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AR function in promoting metastatic prostate cancer

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Abstract

Prostate cancer (PCa) remains a leading cause of cancer-related death in the USA. While localized lesions are effectively treated through radical prostatectomy and/or radiation therapy, treatment for metastatic disease leverages the addiction of these tumors on the androgen receptor (AR) signaling axis for growth and disease progression. Though initially effective, tumors resistant to AR-directed therapeutics ultimately arise (a stage of the disease known as castration-resistant prostate cancer) and are responsible for PCa-specific mortality. Importantly, an abundance of clinical and preclinical evidence strongly implicates AR signaling cascades in the development of metastatic disease in both early and late stages, and thus a concerted effort has been made to delineate the AR-specific programs that facilitate progression to metastatic PCa. A multitude of downstream AR targets as well as critical AR cofactors have been identified which impinge upon both the AR pathway as well as associated metastatic phenotypes. This review will highlight the functional significance of these pathways to disseminated disease and define the molecular underpinnings behind these unique, AR-driven, metastatic signatures.

Keywords

Androgen; Androgen receptor (AR); Prostate cancer (PCa); Castration-resistant prostate cancer; CRPC; Metastasis; Cistrome

1 Overview: prostate cancer development, treatment, and progression

Prostate cancer (PCa) is a leading health concern in the USA with nearly 32,000 deaths attributed to the disease annually [1]. It remains the most common noncutaneous malignancy diagnosed in US men and is the second leading cause of cancer-related deaths [1]. A favorable prognosis is heavy reliant upon early detection of the primary tumor, where localized lesions are effectively treated by radical prostatectomy and/or radiation therapy [2]. Disseminated disease, however, proves much more of a clinical challenge. Upon radiographic evidence of metastatic lesions (most commonly found in local lymph nodes and bone at the earliest stages), patients undergo systemic therapy to thwart cancer growth and progression [3–6]. Unlike other solid tumors, prostatic adenocarcinoma, both at primary and metastatic sites, responds poorly to standard chemotherapeutic intervention [7–9]. Therefore, first-line treatment for this disseminated disease leverages the addiction of these tumors on androgens for both growth and survival [4, 10, 11].

Molecular modeling of PCa has demonstrated that the action of androgens is manifest through activation of the androgen receptor (AR), whose activity is critical for PCa development and progression at all stages of disease [4]. As such, patients with metastatic tumors are treated with androgen deprivation therapy to biochemically dampen AR signaling [5, 6]. Initially, this therapy is quite effective resulting in either stable disease or tumor regression as measured biochemically (via serum prostate-specific antigen (PSA)) or radiographically [6, 11]. However, within 2 to 3 years, progression is often noted despite suppression of testosterone, a stage of the disease known as castration-resistant prostate cancer (CRPC). Herein, the AR signaling axis has been inappropriately reactivated allowing for upregulation of growth and survival pathways necessary for tumor proliferation and metastatic progression [10, 12, 13]. This results in enhanced metastatic tumor burden, and ultimately patient mortality [14]. As a result, there is an intense effort to not only more effectively target AR in CRPC, but to define the molecular pathways governed by AR which are responsible for such lethal metastatic events.

2 Role of AR in disease progression

AR is a large ~110 kDa transcription factor which belongs to the steroid nuclear receptor family, which functions as a ligand-dependent transcription factor necessary for development and function of the prostate [15]. In the normal prostate, AR plays a critical role in the early formation of the prostatic epithelia, directing key developmental programs responsible for proper formation of epithelial architecture and differentiation [15]. Importantly in this context, AR also acts to limit prostatic epithelial growth, which is essential for the development and function of prostatic tissue.

As a function of tumorigenesis, the transcriptional targets of AR are altered, engaging pro-proliferative programs essential for growth of prostatic adenocarcinoma cells [16]. Activation of this pathway is heavily reliant upon circulating androgens, which enter epithelial cells and are rapidly converted to the potent AR ligand 5α-dihydrotestosterone (5α-DHT) by the enzyme 5α-reductase [17]. This facilitates the binding of 5α-DHT to the ligand binding domain (LBD) of AR, shifting the position of helix 12 of the LBD towards the N-terminus [18, 19]. Functionally, this results in enhanced stability of the protein and release from inhibitory heat shock proteins. Agonist-bound AR translocates to the nucleus where it dimerizes and binds to chromatin in a site- and cell type-specific manner [20]. Once bound, AR undergoes a multitude of posttranslational modifications (including phosphorylation, methylation, sumoylation, and acetylation) [21, 22] which facilitate co-factor recruitment and chromatin remodeling [23]. Ultimately, this leads to AR-specific programs critical for the survival and growth of PCa cells [16]. Clinically, the activity of AR can be monitored through the induction of the well-characterized AR target gene *KLK3*, which encodes a secreted protease PSA [6]. Levels of PSA can be monitored in patients serum and used a surrogate for AR activity and associated tumor burden [6]. As such, elevated or sustained increases in PSA levels are used (at least in part) as a marker of PCa development and progression.

2.1 Targeting AR in advanced disease

Work initiated by Huggins and Hodge in the early part of the twentieth century identified the first effective treatment for PCa, androgen deprivation therapy (ADT) [24–26]. To date, ADT still remains the most effective form of treatment for patients with disseminated disease. Standard of care options most often involve chemical castration through use of gonadotropin-releasing hormone (GnRH) agonists which, after an initial surge of testosterone production [27], suppress the secretion of leutinizing hormone (LH) and functionally inhibit testicular androgen synthesis [11, 28]. To prevent tumor growth in response to the testosterone flare, AR antagonists (e.g., biculatamide) are often given in combination with GnRH agonists, a therapeutic strategy known as total or maximum androgen blockade [11]. As a result, circulating testosterone levels often drop to levels seen in castrated males (<0.2 ng/dl [29]), which frequently correlate with a decline in serum PSA levels and stable or decreased tumor burden. Despite this, recurrence is common and within 2–3 years patients begin to experience a resurgence of tumor growth heralded by increases in serum PSA levels (biochemical recurrence), indicative of a reengagement of the AR signaling axis.

At this stage of the disease (metastatic CRPC), tumors have evolved mechanisms to reactivate AR despite continued ADT. As reviewed [4, 10, 30], a multitude of pathways have been described which contribute to inappropriate AR activity in CRPC, many of which involve manipulation of AR itself (amplification, alternative splicing, mutation, and post-translational modifications). Upstream of AR, however, recent evidence also suggests that tumors gain the ability to produce their own androgens, increasing the local androgen pool to sufficient levels to activate the AR axis [10, 31]. Microarray profiling of patient samples confirmed that key genes involved in the conversion of cholesterol derivatives to androgen precursors (CYP17A and HSDD3B2) are induced in response to ADT [32]. Consequently,

direct inhibitors of CYP17 have been developed (orteronel (TAK-700) [13], galeterone (TOK-001) [14]) and currently, abiraterone acetate is approved for the treatment of metastatic CRPC patients who have disease progression despite ADT both pre- and postchemotherapy [33–37]. In clinical trials, administration of abiraterone acetate significantly increased time to biochemical progression and radiographic progression-free survival, indicating that metastatic tumors are still reliant on AR signaling at this stage [34, 35, 38]. As such, some clinical benefit has been seen with the introduction of novel anti-androgens (e.g., Enzalutamide), which extended patient survival by ~3 months [39, 40]. However, once resistant to hormone-directed therapies, taxanes are the next therapeutic option for patients, which, unfortunately only affords a 2–3 months survival advantage (with a high rate of patient morbidity) [8, 9, 41]. As a result, there is a critical need to better define AR-driven networks responsible for lethal tumor phenotypes to more accurately and effectively combat metastatic disease.

2.2 Current strategies for targeting metastatic CRPC

Therapeutic options for treating disease that progresses after hormone therapy are extremely limited (Fig. 1). Taxane-based chemotherapy has proven to be the most effective chemotherapeutic in the management of metastatic CRPC [9, 41]. Preclinical evidence suggests that taxanes inhibit tubulin polymerization, a critical cytoskeletal protein involved in maintenance of cellular integrity and architecture, mitosis, intracellular transport, cell signaling, and gene expression. Docetaxel, a semisynthetic taxane derived from the needles of *Taxus baccata* that reversibly binds to microtubules with high affinity, has been demonstrated to provide a 20–24 % improvement in survival for men with metastatic castrate-resistant prostate cancer [9, 41] and was the first Food and Drug Administration-approved agent for this patient population. Subsequent trials combining various chemotherapeutic or biologic agents to docetaxel have not yielded improved survival. However, preclinical work demonstrated that a second-line taxane, cabazitaxel, had cytotoxicity in cell lines and animal models both sensitive and resistant to docetaxel [36, 41, 42]. While the mechanism of overcoming docetaxel resistance is unclear, clinical evidence has validated the efficacy of this agent as it was shown to improve survival in CRPC patients who had received prior docetaxel [43]. Given the effectiveness in the docetaxel-pretreated patient population, there is currently an ongoing international randomized trial comparing first-line docetaxel versus cabazitaxel (trial #NCT01308567).

In addition to chemotherapy, a new agent was recently approved to treat CRPC of the bone. PCa primarily metastasizes to bone, where tumor growth leads to high fracture rates and is associated with patient morbidity [44, 45]. Clinically, high tumor burden at such metastatic sites correlates with decreased survival time [14], yet few therapeutic options are available which specifically target tumors at these sites. Currently, only Radium-223 dichloride (a radioactive α-emitting isotope which mimics the valence structure of calcium ions) has been developed as an effective treatment strategy for bone-specific metastases [36, 46]. A phase III clinical trial of CRPC patients demonstrated that treatment with Radium-223 significantly reduced first skeletal-related events and prolonged overall survival (~3 months) as compared to placebo [46]. Interestingly, however, it is unknown as to how effective Radium-223 would be in the hormone therapy (HT)-naive setting. Clinical data suggests that

an intact AR signaling axis is critical for the progression of metastatic lesions. Thus it is tempting to speculate that combination ADT and Radium-223 would effectively inhibit PCa progression in hormone-sensitive metastatic disease. Future efforts will likely test this hypothesis to determine if such combinations could be leveraged for patient benefit. While Radium-223 represents a significant step forward in combating metastatic PCa progression, few strategies have been developed which specifically target metastatic programs unique to PCa.

3 Role of the AR in the development of metastatic disease

Given the poor prognosis associated with the development of metastatic prostate cancer and the known role of AR in promoting disease progression, a concerted effort was undertaken to identify the functions(s) by which AR facilitates the metastatic process and/or maintenance of metastatic disease. As will be reviewed herein, translational investigative studies revealed major roles for AR in promoting prometastatic events, through: (1) differential chemokine receptor/ligand function; (2) altered function of tumor-associated AR cofactors (e.g., FOXA1, cyclin D1b, and SWI/SNF); and (3) formation of prometastatic, AR-dependent gene fusions and downstream effectors (e.g., SOX9).

3.1 I. Chemokine and chemokine receptor dysregulation in PCa

Chemokine receptors belong to the G-protein coupled receptor (GPCR) family of proteins. There are over 20 known members of this family each of which contains a generally conserved structure consisting of a seven-pass transmembrane monomer and a soluble cytoplasmic tail [47, 48]. While expression is fairly ubiquitous, these receptors are known to have significant roles in the immune response, development, angiogenesis, and inflammation. Activation occurs upon ligand binding (chemokine family of soluble ligands) which initiates a series of signal transduction pathways responsible for mediating a multitude of cellular responses including actin skeleton rearrangements, calcium signaling, and survival (among others). Given the critical role these pathways play in cancer progression, chemokine receptors are often deregulated in human disease [47–49]. Importantly, as several of these receptors (and their ligands) have been shown to be regulated by AR [50, 51], there is growing interest in determining the relevance of these receptors in mediating metastatic phenotypes in both hormone therapy-sensitive and castration-resistant prostate cancer.

3.1.1 CXCR4—CXCR4 is a 352 amino acid GPCR which is activated though association with its soluble ligand SDF1 (also known as CXCL12) [47]. Upon activation, intramolecular signaling cascades (which vary with cell type) are transmitted through the cell, culminating in cytoskeletal rearrangements which promote migration towards the site of activation as well as transcriptional programs which regulate growth and survival [47]. The prostate gland is one of the few soft tissues which express detectable levels of CXCR4, though it is unclear if it plays a significant role in normal prostatic function [52]. Importantly, however, as a function of tumorigenesis, there is a marked induction of CXCR4 both at the transcript and protein levels [53]. Immunohistochemical analyses of PCa lesions confirm heightened expression of the receptor in both primary and metastatic disease (compared to benign

control), with the highest expression detectable in bone metastases [53]. Of note, tissue-wide interrogation of SDF-1 expression indicated that bone expresses relatively high levels SDF-1 [54]; the bone representing a preferential site for PCa metastatic colonization. This supports the concept that induction of CXCR4 (as a consequence of tumorigenesis) functions to promote metastatic progression via attraction to the high SDF-1 gradient generated in tissues such as bone. While more clinical data is needed to confirm such conclusions, multiple clinically relevant models have demonstrated that CXCR4 is regulated as a function of androgen signaling, and it is likely that the interplay between the two promotes lethal metastatic events.

Multiple AR-positive PCa models have been used to describe enhanced migratory potential of CXCR4 expressing cells towards a gradient generated by SDF-1 [50]. Importantly however, such phenotypes were only observable in the presence of androgen, indicating that CXCR4-dependent metastatic phenotypes required an intact AR signaling axis [50]. Such conclusions are supported by transcriptional evidence which demonstrates robust induction of CXCR4 transcript in response to androgen stimulation [50], which can be reversed though either administration of ADT or direct AR antagonists. Exploration into the underlying mechanisms behind AR-induced CXCR4 expression revealed that the relationship was likely indirect. Chip sequencing analyses from multiple AR-positive cell lines lacked AR binding within a 50 kb window of the transcriptional start site [55, 56]. Such conclusions were confirmed wherein ligand bound AR was unable to promote CXCR4 induction in the presence of protein synthesis inhibitors. Subsequent analysis of AR-regulated transcription factors capable of fostering CXCR4 expression uncovered Kruppel-like-factor 5 (KLF5) as a potential candidate [50]. Indeed, transcript analyses from LNCaP cells revealed a robust and rapid induction of KLF5 transcript upon AR activation. Additionally, expression of exogenous KLF5 in a low-androgen setting was sufficient to promote CXCR4 expression and invasion toward an SDF-1 generated gradient [50]. Collectively, these data demonstrate a tumor-specific AR-driven pathway which promotes invasive phenotypes *in vitro*. While intriguing, it will be critical to determine if AR/KLF5-induced CXCR4 expression is sufficient to promote metastatic phenotypes *in vivo*, and whether or not such pathways are preserved in CRPC tumors. In addition, profiling of the ERG cistrome and transcriptome identified CXCR4 as a putative target of ERG signaling in fusion-positive disease [57]. Given the known metastatic functions of ERG, AR, and CXCR4, it will be important to determine how the interplay between these factors cooperate to promote metastatic disease, and if this pathway offers a novel therapeutic target to thwart metastatic progression in AR-positive, fusion-positive disease.

3.1.2 CX3CR1/fractalkine—While there is an abundance of literature examining tumor-associated androgen signaling and its role in mediating metastatic programs, there is a gap in knowledge as to the potential role of AR in facilitating metastatic events in distal tissues. Though AR is expressed in many tissues, its activity greatly influences bone development [58]. Congruent with this concept, a recent study has identified a novel role for androgen signaling in regulating the cleavage of fractalkine (CX3CL1), a membrane-bound cytokine which is expressed human bone marrow endothelial cells and possesses intrinsic cell adhesive properties [51, 59]. Previous studies utilizing human immune cells which express

the cognate receptor for fractalkine, CX3CR1, showed direct cellular binding to endothelial cells, which likely aided cellular extravasation [60–62]. Analogous *ex vivo* studies utilizing PCa models demonstrated similar binding kinetics to bone marrow endothelial cells, which occurred in a fractalkine-dependent manner [51]. Further investigation determined that, though the induction of extracellular matrix-metalloproteases (e.g., ADAM17), AR signaling can promote the cleavage of fractalkine from bone cells but not bone marrow endothelium [59]. This supports a hypothesis whereby PCa cells bearing CX3CR1, adhere to endothelial bone walls expressing fractalkine, and extravasate towards the soluble fractalkine gradient generated by the AR signaling axis, ultimately resulting in the colonization of the bone microenvironment. Given that first-line therapy for metastatic disease involves androgen ablation, it will be interesting to determine how systemic hormonal therapy affects both fractalkine cleavage and metastatic growth within the bone. Furthermore, as a major mechanism through which PCa tumors evade ADT is through production of their own androgens [10, 13, 32], it would be useful to determine if local production of androgens within CPRC bone metastatic tumors further promotes fractalkine cleavage, bone homing, and subsequent metastatic tumor formation. Future efforts will likely define the clinical relevance of this metastatic cascade in both early and late stages of disease, and determine if targeting this pathway can be leveraged to limit metastatic tumor burden.

3.2 II. AR cofactor deregulation

The discovery of AR-regulated expression of chemokine receptors and ligands represent a significant advance in the search for AR-driven pathways, which could potentially be leveraged to limit metastatic progression. However, it is improbable that a single AR target is responsible for development of metastatic disease. More likely, disease-specific AR-driven networks are causal for invasive phenotypes and development of metastatic lesions. Indeed, recent studies uncovered multiple AR-associated cofactors capable of modulating and "reprogramming" genome-wide AR binding events. Such differential AR signatures have been associated with enhanced metastatic potential *in vitro* and *in vivo*, and have putatively significant implications for the development and progression of AR-driven metastatic events.

3.2.1 FOXA1 controls AR signaling and metastatic events in PCa—The ability of AR to localize to sites of transcriptional regulation upon activation is highly dependent on chromatin state and structure. Repressed, or closed, chromatin is often defined by repressive histone marks (such as H3K9me2) and lacks enrichment for transcriptional regulators, like AR [63–65]. Conversely, active chromatin structures are associated with mono- and dimethylation marks on H3K4 (H3K4me1 or H3K4me2), which are often coupled to sites of active transcription [63–65]. In the context of hormone-dependent tissues, such markers of active transcription are often enriched for binding of the FOXA family of transcription factors, also known as "pioneer factors" that facilitate site-specific chromatin remodeling and subsequent nuclear receptor binding [63, 64, 66–69]. Importantly, genome-wide analysis of the AR cistrome in PCa cells found a high degree of overlap with the FOXA family of proteins (nearly 70 % overlap) [68], consistent with the known role of FOXA factors serving as pioneer factors, which help to define both active and repressed chromatin

states. More specifically, FOXA1 has been demonstrated to be a critical mediator of AR signaling in PCa, as loss of FOXA1 in AR-positive PCa cells, decreased both cell growth and AR signaling (as determined by AR target gene levels) [70, 71]. Moreover, analysis of the AR cistrome in the CRPC line LNCaP-Abl defined a critical role for FOXA1 in mediating AR recruitment to critical cell cycle genes (e.g., UBE2C), which facilitated castration-resistant growth [55]. Consistent with hormone-dependent AR signaling, loss of FOXA1 dramatically decreased growth at this stage indicating that FOXA1 is required for AR-driven proliferative events in both early and late stages of disease [55]. Contrary to what might be expected, knockdown of FOXA1 did not eliminate AR binding across the genome. In fact, depletion of FOXA1 levels resulted in an increase of total AR binding events genome wide (13,505 unique binding events with knockdown) [68, 70], indicating that tumor-associated elevation of FOXA1 levels dramatically restrict the AR cistrome, which likely results in AR-driven signatures responsible for both proliferative and metastatic events. Future efforts will aim to better define the functions of FOXA1 responsible for such divergent AR functions, and the downstream targets responsible for mediating aggressive phenotypes.

Given the knowledge above, it is not surprising that a number of studies have aimed to characterize the expression patterns of FOXA1 in lethal metastatic disease. Interestingly, however, FOXA1 levels have been reported as both elevated or diminished in PCa [71–74]. Such discrepancies could be attributed to potentially novel functions of FOXA1, independent of nuclear receptor signaling. Analysis of the FOXA1 cistrome in Abl cells demonstrated androgen-independent binding of FOXA1 to cyclin E2 regulatory loci, which resulted in increased expression of cyclin E2 and progression through cell cycle [71]. Loss of FOXA1 dramatically reduced cellular growth of CRPC cells, while ectopic expression of cyclin E2 restored proliferative capacity in this context [71], substantiating AR-independent roles of FOXA1 in CRPC-specific proliferation. Conversely, independent studies also reported decreased levels of FOXA1 from metastatic tissue, and demonstrated an association between FOXA1 loss and enhanced metastatic potential [73]. Chip sequencing analyses of the FOXA1 cistrome in LNCaP cells defined a prominent FOXA1 binding site within the intragenic region of the *SNAI2*(Slug) gene, which was associated with low expression of Slug and limited migratory potential [73]. FOXA1 depletion dramatically increased Slug expression, which enhanced the invasive potential of these cells and promoted a dedifferentiation signature [73]. Such data implicate divergent roles of FOXA1 in mediating aggressive phenotypes in a prostate-specific context, and suggest that the functions of FOXA1 in PCa appear to be complex and highly cell type specific. While elevated levels of FOXA1 appear necessary to maintain CRPC-specific growth, depletion of FOXA1 promotes lethal metastatic events associated with patient mortality. Furthermore, recent deep sequencing of patient samples has uncovered mutations within the coding regions of the FOXA1 gene in late stage disease, some of which have the potential to affect FOXA1/DNA interactions and/or function [75–77]. Future efforts will focus on elucidating the context-specific functions of FOXA1, and these newly identified mutants, in promoting aggressive tumor phenotypes, and better define the role of AR in such events.

3.2.2 BAF57 deregulation promotes AR-dependent metastatic events—While pioneer factors like FOXA1 have been shown to play critical roles in defining the AR cistrome, other classes of DNA-associated co-factors have also demonstrated an ability to modulate disease-associated AR binding events. The SWI/SNF complex represents such a class, and functions as a multisubunit chromatin remodeling oligomer, which facilitates context-dependent gene activation or repression. The complex itself consists of a core ATPase (either BRG1 or BRM) and a host of associated cofactors, which help to define genome-wide specificity and tissue-specific gene expression programs [78–80]. Alterations or changes in the subunits that comprise the complex can dramatically affect the genomic localization or targets of SWI/SNF culminating in aberrant transcriptional profiles and cellular function [80]. Consequently, deregulation of SWI/SNF subunits is common in many tumors, including PCa [79, 81, 82]. Clinical evidence suggests that BAF57, a nonessential accessory SWI/SNF subunit is dramatically elevated in metastatic PCa lesions [82]. Furthermore, there is substantial evidence to suggest that BAF57 functions as a direct AR cofactor, enhancing AR-dependent transcriptional regulation and associated growth [81–83]. Microarray profiling of BAF57 upregulation in hormone-dependent cell lines confirmed such conclusions, where BAF57 induction was sufficient to promote ligand-independent AR activity under conditions mimicking castration (as monitored by the clinically relevant targets (*KLK3*, *TMPRSS2*, and *FKBP5*)) [82]. Interestingly, in addition to classical AR targets, gene ontology analysis uncovered a subset of BAF57-regulated genes involved in migration and invasion, specifically integrin alpha 2 (*ITGA2*). Genome-wide interrogation of the *ITGA2* locus uncovered a putative AR binding event in a distal intragenic region, which was co-occupied by both BAF57 and AR (in a hormone-independent fashion) [56, 82]. Further analysis of SWI/SNF occupancy at this site in the presence of elevated BAF57 identified enrichment of BRG1, indicating that SWI/SNF activity is likely required for the AR/BAF57-mediated induction of *ITGA2* under castrate conditions. Consonantly, isogenic models of BAF57 deregulation confirmed elevated levels of integrin alpha-2 at the protein level, which was associated with enhanced migratory and invasive phenotypes *in vitro*[82]. Blocking integrin alpha-2 function at the cell surface with neutralizing antibodies dramatically inhibited the migratory potential of both control and BAF57-expressing cells, suggesting that integrin alpha-2 is required for basal and BAF57-induced phenotypes [82]. Collectively, these data implicate SWI/SNF perturbations in the pathobiology of AR-driven metastatic events in response to hormone therapy, and provide novel targets which could be leveraged for therapeutic benefit. It will be vital to define both the BAF57 and AR cistromes in models of BAF57 deregulation in order to more completely understand the molecular mechanisms that drive BAF57/AR-mediated metastatic phenotypes.

3.2.3 Cyclin D1b alters AR function to promote metastatic events—In addition to BAF57 and FOXA1, cell cycle-specific AR cofactors are also likely to play a role in the development of metastatic disease. Cyclin D1b is a tumor-specific splice variant of the well-characterized cell cycle regulator cyclin D1, which arises due to a failure to splice at the exon4/intron4 boundary of the pre-mRNA, resulting in the incorporation of intron 4 and the introduction of an early stop codon [84]. The resulting protein thus lacks all sequences encoded by the terminal exon (exon5) and harbors a unique 33 amino acid C-terminus encoded by intron 4 [84]. Numerous studies aiming to define the biological and biochemical

consequences of cyclin D1b induction have uncovered novel functions for this splice variant in tumor biology, which differ dramatically from full-length cyclin D1 (cyclin D1a). For example, expression of cyclin D1b but not cyclin D1a was sufficient to promote tumor formation in fibroblasts [85], enhanced the migratory potential of bladder cancer cells [86], and was associated with increased resistance to ER antagonists in breast cancer models [87]. Investigation in PCa-specific models uncovered a unique role for D-type cyclins in modulating AR activity. Consistent with the known role of D-type cyclins as transcriptional modulators, previous reports have described cyclin D1a as a potent regulator of AR, capable of directly inhibiting AR transactivation and subsequent AR activity (as determined by PSA readout) [88–91]. Similar studies assessing the consequences of cyclin D1b expression on AR activity determined that while still able to functionally interact with AR, cyclin D1b was unable to negatively regulate AR function [92]. Such data are congruent with clinical observations aiming to define expression patterns of both cyclin D1a and cyclin D1b in primary disease. Only a small number of tumors scored positive for cyclin D1a (7 %), while nearly 30 % of all primary tumors demonstrated positive staining for cyclin D1b [93]. Further analyses of expression patterns across non-neoplastic and cancerous tissues determined that while there was no induction in cyclin D1a levels as a function of tumorigenesis, there was a marked increase in cyclin D1b levels in cancerous tissues [93]. These data implicate cyclin D1b as a clinically relevant oncogene in PCa, with distinct functions from that of cyclin D1a.

Given the established oncogenic functions of cyclin D1b and the induction of this splice variant in clinical samples, follow-up studies aimed to define the consequence of cyclin D1b on both tumor phenotypes as well as AR activity in PCa. Isogenic models of cyclin D1b induction demonstrated that cyclin D1b-expressing cells harbored enhanced invasive potential which was critically dependent on an intact AR signaling axis [94]. Subsequent microarray analyses defined a large transcriptional regulatory network uniquely regulated by cyclin D1b, which was enriched for genes associated with migration and invasion. Among the most prominently regulated was the transcriptional regulator *SNAI2*, a known AR-regulated gene implicated in metastatic progression PCa [73, 95, 96]. Importantly, induction of cyclin D1b resulted in occupancy of both cyclin D1b and AR at *SNAI2* regulatory loci, which correlated with active marks of transcription (acetylated histone H4) and well as enhanced transcriptional output [94]. Notably, depletion of Slug dramatically inhibited all cyclin D1b-induced phenotypes, indicating that Slug is a critical downstream mediator of cyclin D1b/AR-driven phenotypes *in vitro* [94]. Parallel studies *in vivo* determined that enhanced Slug expression dramatically increased metastatic growth at distal soft tissues (liver and lung) [94], an event that is clinically associated with resistance to chemotherapeutics and a poor prognosis [97]. Finally, analysis of CRPC tumors samples uncovered a strong positive correlation between cyclin D1b and Slug expression, indicating that the cyclin D1b/AR/Slug axis is preserved in human tumors [94]. Taken together, these data suggest that cyclin D1b hijacks the AR signaling axis to promote expression of genes (*SNAI2*), responsible for metastatic progression and ultimately patient mortality. Critical next steps will aim to better define both the cyclin D1b and AR cistrome to better understand the molecular underpinnings of cyclin D1b action on AR-driven metastatic events.

3.3 III. AR-dependent gene fusions and downstream effectors

While there is substantial evidence to suggest that AR cofactors significantly influence AR signaling networks to promote metastatic phenotypes, little is known as to how such factors are induced or regulated in human disease. While future efforts will characterize these pathways to better define the oncogenic cascades responsible for their expression, a class of AR-driven genetic fusions has been identified clinically, and are associated with progression to metastatic disease. As will be discussed, expression of these fusion products and downstream effectors is regulated as a function of AR activity, which can feedback to after the AR cistrome, culminating in an AR transcriptome which fosters invasive disease.

3.3.1 AR-regulated TMPRSS2: ETS fusions—Few genetic aberrations have been associated with the early stages of PCa, which has hindered the ability to stratify prostate cancer into subtypes. However, a chromosomal fusion event, which occurs between the regulatory locus of an AR-responsive gene (TMPRSS2) and the coding region of known oncogenic factors (erythroblast transformation specific, ETS genes) was identified in human prostate cancer samples. While reports vary, it is estimated that nearly half of all primary PCas harbor some form of this genetic fusion [98–103], which in some studies was associated with early onset of disease and a more aggressive, androgen fueled, phenotype [104]. Genetic profiling obtained from primary tumors has identified a multitude of fusion events which occur between the ETS family of transcriptional regulators (ERG, ETV1, ETV4, ETV5, and FLI1) and the regulatory locus of TMPRSS2, the most common of which occurs between the regulatory locus of TMPRSS2 and coding sequence of ERG [102, 103, 105, 106]. While it is still unclear as to how these fusions arise, there is evidence to implicate the AR signaling pathway in their formation [107, 108]. *In vitro* models suggest that upon androgen stimulation, AR facilitates a bridging event between the TMPRSS2 and ETS loci, which fosters DNA recombination at low frequency [107, 108]. Importantly, while the mechanisms behind the fusions initiation are still under investigation, their clinical relevance has been suggested by clinical analyses. In a recent study where treatment involved watchful waiting, patients who were positive for the fusion had more aggressive disease and showed a worse prognosis as compared to fusion-negative patients [109]. Such data indicate that fusions may play a role in the pathogenesis and progression of PCa.

Multiple models of fusion-positive PCa (both *in vitro* and *in vivo*) have been generated in order to define the oncogenic pathways regulated by ERG, which promote aggressive disease. *In vitro*, the VCaP line (derived from a vertebral metastasis) harbors an endogenous fusion between TMPRSS2 and ERG, and is highly responsive to AR activation. While still dependent upon AR signaling for growth and survival, modulation of fusion expression did not affect growth, tumor formation, or survival, yet drastically affected the invasive capacity of these cells [110]. This is congruent with a number of *in vivo* studies which demonstrate that neither induction of ERG or ETV1 alone is not sufficient to promote neoplastic events in the prostate [57, 111]. However, in combination with PTEN deficiency (PTEN +/−) prostate-specific ERG induction promoted aggressive and invasive adenocarcinoma by 8 weeks, with some mice developing poorly differentiated tumors by 12 weeks [57]. Mechanistically, these aggressive phenotypes were attributed to changes in the AR cistrome, which promoted programs of migration and angiogenesis [57]. Furthermore, parallel studies

aiming to define the unique AR cistromes between fusion-positive (VCaP) and fusion-negative (LNCaP) cells confirmed the concept that ERG alters the AR cistrome [112]. While there is some discrepancy between the signatures of these studies, both describe ERG-driven alterations of the AR cistrome, which are associated with enhanced invasive/metastatic potential. The latter also defines an important role for the histone methyltransferase EZH2 in ERG-mediated phenotypes [112]. EZH2 is well characterized for its role in maintaining stem cells in a dedifferentiated state, and has been shown to be elevated in metastatic/advanced disease [113, 114]. In PCa models, ERG-induced expression and activity of EZH2, which resulted in the repression of well-known EZH2 target genes (e.g., CDH1 and DAB2IP) and signatures representative of a dedifferentiated state [115]. Given that EZH2 has recently been implicated as an AR cofactor requisite for CRPC-specific AR signaling [115], it will be critical to determine how ERG, AR, and EZH2 function together to define a genome landscape that promotes lethal metastatic disease.

3.3.2 SOX9: effector of ERG function in PCa—SOX9 belongs to the SOX family of transcription factors that have well-documented roles in the regulation of developmental processes as well as in disease progression [116, 117]. In adult tissues (e.g., liver and pancreas), it contributes to the maintenance of stem-like progenitor cells, promoting programs of dedifferentiation and self-renewal [118–120]. In the developing prostate, SOX9 is required for proper ductal morphogenesis, and loss dramatically impairs prostatic architecture and function [121]. While both SOX9 and AR are required for proper development of the prostate, each is localized to different epithelial compartments in luminal tissue [121]. SOX9 expression is typically limited to the AR-negative/low basal cells, distinct from the AR-positive, SOX9 low, luminal cells [121]. Interestingly, however, AR and SOX9 are coexpressed in a subset of primary PCa [122, 123]. Analysis of a large number of primary PCa tumors identified elevated SOX9 expression in patients who harbored TMPRSS:ERG fusions, a signature which was further enriched in CRPC metastatic tumors [124]. Given the known roles of both ERG fusions and SOX9 in promoting metastatic phenotypes, it was likely that these pathways cooperated to enhance PCa progression and metastasis. Modeling this event using isogenic lines of fusion-positive disease confirmed that androgen-induced ERG expression promoted elevated levels of both SOX9 transcript and protein, while knockdown of either ERG or SOX9 was sufficient to inhibit invasive properties *in vitro* [124]. Importantly however, exogenous expression of SOX9 was unable to promote invasive phenotypes in the absence of androgen, indicating that additional androgen-regulated genes are required for ERG/SOX9-mediated metastatic events [124]. Furthermore, elegant mouse models utilizing prostate-specific inducible SOX9 constructs demonstrated that exogenous SOX9 was sufficient to promote PIN formation and localized invasion in hormone-proficient animals [124]. These phenotypes were further enhanced in a PTEN heterozygous background, indicating that ERG/SOX9-driven pathways cooperate with clinical relevant genetic alterations to promote both PCa progression and invasion. Such data demonstrate that SOX9 is a critical mediator of ERG-driven phenotypes in PCa.

While ERG expression was sufficient to drive SOX9 expression *in vitro* and *in vivo*, the underpinning mechanisms behind such induction remained unclear. ChIP-Seq studies

analyzing the ERG cistrome in fusion-positive models uncovered an enrichment of ERG binding over a 2 kb region proximal to the 3′ UTR of the SOX9 [124]. Interestingly, analysis of the AR cistrome found strong localization of AR within this region, which was lost upon ERG knockdown. Co-occupancy of both AR and ERG was associated with active marks of transcription and enhanced SOX9 expression [124]. Conversely, in fusion-negative models, AR activity decreased expression of SOX9 transcript and protein, and was instead only recruited to an enhancer-like region upstream of the SOX9 gene, implicated in transcriptional repression [124]. These data indicate that ERG can rewire the AR cistrome to promote expression of genes associated with cancer progression and metastasis. Given the knowledge that SOX9-mediated phenotypes require additional AR targets, it will be critical to determine which parallel pathways are governed by AR and SOX9/ERG in order to better define networks which can be exploited for patient benefit.

4 Conclusions and key questions

There is an abundance of clinical data to substantiate the AR signaling axis as a critical driver of metastatic disease at both the hormone therapy-sensitive and castration-resistant states. The findings described herein illuminate multiple pathways governed by AR which are responsible for the induction of prometastatic events, and broaden our understanding of disease-associated AR signatures (Fig. 2). In most cases, expression of critical AR cofactors was responsible for mediating such signatures, in essence rewiring the AR signaling axis to promote programs of invasion, migration, survival, and growth. While identification of these unique programs represents a significant leap forward in the attempt to thwart progression to lethal metastatic disease, there are several gaps in knowledge that have yet to be addressed. *First, despite the identification of numerous AR cofactors associated with metastatic disease (ERG, EZH2, cyclin D1b, BAF57, etc.) PCa still suffers from a lack of biomarkers which are effective at predicting metastatic events.* While most of the cofactors described above have demonstrated the ability to promote prometastatic phenotypes in AR-positive cells, it remains unclear if these markers alone are sufficient to predict metastasis or therapeutic outcome in the clinical setting. Definition of the co-occurrence of each of these cofactors in human disease will be critical to refine clinical signatures which more accurately predict progression to metastatic disease. *Second, how does expression of multiple AR cofactors within a given tumor affect the AR cistrome and downstream biological events?* Given, the heterogeneity of PCa tumors, it is likely that multiple disease-associated AR cofactors are expressed within a given tumor/tumor cell. While current models have examined each of these factors in isolation, critical next steps will aim to build off of clinical knowledge to better define and model the AR interactome, and identify clinically relevant targets and downstream effectors. *Third, what regulates the expression of AR cofactors which promote progression to lethal disease states?* Outside the identification of AR-driven expression of genetic fusions, little is known as to how each of these proteins are regulated. While current emphasis has been directed toward elucidating the downstream consequences of their tumor-associated expression, identification of the upstream oncogenic pathways that mediate their induction could offer novel avenues for therapeutic intervention to curtail development of metastatic lesions. *Fourth, are AR-driven metastatic signatures preserved between hormone therapy sensitive and CRPC tumors?* Genome-wide studies strongly suggest that the AR

cistrome changes during the transition to CRPC, yet few models have been established which have characterized the function of critical AR cofactors and downstream mediators on the AR cistrome in this stage of disease. Uncovering the function of the aforementioned factors in CRPC will help define disease state-specific AR signatures involved in metastatic events at this stage. *Finally, it is time to directly treat the primary tumor in disseminated disease?* It is uncommon for primary tumors to be resected after the detection of distant metastases. Consequently, it remains possible that future distant metastases also originated from this same primary site. Are these primary sites of CRPC tumors as indolent as expected, or are they in essence factories for disseminated tumor cells? While clinical trials directly testing these hypotheses have been proposed, little is known as to the role of primary tumors in advancing metastatic CRPC. Ultimately, elucidation of the sites responsible for such lethal metastatic events could provide novel avenues for intervention to limit the spread of lethal CRPC, dramatically improving patient morbidity and mortality.

In summary, is it clear that the AR pathway is critical for the development and progression to metastatic CRPC. Recent advances have uncovered dramatic changes in the AR cistrome as a function of disease progression, often attributed to altered expression of critical AR cofactors. Such changes have been demonstrated to not only promote the transition to CRPC, but also influence PCa-specific metastatic potential. Further investigation into the pathways downstream of both AR and these cofactors will likely uncover novel targets that can be targeted along side of AR to limit the development of lethal metastatic disease and ultimately enhance patient survival.

Acknowledgments

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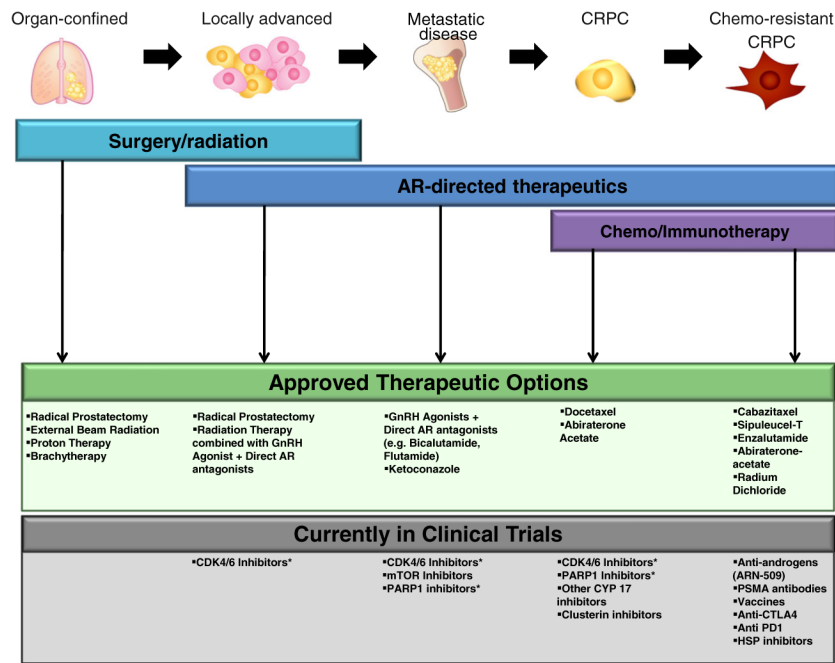**Fig. 1.**
Therapeutic options for patients diagnosed with prostate cancer. Therapeutic options for localized PCa often involve surgery in combination with some form of radiation therapy. In contrast, advanced or metastatic disease leverages the requirement of androgens for tumor growth, utilizing systemic therapy to limit androgen production and directly inhibit AR activity. Once resistant to first-line hormonal therapy, options to limit tumor growth and progression are limited, and consist of second-line hormonal therapy or taxane-based chemotherapeutics. While there are several options which are providing benefit in the chemotherapy-resistant space, a number of clinical trials are currently ongoing which offer promise for earlier intervention to help thwart progression to lethal CRPC [34, 36, 38, 39, 125–129]. Trials can be found online at www.clinicalrials.gov: trial #NCT01171898, NCT01414283, NCT01695044, NCT00694551, NCT00705835, NCT01804465, NCT00113984, NCT01420965, NCT01078662, NCT00471432, NCT01548807, and NCT01546987. *Clinical trials are currently in the planning stages at the University of Michigan and Thomas Jefferson University
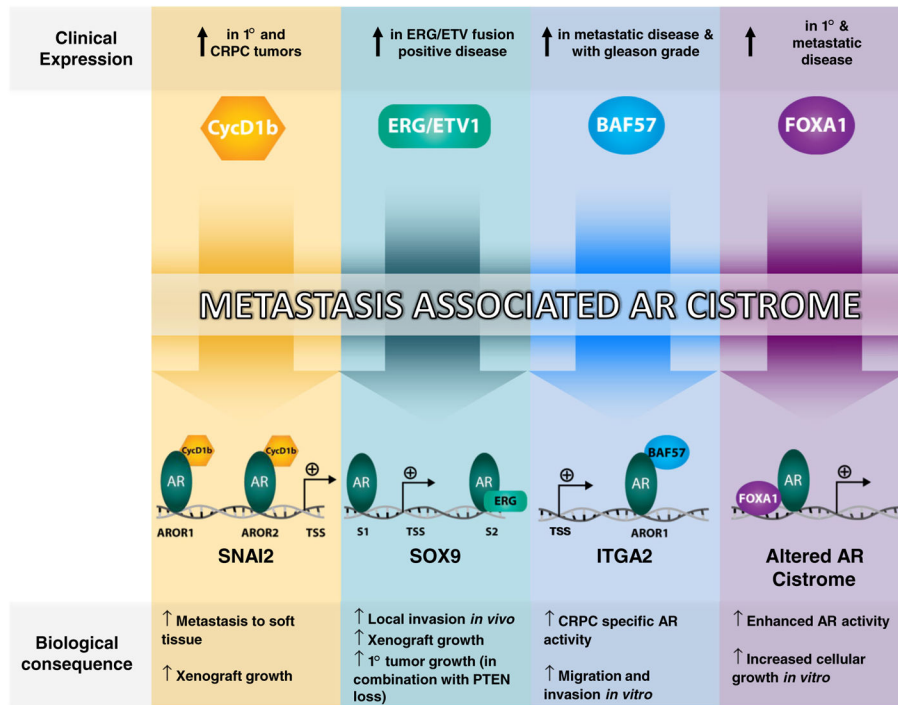
Fig. 2.

Cofactor-induced AR cistromes that promote metastatic phenotypes. A multitude of disease-associated AR cofactors have been identified in both primary and metastatic disease, which can impinge upon the AR cistrome to promote divergent AR binding events, culminating in transcriptional programs responsible for metastatic phenotypes. From left to right; (1) cyclin D1b co-occupied *SNAI2* regulatory loci (*A*ndrogen *R*eceptor *O*ccupied *R*egion 1—AROR1 and AROR2) with AR, promoting both AR occupancy and chromosomal looping, resulting in enhanced Slug expression and metastatic progression (*T*ranscriptional *S*tart *S*ite—TSS) [94]; (2) in fusion-positive disease, ERG binding at a 3′ SOX9 enhancer region (site 2–S2) facilitates AR recruitment to this site, resulting in enhanced SOX9 expression and increased invasion and tumor growth *in vivo* [124]; (3) tumor-associated elevation of BAF57 levels promote AR occupancy within an intronic enhancer of the *ITGA2* gene (AROR1) in a ligand-independent manner, recruiting necessary SWI/SNF subunits to promote *ITGA2* transcription and facilitate invasive and migratory phenotypes *in vitro* [82]; (4) Elevated levels of FOXA1 localize to sites on chromatin and restrict the total number of AR binding events genome wide. AR binding events, in this context, are thus enriched for FOXA1 binding motifs, and are associated with tumor-specific programs of growth and survival [68]