

# A Review of the Pathophysiological Mechanisms Underlying Castration-resistant Prostate Cancer

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**Abstract:** Androgen deprivation therapy or ADT is one of the cornerstones of management of locally advanced or metastatic prostate cancer, alongside radiation therapy. However, despite early response, most advanced prostate cancers progress into an androgen unresponsive or castrate resistant state, which hitherto remains an incurable entity and the second leading cause of cancer-related mortality in men in the US. Recent advances have uncovered multiple complex and intermingled mechanisms underlying this transformation. While most of these mechanisms revolve around androgen receptor (AR) signaling, novel pathways which act independently of the androgen axis are also being discovered. The aim of this article is to review the pathophysiological mechanisms that help bypass the apoptotic effects of ADT to create castrate resistance. The article discusses castrate resistance mechanisms under two categories: 1. Direct AR dependent pathways such as amplification or gain of function mutations in AR, development of functional splice variants, posttranslational regulation, and pro-oncogenic modulation in the expression of coactivators vs corepressors of AR. 2. Ancillary pathways involving RAS/MAP kinase, TGF-beta/SMAD pathway, FGF signaling, JAK/STAT pathway, Wnt-Beta catenin and hedgehog signaling as well as the role of cell adhesion molecules and G-protein coupled receptors. miRNAs are also briefly discussed. Understanding the mechanisms involved in the development and progression of castration-resistant prostate cancer is paramount to the development of targeted agents to overcome these mechanisms. A number of targeted agents are currently in development. As we strive for more personalized treatment across oncology care, treatment regimens will need to be tailored based on the type of CRPC and the underlying mechanism of castration resistance.

**Keywords:** castration resistance, prostate cancer, androgen-deprivation therapy, androgen insensitivity, androgen receptor

## Introduction

Apart from skin cancer, prostate cancer is the most common cancer in American men. A reported 33,330 estimated deaths in 2020 makes it the second leading cause of cancer-related mortality in men in the US. The standard of care treatment for localized disease is prostatectomy or radiation with the goal of removing or destroying the malignant cells confined within the prostate capsule. Whereas for locally advanced, recurrent disease after failure of locoregional treatments and metastatic disease the mainstay of treatment consists of androgen deprivation therapy (ADT). The rationale behind this treatment is to create a state of androgen deprivation that induces the apoptotic death of the androgen-dependent tumor cells as famously theorized by Huggins and Hodges.<sup>1</sup> Unfortunately, despite the early

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efficacy of ADT, after a mean time of 2–3 years prostate cancer patients become refractory to further treatment as the tumors enter into an androgen-liberated state also known as castrate-resistant prostate cancer (CRPC). There are multiple mechanisms implicated in the progression to castration resistance, with the majority involving the androgen receptor (AR). However, there is increasing evidence that the transition from androgen dependence to castrate resistance might rely on alternative growth and survival pathways that either interact with the androgen signaling or are truly AR-independent mechanisms. Interestingly, these driving alterations in pathways may provide important therapeutic targets in androgen-independent prostate cancer. The general aim of this review is to provide an overview of the current evidence regarding both the androgen dependent and independent mechanisms underlying castration resistance.

## Androgen Receptor Physiology

The androgen receptor (AR) gene is located on chromosome X at locus Y11–12 and codes for a nuclear compound with four distinguishable components: the amino-terminal domain (NDT), DNA binding domain (DBD), a hinge region and ligand binding domain (LBD).<sup>2–4</sup> In the absence of ligands, the AR remains in the inactive state, bound to heat-shock proteins (specifically HSP90 chaperone complex) in the cytoplasm. The primary AR ligands are dihydrotestosterone (DHT) and testosterone. Upon ligand-binding, AR shifts its conformation and detaches from the HSP90 complex, transports to the nucleus in a homo-dimerized state and attaches to the androgen response element (ARE) in the promoter region of androgen regulated genes.<sup>3,5</sup>

## Androgen Deprivation Therapy (ADT)

AR signaling is central to prostate cancer (PC) oncogenesis. Early studies by Huggins and Hodges in 1941 demonstrated that castration or estrogen injection reduced PSA level in men with prostate cancer.<sup>1</sup> This observation provided a rationale for ADT which aims to inhibit growth of PC by suppressing serum testosterone to “castrate” levels (defined as <50 ng/mL). ADT was historically achieved with surgical orchidectomy (surgical castration) but can now be performed with medical castration by inhibiting systemic production of androgens via the hypothalamic-pituitary-gonadal (HPG) axis.

Gonadotropin releasing hormone (GnRH) is released from the hypothalamus in a pulsatile manner under physiologic conditions and stimulates the release of luteinizing hormone (LH) from the pituitary, which in turn stimulates testicular Leydig cells to produce testosterone. By contrast, a steady state concentration of GnRH has an inhibitory effect on the pituitary and blocks testosterone production via the HPG axis. Thus, the therapeutic administration GnRH agonists (leuprolide, goserelin, buserelin, triptorelin) and antagonists (degarelix) shuts down androgen production via the HPG axis.

Accounting for approximately 10% of systemic androgen production is the adrenal gland, wherein androgen precursors (pregnenolone, DHEA) are converted to testosterone via adrenal cytochrome P450 pathways. Abiraterone acetate is a selective, irreversible inhibitor of CYP17, and inhibits androgen production in the adrenal glands, testes and prostate-tumor tissue. The STAMPEDE trial showed that addition of abiraterone and prednisolone to ADT in men with locally advanced or metastatic prostate cancer significantly improved overall failure-free survival compared to ADT alone.<sup>6</sup>

Furthermore, combined androgen blockade (CAB) consists of ADT plus antiandrogens (enzalutamide, apalutamide, darolutamide). Antiandrogens interfere directly with AR signalling by blocking binding to androgen ligand, inhibiting nuclear translocation and association with nuclear DNA. A recent study by Sternberg et al showed enzalutamide plus ADT enhanced median overall survival for men with nonmetastatic CRPC.<sup>7</sup>

## Castration Resistance

Early response to ADT is usually phenomenal, with 80–90% of cases of metastatic disease achieving rapid decline in PSA.<sup>8</sup> However, metastatic disease that initially responds to ADT almost always eventually exhibits secondary resistance.<sup>9</sup> Castration resistance can be defined either clinically (as the occurrence of new metastasis or progression of preexisting disease) or biochemically as a 25% increase from the PSA nadir (considering an initial value of  $\geq 1$  ng/mL) in men with serum testosterone of <50 ng/mL, with a minimum rise of 2 ng/mL, which must be confirmed with a second value obtained 1–3 weeks later without radiological evidence of metastases.<sup>10,11</sup>

Understanding of castration-resistant PC (CRPC), previously called hormone refractory PC, has undergone a paradigm shift as elucidation of molecular mechanisms of castration-resistance reveals the continuing role of the androgen axis in the propagation of CRPC.

## AR-mediated Mechanisms of Resistance in CRPC

These mechanisms will be reviewed in this section under the following headings: AR amplification and overexpression, AR splice variants, AR mutations and promiscuity, posttranslational regulation of AR, coactivators or corepressor modification and intratumoral steroid hormone synthesis. Figure 1 illustrates the different AR dependent mechanisms along with potential therapeutic targets.

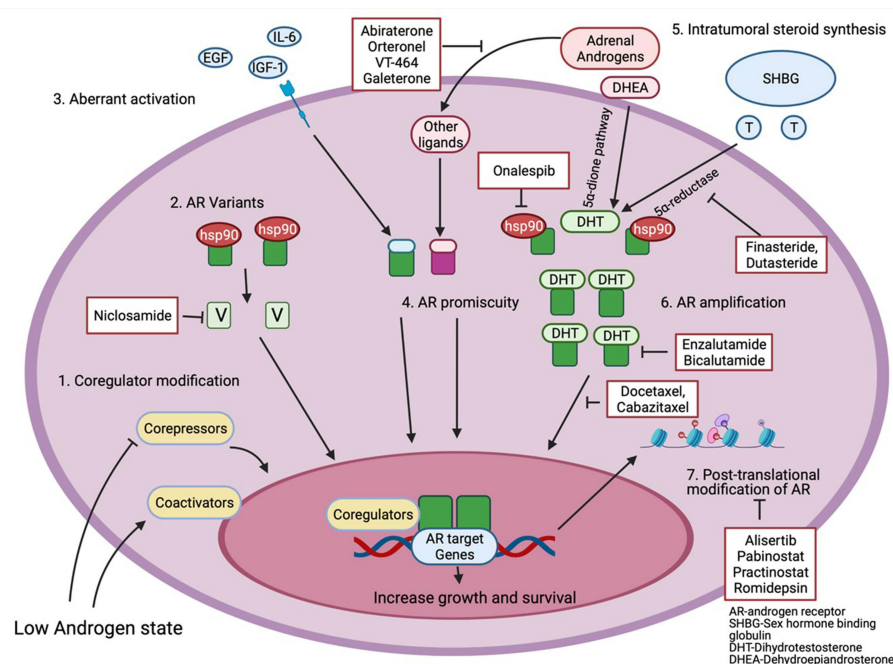
### AR Amplification and Overexpression

Gene amplification is defined as an increase in gene copy number beyond the normal diploid copy number. AR amplification (Xq11-q13 region) is present in 20–31% of patients with CRPC.<sup>12–14</sup> AR amplification was present in none of the benign prostatic hyperplasia (BPH) samples, only 2% of the primary PC tumor and 23.4% of CRPC tumors on FISH analysis.<sup>13</sup> Evaluation of samples from the same case have shown that AR gene amplification only occurs when the tumor enters a castrate-resistant state and is associated with a twofold increase in AR mRNA levels.<sup>14,15</sup> Chen et al compared AR protein levels in CRPC samples to multiple isogenic tumor xenograft models using microarray-based profiling and found increased AR expression in CRPC cells.<sup>16</sup> In addition, CRPC is also marked by enhanced AR protein stabilization due to

posttranslational modifications<sup>17</sup> and interaction with protein chaperones, for example, the heat shock protein (HSP) family.<sup>18</sup> The end-effect of these mechanisms is to empower PC cells with enhanced sensitivity to minimal levels of circulating androgens allowing them to recommence androgen-mediated growth despite being in an androgen-depleted milieu.

### Splice Variants

AR splice variants encode a modified form of AR protein with an absent C-terminal LBD but an intact N-terminal domain and a partial or complete DBD which is still functionally capable of interacting with DNA and AR co-receptors.<sup>19,20</sup> Thus, despite being unable to bind ligands, these truncated proteins are constitutively active as transcription factors, promoting expression of target genes activating downstream AR signaling pathways.<sup>9</sup> Out of the seven AR variant transcripts described in literature (ARV1–ARV7), AR-V1 and AR-V7 are most often expressed in CRPC.<sup>19</sup> AR splice variants are of particular interest in CRPC with resistance to enzalutamide and abiraterone, which occurs as primary resistance in 20 to 40% of cases and secondary resistance in virtually all cases despite initial response.<sup>21–23</sup> A plausible mechanism of resistance is suggested by the fact that AR splice variants lack an LBD and both enzalutamide and abiraterone



**Figure 1** Androgen receptor mediated mechanisms of castration resistance in prostate cancer along with potential therapeutic targets. Made with Biorender.com.

exert their action via binding to the LBD. Multiple therapeutic agents targeting AR variants by enhancing their degradation (eg niclosamide or selective AR degraders such as UT-155 and UT-69), inhibiting their synthesis (eg onalespib, quercetin, thailanstatins), binding to N-terminal domain (eg EPI-001) or DBD (eg VPC-14,449) or impeding the action of AR variant coactivators are currently under investigation.<sup>24</sup>

## Mutations

Another mechanism of castration resistance in prostate cancer is gain of function mutations in the AR. As with splicing variants, clinically relevant point mutations predominantly affect the ligand-binding domain. However, rather than conveying constitutive activation, AR gain of function mutations have been shown to induce enhanced sensitivity to low levels of androgens, affinity for non-androgen ligands (glucocorticoids, progestins, estrogens, dehydroepiandrosterone) and even antagonist-to-agonist switching.<sup>5,25–27</sup> One such mutant is AR T877A which not only binds to androgens, but also to other hormones such as estrogen and progesterone as well as AR antagonists.<sup>5,25,27</sup> AR T877A has only been identified in CRPC patients, but not hormone-naïve patients, supporting the role of T877A in developing castrate-resistance following prolonged exposure to ADT<sup>28,29</sup> In fact, Suzuki et al found that expression of this mutant in CRPC patients was associated with a remarkable fall in prostate-specific antigen after withdrawal of antiandrogen therapy.<sup>27</sup> In a similar fashion, the AR-LBD missense mutation (F876L) results in agonists activity from the AR antagonists ARN-509 and enzalutamide.<sup>30</sup>

## Posttranslational Regulation of AR

Posttranslational regulation of AR represents an important molecular mechanism for enhancing AR signaling—these processes include acetylation, methylation, phosphorylation, SUMOylation and ubiquitination.<sup>31</sup> The functions of these processes include: dictating AR protein stability and degradation, intracytosolic AR localization, controlling transcriptional function of AR and modulating the expression of AR target genes expression.<sup>32</sup> The role of phosphorylation, methylation and acetylation is generally to augment the transcriptional function of AR with only a small number of the 19 phosphorylation sites of AR inhibiting transcriptional activity.<sup>32</sup> Protein kinase A, B and C, mitogen-activated protein kinase, activated CDC42 kinase 1 and src-family kinases have all been

incriminated in AR phosphorylation.<sup>33</sup> The phosphorylation of AR at serine 81 (S81), located in the N terminal domain of AR, regulates AR protein stability, cellular activation and transactivation.<sup>32</sup> S81 is the most highly phosphorylated site on AR with phosphorylation occurring in response to androgens. Phosphorylated S81 is decreased after castration corresponding with loss of AR activity, but is highly expressed in CRPC tumors with one study suggesting its association with AR resurrection in CRPC.<sup>34</sup> A number of cyclin-dependent kinases are involved in phosphorylation of S81, with inhibition of these kinases leading to a reduction in the process.<sup>32</sup> Phosphorylation of serine 308 (S308) and serine 791 (S791) negatively regulates the transcriptional activity of AR.<sup>32,35</sup> Ubiquitination also promotes AR transcriptional activity.<sup>33</sup> It was found that Siah2, a E3 ubiquitin ligase contributes to CRPC, by regulation of androgen receptor activity with Siah2 inhibition promoting PC (PC) regression following castration.<sup>36</sup> Finally, SUMOylation, which leads to binding of small ubiquitin like modifiers (SUMO) to AR, generally results in repression of AR transcriptional activity.<sup>33</sup> When chemical castration fails, targeting the above pathways of AR posttranslational modification may represent a promising treatment strategy for CRPC.

## Coactivators or Corepressor Modification

AR's interaction with coregulators is key in the development of CRPC. AR acts as a transcription factor in collaboration with a number of “coactivators” and “corepressors” which control its transcriptional activity.<sup>37</sup> Greater than 150 molecules are known to influence AR's transcriptional activity.<sup>38</sup> These coregulators have a wide variety of roles: as previously mentioned, they modulate other proteins through phosphorylation and epigenetic alterations, act as molecular drivers, interfere with RNA splicing and stimulate transcriptional pathways.<sup>39,40</sup> Critically, these coregulators facilitate transcriptional function of AR even under an androgen-depleted milieu and have thus been implicated in development of CRPC.<sup>37</sup> CBP/P300, p160/SRC, and GATA2 are examples of coactivators of AR.<sup>41–43</sup> Interplay with a coactivator results in aberrant stimulation of AR, leading to heightened transcriptional response and consequently increased PC growth.<sup>39</sup> When inhibited, AR expression is decreased and *in vitro* and *in vivo* studies, the use of small molecule inhibitors of these coactivators leads to reduction in PC growth.<sup>42–44</sup> Components of the steroid receptor coactivator family (SRC) are heavily expressed in CRPC.<sup>38,45</sup>

Phosphorylation of SRC1 by a number of different mechanisms enables it to activate AR transcription in the absence of androgens.<sup>46</sup> Corepressors, such as NcoR 1 and NcoR 2 have the opposite effect on AR transcription activity. A preclinical study found that following treatment with bicalutamide, an antiandrogen, NCoR recruitment was reduced or absent in cells with increased AR receptor protein.<sup>16</sup> Corepressors are frequently decreased in CRPC.<sup>47</sup> This imbalance of control, arising from increased activity of coactivators and reduced influence of corepressors leads to a progrowth state, which is androgen independent—in essence facilitating development of CRPC.

Ligand independent activation of AR signaling can also be achieved by interactions with other cytokines, growth factors and hormones. In an androgen-depleted state, IL-6 can potentiate the existing androgens as well as self-activate AR at about half of its functional capacity.<sup>48,49</sup> Insulin like growth factor-1 (IGF-1), epidermal growth factor (EGF) and keratinocyte growth factor (KGF) have also been found to directly activate the AR in the absence of androgens<sup>50</sup> with use of bicalutamide completely inhibiting activation of AR by these factors.<sup>50</sup> In addition, elevated IGF binding protein corresponds to faster progression to CRPC and lower survival in men with metastatic PC.<sup>51</sup>

## Intratumoral Steroid Hormone Synthesis

PC employs intracrine androgen biosynthesis as another method to allow growth in androgen depleted conditions. Hormonal therapy which lowers circulating testosterone and thus depletes intraprostatic/intratumoral DHT (dihydrotestosterone) (which has a much more potent effect on AR) has been the mainstay treatment of advanced PC for many years.<sup>52</sup> PC cells contain all the necessary components for androgen biosynthesis, with levels of many key enzymes being increased in recurrent PC.<sup>53</sup> The source of androgens in prostatic tissue after ADT involves intracrine production within the gland, converting adrenal androgens to DHT. It was found that DHT measurements in prostatic samples after androgen deprivation therapy remained at 25% the amount measured before anti-hormone treatment.<sup>54</sup> Montgomery et al found that this intracrine steroidogenesis occurs not only in primary PC tumors, but also in distant metastases.<sup>55</sup> Moreover, CRPC metastatic sites are marked by an augmented expression of steroidogenic genes such as *CYP17A1-19A1*, *HSD3B1-17B3*, etc.<sup>55</sup> The alternate 5 $\alpha$ -dione pathway is the major route for DHT synthesis in CRPC. This pathway catalyzes the

conversion of dehydroepiandrosterone (DHEA) and androstenedione to DHT while bypassing testosterone completely.<sup>52</sup> Enzalutamide is an AR antagonist. Enzalutamide resistant cell lines were found to have increased expression of genes involved in androgen biosynthesis.<sup>56</sup>

## Nonandrogen-mediated Mechanisms of Resistance FGF Signaling

The fibroblast growth factor (FGF) pathway is central to organogenesis and oncogenesis as it cross-links the epithelial and mesenchymal compartments.<sup>57</sup> The function of the interaction between these two domains is to mediate the regulatory effects of androgens and, therefore, prostatic development, function and tissue homeostasis.<sup>58,59</sup> The FGF axis might be implicated in the reciprocal communication between epithelium and mesenchyme via a paracrine crosstalk through a diverse set of ligands and receptors, ultimately leading to the proliferation and differentiation of both the epithelium and the stroma.<sup>60,61</sup>

FGF ligands signal through four analogous tyrosine kinase receptors, appointed FGFR1 to 4, piloting the subsequent induction of myriad signal transduction channels, such as the Erk, MAPK, and PI3K-Akt pathways.<sup>57</sup> Alternative splice variants of FGFRs are distributed between the epithelium and the stroma and have very different binding and mediating capacities. In fact, FGF7 and FGF10, expressed exclusively by the mesenchyme in benign prostate tissue, can induce prostate organogenesis independently of the AR, with mitogenic action selective for prostate epithelium but not the stroma.<sup>62,63</sup> Both ligands usually bind preferentially to the FGFR2 IIIb isoform (FGFR2b), while FGF7 also has affinity for the IIIb splice variant of FGFR.<sup>57,64</sup>

Conversely, a disruption of the tissue and cell type specific expression of the FGF family leads to alterations in the homeostatic balance that promotes neoplastic transformation, often found in prostate tumors. In further detail, autocrine expression of FGF6 and FGF8 is present in the majority of PCs, with increased expression of FGFR1 typically identified in poorly differentiated prostate adenocarcinoma.<sup>65</sup> Moreover, the abovementioned FGFRIII-b isoform is often lost or alternatively spliced to FGFR2IIIc in epithelial cells during progression to malignancy. The latter isoform does not recognize FGF7 and instead binds strongly to FGF2, a component of the

FGF family incriminated in the progression and spread of solid cancers.<sup>66–68</sup> In the study by Naimi et al, it was noted that FGFR2IIIb isoform was drastically reduced in 60% of androgen responsive tumors and 90% of castrate resistant tumors with a definite association between a decreased FGFR2(IIIb) expression and Gleason score.<sup>68</sup> In addition, the switch from epithelial-type FGFR2IIIb to mesenchymal-type FGFR2IIIc, along with ectopic FGFR1 expression, has been hypothesized to be a manifestation of an epithelial-to-mesenchymal transition (EMT), a phenomenon strongly linked with tumor progression and, more specifically, sarcomatoid carcinoma.<sup>69–72</sup> Lastly, as observed by Memerzadeh et al in their model, it appears logical to assume that the ability of FGF-10 to help normal prostate cells thrive in an androgen-starved environment translates into an FGF10-induced survival of prostatic cancer cells through the activation of a paracrine mesenchymal AR signaling, despite castrate levels of androgen.<sup>60</sup>

## TGF- $\beta$ Pathway

The transforming growth factor- $\beta$  (TGF- $\beta$ ) family comprises TGF- $\beta$ s, bone morphogenetic proteins (BMPs), activins and related proteins. There are three different TGF- $\beta$ -secreted cytokines,  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3, all of which operate through the same receptor signaling systems.<sup>73</sup> Their role in the regulation of cellular functions is a complex one and highly dependent on the cell context. In normal prostate cells, TGF- $\beta$  signaling has been known to have an inhibitory effect on proliferation and differentiation linked to castration-induced apoptosis.<sup>74</sup> Paradoxically during tumorigenesis, TGF- $\beta$ s, and particularly TGF- $\beta$ 1 and TGF- $\beta$ 3, can instead have oncogenic activities by stimulating angiogenesis,<sup>75</sup> suppressing the immune system,<sup>76</sup> and stimulating invasion and metastatic potential.<sup>77,78</sup>

TGF- $\beta$ s exerts their action through a well-documented SMAD-dependent mechanism, as well as a SMAD-independent one, through mediators such as and PI3K/AKT23, mitogen-activated protein kinase (MAPK), LIM kinases and transforming protein RhoA.<sup>79,80</sup> Interestingly, these different mediators of the TGF- $\beta$  messaging cascade might explain the dual nature of TGF- $\beta$  multifunctional cytokines. The SMAD-dependent pathway likely has an anti-oncogenic effect in initial stages of PC and then switches towards nonSMAD pathways which at as pro-oncogenic in late stages of the disease.<sup>81</sup> Alteration in the activation of the

latter is associated with acquired resistance selective for TGF- $\beta$ 1's antiproliferative effects,<sup>82,83</sup> while maintaining sensitivity for its inducement toward extracellular matrix (ECM) disintegration and EMT. As discussed by Jones et al, the abnormally high level of TGF- $\beta$  is generally linked with a concomitant absence of the T $\beta$ RI and T $\beta$ RRII receptors, which are key in conserving the physiologic role of TGF- $\beta$  pathway.<sup>81</sup> Their loss during tumor progression permits the cells to escape the growth constraints normally imposed by TGF- $\beta$  and shifts the balance toward a pro-oncogenic outcome. Moreover, studies like the one conducted by Song et al showed how the loss of T $\beta$ RRII is key in the androgen-mediated regulation of TGF- $\beta$  signaling in PC cells.<sup>84</sup> In fact, DHT stimulation can interrupt the TGF- $\beta$  messenger cascade by downregulating the synthesis of T $\beta$ RRII via Sp1/3's attachment to the *T $\beta$ RRII* promoter region, affecting its transcriptional and possibly translational control.<sup>84</sup> Notably, the TGF- $\beta$  promoter was shown to contain six androgen-response elements which have a strong foothold in the androgen regulation of TGF- $\beta$ 1 transcription. According to the growing evidence, the AR signaling pathway might be constitutively activated in advanced prostate carcinoma, probably by AR imitating the configuration of ligand-activated AR in the absence of androgen. Ultimately, this androgen-independent activation of the AR pathway may suppress TGF- $\beta$  messaging by inhibiting the genesis of T $\beta$ RRII, constituting a possible mechanism operating in androgen refractory PC. In addition, the loss of tumor suppression PTEN might promote liberation from androgen by inhibiting the activation of SMAD3 by an AR-independent mechanism by sequestering SMAD3 from T $\beta$ RI<sup>85,86</sup>

## RAS/MAPK Pathway

The RAS-MAPK pathway is a downstream mediator to the cellular responses to different growth signals and is often deregulated in human cancer, leading to a cascade of successive phosphorylation steps that culminates in the activation of mitogen-activated protein kinases (MAPKs).<sup>87,88</sup> Both the overexpression and mutational activation of c-Ras are well-known agents in human oncogenesis and correlate with disease progression.<sup>89</sup> Similarly, elevated levels of activated MAP kinase are associated with an increasing Gleason score and prostate tumor stage<sup>90</sup> and ERK1/2 activation is essential in causing RAF-induced AR suppression, supporting MAPK signaling in PC advancement.<sup>89,91</sup>

Overall, the RAS/MAPK signaling was observed to be activated in 43% of primary PC samples and 90% of metastatic samples.<sup>92</sup> Activation of the MAPK signaling via *B-Raf* (V600E) expression can independently induce basal (p63+) cell proliferation and expression of EMT markers<sup>93</sup> that result in abnormal proliferation and basaloid hyperplasia. In particular, the conditional loss of function of *PTEN* combined with the activation of *B-Raf* resulted in prostate tumors that were innately resistant to castration and widely metastatic, as observed by Wang et al in mouse models.<sup>94</sup> Moreover, this pathway has a substantial role in promoting CRPC metastasis in DU145 PC cells and, conversely, treatment with a MEK inhibitor or knockdown of either ERK1 or ERK2 reduced these cells' sphere-forming ability.<sup>89,95</sup> In particular, DU145 cells, with known low metastatic potential, were employed in a xenograft metastasis model by Yin et al to demonstrate how the expression of Ras-mediated effector pathway differentially stimulated metastasis in different organs, with *RasV12G37* expression inducing bone metastasis.<sup>96</sup> Further preclinical evidence supports that this pathway can lead to cooperation with other pathways previously mentioned, such as the TGF and FGF signaling—toward the progression of LNCaP cells to hormone-refractory disease, rendering them hypersensitive to low levels of androgen. Interestingly, the study conducted by Bakin et al proved how the dominant negative form of *Ras* (*RasN17*), and thus the attenuation of Ras signaling, leading to the restoration of androgen responsiveness in castrate resistant C4-2 PC cells.<sup>97</sup> In this study, the expression of *RasN17* in combination with bicalutamide leads to a nearly complete growth inhibition of RasN17 cells, with the inhibition of MAPK signaling in the highly tumorigenic LNCaP cell line causing tumor regression in surgically castrated mice. This suggests that the MAPK arm of Ras signaling may be an appropriate target for treatment of CRPC. Likewise, it is plausible to assume that it can be cotargeted with the PTEN/PI3K/AKT axis, given its conjunct action in upregulating *c-myc38*, to prevent the development of metastatic PCs.

## Wnt- $\beta$ Catenin Pathway

The canonical Wnt/ $\beta$ -catenin signaling pathway, which is known to play a central role in embryogenesis, has emerged as a culprit in progression to late-stage PC as well as correlating with aggressive disease, higher Gleason score and PSA levels.<sup>98</sup> The role of the active Wnt/ $\beta$ -catenin pathway in PC progression is manifold and

involves neuroendocrine differentiation, induction of epithelial to mesenchymal transformation, bone metastasis, resistance to androgen deprivation therapy and complex interaction with other molecules/pathways such as the PTEN, PI3K and mTOR.<sup>99–102</sup>

Under physiologic conditions,  $\beta$ -catenin exists as a component of the E-cadherin-based cell surface adherens junction.<sup>103</sup> Upon disruption of the cell surface complex,  $\beta$ -catenin is released into the cytoplasm where it is sequestered and phosphorylated by APC complex which marks it for ubiquitination. However, in the presence of activated Wnt signaling pathway, this  $\beta$ -catenin molecule escapes degradation and translocates into the nucleus where it interacts with TF/LEF-1 and upregulates the transcription of several oncogenes such as *Myc* and *cyclin D1* amongst others.<sup>104</sup> Moreover,  $\beta$ -catenin acts as a coactivator of the androgen receptor, the two colocalizing into the nucleus, where  $\beta$ -catenin aids in androgen-independent transcription of AR target genes<sup>105</sup> Interestingly, AR overexpression in CRPC is seen to co-exist with an activated Wnt/ $\beta$ -catenin signaling pathway, whereas this pathway is inhibited in the presence of normal levels of androgen or AR agonists.<sup>106</sup> In addition, the expression of lymphoid enhancer binding factor-1 or LEF-1, the central effector protein in Wnt/ $\beta$ -catenin signaling pathway, is augmented by a 100-fold in androgen-independent LNCaP variant cells and is associated with AR overexpression. Alternatively, the absence of LEF-1 is shown to downregulate AR expression and decrease the invasiveness of androgen-independent PC cells.<sup>107</sup>

Wnt/ $\beta$ -catenin pathway plays a two-pronged role; it helps sensitize PC cells to minimal levels of androgen and abets AR signaling in the castrated state. However, once PC cells lose their prostatic differentiation, Wnt/ $\beta$ -catenin signaling switches its role to downregulate AR expression and promotes neuroendocrine differentiation.<sup>108,109</sup> The latter is achieved by a cross-communication between Wnt and Akt, whereby activated Wnt pathway phosphorylates Akt resulting in proteosomal degradation of AR.<sup>110</sup> Ciarlo et al have proposed a model wherein Akt activation results in hn-RNP and  $\beta$ -catenin phosphorylation and their transcriptional activities subsequently induce NE differentiation.<sup>111</sup> Neuroendocrine PC is characterized by heightened expression of several downstream targets of the Wnt signaling pathway such as *FOXA2*, *SOX2*, *CD44*, amongst others.<sup>112–114</sup> Yang et al demonstrated that LNCaP cells cultivated in an androgen-devoid medium

and transfected with  $\beta$ -catenin differentiated into NE cells characterized by increased expression of neuron-specific enolase and chromogranin-A.<sup>115</sup>

Another seminal impact of Wnt signaling is its role in inducing epithelial to mesenchyme transformation whereby PC cells shed their epithelial characteristics and gain metastatic potential. This is achieved by a multitude of events such as upregulation of N-cadherin coupled with downregulation of E-cadherin, induction of transcription factors such as TWIST, SNAIL and SLUG, and expression of matrix metalloproteinases that degrade extracellular matrix and aid in invasiveness.<sup>116–119</sup> Further, Zhang et al have attributed the self-renewal properties of cancer stem cells, at least in part, to Wnt/ $\beta$ -catenin signaling in high TERT expression PC cells.<sup>120</sup> Given the multilayered involvement of the Wnt/ $\beta$ -catenin pathway in PC progression, small molecular inhibitors targeting a combination of mechanisms that activate this pathway are emerging as promising therapies.

## Hedgehog Signaling Pathway

Another developmental signaling pathway that is central to organogenesis and homeostasis, the hedgehog (Hh) pathway, has been incriminated in propagation of CRPC. Hh pathway is active in urogenital sinus epithelium, the site of prostate development during embryogenesis and is reactivated during carcinogenesis.<sup>121</sup> When Hh proteins binds to its receptor ie, patched (PTCH), a G-protein coupled like receptor called smoothened (Smo) is unsuppressed and leads to stimulation of Gli transcription factors culminating in overexpression of culprit genes.<sup>122</sup> The Gli family proteins also interact with AR at its Tau5 domain and induce AR transcription in an androgen-deprived state. Shaw et al have demonstrated augmented expression of Hh signaling proteins in androgen independent LNCaP cell lines which were successfully suppressed by cyclopamine, a known Hh signaling inhibitor.<sup>123</sup> They also exhibited increased expression of PTCH in circulating tumor cells derived from patients with CRPC as compared to normal individuals. Moreover, the levels of PTCH were strongly associated with the length of exposure to androgen-deprivation therapy.

Hh signaling has a multifaceted role in metastasis including promotion of angiogenesis by upregulating angiopoietin-1 and downregulating angiopoietin-2,<sup>124</sup> inducing epithelial to mesenchymal transition as well as rendering stemness as characterized by expression of stem cell markers like nestin and Bmi-1.<sup>125</sup> Hh pathway is both

necessary and sufficient for CRPC growth and offers an attractive therapeutic target. Itraconazole, an SMO inhibitor, has been shown to inhibit Hh signaling in CRPC and clinical trials assessing the role of Hh inhibitors vismodegib and sonidegib are underway.<sup>126</sup>

## Role of Cell-surface Adhesion Molecules (CAMs)

When it comes to cancer progression, cell surface adhesion molecules go above and beyond their basic function of cell-cell attachment and serve a critical role in cellular proliferation, motility, invasiveness and EMT as their interactions with extracellular matrix become dysregulated. Integrins are one such family of CAMs that stand out with respect to differential expression in different stages of PC progression. Out of the 24 sub-units of the integrin family (18  $\alpha$  and 8  $\beta$ ); the  $\alpha$ Ib,  $\beta$ 1,  $\beta$ 3, and  $\beta$ 6 subunits and their heterodimer forms such as  $\alpha$ V $\beta$ 6,  $\alpha$ V $\beta$ 3,  $\alpha$ 3 $\beta$ 1,  $\alpha$ 6 $\beta$ 1 are selectively overexpressed in invasive carcinomas whereas most other phenotypes are lost.<sup>127,128</sup> The  $\alpha$ 6 $\beta$ 1 integrin acts as a laminin receptor and advances cellular migration whereas the  $\alpha$ 6 $\beta$ 4 integrin which strengthens the hemidesmosomal bonds between ECM and cellular skeleton is often lost in the progression from intra-epithelial neoplasm to invasive cancer.<sup>129</sup> Quaglia et al have demonstrated that  $\alpha$ V $\beta$ 3 integrin-rich extracellular vesicles originating from PC cells induce neuroendocrine differentiation whereas the  $\alpha$ V $\beta$ 6 integrin is limited to areas of adenocarcinoma and is upregulated in areas of bony metastasis.<sup>127</sup> Similarly, Lu et al have discovered that  $\alpha$ V $\beta$ 6 is involved in androgen-independent nuclear localization and signal transduction of androgen receptor via JNK-1 pathway.  $\alpha$ V $\beta$ 6 also results in overexpression of survivin which in turn supports the growth of detached  $\alpha$ V $\beta$ 6-expressing PC cells and leads to a vicious cycle of CRPC propagation.<sup>130</sup> With this in mind, multiple integrin inhibitors including monoclonal antibodies such as abituzumab (anti-  $\alpha$ v antibodies), etaracizumab (anti-  $\alpha$ v $\beta$ 3 antibody), camptothecins and statins that target  $\alpha$ v $\beta$ 3, aptamers against  $\alpha$ 6 $\beta$ 4 as well as RGD sequence containing peptides as well as nonpeptides that bind to integrins, are being developed as therapeutic targets.<sup>131</sup>

Besides integrins, another member of the cadherin superfamily, protocadherin 7, has been shown to be amplified in CRPC and modulates phosphorylation of ERK, Akt and RB thereby powering tumor propagation.<sup>132</sup> Epithelial cell adhesion molecule (EpCAM) has also been associated



with metastatic progression in CRPC by fueling the PI3K/Akt/mTOR pathway.<sup>133</sup> Furthermore, overexpression of the  $\beta$  subunits of the lesser known voltage sensitive sodium channel-associated cell adhesion molecules is yet another mechanism that makes PC cells more adherent to vitronectin and aids in invasion through extracellular matrix.<sup>134</sup>

## Role of G-protein-coupled Receptors (GPCR)

Increased expression and activation of GPCRs and their ligands is linked to tumor growth in PC.<sup>135</sup> Many of the GPCRs make up the hypothalamus-pituitary-gonadal (HPG) axis, which is central to the pathophysiological progression of PC. The key hormone-responsive GPCRs involved in the regulation of PC are gonadotropin-releasing hormone receptor (GnRHR), luteinizing hormone receptor (LHR), follicle stimulating hormone receptor (*FSHR*), relaxin family peptide receptor (RXFP), growth hormone secretagogue receptor (GHSR), and kisspeptin receptor (GPR54).<sup>136</sup>

While GnRHR plays a pivotal antitumor role, such is not the case with other receptors in the HPG axis. The Gs protein coupled LHR has been implicated in in-situ steroid production within androgen sensitive as well as insensitive PC cells via the cAMP/PKA/ERK1/2 pathway that stimulates the function of target genes such as *CYP17A1*, *STAR* and *AKR1C1-C3*.<sup>137</sup> Moreover, LHR also prevents apoptosis by upregulation of Bcl and Bcl-X<sub>L</sub> while decreasing the expression of Bad and Bax via the PI3K/Akt cascade.<sup>138–140</sup> Similarly, the *FSHR* also promotes steroidogenesis, but additionally participates in the progression of bone metastasis by positively modulating osteoclastic activity as well as promoting angiogenesis.<sup>141</sup> Another GPCR—the relaxin receptor stimulates cellular proliferation, invasion, and blood vessel formation in CRPC via the PI3K/Akt/ $\beta$ -catenin pathway.<sup>142,143</sup> Akin to GnRHR, the kisspeptin receptor also suppresses tumor growth via Gq/phospholipase-C/calcium-mediated signaling<sup>144</sup> whereas the GHSR plays a biphasic role by inhibiting cell growth at higher concentration and promoting cellular proliferation at lower levels.<sup>145</sup> Besides, activation of GPCRs by other ligands such as endothelin and bradykinins have also been implicated in CRPC progression.<sup>146,147</sup>

## The JAK-STAT Pathway

Cell proliferation, migration, and other major cellular functions are highly regulated via cytokines. Among cytokines, interleukin-6 (IL-6) is of particular interest

for PC development. IL-6 levels have been shown to be higher in PC patients compared to their counterparts.<sup>148,149</sup> IL-6 plays a role in regulating the immune response as well as cell growth via activation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway and cross-talk with MAP kinase, PI3K, and other cellular pathways.<sup>150</sup> Both *BRCA1* and AR can bind to *STAT3* in PC cells and activate the downstream JAK/STAT channel upregulating target genes such as VEGF, Bcl-2, Bcl-x<sub>L</sub>, survivin and Mcl-1, acting as a retardant for apoptosis and enabling angiogenesis.<sup>151</sup> IL-6 also works as an autocrine growth factor that promotes PC cell growth.<sup>152,153</sup>

From the immunological perspective, the JAK/STAT pathway is vital to PD-L1 expression as an upstream regulator. It is well-known that overexpression of PD-L1 allows tumor cells to evade cell death by T-cell immune responses.<sup>154,155</sup> In addition, the antitumor pathway of natural killer (NK) cells can also be inhibited via PD-L1 expression by blocking NKG2D ligands on the cell's surface. In CRPC, there is increased resistance to natural killer (NK) cell-mediated cytotoxicity as IL-6 dysregulates PD-L1 and NKG2D ligand levels.<sup>156</sup> In fact, blocking the JAK/STAT pathway has shown to confer increased susceptibility to NK-cell mediated death in CRPC cells.<sup>157</sup>

## Role of MicroRNAs

MicroRNAs (miRNAs) are small noncoding 21 to 23 nucleotide base pair RNA molecules that act as transcriptional and posttranscriptional regulators of gene expression via base-pairing with messenger RNAs.<sup>158</sup> About 60% of all human genes are regulated by miRNAs and miRNAs interact with a number of the pathways discussed above.<sup>159</sup> In prostate cancer, miRNAs have demonstrated their value in assisting in diagnosis of recurrence, monitoring progression, and predicting treatment response.<sup>157,159,160</sup> Their roles can be divided by their associations with cell proliferation, apoptosis, invasion and metastasis, epithelial–mesenchymal transition, cancer stemness, and AR status.<sup>161</sup> Upregulation of *miR-96*, *-182*, *182\*–183*, *-375*, *32*, *-26a*, *-181a*, *-93*, *-196a*, *-25*, *-92* and *let-7i* and downregulation of *miR-16*, *-31*, *-125b*, *-145*, *-149*, *-181b*, *-184*, *-205*, *-221* and *-222* have been confirmed in prostate cancer tissue.<sup>162,163</sup> It has also been found that miRNA expression alterations occur during the development of CRPC.<sup>164</sup> In a study analyzing the differential expression of seven miRNAs (*miR-221*, *-222*, *-23b*, *-27b*, *-15a*, *-16-1*, and *-203*) across different types of

benign prostate and prostate cancer samples, found that ~90% of the analyzed metastatic CRPC tumors could be characterized by the increased miR-221/-222 expression and the downregulated miR-23b/-27b expression.<sup>164</sup> In addition, as previously mentioned AR splice variants increase in expression during progression to CRPC. AR-V7 is the most common AR splice variant with resistance to enzalutamide and abiraterone because of androgen-independent proliferation of prostate cancer cells associated with high levels of AR-V7 in circulating prostate cancer cells.<sup>165</sup> Several miRNAs (*miR-21*, *-31*, *-34a*, *let-7c*, *-124*, *-205*, *-185*, *488\** and so on) have been reported to regulate

AR and AR splice variants expression.<sup>161</sup> In addition, another miRNA miR-125b has been found to directly target previously mentioned AR corepressor NcoR 2, subsequently activating AR signaling.<sup>166</sup> miRNAs may also have therapeutic implications. Lin et al identified two microRNA mimics—miR-217 and miR-181b-5p, which substantially enhance the docetaxel and cabazitaxel sensitivity of CRPC cell lines.<sup>167</sup> An observational prospective study investigating the plasmatic levels of miRNA according to AR-V7 mutational status in metastatic CRPC patients receiving standard of care therapy is currently recruiting (NCT04188275).

**Table 1** Nonandrogen-Dependent Mechanisms in CRPC

Pathway	Mechanisms for Castrate Resistance	Potential Therapeutic Agent	Ongoing/Recent Clinical/Preclinical Studies
FGF pathway	Overexpression of <i>FGFR1</i>	Dovitinib (FGFR inhibitor)	Phase II study of dovitinib in patients with CRPC after failure of docetaxel based chemotherapy (NCT01741116) <sup>168</sup>
	Loss of <i>FGFR3</i> or its alternative splicing to <i>FGFR3IIIc</i> facilitates EMT		
	Enhanced <i>FGF10</i> expression leads to paracrine mesenchymal AR signaling		
TGF- $\beta$ pathway	Overexpression of TGF- $\beta$ I and TGF $\beta$ III	Galunisertib (T $\beta$ RI inhibitor)	Phase II study of a combination of galunisertib and enzalutamide in metastatic CRPC (NCT02452008) <sup>169</sup>
	Loss of expression of T $\beta$ RI and T $\beta$ RII receptors		
	Blocked <i>SMAD3</i> inactivation		
RAS/ MAPK pathway	MAPK induced c-Myc expression	Trametinib (MEK 1/2 inhibitor)	Phase II study of trametinib in metastatic CRPC that has progressed on either enzalutamide or abiraterone acetate. (NCT02881242) <sup>170</sup>
	RalGEF pathway activation		
Wnt- $\beta$ catenin signaling pathway	$\beta$ catenin acts as a coactivator of AR	1. CWP232291, a small molecule $\beta$ -catenin inhibitor 2. Foxy-5—mimicker of Wnt 5 a protein	1. Preclinical study confirmed antitumor activity of small molecule $\beta$ -catenin inhibitor in prostate cancer cell lines and primary cells derived from CRPC patients <sup>171</sup> 2. Ongoing phase 1b dose-escalating study of Foxy 5 in metastatic breast, colon and prostate cancer (NCT02655952) <sup>172</sup>
	Induction of <i>FOXA2</i> , <i>SOX2</i> and <i>MYCN</i> facilitates neuroendocrine differentiation		
	Induction of transcription factors such as <i>SNAIL</i> , <i>SLUG</i> and <i>TWIST</i> , downregulation of E-cadherin and upregulation of N-cadherin promotes EMT		
Hedgehog signaling	Disinhibition of smoothened (Smo) leading to upregulation Gli family proteins (Gli 1&2)	Vismodegib (Smo antagonist)	A pharmacodynamic study of vismodegib in men with metastatic CRPC (NCT02115828) <sup>173</sup>
	Gli family proteins interact with AR at its Tau5 domain		
	Promotion of angiogenesis by upregulating angiopoietin-1 and downregulating angiopoietin-2		
JAK/STAT pathway	Overexpression of IL-6	Galiellactone ( <i>STAT3</i> DNA-binding inhibitor)	Preclinical study showed that galiellactone inhibits the <i>STAT3</i> /AR signaling axis and suppresses enzalutamide-resistant prostate cancer <sup>174</sup>
	Constitutive activation of <i>STAT3</i> and <i>STAT5</i> that induce anti-apoptotic genes.		
	<i>STAT3</i> also colocalizes with AR and induces transcription of AR target genes		

## Conclusion

The natural evolution of PC growth inevitably leads to an androgen ignorant state. At present, CRPC remains a lethal form of prostate cancer with no available treatment to effectively increase patient survival. The development of CRPC entails both androgen-dependent and androgen-independent growth signaling pathways. Understanding such mechanisms and their interaction is of the utmost importance to develop targeted therapies directed against the various pathways that subvert normal restraint on cell growth. While there have been significant advances in our comprehension of AR dependent mechanisms as well as large trials to optimize ADT, a better grasp of the numerous alternative oncogenic pathways is necessary. As emphasized in this review, the latter are complex pathways that act independently, whilst also interacting with each other as well as the AR and may very well represent future targets of treatment once better explored (summarized in Table 1). To be successful in targeting critical pathways driving hormone-independence, therapeutic measures will need to be personalized and tailored based on the type of CRPC and the underlying castration resistance mechanism.

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## Disclosure

Dr Zach Dovey reports he is Medical Director and owner of stock by certificate of shares from MedTech Holdings Ltd, outside the submitted work. The authors report no other conflicts of interest in this work.

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