Furthermore, mean apoptotic indices increased from 7.1 to 21.2%. This pharmacodynamic data indicates that OGX-011 is biologically active in humans

and identified 640 mg as the optimal biologic dose for Phase II trials. Plasma pharmacokinetic parameters have been similar across phase I studies including when OGX-011 was combined with chemotherapy and decreases in serum sCLU have been consistently observed [65, 66].

A phase II study in chemo-naïve, metastatic CRPC randomized 81 patients to either docetaxel-OGX-011 or docetaxel-alone [67]. The median cycles delivered for docetaxel-OGX-011 was 9 compared to 7 for docetaxel-alone. There was evidence of biologic effect with 18% decrease in mean serum sCLU in patients treated with docetaxel-OGX-011 versus 8% increase in controls ($P=0.0005$). Median overall survival on the docetaxel-OGX-011 arm was 23.8 months, ~7 months longer than those receiving docetaxel-alone (16.9 months) [68]. Multivariate analysis of factors associated with improved overall survival identified ECOG performance status of 0 (Hazard Ratio (HR) = 0.28, $P<0.0001$) and treatment assignment to the docetaxel-OGX-011 treatment arm (HR = 0.49, $P=0.012$) [68]. Another Phase II trial of docetaxel-recurrent CRPC randomized 42 patients to receive either docetaxel or mitoxantrone combined with OGX-011, to test whether OGX-011 could reverse docetaxel resistance or improve mitoxantrone efficacy in a chemo-resistant population [69, 70]. PSA declines of ≥30% were seen in 55% of docetaxel-OGX-011 patients and 32% of mitoxantrone-OGX-011 patients. Pain responses were also seen in >50% of patients and after a median follow-up of 13.3 months, 60% of patients were alive in both arms. These results are also of interest considering PSA response rates of <20% and median survival <12 months is usually reported in patients with docetaxel-resistant CRPC receiving second-line chemotherapy [71], supporting further studies second line indications for CRPC. Given the signals of anticancer activity in these two CRPC trials, randomized phase 3 studies have begun comparing docetaxel with or without OGX-011 in chemo-naïve patients (http://ClinicalTrials.gov: NCT01188187) and cabazitaxel with or without OGX-011 in chemo-recurrent patients (http://ClinicalTrials.gov: NCT01083615).

A phase II trial of 85 patients with non-small cell lung cancer (NSCLC) treated with combined OGX-011 and gemcitabine–cisplatin chemotherapy [66, 72] reported an objective response rate of 23% and median overall survival of 383 days with 58% surviving >1 year. This overall survival data was considered as a clinically significant signal compared to prior clinical trials data with chemotherapy alone, justifying Phase III studies in NSCLC that opened in 2012 (http://ClinicalTrials.gov NCT01630733).

## 20.9 Conclusion

In summary, sCLU is a stress-activated molecular chaperone that activates cytoprotective transcriptional and signaling pathways involved in acquired treatment resistance. sCLU correlate with poor prognosis, and preclinical and randomized clinical data using OGX-011 is associated with target suppression and anticancer activity. Further studies of OGX-011 in randomized phase III trials are ongoing.

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# Index


**A**
Abiraterone, 200, 247
Acute myeloid leukemias (AML), 323
Aflibercept, 288
Allosteric signaling pathway, 33–34
Ambros, V., 298
Andreu-Vieyra, C., 209
Androgen-dependent prostate cancer (ADPC), 54, 310–311
Androgen deprivation therapy (ADT), 128
Androgen receptor (AR)
  activation and repression, 12, 13
  activation function, 11–12
  Akt activation and AR regulation, 179
  allostery, 33–34
  androgen-stimulated CWR22 cell, 175
  ARE
    definition of, 27–28
    DNA motifs, human genome, 27
    optimal hexamer motif, 28
    position-specific probability matrix, 30, 32
    selective ARE, 28–29, 31
    sequence logos, 28
    TMPRSS2, 30–31
    Zn-finger module, 29–30
  caveolin-1, 172–173
  cell cycle inhibitor, p21, 174
  cell signaling, 16–17
  chromatin looping (*see* Chromatin looping)
  chromosomal rearrangement, 171
  coactivators
    cointegrators, 15
    DEAD box RNA helicase p68, 15
    GTF, 12–14
    p160, 14
    SRC-1, 15
    SRC-3, 14
    TIF2, 14
  coregulator interaction and target cell signaling pathway, 18
  co-regulatory protein, 172
  corepressors, 15–16
  and cyclin A1, 179–180
  cyclin D1, 173
  DBD
    ARE, 24
    carboxyterminal extension, 26
    hinge mutation, 26
    intranuclear mobility, 26–27
    and LBD, 25
    nuclear translocation, 25–26
    *vs.* steroid receptor, 24
    Zn finger, 25
  DNA sequence, activity, 34
  EMT, 178–179
  epigenetic aberration, 168, 170–171
  gene up- and down-regulation, 180
  high-throughput genome analyses, 174–175
  ligand-independent AR-mediated gene expression, 176
  LNCaP cells, androgen/AR signaling, 169
  miRNA, 300–301
  M-phase cell cycle, 175–176
  negative regulation, 177
  non-genotropic action modulation, 168
  oligoarray and ChIP display analysis, 176–177
  orthotopic xenograft mouse model, 169–170
  PCa proliferation and metastasis, 173, 174



Z. Wang (ed.), *Androgen-Responsive Genes in Prostate Cancer: Regulation, Function and Clinical Applications*, DOI 10.1007/978-1-4614-6182-1,

Androgen receptor (AR) (*cont.*)
pes-ARKO-TRAMP mouse model, 169
physiological process, 24
polyQ, 174–175
prostate cancer, 18
castration resistant, 300
epithelia, 300
prostate regeneration mouse model system, 169
prostate *vs.* metastatic tumors, 178
PTEN/PI3K pathway, 176
SPARKI model, 33–34
structure of, 11–12
TMPRSS2:ERG gene fusion, 177
transcription regulation, 12, 13
Androgen receptor occupied regions (ARORs)
CTCF-FOXA1complex, 78
de novo motif analysis, 74, 76–77
DHT-treated LNCaP cell profiling, 74
DNaseI hypersensitivity, 78
FOXA1, 74–75
H3K9,14ac, 74, 75
overlapping AcH, 74, 75
PCa progression and differentiation, 75, 78
Androgen-responsive genes (ARGs)
androgen ablation therapy, 148, 155
benign *vs.* malignant prostate, 155–156
biomarkers, 148–149
cell–cell interaction and cell adhesion, 160
and CRPC, 157, 159
CWR22-R1 xenograft, 153–154
diagnostic biomarker, 160–161
differential gene expression analyses, 157–159
disease stage change, 160–161
gene expression analysis
androgen-responsive *vs.* castrate-resistant tumor, 154–155
biphasic proliferative effect, LNCaP, 150
cell line, advantages, 150–151
CRPC, 155
hormonal therapy, 155
LNCaP cell, 149–150
methods of, 151–152
RNA extraction manipulation, 152
rodent *vs.* human prostate, 151
sample collection, 151
goserelin and bicalutamide treatment, 156–157
human prostate cell line, 152–153
LNCaP Hollow Fiber model, 154
prostate and androgen, 148
*PSA/KLK3*, 149
steroidogenic enzyme *vs.* androgen-sensitive tumor, 160
target androgen receptor signaling, 148–149
*TMPRSS2-ERG* fusion gene, 156, 159
Angiogenesis
ADT, 287–288
aflibercept and tasquinimod, 288
anastomosis, 286
angiogenic switch, 286
EAF2, 288–289
EAF2/ELL collagen axis, 292
HIF, 289–290
p53 and EAF2 cotransfection, 290–291
prostate tumor, 286
TSP-1, 287
tumor invasiveness and metastasis, 287
VEGF, 286–287
VHL pathway, 291
Armstrong, K., 177

**B**
Barbieri, C.E., 14
Best, C.J., 154, 155, 158
Bolton, E.C., 55
Bone morphogenetic protein (BMP), 305
Brown, M., 74

**C**
Cabazitaxel, 247
*Caenorhabditis elegans*, 289, 298
Cai, C., 172
Cancer of the Prostate Risk Assessment (CAPRA), 311
Canonical Mitogen Activated Protein Kinase (MAPK) signaling, 17
Carver, B.S., 176, 177
Castration-resistant prostate cancer (CRPC), 54, 300, 310–311
ADT, 214
AR activation, 341–342
AR-Vs
abiraterone and MDV3100, 222–223
and AR-FL signaling, 218
AR-V7 nuclear staining, 218, 219
AR-V7 UP and AR-FL UP gene set, 220–222
cell-type-specific regulation, 222
functional diversity, 215–216

genome-wide copy number analysis, 217
human AR-V, characteristics, 214, 215
lethal mitotic phenotype, 222
ligand-independent AR signaling, 214, 219–220
LNCaP95 cell, 218–219
NMD mechanism, 216–217
posttranscriptional splicing, 217–218
RT-PCR, 216–217
SLASR, 214–215
secretory clusterin
adverse prognosis, 346
apoptosis, 345
migration, invasion, and metastasis, 346
OGX-011, 347–348
protein homeostasis, 344–345
structure, 342–344
therapeutic efficacy, 223
β-Catenin
androgen-mediated prostate cell growth and tumor formation, 116–117
AR transcriptional complex, 115–116
cellular localization and expression, 114–115
chromatin immunoprecipitation assay, 116
Cell cycle
androgen regulation, 228
AR and androgen, 233–234
bell-shaped response, 228
CDKi, 229
E2F1, 231
EZH2, 231–232
p300, 233
$p21^{CIP1}$, 233
$p27^{KIP1}$, 230–231
proliferating cell nuclear antigen, 232
prostate cancer incidence and aging, 234
PTEN tumor suppressor, 234
S and M phase, 228–229
SKP2, 229–230
testosterone and DHT, 228
UBE2C, 232
Chandran, U.R., 154, 158
Chang, C., 170
Chen, C.D., 201, 208
Chen, H., 175
Chen, L., 177
Chromatin conformation capture assay, 57–58
Chromatin immunoprecipitation (ChIP) assay, 27–28
Chromatin Interaction Analysis by Paired End Tag (ChIA-PET) sequencing, 58
Chromatin looping
ADPC, 54
androgen-dependent gene transcription, 54
AR coactivator, 60–61
AR-mediated looping, 59
ChIA-PET sequencing, 58
ChIP-on-chip analysis, 55
chromatin conformation capture assay, 57
CRPC model, 54–55
CTCF, 61–63
DNA-binding collaborating factor, 60
enhancer-promoter interaction, 56–57
gene ontology analysis, 56
Hi-C, 58
histone posttranslational modification, 63–64
interchromosomal AR-mediated chromatin interaction, 64
LNCaP and abl PCa model, 55
PCa development and progression, 64
sequencing analysis, 57
transcription factor-mediated chromatin looping, 59
VCaP and LNCaP cell, 55–56
Chronic lymphocytic leukemia (CLL), 299
Clegg, N., 152
Clusterin. *See* Secretory clusterin (sCLU)
Coetzee, S.G., 72, 84
CRPC. *See* Castration-resistant prostate cancer
Culig, Z., 202

**D**

Dehm, S.M., 128, 151, 152, 214, 216
Dekker, J., 57
Denayer, S., 31
Deoxyribo nucleic acid-binding domain (DBD)
ARE, 24
carboxyterminal extension, 26
hinge mutation, 26
intranuclear mobility, 26–27
and LBD, 25
nuclear translocation, 25–26
*vs.* steroid receptor, 24
Zn finger, 25
Desai, S.J., 176
DiGeorge syndrome critical region gene 8 (DGCR8), 299
Docetaxel. *See* Taxotere
Drosophila planar cell polarity (PCP) pathway, 112

**E**
Electrophoretic mobility gel shift assay (EMSA), 233
Ellinger, J., 171
Encyclopedia of DNA Elements (ENCODE), 129–130
Endoplasmic reticulum (ER) stress, 345
Epigenetic silencing mechanism, 133–134
Epithelial-mesenchymal transition (EMT), 178–179, 324, 346
  abiraterone, 247
  ADT, therapeutic failure, 240
  androgen/AR signaling, 241
  androgen-modulated EMT effect, 250
  AR
    aberrant signaling pathways, 245
    FLNA, 243, 245
    intermolecular homo-dimerization, 245
    nuclear translocation protocol, 243
    prostate growth and functional interaction, 257–258
    proteomic and microassay analyses, 248–249
    stromal-epithelial interaction, 248
    structure, 241–243
    TMPRSS2: ERG gene fusion, 258
    translocation, 241, 244
  biological process, 249
  cabazitaxel, 247
  cadherin switching, 250–251
  CRPC, 240–241
  EZH2, 253
  MDV3100, 248
  migratory/invasive property, 249–250
  Notch (Hi)-Jagged AR, 257
  PSA screening, 240
  Snail, 251, 252
  taxotere and paclitaxel, 246–247
  TGF-β, 255–256
  Wnt signaling and AR, 254–255 (*see also* Wnt signaling pathway)
  ZEB1 feedback loop, 251–253
Estrogen receptor alpha (ERα), 304
Ewing's sarcoma (EWS), 323

**F**
Fang, Z., 173
Filamin-A, 243, 245
Fleischmann, A., 170
Fluorescence in situ hybridization (FISH), 61
Fluorescent Recovery After Photobleaching technology (FRAP), 26–27
Fondell, J.D., 173

**G**
Garber, M., 139
Gene expression profiling
  androgen-responsive *vs.* castrate-resistant tumor, 154–155
  biphasic proliferative effect, LNCaP, 150
  cell line, advantages, 150–151
  CRPC, 155
  hormonal therapy, 155
  LNCaP cell, 149–150
  methods of, 151–152
  RNA extraction manipulation, 152
  rodent *vs.* human prostate, 151
  sample collection, 151
  steroidogenic enzyme *vs.* androgen-sensitive tumor, 160
  *TMPRSS2-ERG* fusion gene, 156, 159
Gene set enrichment analysis (GSEA), 219–220
Gioeli, D., 26
Guo, Z., 215

**H**
Handoko, L., 62
Hatley, M.E., 308
Heemers, H.V., 205
Histone acetyl transferases (HATs), 323
Histone deacetylases (HDACs), 323
Hodges, C.V., 112, 200, 214, 240
Holzbeierlein, J., 154–158, 161
Hormone-refractory prostate cancer. *See* Castration-resistant prostate cancer (CRPC)
Hornberg, E., 222
Horoszewicz, J.S., 202
Huggins, C.B., 71, 112, 200, 214, 240
Hu, R., 214, 215
Hu, S., 33

**I**
Insulin-like growth factor (IGF) signaling pathway, 117–118
Interleukin-6 (IL-6)
  anti-IL-6 monoclonal antibody siltuximab, 194
  AR in vivo upregulation and mutation, 190
  cancer cell line, disease pathogenesis, 191
  DU-145 cell, 192
  JAK/STAT signaling pathway, 190–191
  LAPC-4 and MDA PCa 2b xenograft, 192–193

ligand-independent and synergistic activation, AR, 191
p300 and SRC-1, 193
PCa proliferative response, 193
PIAS1, 194
proliferation and apoptosis, 190–191
prostate cancer therapy, 195
PSA regulation, 192
SOCS-3, role, 193–194

**J**
Jalava, S.E., 301
Jariwala, U., 176
Jia, L., 55, 171
Jin, F., 173

**K**
Katayama, S., 133
Kerkhofs, S., 31
Khalil, A.M., 134
Klein, K.A., 202
Kokontis, J., 203
Korenchuk, S., 202

**L**
Lee, S.O., 169
Lee, Y.G., 202
Lehmusvaara, S., 156
Li, C., 174
Lin, H.K., 176
Li, T., 309, 310
LNCaP Hollow Fiber model, 154

**M**
Makkonen, H., 208
Mallik, I., 173
Marques, R.B., 178
Massie, C.E., 55, 56, 204–207
Matrix metalloproteases (MMP), 308
Medina, P.P., 308
Mercer, T.R., 134
Metzger, E., 170
MicroRNAs (miRNAs)
androgen receptor, 300–301
biogenesis and function, 298–299
*Caenorhabditis elegans*, 298
human disease, 299
LIN-14 protein, 298
miR-21
AP-1 transcription factors, 304
BTG2 and CDK6, 307
epigenetic forces, 304
IL-6/STAT3 pathway, 304
inflammatory pathways, 308–309
matrix metalloproteases, 308
Microprocessor complex, 304–305
NCAPG, 307
non-spliced pri-miR-21 transcripts, 304
PCBP1, 308
PDCD4, 306–307
in prostate and PCa, 309–311
TGFbRII and SKI, 307
TPM1, 308
transgenic mouse model, 308
VMP1 and pri-miR-21, 302–303
Mostaghel, E.A., 155–158, 160
Mulholland, D.J., 176

**N**
National Center for Biotechnology Information (NCBI), 129, 130
Negative androgen-response element (nARE)
androgen-driven transcription, luciferase assay, 44, 46
antagonist-mediated repression, 40
AR-mediated gene expression, 40
AR-mediated transcriptional activity, 41–42
ChIP-chip, 40
*cis*-element insertion, luciferase reporter, 41, 42
Ets and HRE element, 49
gel shift, AR DBD protein, 48
histone amino-terminal modification, 48
knock-in mutation, *TGF-β1*, 48–49
luciferase assay, 41, 42
masipin promoter sequence, 47–48
nucleotide mutation, 45, 47
pARE consensus, 44–45, 47
PSMA promoter/enhancer, 43
synergistic function, 44, 45
synthetic promoter and transient transfection assay, 48
*TGF-β1* promoter, 42–44, 46
XBE, 40–41
Nelson, P.S., 152
Niu, Y., 170, 179
Noncoding ribonucleic acid (ncRNA)
androgen regulated noncoding RNAs, 135
growth-arrest-specific 5, 135
junk DNA, 132
lincRNAs, 134
lncRNA, 133

Noncoding ribonucleic acid (ncRNA) (*cont.*)
miRNA, 132–133
p15as, 134
PCAT, 136
pseudogene, 135
small RNA sequencing, 133
*ZEB2*, 134
Non-small cell lung cancer (NSCLC), 348
Noss, K.R., 43
Notch signaling pathway, 257

**O**
Orchiectomy, 71
Orthotopic xenograft mouse model, 169–170
Östling, P., 301

**P**
Pes-ARKO-TRAMP mouse model, 169
Phosphatidylinositol 3-kinase (PI3K)/AKT pathway, 118
Plasminogen activator inhibitor type1 (PAI-1), 323
Poly(rC)-binding protein 1 (PCBP1), 308
Polycomb group II (PcG II), 323
Prescott, J., 177
Programmed cell death protein 4 (PDCD4), 306–307
Prostate cancer (PCa)
abiraterone, 200
age-related macular degeneration disease, 79
androgen-signaling pathway, 112
apoprotein, 98
AR, 96 (*see also* Androgen receptor)castration *vs.* castration + AR antagonist, 71–72
ChIP-seq, 73
CRPC, 72–73
deep sequencing technology, 73–74
MDV3100, 73
regulation, 71
ARE-mediated gene activation, 97–98
ARORs, 74
AR overexpression
ADT, 210
androgen-repressed gene, 204
ARBS map, 209–210
ARBSs, LNCaP-derived cell line, 205, 206
CAMKK2, 204
cell line model, 201, 202
ChIP-seq, 205
chromatin binding assessment, 205, 207
chromatin remodeling, 208–209
coregulator's expression, 204–205
CPRC, androgen signaling pathway inhibition, 210
feedback loop, 201
gene amplification, 200–201
hormone-refractory xenograft, 201, 203
LNCaP *vs.* VCaP, 205, 207
LuCaP69, ChIP-seq analysis, 207–208
M-phase cell-cycle gene, 203
transcriptional program, 209
β-catenin (*see* β-Catenin)
chromatin looping putative mechanism, 99–100
correlated SNP, GWAS, 89–91
CRPC, 96
DHT, castration, 200
E-cadherin, 119–120
enhancer activity, dual luciferase reporter assay, 85, 87–88
ERG oncogenic activation
acute myeloid leukemias, 323
African American and Caucasian American patients, 322–323, 331
*C-MYC* expression, 323–324
DNA damage-sensing repair pathways, 324
Ewing's sarcoma, 323
HATs and HDACs, 323
inflammatory pathways, 324
matrix metalloproteinases, 323
*OPN* gene, 323
*PAI-1* genes, 323
PcG II, 323
*TMPRSS2–ERG* gene fusion, 320–322, 325–326
transgenic mice models, 324–325, 332
WNT signaling pathways, 324
ERG oncoprotein detection, 329–330
ERG-targeted therapeutic strategies, 330–331
ETS transcription factor, AR signaling axis, 326–329
FoxA1, 100
FunciSNP, 83–85
FunciSNP heatmap, 85, 86
genetic risk factor, 78–79
gene transcription, 99
genome SNPs, overlapping, 89
GWAS
PCa, 83–85

SNPs, 71, 72
tagSNP, 79–82
HapMap project, 83
human genome project, 86–87
IGF-1 signaling pathway, 117–118
ligand-insensitive gene activation, 96–98
linkage disequilibrium, 79
miR-21 (*see* MicroRNAs (miRNAs))
pathogenesis, 120–121
PI3K/AKT and PTEN, 118
SNPs, 79, 83
TCF/LEF and AR interaction, 119
tethering protein, 98–99
tetherome
androgen ablation, 105–106
androgen/AR signaling, 105
AR binding domain, 105
bimodal action, 105
C/EBPα, 101–102
Elk1, 100–101
Elk1-AR interaction, 106
gene regulation, 103, 104
HoxB13, 102
ligand sensitivity and AR dimerization, 103–104
TMPRSS2 and ETS protein, 102–103
Wnt signaling pathway (*see* Wnt signaling pathway)
Prostate-specific antigen (PSA), 309

**R**
Rajput, A.B., 177
Reis, E.M., 135
Rett syndrome, 57
Ribonucleic acid (RNA) transcript profiling
ADT and CRPC, 128
alternative protein-coding transcript, 130
androgen- responsive gene, 138–139
ENCODE, 129–130
microarray protocol and limitation, 128–129
multitasking genomic loci
alternative RNA splicing, 136
*HOXC*, 136, 137
*p53* mRNA, 136, 138
*PTENP1*, 138
SRAPprotein, 136
NCBI RefSeq transcript, 129, 130
ncRNA (*see* Noncoding ribonucleic acid)
RNAseq, 129
SLC45A3-ELK4, chimeric transcript, 131, 132
transcript splicing, 130–131
3’ UTR, 131
Riegman, P.H., 173
RNA-induced silencing complex (RISC), 299
Romanuik, T.L., 159
Ruvkun, G., 298

**S**
Sahu, B., 205–208
Saramäki, O.R., 202
Secretory clusterin (sCLU)
adverse prognosis, 346
apoptosis, 345
migration, invasion, and metastasis, 346
OGX-011, 347–348
protein homeostasis, 344–345
structure, 342–344
Selective linear amplification of sense RNA (SLASR), 214–215
Serum response factor (SRF) signaling
ADT, 269–270
androgen-responsive gene signature, 276
AR
coactivators, 271–272
PCa therapy, 272, 274
steroid synthesis pathway, 271
structure and function, 270–271
*TFBS*, 272, 273
transcriptional program, 270
cellular and physiological function, 274–275
Cox proportional hazard model, 277
feed-forward mechanism, 274
gene set analysis, 277
indirect action mechanism, androgen, 275–276
MAP kinase and RhoA signaling, 274–275
mRNA expression dataset, profiling study, 276–277
PCa model system, 280–281
phosphorylation, 278
RhoA
actin/MAL signaling axis, 278–279
AR-RhoA-SRF signaling axis, 280, 281
effector, 278
GTPase activity, 279–280
immunohistochemical analysis, 279
siRNA-mediated silencing, 278–279
Sharma, A., 201

Shen, J., 311
SPecificity affecting AR Knock In (SPARKI) model, 33–34
Splinter, E., 57
Stanbrough, M., 158
Sun, S., 215

**T**
Takayama, K., 174, 207
Tamura, K., 154, 155, 159, 177
Tasquinimod, 288
Taxotere, 246–247
Taylor, B.S., 16, 204, 208
Testosterone-repressed prostate message 2 (TRPM-2), 343
Tetherome
  androgen ablation, 105–106
  androgen/AR signaling, 105
  AR binding domain, 105
  bimodal action, 105
  C/EBPα, 101–102
  Elk1, 100–101
  Elk1-AR interaction, 106
  gene regulation, 103, 104
  HoxB13, 102
  ligand sensitivity and AR dimerization, 103–104
  TMPRSS2 and ETS protein, 102–103
Tindall, D.J., 151, 152
Titus, M.A., 200
Tomlins, S.A., 202
Transforming growth factor-β (TGF-β), 255–256, 305
Tropomyosin alpha-1 chain (TPM1), 308
Truica, C.I., 115

**U**
Urbanucci, A., 206

**V**
Varambally, S., 158
Vascular endothelial growth factor (VEGF), 286–287
Velasco, A.M., 152
Veldscholte, J., 202
Von Hippel-Lindau disease, 289–290

**W**
Waltering, K.K., 202, 301
Wang, Q., 55, 61, 134, 174, 175, 204–206
Welsbie, D.S., 171
Wissmann, C., 113
Wissmann, M., 171
Wnt signaling pathway
  and androgen interaction, 113
  canonical signaling pathway, 112
  Frizzled receptor, 113–114
  Gleason score, 113
  LNCaPcell, 113
  noncanonical signaling pathway, 112
  transmembrane Frizzled protein, 112
  Wnt-3a CM, 114

**Y**
Yamagata, K., 305
Yamane, K., 171
Yang, X., 215
Yang, Y., 307
Y-box binding protein-1 (YB-1), 343, 344
Yu, J., 134, 159, 205–207, 209

**Z**
Zhang, H.L., 310
Zhang, X., 217
Zhao, B., 57
Zhao, J.C., 204, 208
Zhu, M.L., 178