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The Importance of Non-Nuclear AR Signaling in Prostate Cancer Progression and Therapeutic Resistance

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Abstract

The androgen receptor (AR) remains the major oncogenic driver of prostate cancer, as evidenced by the efficacy of androgen deprivation therapy (ADT) in naïve patients, and the continued effectiveness of second generation ADTs in castration resistant disease. However, current ADTs are limited to interfering with AR ligand binding, either through suppression of androgen production or the use of competitive antagonists. Recent studies demonstrate 1) the expression of constitutively active AR splice variants that no longer depend on androgen, and 2) the ability of AR to signal in the cytoplasm independently of its transcriptional activity (non-genomic); thus highlighting the need to consider other ways to target AR. Herein, we review canonical AR signaling, but focus on AR non-genomic signaling, some of its downstream targets and how these effectors contribute to prostate cancer cell behavior. The goals of this review are to **1**) re-highlight the continued importance of AR in prostate cancer as the primary driver, **2**) discuss the limitations in continuing to use ligand binding as the sole targeting mechanism, **3**) discuss the implications of AR non-genomic signaling in cancer progression and therapeutic resistance, and **4**) address the need to consider non-genomic AR signaling mechanisms and pathways as a viable targeting strategy in combination with current therapies.

Keywords

Androgen Receptor; non-genomic signaling; prostate cancer; therapy

Introduction

Prostate cancer is a leading cause of cancer mortality in men in the U.S. [1]. The androgen receptor (AR) still remains the major oncogenic driver of prostate cancer, whether it be localized, castration resistant, or metastatic disease. Several lines of evidence support this AR dependence. First, androgen deprivation therapy (ADT) is wholly and dramatically effective at putting patients with naïve tumors into remission [2]. Second, even though these same patients will regress as their tumors become resistant to ADT, AR is retained and still

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highly expressed in those tumors, often amplified or mutated [3–5]. Third, treatment of these patients with more potent second generation ADTs, such as Enzalutamide or Abiraterone, leads to further suppression of the resistant tumors, even if it is ultimately not curative [6]. Fourth, laboratory studies demonstrate that genetically inducing the loss of AR in castration-resistant tumor cell lines, results in the death of these cells [7–9]. Recent evidence indicates that one resistance mechanism to second generation ADTs, is the generation of constitutively active AR splice variants that no longer depend on androgen [10]. A second resistance mechanism may involve AR function in the cytoplasm. Enzalutamide inhibits AR nuclear translocation [11] leading to cytoplasmic localized AR and elevated Src signaling [12]. Elevated Src activity, a known AR cytoplasmic target [13–15], is associated with a subset of Enzalutamide-resistant tumors [16]. These findings necessitate the re-evaluation of cytoplasmic AR functions, sometimes referred to as non-genomic signaling, and the implications for relying too heavily on targeting only the nuclear functions of AR.

I. The Androgen Receptor and Canonical Transcription

AR is a member of the nuclear steroid receptor family [17]. It is encoded by a single copy gene, nearly 90 Kb in size, and located on the q-arm of the X chromosome (Xq11.2). Once transcribed and translated, AR has a molecular weight around 80–110 kDa. AR, like other members of the steroid nuclear receptor family, is characterized by four functional domains. AR has an N-terminal transactivation domain (AF-1), a DNA binding domain (DBD) with two zinc fingers motifs and AR dimerization domain, a hinge region, and a ligand binding domain (LBD) containing a second activation domain (AF-2) [18–20]. AF-2 is involved in protein-protein interactions that aid in recruitment of co-activators that contain LXXLL motifs [21]. AR also has a poly-glutamine tract at residues 448–472 that varies in length between individuals [22]. This poly-glutamine stretch ranges from 8–31 repeats in normal individuals; however, expansion of this poly-glutamine tract is reported to cause spinal and bulbar muscular atrophy (also known as Kennedy's Disease) [23]. Conversely, if the poly-glutamine stretch is shortened, AR is reported to be more transcriptionally active [24]. The hinge region possesses a nuclear localization sequence (NLS) which is exposed upon androgen binding [25, 26]. The LBD is composed of twelve alpha-helices and upon androgen binding, helix 12 becomes stabilized. This leads to the formation of a hydrophobic binding pocket for ligands such as androgen or other proteins that have the preferred FXXLF motif [22]. The LBD, in the absence of ligand, aides in keeping AR localized within the cytosol where it is kept in an inactivate conformation by heat shock proteins (Hsp) including Hsp 90, Hsp 70, Hsp 56, and p23. Circulating testosterone is delivered to AR-positive cells attached to sex hormone binding globulin (SBHG), and is then reduced intracellularly to dihydrotestosterone (DHT) by 5α-reductase (Figure 1). When DHT binds the LBD of AR, it displaces Hsps. AR then homo-dimerizes, is phosphorylated, undergoes a conformational change which exposes the nuclear localization sequence (NLS), and translocates to the nucleus.

Once AR is in the nucleus, dimeric AR binds to androgen response elements (AREs) in the promoters or enhancers of target genes. ARE sequences are usually 6 base pair long "half site" direct or inverted repeats separated by 3 base pairs [18, 27, 28]. There are two classes of AREs; class I possess typical guanine residues and class II has atypical sequences and

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features that confer synergistic transcriptional activity to AR [28]. Interestingly, AR is also reported to bind upstream of promoters and enhancers of genes that do not possess a putative AREs [29, 30]. Once AR binds AREs, it recruits co-activators, co-repressors, and components of the pre-initiation complex [22, 31], which can either activate or repress transcription.

II. AR Function in Normal versus Cancerous Prostate

The human prostate gland is composed of epithelial-lined secretory ducts embedded in a smooth muscle-enriched stroma [32, 33]. The epithelium contains two cell types: a basal epithelium overlaid with luminal cells (Figure 2). AR is expressed within the luminal layer and in the stroma, where it has distinct roles. AR expression in the luminal cells is dispensable for gland development; however, it is required for the secretory function of luminal cells [20, 32–35]. In normal prostate epithelium, the primary function of AR is to induce expression of genes required to promote terminal differentiation, suppress proliferation, and promote secretion [36–40]. In contrast, proper gland development also requires AR expression in the stroma compartment. In the stroma, AR is thought to promote the production of growth factors, such as andromedins [41] or keratinocyte growth factor (KGF) [42], required for differentiation of the luminal cells [20, 34].

During prostate cancer development, the distinction between basal and luminal cells is lost, accompanied by a complete loss of discernable basal cells (Figure 2). The molecular basis for this apparent loss is unknown, but may involve some differentiation aspects related to AR. What results are tumor cells with markers and functions derived from both basal and luminal cells. For instance, prostate tumors often co-express both basal and luminal keratins, K5 and K8 respectively. They co-express basal cell integrins and luminal AR [34, 43–45]. One idea is that the tumor cells arise from a transient amplifying cell population or differentiation intermediate where these molecules may normally be transiently co-expressed [45].

But by far the most striking aspect of prostate cancer, is the change in how AR behaves. While dispensable for survival and proliferation within normal prostate epithelium, it is absolutely required for prostate cancer survival and proliferation. The basis for this mechanistic switch is essentially unknown. However, the answer may lie in part with the relatively common occurrence of AR-regulated cancer-specific gene fusions. The first described fusions involved the juxtaposition of the 5′ ARE-containing promoter from the AR target gene, transmembrane protease serine 2 (TMPRSS2), to the coding sequence of several Ets family members including Erg, ETV1, ETV4, ETV5 and FLI1 [46, 47]. Therefore, the fusion leads to the production of Ets transcription factors, which potentially drive proliferation and suppress differentiation, under the control of AR. This could then confer on AR the ability to promote cell survival and proliferation in part by differentially targeting transcription to a new set of genes through Ets-like proteins [48–50].

However, not all tumors harbor these fusions [46]. Whole genome chromatin immunoprecipitation studies (ChIP-Seq) that interrogated sites of AR binding in tumor cells identified many targets not found in normal cells suggesting AR itself may bind aberrant

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targets in cancer cells [29, 51]. When combined with expression data, it was these off-target AR genes that more highly correlated with cancer pathogenesis [52].

One of the most well characterized AR target genes is PSA. PSA is a serine protease [53] secreted into the ducts of normal prostates, which can also be detected in the serum of men. Because AR signaling is dramatically increased in prostate cancer, detection of elevated levels of PSA in serum is a reflection of elevated activity in the gland and forms the basis for the PSA test. However, it can also reflect elevated activity due to other pathologies including prostatitis and BPH, limiting its specificity for cancer. Because elevated PSA is not required for tumorigenesis, but rather a bystander event, its levels may not truly reflect tumor burden or disease severity. This limited predictive value has caused the United States Preventative Task Force in 2012 to recommend against using the PSA test stating that PSA testing may lead to over-diagnosis and over-treatment [54]. Thus, understanding and discovery of improved biomarkers remains a critical need in the field of prostate cancer research.

III. Androgen Receptor Antagonists

Because most prostate tumors are exquisitely dependent on AR, inhibiting AR is the primary and most effective therapeutic strategy. Current ADTs are designed to prevent AR function by blocking androgen binding to AR. This is accomplished through three primary mechanisms. The first is to reduce androgen levels in the body or tissues using chemical castration agents such as luteinizing-hormone releasing hormones (LHRH) agonists or antagonists (i.e. leuprolide or abarelix). These function primarily by down-regulating the LHRH receptor and preventing androgen synthesis in the hypothalamus. Newer antagonists, abiraterone acetate, VT-464, and TAK-700, inhibit 17 α-hydroxylase/C17,20 lyase (CYP17A), an enzyme expressed in testicular, adrenal, and prostatic tumor tissue and is responsible for converting pregnenolone into androgen [55–59]. Another part of the androgen synthesis pathway is the conversion of androstenedione, a relatively weak androgen, into testosterone [60]. This reaction is catalyzed by aldo-keto reductase family 1 member 3 (AKR1C3) (also known as HSD17β) [60]. AKR1C3 inhibitors are currently under development [61]. A second mechanism to block androgen is to target 5-α reductase with finasteride and dutasteride, which blocks the intracellular conversion of testosterone to its higher binding affinity derivative DHT [62, 63].

The third approach is to block androgen binding to AR using competitive antagonists (i.e. bicalutamide, flutamide). However, these inhibitors have limitations. These include a 30-fold weaker binding affinity for AR LBD than DHT [64]. Also, a known AR T887A mutation allows these antagonists to act as agonists. Furthermore, they do not prevent AR nuclear translocation [65–68]. Combined targeting of circulating androgens and use of competitive antagonists, are commonly prescribed during ADT. ADT generally causes prostate cancer remission in 80–90% of patients, resulting in a median progression-free survival of 2.5–3 years [2, 69–71]. However, after this remission period, the cancer becomes castration resistant [2] and AR is still found to be persistently active during this phase of the disease. Given this notion of AR still being active and its ability to elude current competitive antagonists, next-generation AR antagonists that are non-competitive or have a greater AR

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affinity, were developed [72, 73]. One of these is enzalutamide (aka MDV3100) which, unlike bicalutamide, prevents AR nuclear translocation [73]. While initially effective, many responders later develop resistance after 47 weeks [6].

IV. Mechanisms that Confer Resistance to Anti-androgen Therapy

Despite attempts to improve therapeutic targeting of AR, resistance still remains a major obstacle. The over-expression or amplification of AR in 20–30% of castration-resistant cases [3–5] as well as gain-of-function point mutations have long been recognized as major resistance mechanisms [74]. AR mutations are observed in approximately 10–25% of CRPC [75, 76]. Amplified AR expression results in increased sensitivity to lower levels of androgen [77] and mutations often result in increased ability to bind other steroids, such as corticosteroids or estrogen. Some mutations convert antagonists to agonists [68, 78].

While enzalutamide displayed effectiveness initially in patients who failed standard ADT, many responders later developed resistance [6]. Several mechanisms for this resistance have been reported. One mechanism is a mutation in the LBD of AR which converts phenylalanine 876 to leucine (F876L) [16]. Another is emergence of AR splice variants lacking the LBD, creating a constitutively active AR [10, 79]. Finally, enzalutamide resistance also correlates with increased Src signaling.

The idea that tumor cells can make their own androgen, and thus elude the standard LHRH ADT treatment was recently demonstrated [32]. Androgens can be synthesized *de novo* from cholesterol via enzymatic steps that are catalyzed by cytochrome P450 (CYP) members [60, 80]. Cholesterol undergoes a cleavage reaction by the enzyme desmolase (CYP11A1) to convert it into pregnenolone that can be further converted into progesterone by 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) [60]. After these steps, pregnenolone or progesterone can be further converted into 17-OH pregnenolone or 17-OH progesterone by CYP17A1 [80]. These are further converted into the metabolic intermediates dehydroepiandrosterone (DHEA) or androstenedione respectively, which are converted to testosterone and then reduced to DHT by 5α reductase [80]. These enzymes are upregulated in CRPC to overcome the lack of circulating androgen. Further selection and dependency on this pathway was demonstrated by the recent discovery of a mutation in the androgen-synthesizing enzyme, 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) at residue N367T. This mutation confers resistance to poly-ubiquitylation leading to an accumulation of DHT [81]. It is unclear whether this mutation elicits resistance to abiraterone and other therapies. Enhanced androgen production can also be achieved by elevated expression of both type 1 and 2 5-α reductase [82]. Under normal conditions, type 1 5-α reductase is expressed in various cell types such as fibroblasts and skin cells while type 2 5-α reductase is expressed in prostate. However, both isozymes of 5-α reductase are expressed in prostate cancer [63].

AR regulates transcription by recruiting various co-activators such as p300-CBP and p160 [83, 84]. These are often over-expressed in prostate cancer resulting in increased AR transcriptional activity. The p160 co-activator, SRC-3, is degraded by the E3 ubiquitin ligase adaptor speckle-type poxvirus and zinc finger (POZ) domain protein (SPOP) [85]. However, in patients SPOP has a missense mutation in its substrate binding domain causing it not bind

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to SRC-3 and stabilizing SRC-3 [86, 87]. When *SPOP* is found mutated, DEK a SPOP substrate and onco-protein [88], was found to be up-regulated and contributed to tumor cell invasion [89].

Most recently, AR splice variants were discovered where exons 5 to 7 were deleted resulting in loss of the LBD [90]. This AR-variant (AR-V) is capable of nuclear translocation, ARE binding, and can activate AR target genes in the absence of androgen. Typically AR-V is detected in tumors that also express full length AR [91]. It is controversial as to whether AR-V function is dependent on full-length AR [90–92]. AR-V is detectable in castration resistant tumors and its expression is associated with shorter patient survival times and with enzalutamide resistance [79, 93–95]. Splice variant 7 of AR (ARV-7) has also drawn a lot of attention given its clinical significance in CRPC [10, 96].

AR can be activated by other signal transduction pathways, including growth factors, MAPK, Src, PKC, and PI3-K/Akt [97–101]. AR interaction with signaling scaffold proteins can lead to AR activation in the absence of ligand [102]. Receptor for activated C kinase 1 (RACK1), a protein kinase C (PKC) anchoring protein, was shown in a yeast two hybrid screen to be an AR interacting protein [103]. *In vitro*, RACK1 promotes AR nuclear translocation upon PKC activation in the absence of androgen [103]. Non-receptor tyrosine kinase Src can phosphorylate AR at Y534 within the hinge region of AR leading to increased AR translocation to the nucleus and enhanced transcriptional activation.

For all of these resistance mechanisms, there are currently no effective alternative therapies. Nor will their inhibition be accomplished by continuing to focus on agents that target exclusively the androgen component. A better understanding of these resistance mechanisms, how to measure or predict their occurrence, combined with a multi-targeted approach will be required before we can hope to fully eradicate castration-resistant prostate cancer.

V. Non-Nuclear AR Signaling

All of the therapy resistance mechanisms outlined above, largely focus on the nuclear transcriptional activity of AR. However, the use of newer therapies that displace AR from the nucleus into the cytoplasm may have unintended consequences as AR has known functions in the cytoplasm, which is often referred to as non-genomic signaling [104–107]. A primary characteristic of non-nuclear signaling is the rapidity with which it occurs. While still in the cytosol steroid receptors can undergo several protein-protein interactions within seconds to minutes after stimulation with steroids [108]. This ability is not limited to AR as it has been reported for several other steroid receptors including estrogen, glucocorticoids [109], thyroid hormone [110], and progesterone [111]. Signaling molecules activated by AR and other steroid receptors in a non-genomic fashion include Src family kinases (SFK), Ras, MAPK, Akt, L-type calcium channels, PKC, PLC, EGFR, and other second messenger proteins [112–117]. Furthermore, the cytoplasmic function of AR is observed in non-prostate cells, such as fibroblasts, where the levels of AR are relatively low, and AR translocation in response to androgen is not observed [118].

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The most well studied signaling molecule activated by steroid receptors is Src. Src is a cytosolic 60 kDa protein composed of seven functional domains: a myristoylated amino-terminus, a unique domain specific to each SFK member, a Src homology domain 3 (SH3), Src homology domain 2 (SH2), a linker region, a catalytic domain, and a COOH-terminal tail [119, 120]. The myristic acid moiety allows Src to localize to the inner membrane. When in its closed inactive conformation, the SH2 domain binds to its tyrosine phosphorylated Y530 tail and the SH3 domain binds to PXXP sequences within the linker region [119]. Upon activation, de-phosphorylation of the Y530 tail facilitates Src unfolding and activation loop phosphorylation site Y419 opens the catalytic domain. Further stabilization of activated Src or an alternative mechanism for activation can be enhanced by SH3-mediated binding to PXXP motifs in associated molecules. Although mutations in the C-terminal Y530 domain of Src generates a potent oncogene, such mutations are rare in human cancers. Nonetheless aggressive tumors have elevated Src tyrosine phosphorylation gene signatures that correlate with poor outcomes, including prostate cancer [12]. The mechanisms that lead to aberrant Src signaling in tumors is not completely clear, but growth factor signaling or elevated Src expression are known stimulators. Several SFKs are reportedly elevated in prostate cancer. Src is over-expressed in some prostate cancer cell lines as is a related member Abl [121, 122]. Other elevated SFKs in prostate cancer include Fyn and Yes [123, 124]. The SFK member FGR is significantly up-regulated in tissues from patients with castration-resistant disease [125]. Furthermore, elevation in SFK expression or activity is often correlated with poorer outcomes [125]. Thus, SFKs may play an important role in driving lethal prostate cancer, particularly in castration-resistant disease.

Within steroid-dependent tumors, some of the elevation in Src activity likely occurs through interactions with the receptors. While several steroid receptors can activate Src, the mechanisms involved appear to be different. For ER, Src can bind directly via its SH2 domain to tyrosine-phosphorylated ER. However, further studies demonstrate that ER-α and ER-β bind to LXXLL motifs in the scaffold protein modulator of non-genomic actions of the estrogen receptor (MNAR/PELP1) to interact with Src while localized to the cytosol [126, 127]. This ER-α-MNAR-Src complex leads to Src activation. For AR, the PXXP sequences within the AR linker domain mediate binding through the Src SH3 domain [5, 14, 128]. AR can also form a tertiary complex with MNAR/PELP1 and Src [129] (Figure 3). Initially, Src is inactive within this complex. However, when AR binds Src, this leads to the activation of Src in this complex (AR/MNAR/Src) and the subsequent activation of a downstream effector, MEK [129]. This complex is androgen dependent in LNCaP cells, but is constitutively active in a castration-resistant LNCaP derivative cell line, C4-2.

Another signaling molecule commonly activated by steroid receptors in a non-genomic fashion is MAPK [130]. Androgen stimulation of LNCaP or PC3 cells stably re-expressing wildtype AR for 2–60 minutes increased Erk-1/2 phosphorylation [130]. In another study, androgen activated Raf and Erk-2 within 2–5 minutes [14]. This was abrogated using either Casodex or Src inhibitor PP1 [14]. Introduction of AR into COS cells and subsequent androgen stimulation led to a complex of AR and Src [14]. Similar studies assessing non-genomic actions of AR and ER in an osteocytic cell line MLO-Y4 or COS cells highlighted the rapidity of steroid signaling and identified specific interactions between AR or ER and Src [14, 128].

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Calcium signaling is a major player in prostate cancer progression [131]. Several studies demonstrated that non-genomic actions of AR can lead to increased intracellular calcium [132, 133]. Different mechanisms have been proposed, one involving a GPCR and another involving EGFR [134, 135]. Non-genomic action of AR on calcium signaling is also observed in other calcium-regulated cells, including neurites, smooth muscle, and heart [136–138].

VI. Functional Consequences of Non-Nuclear AR Signaling

Proliferation

Many different events are activated by AR signaling, including proliferation. AR transcriptional activity plays an important role in G1/S-phase transition [139] and castration-resistant prostate cancer [140]. However, non-genomic signaling also influences cell proliferation (Figure 3). In quiescent NIH3T3 fibroblasts, androgen induces S-phase entry through AR association with Src, which stimulates phosphatidylinositol 3-kinase (PI3-K). NIH3T3 cells express relatively low levels of AR and these events occur in the absence of AR nuclear localization [118]. Inhibition of Src in LNCaP cells decreased AR-dependent, but androgen-independent cell proliferation induced by IL-8 [141].

Cell Survival

Non-genomic AR signaling also promotes tumor cell survival. One example is the ability of AR to activate PI3-K [142]. Vas deferens epithelial cells and PC3 cells made to stably express AR and stimulated with androgen for 10–30 minutes induced p-AKT^{Ser473} activation. This activation was blocked by PI3-K inhibitor LY294002 or AR antagonist bicalutamide [142]. Another study demonstrated that stimulating PC3-AR cells for 10–30 minutes induced phosphorylation of Forkhead protein FKHR-L1 at Ser256 and Bad at Ser112; both involved in promoting cell survival [143]. In this study, an interaction between AR and the PI3-K p85α subunit was detected. Further reports demonstrated that non-genomic AR triggered Src-MEK-1/2-cAMP-response element binding protein (CREB) activation. This pathway stimulated prostate tumor cell proliferation and enhanced survival capabilities [129] (Figure 3).

Invasion and Metastasis

Many of the signaling pathways activated by steroid receptors are known regulators of cell migration, invasion, and metastasis. So while AR-dependent non-genomic regulation of cell proliferation and survival are well established, its ability to control invasion and metastasis is not fully characterized. Since metastatic spread of cancer decreases the quality of life and ultimately leads to death, it is critical to understand the mechanisms that AR uses to drive metastasis.

Src plays a well-known role in regulating cell migration and invasion through phosphorylation of many substrates including p130Cas, Shc, FAK, p190RhoGAP, paxillin, and CDCP1 [144, 145]. Clinically, Src is implicated in driving bone metastasis. The majority of breast cancers that metastasizes to the bone possess active Src [146], and a Src signature is detected in breast cancer bone metastasis [147]. In *in vivo* studies using murine

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models of breast cancer, inhibition of Src decreased the size of metastatic tumors and delayed the appearance of bone metastasis [148, 149]. In prostate cancer models, inhibition of Src decreased prostate cancer cell adhesion, migration, and invasion, and targeting Src and Abl with dasatinib attenuated lymph node metastasis of orthotopic PC3 xenografts [13, 150–153].

The ability of non-genomic steroid signaling to facilitate migration, invasion, and metastasis was initially identified in breast cancer and later shown to facilitate movement of cholonic smooth muscle [154]. While correlations between elevated AR and Src activity and invasive migration were reported for prostate cancer, the mechanisms by which this occurred were not examined. A recent study demonstrated that the ability of AR to enhance invasion of prostate cancer cell lines occurred independently of its nuclear localization and was dependent on Src [13]. The study further identified matriptase activation by Src to be involved in promoting non-genomic AR prostate cancer cell invasion [13]. Specifically, the initial cleavage and shedding of minto the medium occurred within 20 minutes of androgen stimulation, required Src and AR, and occurred in cells expressing a non-nuclear form of AR (Figure 3). Interestingly, 24 hours later new matriptase mRNA was induced, which was shown to be dependent on nuclear AR [155]

VII. Clinical Importance of Targeting Non-genomic AR Signaling

Treatment of tumor cells with enzalutamide leads to high levels of cytosolic AR [12]. This has been tied to increased Src activation and cell motility and may be a plausible reason for recurrent disease. In the cytosol AR can activate Src and Src can phosphorylate AR at Y534 [156]. Interestingly, Y534 phosphorylation sensitizes AR to low levels of androgen [156], creating the possibility that cytoplasmic AR may stimulate signaling independent of ligand. As highlighted earlier, non-genomic signaling can also activate the MAPK signaling pathway [130]. Thus, non-genomic AR could increase MAPK signaling, which is coordinately dysregulated along with Akt signaling in advanced prostate cancers [157–159]. Additionally, enhanced Ras signaling reduces androgen dependency and Ras/MAPK activation is associated with metastatic lesions [160, 161].

These findings demonstrate that after ADT, AR non-genomic substrates may still need pharmacological inhibition and targeting these non-genomic signaling pathways could offer additional therapies. Indeed Src, PI3-K, and MAPK signaling inhibitors are clinically available. However, given that L-type calcium channels can be stimulated by AR, and the possibility of blocking their activity offers another potential therapeutic approach [132, 133]. Targeting other AR non-genomic downstream targets such as matriptase or MNAR may also be sagacious avenues worth scientific exploration.

However, clinical trials clearly indicate that attempting to target Src, PI-3-K, or Ras/MAPK in isolation is not effective in prostate cancer patients. The continued presence of AR-genomic signaling in this context still needs to be addressed and allows ‘escape’ from these targeted therapies. Thus, combination therapies in which enzalutamide-mediated shuttling of AR into the cytoplasm to prevent nuclear AR action, reduction in tumor cell synthesis of androgens with abiraterone to lower androgen levels, while also targeting Src, MAPK,

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calcium channels, or other targets to block non-genomic signaling may be required to effectively reduce the oncogenic dependency driven by AR.

VIII. Emerging Alternatives for Targeting AR

Current ADTs are designed to prevent AR signaling by blocking androgen binding to AR or by blocking circulating androgens. However, there are no FDA approved antagonists available that currently target cytosolic AR or the AR-V splice variant. One alternative strategy to target AR is to promote its destruction, either through inhibition of Hsp90 [162] or by introduction of an AR mRNA hammerhead ribozyme [163]. Targeting the DNA-binding domain (DBD) would also be plausible, as it would occlude AR from binding to ARE sequences and suppress genomic transcription.

Emerging methodologies to target the N-terminus of AR, independently of ligand, are currently being investigated. These agents include niphatenone B, Sintokamide A, EPI-506, and EPI-001. Each of these targets the AF-1 domain of AR in the N-terminus and prevents AR from binding to AREs [164–166]. Each of these pharmacological agents demonstrate promising results *in vitro* and *in vivo* by repressing androgen/AR induced transcription. The greatest benefit, is that they would be effective in cells harboring the AR-V or other splice variants lacking the LBD.

Finally, although rare, there is a subset of prostate tumors that express very low levels or do not express AR at all due to a number of possible mechanisms including methylation [167, 168]. In these cases, targeting AR will not be effective no matter how it is done. However, in these AR negative tumors, several downstream signaling cascades remain expressed or constitutively active, that may represent effective therapeutic targets. As we improve AR targeting at multiple levels, we may find that the frequency at which non-AR tumors arise increases. Thus, this last final challenge of defining the non-AR pathways that keep these tumors alive will remain when all the others have been solved.

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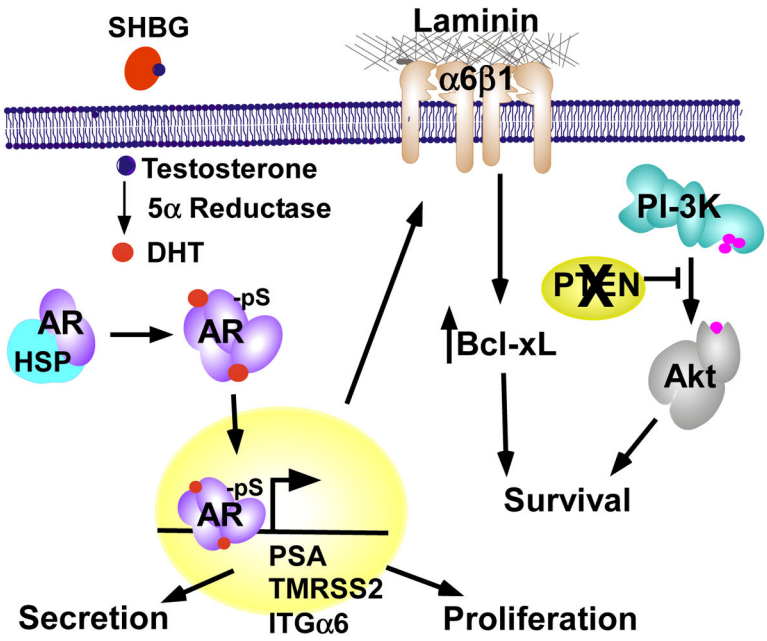

Figure 1. Canonical androgen receptor-mediated transcription in tumor cells

Circulating testosterone is delivered to AR positive cells attached to sex hormone binding globulin (SHBG), which is then reduced intracellularly to dihydroxytestosterone (DHT) by 5α-reductase. When DHT binds AR, it displaces heat shock protein (Hsps). AR then homo-dimerizes, is phosphorylated (pS), and translocates to the nucleus. Dimeric AR binds to androgen response elements in the promoters or enhancers of target genes to enhance or suppress their transcription. Targets include PSA and TMPRSS2 which are secreted into the lumen, integrin α6 (ITGα6) which promotes survival through adhesion to laminin, and cell cycle regulators that promote proliferation.

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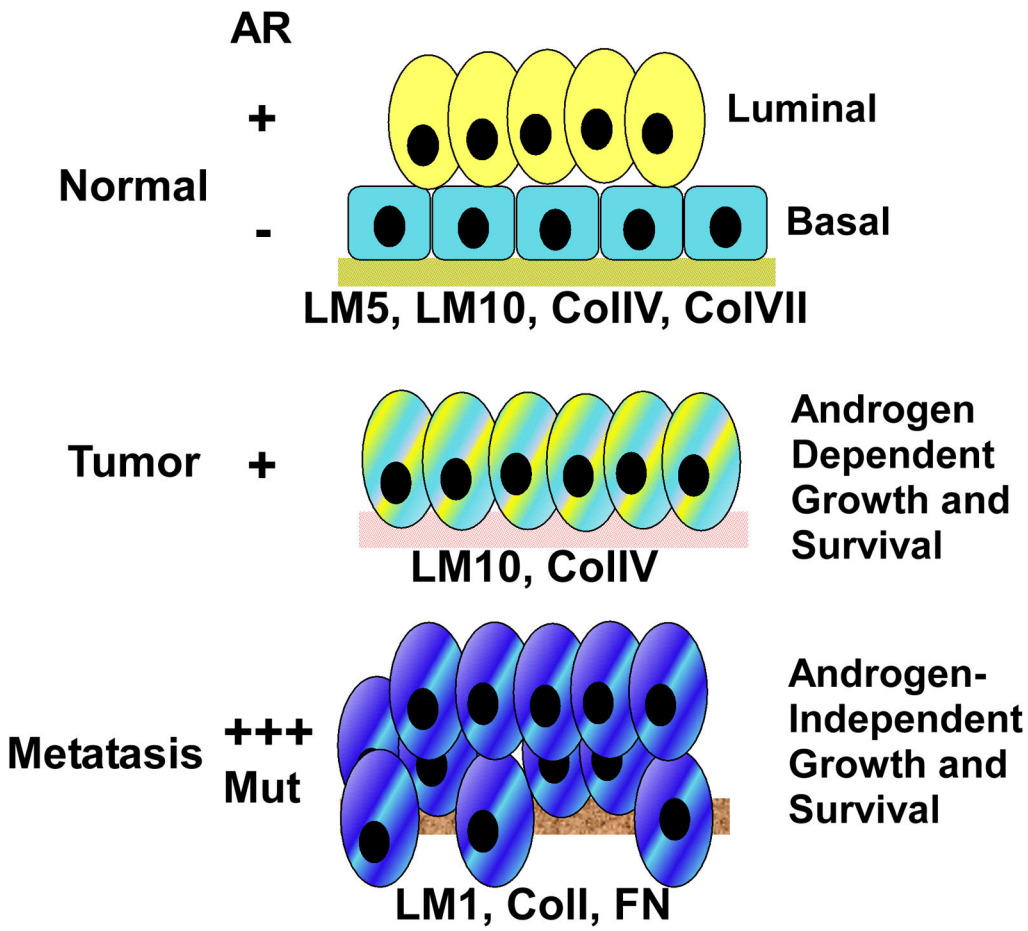

Figure 2. The journey of a normal prostate to prostate cancer

The normal human gland consists of a basal cell layer bound to extracellular matrix (ECM) via integrins with a luminal layer on top expressing AR. AR functions in these cells to suppress growth and promote secretion. Basal cells are lost and ECM is altered in prostate tumors and the luminal-like cells co-express AR and integrins. AR drives androgen-dependent growth and survival of the tumor. Metastatic tumors become castration-resistant following ADT, which is accompanied by increased AR expression or mutational activation. Cells are invasive and the ECM composition is dictated by the metastatic site. Tumors are still dependent on AR for growth and survival, but no longer need physiological levels of androgen.

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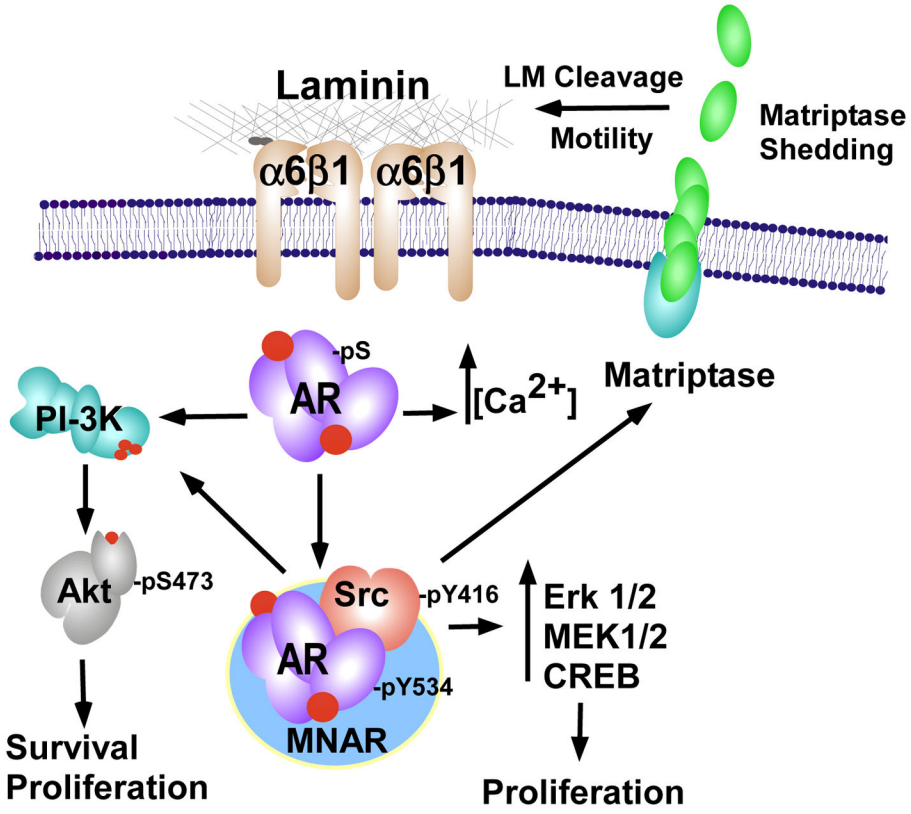

Figure 3. Non-genomic AR signaling

Within the cytoplasm, active AR (mediated by androgen or constitutive kinase phosphorylation) binds the NMAR scaffold complex with Src. Src is activated by AR, and in turn AR is phosphorylated by Src (pY534). This complex can activate the MAPK/CREB pathways to promote proliferation. Src can also stimulate cell migration and Matrigel invasion through activation and shedding of Matriptase. PI3-K can be activated by AR either through Src or independently, leading to enhance proliferation and survival. AR can also independently stimulate intracellular calcium stores ($[Ca^{2+}]$).