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

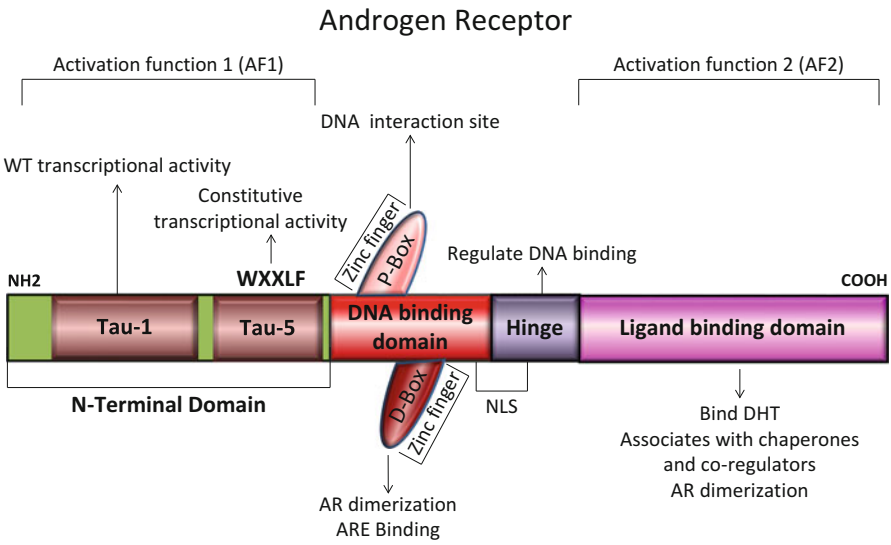

Fig. 15.1 Androgen receptor structure: schematic diagram of protein domains and functions

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

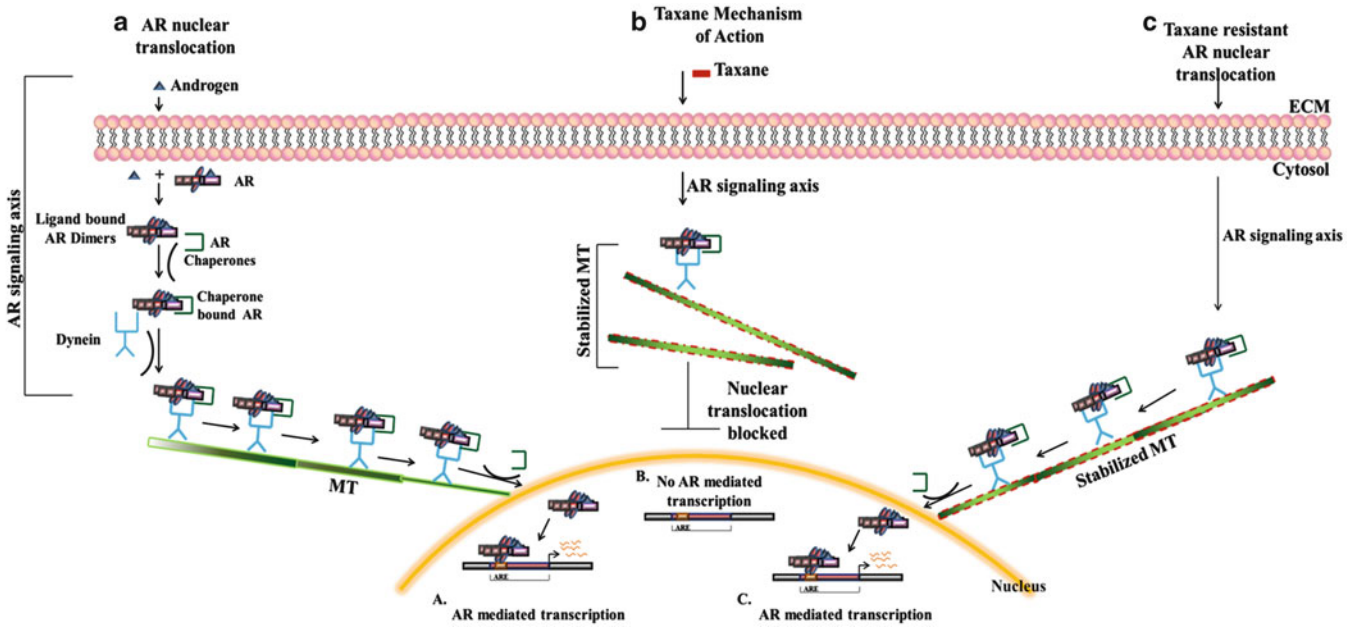

Fig. 15.2 Targeting AR translocation to the nucleus in prostate cancer. (**a**) After binding of cognate ligand, molecular handling by super-chaperone complexes, dimerization, phosphorylation, and association with dynein motor proteins onto microtubules (MT), AR translocates to the nucleus to mediate 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

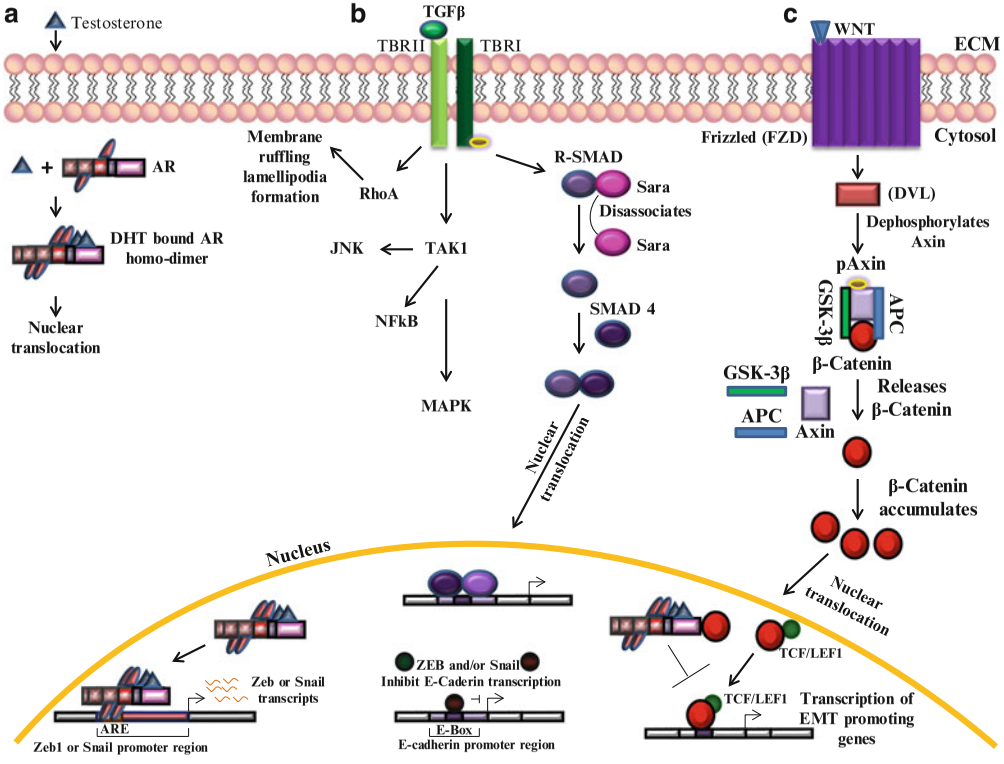

Fig. 15.3 Role of AR in prostate cancer and EMT (**a**) Cadherin switching, Zeb1 feedback loop, and expression of Snail2 are modulated by the crosstalk with Androgen Receptor (AR). (**b**) TGFβ signaling can induce EMT-associated changes in Prostate cancer via Smad-dependent and -independent pathways 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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