

Androgen receptor-mediated non-genomic regulation of prostate cancer cell proliferation

Ross S. Liao, Shihong Ma, Lu Miao, Rui Li, Yi Yin, Ganesh V. Raj

Department of Urology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, USA

Correspondence to: Ganesh V. Raj, M.D., Ph.D. Department of Urology, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9110, USA. Email: ganesh.raj@utsouthwestern.edu.

Abstract: Androgen receptor (AR)-mediated signaling is necessary for prostate cancer cell proliferation and an important target for therapeutic drug development. Canonically, AR signals through a genomic or transcriptional pathway, involving the translocation of androgen-bound AR to the nucleus, its binding to cognate androgen response elements on promoter, with ensuing modulation of target gene expression, leading to cell proliferation. However, prostate cancer cells can show dose-dependent proliferation responses to androgen within minutes, without the need for genomic AR signaling. This proliferation response known as the non-genomic AR signaling is mediated by cytoplasmic AR, which facilitates the activation of kinase-signaling cascades, including the Ras-Raf-1, phosphatidylinositol 3-kinase (PI3K)/Akt and protein kinase C (PKC), which in turn converge on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) activation, leading to cell proliferation. Further, since activated ERK may also phosphorylate AR and its coactivators, the non-genomic AR signaling may enhance AR genomic activity. Non-genomic AR signaling may occur in an ERK-independent manner, via activation of mammalian target of rapamycin (mTOR) pathway, or modulation of intracellular Ca^{2+} concentration through plasma membrane G protein-coupled receptors (GPCRs). These data suggest that therapeutic strategies aimed at preventing AR nuclear translocation and genomic AR signaling alone may not completely abrogate AR signaling. Thus, elucidation of mechanisms that underlie non-genomic AR signaling may identify potential mechanisms of resistance to current anti-androgens and help developing novel therapies that abolish all AR signaling in prostate cancer.

Keywords: Androgen receptor (AR); ERK MAP kinases; cell proliferation; non-genomic signaling; prostate cancer



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Introduction

Androgen receptor (AR) is a classic steroid hormone receptor that is critical for prostate cancer development and progression. In its unbound conformation, AR is located primarily in the cytoplasm in complex with heat shock proteins, cytoskeletal proteins, and other chaperones (1-5). These proteins also enable modulation of AR conformation for efficient ligand binding (6,7). When androgen binds AR, AR forms a homodimer, undergoes a conformational change, and interacts with additional proteins that facilitate its nuclear translocation (8-10). Once in the nucleus,

AR binds to the androgen response elements (AREs) on promoter/enhancer regions, recruits coregulators, and forms the transcriptional machinery for AR-regulated gene expression (10). This AR-signaling pathway, known as the genomic pathway, relies on AR nuclear translocation and AR-DNA binding for cell proliferation. The genomic pathway is thought to occur over several hours and is characterized by increased expression of specific AR-regulated genes (*Figure 1*).

However, studies have shown a rapid and reversible AR signaling that occurs within minutes and results in regulation of prostate cancer cell proliferation (11-13). This

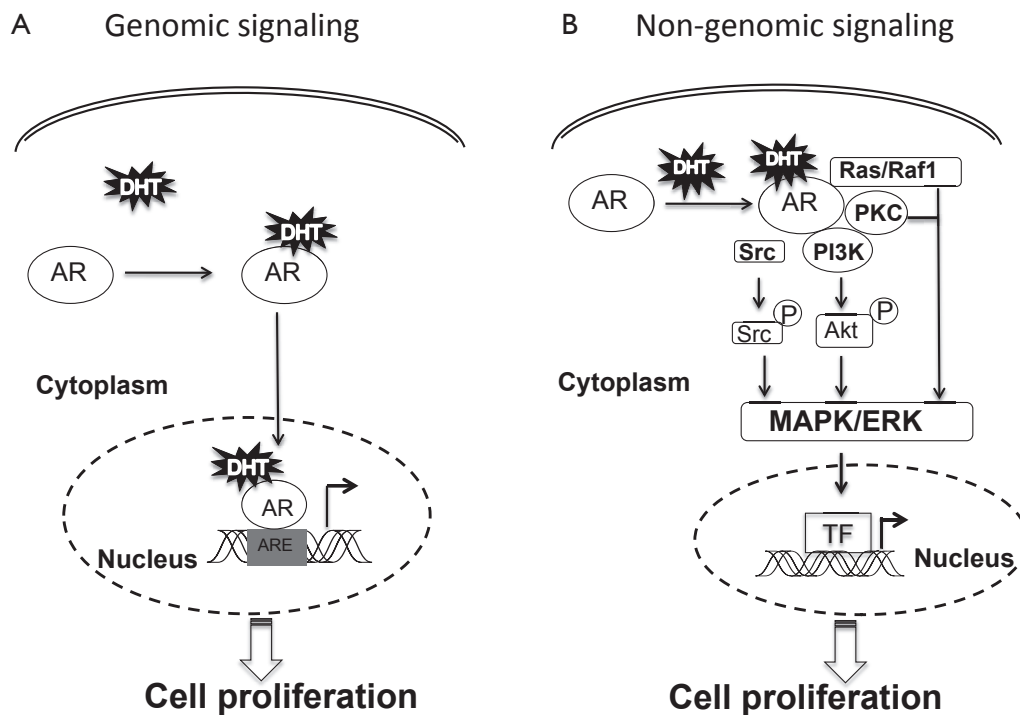


Figure 1 Genomic and non-genomic AR signaling in prostate cancer cells. (A) Genomic AR signaling. After binding with the activated form of androgen, 5α -dihydrotestosterone (DHT), AR undergoes a conformational change and translocates to nucleus. In the nucleus, AR binds to the androgen response elements (AREs) on promoter/enhancer regions, recruits coregulators, and forms the transcriptional machinery for AR-regulated gene expression; (B) Non-genomic signaling. Activated AR in the cytoplasm can interact with several signaling molecules including the phosphatidylinositol 3-kinase (PI3K)/Akt, Src, Ras-Raf-1, and protein kinase C (PKC), which in turn converge on mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) activation, leading to cell proliferation.

AR-signaling pathway, known as the non-genomic pathway, requires neither AR nuclear translocation nor AR-DNA binding. Instead, cytoplasmic AR signaling may function through mitogen-activated protein kinase (MAPK) signaling cascades, converging on extracellular signal-regulated kinase (ERK) activation (14,15). Treatment of AR-positive prostate cancer cells with 5α -dihydrotestosterone (DHT) leads to increased ERK-1/2 phosphorylation within 5 minutes in a dosage-dependent manner (13) (*Figure 1*).

While non-genomic AR signaling has thus far been shown to primarily require MAPK/ERK activation, cell signaling can also occur without ERK activation. Non-ERK pathways involve activation of mammalian target of rapamycin (mTOR) via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway or involvement of plasma membrane, G protein coupled receptors (GPCRs) and the sex hormone binding globulin receptor (SHBG) that modulate intracellular Ca^{2+} concentration and cyclic adenosine monophosphate (cAMP) levels, respectively (16,17).

In addition, non-genomic AR signaling may be mediated by a membrane-bound AR that can regulate intracellular Ca^{2+} concentration and membrane ion channels (18,19). Studies with bovine serum albumin (BSA)-bound DHT, a compound that is unable to penetrate the plasma membrane, show a dose-dependent suppression of the PI3K and MAPK pathways (20). These data indicate that non-genomic AR signaling may suppress proliferation via membrane-bound AR (21) or activate proliferation via cytoplasmic AR.

Finally, recent data indicates that the non-genomic AR signaling may regulate genomic AR signaling and that the non-genomic and genomic AR signaling may work together to coordinate gene regulation in prostate cancer cells. In this manuscript, we provide a comprehensive review of non-genomic AR signaling with an emphasis on the established role of MAPK/ERK in prostate cancer cell proliferation. Clinically, understanding of these non-genomic AR signaling pathways is important, as they may represent potential mechanisms of resistance to AR antagonists.

AR antagonists including casodex and flutamide have no effect on non-genomic AR signaling, as evidenced by ERK phosphorylation in the presence of these drugs (13). Thus, elucidation of non-genomic AR signaling pathways may enable development of novel agents to inhibit all forms of AR signaling in prostate cancer.

ERK-1/2 mediated non-genomic AR signaling

The MAPK/ERK signaling cascade is important in regulating diverse biological functions including cell survival, motility, and proliferation, which are essential to prostate carcinogenesis (22). Aberrant activation of kinases in this pathway is frequently reported in human cancer (23-25). Studies of DHT-responsiveness in prostate cancer cells in our lab and others show evidence of ERK-1/2 phosphorylation within 1-2 minutes of DHT treatment and peak levels of ERK-1/2 phosphorylation within 5-10 minutes. Activated ERK-1/2 then translocates to the nucleus and directly interacts with and phosphorylates transcription factors (TFs), such as nuclear ETS domain-containing Elk1 (26-28). Elk1 transcriptionally regulates immediate early genes (IEGs) such as *c-fos* (26,29), which coordinately regulates the expression of several genes involved in cell proliferation (26,27). This response is AR-dependent as no effect was observed in AR-negative PC-3 prostate cancer cells (13). Thus, while ERK phosphorylation occurs within minutes and serves as a measurable response of non-genomic activation, the molecular processes involved in cell proliferation occur over several hours and days.

DHT-induced ERK activation in prostate cancer cells may be mediated via multiple pathways, including the PI3K/Akt, Ras-Raf, and protein kinase C (PKC) pathways. Extensive evidence suggests AR associates with plasma membrane lipid rafts that facilitate AR activation of these pathways (30). AR activation of the PI3K/Akt pathway involves direct interaction of AR with the p85 α regulatory subunit of PI3K (31), while the activation of the Ras-Raf pathway may involve the sequential activation of Ras, Raf and MEK kinases and may be dependent on the formation of an AR-Src complex (29,32). Importantly, Src or scaffolding proteins like proline-, glutamic acid-, and leucine-rich protein-1 (PELP1) may modulate the interaction of AR with Akt (31,33). PKC activation of ERK may involve modulation of intracellular Ca²⁺ concentration (13). Furthermore, ERK and Src are calcium-dependent kinase cascades suggesting AR could directly regulate them via mobilization of intracellular Ca²⁺ levels (32,34). Each of

these AR signaling pathways can result in ERK activation and represent redundancy that ensures a proliferative response to DHT. Further, crosstalk between AR-mediated signaling cascades suggests a complex network of signals that converge on ERK phosphorylation. Each of these pathways is individually discussed in detail below.

PI3K/Akt/PTEN pathway

Ligand binding induces AR to directly interact with the p85 α regulatory subunit of PI3K, resulting in the activation of the PI3K/Akt pathway (31). PI3K phosphorylates Akt (also known as PKB), a subfamily of serine-threonine protein kinases. Akt expression is frequently observed to be elevated in human prostate, ovarian, and breast cancers (35-37). The PI3K/Akt pathway activates the MAPK/ERK cascade and is regulated by phosphatase and tensin homolog (PTEN). PTEN, a protein phosphatase that dephosphorylates phosphatidylinositol (3,4,5)-triphosphate (PIP3) thereby inhibiting PI3K induced activation of Akt (38,39), is one of the most commonly lost tumor suppressors in prostate cancer (40-42). PTEN loss of function often results in constitutively active Akt and may result in chronic activation of the proliferative genes.

The PI3K/Akt pathway activates mTOR and forkhead box protein O1 (FOXO1) and the MAPK/ERK cascade. Kinase inhibitors and dominant negative mutants of PI3K disrupt DHT-mediated activation of ERK and have supported a central role for the PI3K/Akt pathway in non-genomic AR signaling (13). Further, DHT-mediated activation of PI3K/Akt is AR-dependent (31,32,43).

Src pathway

Several studies have also implied the importance of Src in AR activation of kinase signaling cascades (17,32,43). In its inactivated conformation, interaction of the Src homology 2 (SH2) and Src homology 3 (SH3) domains causes autoinhibition of Src. AR interacts with the SH3 domain of Src relieving its autoinhibition and resulting in Src activation of the adaptor protein, Shc, a known upstream regulator of the MAPK pathway (44-46). AR-Src complexes may be noted in immunoprecipitation assays resulting in activation of Shc (29,43). Inhibition of the Src/MAPK pathway decreases DHT-induced ERK-1/2 phosphorylation (47).

In addition to Src-mediated direct activation of the ERK1/2 signaling cascade, Src may also activate the expression of receptors such as the insulin-like growth

factor 1 receptor (IGF-1R) (47). Activated AR can also directly regulate IGF-1 gene expression as the IGF-1 promoter contains two AREs (48). However, data from quantitative RT-PCR studies shows expression of IGF-1R may be independent of AR-DNA binding. While the exact mechanism is not clearly elucidated, induction of IGF-1R expression appears to depend on Src/MAPK activation. Inhibition of the Src/MAPK pathway decreases IGF-1R expression and decreases ERK1/2 phosphorylation (47). IGF-1 signaling has been shown to promote prostate cell proliferation, migration, and tumor angiogenesis, resulting in prostate carcinogenesis and cancer progression (49).

Further, IGF-1 signaling can subsequently activate the PI3K/Akt pathway in prostate cancer cells (49). Increased IGF-1R binding of IGF-1 results in activation of the PI3K/Akt pathway, which can then regulate the action of proteins like mTOR and FOXO1. These processes activate multiple pathways including the Src/MAPK pathway early, subsequently IGF-1 pathway and later the PI3K/Akt pathway being temporally activated, ensuring a robust proliferation response to DHT.

Ras-Raf pathway

The Ras-Raf pathway is comprised of the Ras family of small GTPases and their downstream interaction of Raf kinase proteins. The Ras-Raf pathway is part of the larger Ras-Raf-MEK-MAPK-ERK signaling cascade that ultimately results in phosphorylation of the kinases ERK-1/2 (25,50,51). Dominant negative constructs of Raf-1 abrogated the DHT-induced ERK-1/2 but also reduced basal activity, which may have been present from residual hormone in the culture medium (13).

PKC pathway

Studies with PKC inhibitors indicate that AR utilizes PKC as a mediator of MAPK/ERK pathway activation (13,17,52). PKC kinase activity is regulated by both modulation of intracellular Ca^{2+} concentrations and diacylglycerol (DAG) binding to PKC itself (53). Mechanisms of non-genomic AR-mediated regulation of Ca^{2+} concentration appear to be cell-type dependent (11,54). Ca^{2+} could be released via internal stores and/or through influx from extracellular space. Interestingly, these mechanisms may not be blocked by AR antagonists (54). The etiology for cell type differences may indicate a role for cell type-specific AR cofactors (54). These findings also hint at the association

of cytoplasmic AR or membrane-bound AR with plasma membrane receptors such as GPCRs or ion channels that may modulate intracellular Ca^{2+} ion concentration resulting in PKC activation.

Plasma membrane lipid rafts

AR in the plasma membrane may mediate DHT-induced activation of the PI3K/Akt, Ras-Raf, and PKC pathways (30). Non-cytoplasmic AR may be localized to the membrane and/or specialized liquid-ordered micro-domains within the lipid bilayer of the plasma membrane that are enriched with sphingolipids, caveolins, Src family kinases, G proteins and signaling mediators called "lipid rafts" (55,56). Several observations support the existence of and role for non-cytoplasmic AR in mediating non-genomic AR signaling. First, AR has been detected in the membrane and in lipid rafts. Cell membrane binding sites for androgens have been identified in several different cell types including rat osteoblasts (57), rat vascular cells (58), murine RAW 264.7 and IC-21 macrophages (19,59), murine splenic T lymphocytes (18), human prostate cancer cells (20,60), as well as in human prostate carcinoma cells (61). In PC3-AR cells, both AR and EGFR are found within plasma membrane lipid rafts (62). Co-localization of AR with caveolin-1 was found within lipid rafts of human aortic endothelial cells in response to testosterone treatment (33). Studies, using DHT-BSA, a large plasma membrane-impenetrable compound, showed binding of DHT to the membrane (18,19,63). Secondly, AR has been detected in complexes with multiple members of the lipid rafts. In caveolin-rich lipid rafts, a direct interaction was noted between caveolin-1 and a conserved nine-amino acid motif in the ligand-binding domain (LBD) of AR (30,64). AR forms a DHT-sensitive complex with the serine-threonine kinase Akt1 in caveolin-negative lipid rafts and mediates a non-genomic signal independently of Src and PI3K to activate Akt (65). Thirdly, androgens induce a rapid rise in intracellular Ca^{2+} concentration that can be blocked by pertussis toxin or by phospholipase C (PLC) inhibitor indicating a membrane-binding site for androgens that may be in close association with a GPCR (57,59,66). Finally, DHT-induced signaling cascades namely the Ras-Raf (67,68), MAPK/ERK (69,70), adenylyl cyclase (71), and PI3K(72), pathways have been shown to be enriched in lipid rafts. While specific membrane receptors, GPCR30 and membrane progesterone receptor (mPR), have been identified for estrogen and progesterone,

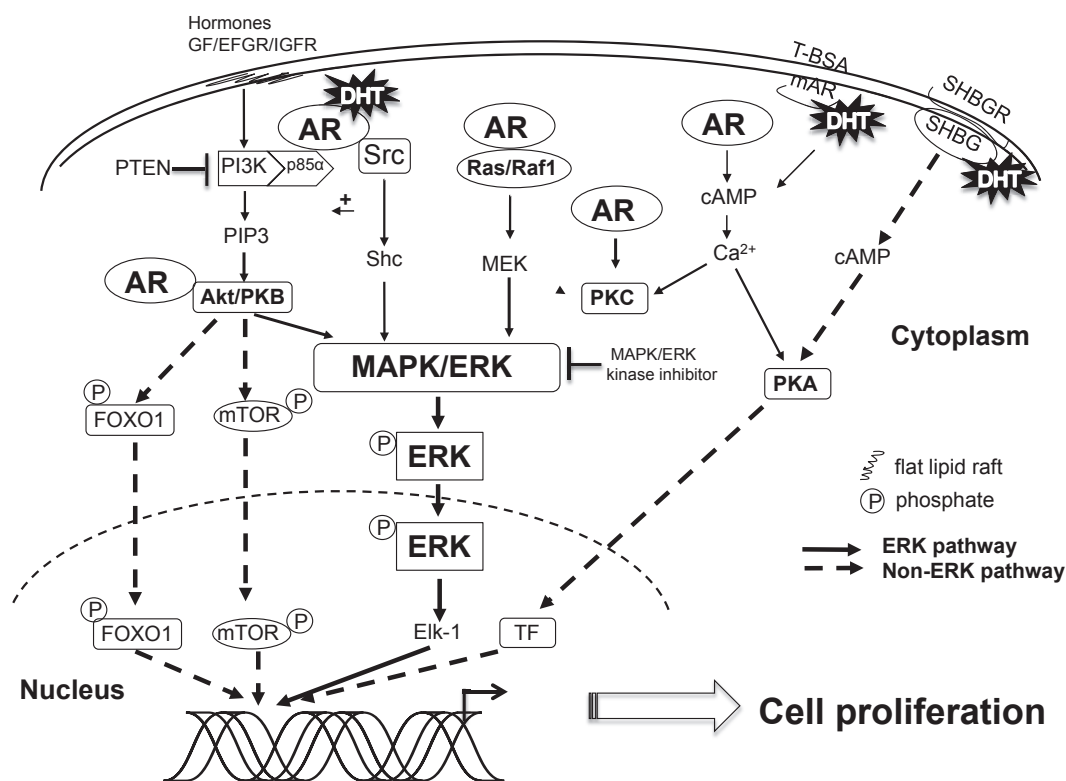


Figure 2 ERK and non-ERK mediated non-genomic AR signaling. ERK mediated non-genomic AR pathways are highlighted in solid line arrows. (I) AR interacts directly with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K) and activates Akt pathway. (II) AR interacts with Src resulting in Src activation of the adaptor protein, Shc, a known upstream regulator of the MAPK pathway. (III) AR interacts with Ras-Raf leading to sequential activation of Ras, Raf1 and MEK kinase converging on the phosphorylation of ERK. (IV) AR also utilizes PKC as a mediator of MAPK/ERK pathway activation. PKC kinase activity can be regulated by intracellular Ca²⁺ concentrations. Intracellular Ca²⁺ concentration may be modulated through plasma membrane G protein-coupled receptors (GPCRs), the sex hormone binding globulin receptor (SHBGR) and by a membrane-bound AR via up-regulation of cyclic adenosine monophosphate (cAMP) levels. Activated MAPK/ERK translocates to the nucleus, directly interacts with and phosphorylates transcription factors (TFs), such as Elk1, which coordinately regulates the expression of several genes involved in cell proliferation. Non-ERK mediated non-genomic AR pathways are highlighted in dash line arrows and include (V) PI3K/Akt/mTOR or (VI) forkhead box protein O1 (FOXO1) pathway activation. Akt activation may result in FOXO1 phosphorylation resulting in its retention in the cytoplasm and subsequent degradation. In addition, AR interacts with FOXO1 and impairs FOXO1 DNA binding ability and its ability to mediate pro-apoptotic pathways. (VII) Non-ERK signaling can also occur through activation of kinases such as protein kinase A (PKA), whose activation is regulated by intracellular Ca²⁺ concentration.

respectively (73,74), to date, a membrane AR has not yet been purified or cloned. A clear understanding of the functional importance of non-cytoplasmic AR-mediated (membrane-bound or lipid raft) non-genomic signaling is lacking.

NonERK-mediated non-genomic AR signaling

Non-genomic AR signaling may occur without ERK participation through either PI3K/Akt/mTOR pathway activation or changes in intracellular Ca²⁺ concentration

that result in activation of kinases such as PKA. For example, AR interaction with its p85α PI3K regulatory subunit may induce Akt-mediated phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP₂) to generate the second messenger PIP₃ (75,76), which then activates downstream signaling pathways important in cell proliferation (77). Alternatively, AR-directed Akt activation may result in FOXO1 phosphorylation resulting in its retention in the cytoplasm and subsequent degradation (78,79). In addition, liganded AR physically interacts with FOXO1 and impairs FOXO1-DNA binding ability and its

Crosstalk between AR genomic and non-genomic pathways

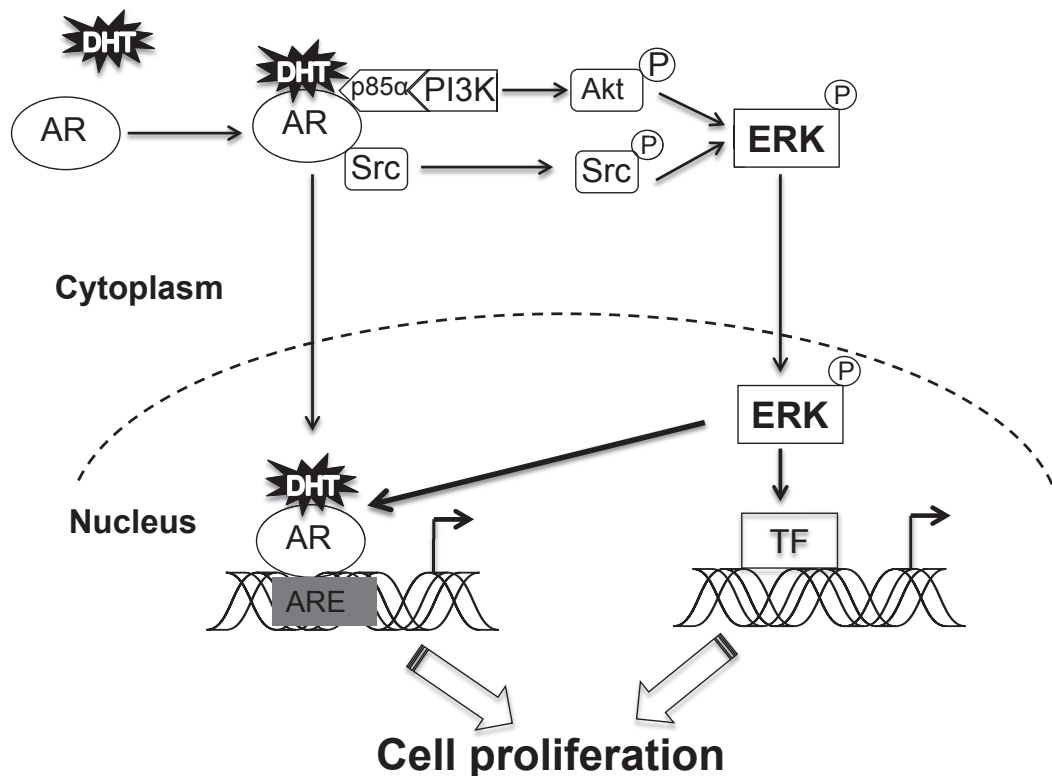


Figure 3 Crosstalk between genomic and non-genomic AR pathways. Non-genomic AR signaling may regulate genomic AR signaling. ERK has been shown to enhance AR transcriptional activity through the direct phosphorylation of AR and its coregulators. The non-genomic and genomic AR signaling may work together to coordinate gene regulation in prostate cancer cells.

ability to mediate pro-apoptotic pathways. Finally, increased intracellular Ca^{2+} concentration and increased level of cAMP induced by GPCR or SHBGR also activate protein kinase A (PKA) (67,68), which then enhances prostate cancer cell proliferation (17,69).

Critically, each of these pathways is also capable of activating ERK1/2. This suggests that a number of signaling cascades may be activated in tandem with the MAPK/ERK pathway to induce proliferation (Figure 2). Crosstalk between these pathways further amplifies the signal and ensures that the cell responds to androgenic stimulation.

Crosstalk between genomic and non-genomic pathway

ERK has been shown to enhance AR transcriptional activity through the direct phosphorylation of AR and its coregulators (13). This autocrine loop could present a

non-genomic mechanism to control AR transcriptional activity and could be important in cell adaptation to low androgen environments.

Non-genomic AR signaling mediated by induction of cAMP and PKA activation may involve SHBGR (80,81). DHT-SHBG also enhances AR transcriptional activity via phosphorylation of AR and AR coregulators facilitating their binding to AR (17). Thus, PKA can enhance prostate cancer cell proliferation and AR transcriptional activity even at very low levels of androgen (17,67,82). Thus, some of the non-genomic AR actions mediated by second messenger activation may influence the AR genomic responses (54) (Figure 3).

Implications in prostate cancer

Second-generation anti-androgens including MDV3100 and ARN-509 competitively target the activation of

AR, its nuclear translocation and its genomic activity (68,69). However, non-genomic AR signaling that functions through cytoplasmic AR may still be active in MDV3100 treated prostate cancer cells. Prior studies have indicated that DHT-mediated non-genomic activation of ERK-1/2 in prostate cells are insensitive to anti-androgens specifically hydroxyflutamide and casodex (13). Thorough evaluation of the non-genomic AR axis is mandatory in assessing the effect of drugs targeting AR signaling in prostate cancer (13).

Conclusions

The existence of rapid, non-genomic AR signaling is incontrovertible. AR non-genomic regulation functions through the activation of intertwined complex signaling cascades resulting in expression of proliferative genes and responses. Non-genomic AR signaling may act to modulate genomic AR signaling and enable a coordinated, sustained and vigorous response to androgenic stimuli. Non-genomic AR signaling may represent a potential mechanism of resistance to anti-androgens.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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