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Chapter 20

Prostate Cancer Progression to Androgen Independent Disease: The Role of the PI3K/AKT Pathway

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Additional information is available at the end of the chapter

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1. Introduction

1.1. The androgen receptor and CaP progression

The development and progression of prostate cancer (CaP) is largely dependent on the dysregulation of the androgen/androgen receptor (AR) signaling pathway; though, the mechanism of CaP progression remains elusive. Initial treatments for CaP included prostatectomy or radiation to destroy cancerous cells [1]. However, these treatments were not curative and more often than not there were recurrences and metastases of the cancer. Mainstay treatments that target the androgen/AR pathway through anti-androgen and androgen ablation therapies have been promising; yet again, these therapies seem to fail as the tumor progresses. This suggests that the androgen/AR dependence of CaP cells vary over time such that alterations in androgen availability, AR sensitivity and receptor promiscuity fuel a more aggressive CaP.

Approximately 80-90% of CaPs are originally androgen dependent (AD) at diagnosis [2]. Androgens stimulate the proliferation and inhibit the apoptosis of cells, thus implicating that CaP cells require a certain level of androgens to maintain their proliferation and survival [1]. This is primarily the reason why androgen ablation therapy is initially successful—it removes the stimulation these cells require for proliferation, ultimately causing the regression of the tumor. However, over time patients often fail androgen ablation therapy as the tumor becomes a more lethal androgen independent (AI) or castration resistant form. There is no effective therapy for AI-CaP.

The prostate requires androgenic steroids for development and function. Testosterone is the main circulating androgen and is secreted from the testes as well as the adrenal glands (adrenal

steroid conversion). Once in the blood stream, the majority of the testosterone binds to albumin and sex-hormone-binding globulin (SHBG) while a small fraction is freely dissolved within serum. Within the prostate, testosterone is converted to a derivative, dihydrotestosterone (DHT), by 5-alpha-reductase. DHT is a more potent and active form of testosterone and has a greater affinity for the AR relative to testosterone. Testosterone and DHT bind to the AR and causes its nuclear localization, transcriptional activation and its interaction with co-regulators/co-activators to mediate AR-directed gene transcription [2].

The AR is required for the development of prostate carcinogenesis from early prostate intraepithelial neoplasia (PIN) to organ-confined or locally invasive primary tumors [3]. As a member of the steroid-thyroid-retinoid nuclear receptor superfamily of proteins, the AR is in its inactive form within the cytoplasm, bound to heat shock proteins (HSP) [4-7] and components of the cytoskeleton [7,8], preventing AR nuclear localization and transcriptional activation. The binding of DHT or testosterone causes a conformational change leading to the dissociation of the AR from the HSPs and its subsequent phosphorylation [1, 9]. Once ligand bound, the AR is stabilized within the cytoplasm and translocates to the nucleus. The androgen-AR complex is in a conformational state to now homodimerize within the nucleus and bind to androgen response elements (AREs) in the promoter region of target genes [1] such as prostate specific antigen (PSA), a routine biomarker for prostate cancer diagnosis and progression [7, 10] and, probasin, a prostate-specific gene that has been exploited as a marker of prostate differentiation [11]. The AR has both a cytoplasmic and nuclear distribution, and shows a certain degree of trafficking either to or from the nucleus [12]. There are varying reports on the subcellular distribution of the AR in different cell types; however, this two-step model for steroid hormone receptor activation is a clear representation of ligand activated translocation and the observed focal accumulations of the AR within the nucleus [12].

1.2. AR structure and function

The AR gene is located on the X chromosome (q11-12), and contains eight exons that produce a protein of approximately 920 amino acids [7]. Exon 1 codes for the N-terminal domain (NTD), exons 2 and 3 translate into the central DNA binding domain (DBD) which contains two zinc fingers for specific binding of DNA sequences [1], and exon 4 to 8 code for a hinge region and a conserved C-terminal ligand binding domain (LBD).

The NTD (1-558) is a poorly conserved region that houses important sequence motifs for AR conformation and activity [7]. There are three regions of tri-nucleotide repeats, which include poly-glutamine (Q) and poly-glycine tracts [7, 13]. The poly-Q tract is encoded by a polymorphic CAG repeat [14]. The length of the repeats inversely affects the stability of the AR-NTD and C-terminal LBD interaction, and, AR expression and activity [7, 15, 16]. CAG tri-nucleotide repeats can vary between 11 and 31 repeats; less than 18 repeats are thought be an indicator of CaP risk.

The NTD also contains the transcriptional activation function-1 (AF1) comprising two transcriptional activation units (TAU): TAU-1 and TAU-5. The AF1 subdomain of the AR is the predominant site for transactivation, where TAU-1 is required for ligand-dependent transcription of the AR; TAU-5 is responsible for the majority of the constitutive activity

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associated with the NTD, and the recruitment of the Steroid Receptor Co-activator (SRC)/p160 family of co-activators. For example, TIF2 (Transcriptional Intermediary Factor 2), SRC-1, and GRIP-1 are members of the SRC/p160 family which increase AR transcription through their interactions with the NTD and DBD [4, 5, 7, 17]. These co-activators also recruit other co-regulators such as histone acetyl transferase (HAT) activity containing enzymes such as cAMP response element binding protein (CREB)-binding protein (CBP)/p300 and p300/CBP-associated factor (p/CAF) to initiate chromatin remodeling [7, 18] in preparation for DNA transcription [7, 19].

The LBD folds into 12 helices to form the ligand binding pocket. Interaction of ligands to the LBD promotes AR stability by the formation of the C-terminal transcriptional activation function -2 (AF2) domain and the subsequent interactions between the NTD/LBD [7]. The NTD interacts with the LBD through its sequence motifs ^{23}FQNLF^{27} and ^{433}WHTLF^{437} [5, 7, 20], while co-activators/co-regulators (E.g. SRC/p160 family of co-activators) bind to the LBD by a highly conserved consensus sequence LXXLL (L is Leucine and X is any amino acid) motif (also known as the NR box) [7]. The LBD LXXLL binding region primarily serves to recruit LXXLL motif containing co-activators/co-regulators and structurally enables the NTD FXXLF containing region to interact with the LBD [7]. The LXXLL motifs of such co-regulators form a two-turn amphipathic α-helix which binds to the hydrophobic cleft of the LBD (specifically AF2) [21].

The LBD AF2 domain is comprised of helices 3, 4, 5 and 12 [22]. The ligand binding pocket is formed by helices 3, 5, and 10. Helix 12 is thought to lie across the ligand binding pocket and stabilize the ligand-AR interaction and increase ligand-activated transcription. The AR NTD and C-terminal domain (CTD) interaction in conjunction with Helix 12 serve to stabilize agonist ligand binding and receptor transcriptional activity [23]. Furthermore, the interaction of AR-interacting proteins or co-regulators such as androgen receptor co-activator, ARA70, (which binds to both the AR-DBD and AR-LBD) can increase the receptivity of the AR-LBD to other activating ligands such as hydroxyflutamide (non-steroidal anti-androgen) and estrogens [7, 24-26]. However, it was shown that the AR NTD and CTD interaction was not absolutely required for transcriptional activity. For example, ligands used at high concentrations and peptides that blocked the NTD and CTD interaction did not absolutely inhibit transcriptional activity of the AR [23, 27, 28].

The AR is opposed by co-repressors which inhibit its transcriptional activation. Nuclear receptor co-repressor (NCor) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) disrupts the NTD-LBD interaction and the binding of SRC/p160 co-activators [7]. NCor and SMRT are able to recruit histone deacetylases (HDAC) to promote the repackaging of DNA and prevent the binding of transcriptional machinery, activators, and receptors [7, 29]. However, NCor requires the presence of a ligand (agonist or antagonist) whereas SMRT is able to mediate its effects in the presence or absence of ligands [7, 29-31]. The LBD also houses the nuclear export signal (NES) (amino acids 742-817) and the nuclear localization sequence (NLS), found at the junction between the hinge region and DBD (50 amino acids, 625-676) [7]. Upon ligand binding the NES becomes inactive and the NLS is bound by co-activators such as Filamin-A and importin-α. These interactions direct the nuclear localization of the AR [6, 7, 26, 31, 33, 34, 35]. Upon the loss of ligand interactions, the NES co-ordinates the shuttling of

the AR to the cytoplasm where AR can tether to cytoskeletal proteins to again prepare for ligand binding [5,7].

The DBD (559-624) is comprised of two zinc fingers domains created by three α-helices and a 12 amino acid C-terminal extension [1]. The first zinc finger contains a P-Box motif for specific nucleotide interactions and the second, a D-Box motif which functions as a DBD/DBD site for receptor homodimerization [7]. It is thought that Lysine (Lys;K) 580 and Arginine (Arg;R) 585 in the first zinc finger bind respectively to the second and fifth nucleotide pairs in the first ARE repeat: GGTACA [22, 36-39]. The second zinc finger stabilizes the binding complex by making hydrophobic interactions with the first zinc finger and contributes to the specificity of receptor DNA binding [22, 39]. Due to the similarity of the hormone response elements (HREs) of the nuclear receptor family, there is an overlap of nucleic acid sequences in which these receptors can bind. Steroid receptors recognize a palindromic sequence spaced by three nucleotides [40]. The AR, glucocorticoid, mineralcorticoid and progesterone receptors recognize the 5′-TGTTCT-3′ core sequence [40]. However, it has been found that ARs can also recognize specific AREs that consist of two hexameric half-sites separated by 3 base pairs [41-45]. Although ligand specificity brings about hormone specific responses, the specificity of hormone receptors has been questioned as each receptor can bind to similar or the same sequence [45]. It is thought that protein-protein interactions play a role in discriminating AR and other steroid mediated effects [46, 47] to enable ARE dependent gene transcription rather than the activation of other HREs.

1.3. AR and post translational modifications

Despite the AR's role in genomic upregulation of androgen dependent gene transcription, its activation can signal through alternative means at the plasma membrane and cytoplasm (referred to as non-genomic signaling) [1]. For example, the AR can trigger intracellular calcium release and the activation of protein kinases such as the Mitogen Activated Protein Kinases (MAPK), Protein Kinase A (PKA), AKT and PKC [7]. Phosphorylation of the AR by MAPK, JNK, AKT, ERK, p38, increases AR response to low level of androgens, estrogens, and anti-androgens as well as enhances the recruitment of co-activators [7]. Furthermore, the AR itself is a downstream substrate for phosphorylation by receptor-tyrosine kinases and G-protein coupled receptor signaling. The phosphorylation of AR is mediated by the recruitment of kinases in the presence or absence of androgens. Phosphorylation at Serine (Ser) residues, Ser80, Ser93, and Ser641 is thought to protect the AR from proteolytic degradation [7, 48]. Alternatively, AR degradation is regulated by the phosphorylation of specific residues recognized by E3 ubiquitin ligase. For example, MDM2 E3 ubiquitin ligase promotes polyubiquitylation of the AR by recognizing AKT dependent phosphorylated serine [3,49]. Moreover, transactivation of the AR largely relies upon the phosphorylation of Ser213, Ser506, and Ser650 [7]. Phosphorylation of the AR is required for its effects within the nucleus and the AR should remain hyperphosphorylated to mediate its transcriptional role [3]. Studies have also shown constitutive phosphorylation of the AR at Ser94 as well as on other serine residues such as Ser16, 81, 256, 309, and 424. The loss of phosphorylation results in the loss of transcriptional activity and nuclear localization [3, 50-52]. Specifically, Yang et al., (2005) demonstrated that

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dephosphorylation of AR at the NTD by protein phosphatase 2A (PP2A), resulted in the loss of AR activity.

The AR receptors can also be acetylated, and sumoylated. These types of post translational modifications have also been shown to affect receptor stability and activity. The KXKK motif of the hinge region is a site for acetylation. Mutations of lysine to alanine reduced the transcriptional activity of AR by favoring NCoR interactions [3, 53]. Sumoylation of the AR is hormone dependent and competes with ubiquitination of lysine residues. Sumoylation is thought to repress AR activity. Disruption of sumoylation on Lys386 and Lys520 resulted in an increase in AR transactivation [3, 54].

1.4. AR in CaP progression

The efficacy of many CaP treatments is often temporary, as CaP cells often become refractory to hormone ablation therapies. The current therapeutics are largely targeted towards the inhibition of AR activation, such as anti-androgens, chemical castration (treatment with gonadotropin releasing hormone (GnRH) super agonists to inhibit testosterone secretion from the testes), or surgery (orchidectomy) [7]. AI-CaP or castration resistant CaP is thought to occur due to the androgen deprivation therapies as they may induce altered protein activity and expression in the cancer cells. Despite androgen blockade in AI-CaP patients, expressions of AR target genes such as PSA remain high. Furthermore, hormone refractory CaP continues to rely on AR expression, suggesting that the AR is necessary to maintain proliferative and anti-apoptotic effects. Therefore, CaP acquires the phenotype of oncogenic addiction to the AR for its continued growth and resistance to therapy. The progression of CaP from an hormone sensitive AD to a hormone resistant AI state is likely due to mechanisms involving alterations in AR expression, amplification, mutations, and/or AR activity.

AR mutations in primary CaP are relatively low when compared to metastatic CaP where frequencies are as high as 50% [1, 55-57]. Germline or somatic mutations of the AR leads to AR overexpression and hypersensitivity due to point mutations and promiscuous mutant AR proteins. Germline mutations of the AR are rarely found. Familial inheritance of CaP with at least two first degree relatives account for 20% of cases and transmission compatible with Mendellian inheritance is described to be 50% of the cases observed [3]. Genetic susceptibility seems to be more significant in patients <55 years old [3]. Recently, a R726L mutation was reported in only Finnish patients with sporadic or familial CaP [3, 58, 59]. Genomic alterations to the AR have been found in both non-coding and coding sequences such as polymorphisms of CAG and GGC repeats, single nucleotide polymorphisms, as well as silent and missense mutations [3, 58, 60, 61]. Koochekpour et al., (2010) screened 60 CaP patients of African-American and Caucasian families with a history of familial CaP. Using exon-specific PCR, bi-directional sequencing and restriction enzyme genotyping, they found that one African-American family had a novel germline AR misssense mutation (exon 2 of DBD A1675T; T559S) in three siblings with early onset CaP. This mutation was transmitted in an X-linked pattern and located at the N-terminal region of the DBD. Koochekpour et al., (2010) reason that the location of this particular mutation likely affected AR ligand binding.

Somatic mutations are largely single base substitutions: 49% at the LBD, 37% at the NTD, and 7% at the DBD [3]. For those CaP that harbor gain of function mutations the result is primarily an increase in ligand promiscuity. The AR is activated by testosterone and DHT; however, mutations in the LBD make the AR less stringent of its partners. For example, in LNCaP cells, a Threonine (Thr; T) to Alanine (Ala;A) mutation (T877A) caused the expansion of ligand binding activity [1, 8]. This mutation permitted AR activation by androgens, estrogens, progesterones as well as the non-steroidal antagonist, flutamide. A study by Gaddipati et al., (1994) found that 25% of patient metastatic tumors had a T877A mutation. Patients that were treated with flutamide often experienced a worsening of symptoms over time. Once flutamide was withdrawn, patients tended to do better. Interestingly, some patients also experienced a rise in serum PSA levels upon flutamide treatment. Taplin et al., (1999) studied patients that were on flutamide treatment relative to those that were not given this particular treatment. Tumor cells that had the T877A mutation increased in proliferation while patients who were not treated with flutamide harboured different mutations of the AR that were not activated by flutamide. Therefore, there seems to be a strong selective pressure for AR mutants arising from flutamide treatment such that discontinuation of flutamide resulted in tumor regression before growth resumed again. Other mutations such as the H874Y (Histadine to Tyrosine) mutation in the CWR22 cell line have been found to affect co-activator interactions by altering the conformation of Helix 12 of the LBD. Helix 12 regulates co-activator binding and creates a specific groove with helices 3, 4, and 5 [63-67]. Helix 12 rotates over the ligand binding pocket and assumes favorable or unfavorable positions depending on agonist or antagonist binding, respectively. Helix 12 mutations have also been detected in CaP patients, such as Q902R (Glutamine to Arginine), and M894D (Methionine to Aspartic Acid) (an androgen insensitive mutation) [56, 67, 68]. The importance of Helix 12 and the NTD-LBD interaction for AR activity is underscored by the fact that spontaneous mutations in Helix 12, NTD, and LBD caused either complete or partial androgen insensitivity [67, 68]. Additionally, a L701H mutation was also identified in conjunction with the T877A mutation in MDA CaP 2a cell lines [1, 69]. L701H mutation alone decreased the ability of AR to bind DHT, but increased binding of other non specific adrenal corticosteroids. The presence of the T877A mutation together with L701H potentiated this interaction by more than 300% as both mutations were located within the LBD [1, 70]. Hence, the susceptibility of the AR to minimize its ligand specificity in AI-CaP makes AR dependent disease progression difficult to treat. On the other hand, other anti-androgens such as Casodex (bicalutamide) do not seem to have the same response to T877A AR [1]. Novel truncated AR mutant, mRNA splice variants and mutant AR lacking exon 3 (coding for C-terminal portion of the DBD) tandem duplication have also been found in the 22RV1 cell line (AI-CaP), derived from the CWR22R cell line [3, 71]. Furthermore, an important study by Han et al., (2001) demonstrated that prostate tumors from a genetically engineered mouse model upon androgen ablation resulted in AR gene mutations within AR NTD. Specifically, amino acid substitution A229T and E231G (Glutamic Acid to Glycine) within the AR NTD signature motif: ARNSM (Ala-Arg-Asn-Ser-Met), increased ligand independent basal activity, whereas, E231G increased responsiveness to androgen receptor co-activator ARA160 and ARA70. The ARNSM motif is unique to the AR and the most highly conserved region of the AR NTD.

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Another possible mechanism for the progression of AI disease is mediated by AR amplification. Overexpression of the AR causes hypersensitivity of the AR under low levels of androgens. Visakorpi et al., (1995) were the first to show that the AR was amplified in 305 hormone refractory tumors subsequent to androgen ablation therapy. Although these tumors were clinically presenting as AI-CaP, there was increased levels of the AR, and, continued proliferation of the tumor still required androgen. This suggested that some AR amplified tumors may require the presence of residual androgens that remain in the serum after monotherapy [1, 74]. Similarily, mouse models of CaP progression characterized by high expression of AR, increased AR stability, and AR nuclear localization, had hypersensitive tumor growth promoting effects upon DHT administration. DHT concentrations of 4 orders of magnitude lower were able to stimulate growth relative to DHT levels required for AD LnCaP cell proliferation [1, 75].

Although AR gene amplification and hypersensitivity serves to be a sound model for AI-CaP progression, the AR may be activated by alternative means including activation by co-regulators, increased androgen production, and/or intermediary downstream signaling pathways. Greater levels of co-activator expression such as SRC-1, ARA70, and TIF2 were demonstrated to be elevated in CaP and correlated with increased CaP grade, stage, and decreased disease free survival. For example, Cdk-activating phophatase B, an identified co-activator of the AR was overexpressed and also highly amplified in tumors with high Gleason scores [3]. Local production of androgens within the prostate can also increase AR transactivation by compensating for decreased serum testosterone resulting from androgen ablation therapy. Studies have shown that serum testosterone levels can decrease 95%, contrasting the DHT levels within prostate tissue which only reduce by 60% [1, 76]. Locke et al., (2008) demonstrated that there was de novo and organ synthesis of androgens in LNCaP xenograft mouse models, suggesting that CaP cells had steroidogenic properties that enable them to survive in androgen depleted environments. Moreover, this was also indicative of greater levels of intratumoral 5-alpha-reductase activity. It is likely then, that during AI-CaP disease progression, there is a switch in androgen source whereby testicular androgens are replaced by prostatic androgen. Bennett et al., (2010) have deemed this as 'androgen self-sufficient'. There is also a hypothesis that conversion of adrenal steroids can sustain the androgen signal by supplying adrenal androgens such as DHEA and androstenedione [78]. After castration, adrenal androgens could account for as much as 40% of the total DHT in the prostate [76, 78].

Hormone receptors that are activated by ligand independent mechanisms are known as 'outlaw' receptors [1]. Certain growth factors such as Insulin Growth Factor (IGF)-1, Keratinocyte Growth Factor (KGF), and Epidermal Growth Factor (EGF) have been demonstrated to activate AR and induce the expression of AR target genes. Culig et al., (1994) showed that there was a 5-fold increase in PSA levels in LNCaP cells upon IGF-1 stimulation. Moreover, the addition of Casodex abolished the activation of the AR by IGF-1, KGF and EGF, indicating that the LBD was necessary for this activation. Overexpression of these growth factors has been observed in CaP; however, it is unclear whether it is the AR pathway or indirect downstream effects that are mediating tumorigenesis. In fact, patients with AI-CaP can fail Casodex therapy suggesting that other mechanisms are in play for ligand independent activation of the AR.

Furthermore, patients who received androgen ablation therapy have tumor cells that overexpress growth factor receptors, the receptor tyrosine kinases. Craft et al., (1999) demonstrated that an AI-CaP cell line, generated from xenografts implanted in castrated mice, consistently overexpressed Her-2/neu (from the EGF receptor family of receptor tyrosine kinases) [1]. Interestingly, AD-CaP cell lines could also be converted to AI-CaP cells by overexpressing Her-2/neu. This pathway was not blocked by Casodex, which indicated that the LBD of the AR was not necessary to transduce the effects of Her-2/neu. Although Trastuzumab (Herceptin) is used primarily to treat breast cancer, Herceptin had anti-proliferative effects on AD- and AI-CaP xenografts when combined with the chemotherapeutic drug paclitaxel. Yeh et al., (1999) believe that Her-2/neu activated AR via the MAPK pathway, as inhibitors of MAPK decreased HER-2/neu mediated activation of the AR. In effect, a positive feedback loop is created where the AR can activate kinases and in turn, where kinases can activate the AR through its phosphorylation (in the presence or absence of ligand), regardless of the varying levels of androgens [1].

The AR pathway is thought to be in interplay with other signaling pathways. AR activation due to cross regulation by receptor tyrosine kinases and their downstream effectors provides alternative and sustained routes for AR activation despite androgen depletion. Currently, there has been accumulating evidence that the phosphatidylinositol 3-kinase (PI3K)-AKT pathway plays a significant role in CaP tumor progression. The cross-regulatory mechanism by which the PI3K/Akt pathway modulates the expression and activity of AR is a novel area of study. Growing evidence continues to support the increased role of the PI3K/Akt and AR signaling pathways in mediating the progression of CaP to castrate resistant disease.

2. Phosphatidylinositol 3-kinase (PI3K)-AKT pathway: A brief overview

Evidence has largely supported the phosphatidylinositol 3-kinase (PI3K)-AKT signalling pathway as a key regulatory system essential to mammalian cell proliferation, survival, and metabolism. The gain- or loss-of-function of components of this pathway lead to neoplastic transformation in a wide spectrum of human cancers, including CaP. Briefly, the canonical PI3K/AKT pathway is activated by mitogenic growth factor stimulation of receptor tyrosine kinases (RTKs), the most common RTKs include Epidermal Growth Factor Receptor (EGFR, ERBB1), Her2 (EGFR-2, ERBB2), KIT, PDGFRα, and MET. Receptor activation causes RTKs to dimerize and undergo autophosphorylation at tyrosine residues and enables interaction with Src Homology 2 (SH2) domain-containing molecules. The signal then becomes transduced, through the oncogene, RAS, and ultimately leads to the conversion of membrane phosphatidylinositol-bis-phosphate ($PI(3,4)P_2$; PIP_2) to phosphatidylinositol-tri-phosphate ($PI(3,4,5)P_3$; PIP_3) by PI3K. The presence of PIP_3 mediates the recruitment of AKT (also known as PKB) to the plasma membrane and its subsequent phosphorylation by 3-Phosphoinositide- Dependent protein Kinase (PDK) 1 and PDK 2 at Threonine 308 (T308) and Serine 473 (S473), respectively. Activated AKT or phosphorylated AKT (P-AKT) is the central effector of many downstream signaling pathways regulating protein synthesis, cell cycle, cell death, cell growth, and cell survival [summarized in Reference 82]. The loss and/or mutation of the tumor suppressor

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protein and negative regulator of the PI3K/AKT pathway, Phosphatase and Tensin homolog deleted on chromosome TEN (PTEN), is a common event in various cancers, causing the constitutive activation of PI3K/Akt signalling. PTEN, a dual protein and lipid phosphatase, dephosphorylates PIP_3 to PIP_2, hence, buffering the proliferative and transformative effects of the PI3K. This review will primarily focus on the most studied canonical PI3K/AKT pathway.

2.1. Phosphatidylinositol 3-kinases

The PI3Ks are enzymes that are grouped into three classes (I-III). Most members of this family are bound to regulatory subunits which determine its specificty and function [83-85]. Class I PI3Ks are subdivided into IA and IB and are members to the canonical PI3K/AKT pathway. They are heterodimeric serine and threonine kinases comprising a catalytic subunit, p110, and a regulatory subunit encoded by the PIK3CA and PIK3R1 genes, respectively [83]. The four isoforms of p110 (α-δ) and their regulatory subunits have distinct structure-function domains and specificity. For p110α, β, and δ the most commonly associated regulatory subunit has been identified as p85 [83]. Specific isoforms of the p85 adaptor subunit (p85α, p85β, p50α, p55α, or p55γ) facilitate the interaction with RTKs as well as the p110 catalytic domain isoforms [86]. The p85 subunit directly associates with active RTKs through the physical interaction of its SRC homology 2 (SH2) domain at phosphotyrosine residues of RTKs [87]. The consensus sequence has been identified to be YXXM [87]. In particular, Class IA PI3Ks' p85α subunit encodes an adaptor-like protein that has two SH2 domains and an inter-SH2 domain that binds constitutively to the p110 catalytic subunit [87]. The two splice variants (p55α and p50α) retain such regions but lack an amino terminal SH3 domain (mediates the binding of proline rich sequences) and a breakpoint cluster region (BCR) homology domain (a protein-protein interaction motif) [87]. The p110 isoforms (α, β, δ) have the same basic structure, including a kinase domain and a C2 domain for membrane anchoring [87].

Class I PI3Ks, once activated by RTKs (Class IA) or G-protein-coupled receptors (GPCRs, [Class IB]), have preferred substrates, in particular, the non-phosphorylated phosphatidylinositol (PI), inositol monophosphate (PI(4)P), and phosphatidyl-bis-phosphate ($PI(4,5)P_2$), and mediate the addition of a phosphate group in the D-3 position of the inositol ring to generate PI(3)P, PIP_2, and PIP_3, respectively [83, 88]. PIP_3 is a potent second messenger in the cell and the predominant arbitrator of PI3K signalling. Class IA PI3K p110α domain isoform is the most mutated amongst cancers, causing the kinase to be more active [86, 89, 90] and perpetuating a constitutively active PI3K pathway. Class II and III PI3Ks, on the other hand, are less studied and are recognized for their involvement in membrane trafficking and receptor internalization, and, vesicle trafficking, respectively [91-93]. PI3Ks within Class II generate $PI(3,4)P_2$ from PI(3)P and can also produce PI(3)P from PI. However, they cannot recognize $PIP(4,5)P_2$ as substrate to produce PIP and PIP_2. Class II PI3Ks use only PI to convert it to PIP [83, 94]. Furthermore, unlike Class I PI3Ks, Class II PI3Ks do not require a regulatory subunit but comprise three distinct isoforms to mediate their functions. Class IA PI3K will be discussed in this review and will be referred to as PI3K unless otherwise stated.

PI3K activity is normally strictly regulated within the cell by growth factor-receptor interactions [95]. As such, the majority of the PI3K is inactive in the cytosplasm and remains removed

from its plasma membrane substrates. Moreover, only a small fraction of these PI3Ks become activated upon growth factor stimulation [95]. Currently, it is thought that there are pre-formed inactive p85-p110 complex present in the cytoplasm, whereby ligand mediated activation of kinase activity and transphosphorylation of RTK's cytoplasmic tail recruits p85-p110 complexes to the receptor by the SH2 domain of p85 [87]. This brings PI3K in close proximity to its lipid substrates; moreover, it is reasoned that the RTK-p85 interaction may remove an inhibitory effect of p85 on p110 kinase activity [87, 96]. This is thought to involve conformational changes in the p85-p110 complex through the SH3 and BCR domains.

Mutations have now been identified in the genes coding for the p110 and p85 subunits which have shed light on the pathology of metabolic diseases and cancer [83, 93]. These mutations occur at a frequency of 5-25% in common cancers such as breast, endometrium and large intestine [83]. Activating mutations or 'hot spots' of PIK3CA occur at a frequency of 80% and are located in the PI3K catalytic kinase domain, H1074 and the helical domain, E542 and E545 [93]. Both mutations have been demonstrated to drive transformation *in vitro* [93, 97]. As a result, the lipid kinase activity is increased [83, 89, 98-102], downstream signalling no long requires upstream growth factor stimulation, and increased oncogenic potency. Expression of these hot spot mutants induced oncogenic transformation in avian and mammalian cell culture and transgenic expression of H1047R p110α in mouse models induced adenocarcinoma of the lung [83, 103]. As such, hot spot mutations then can be suggested to function as drivers of cellular transformation to a more oncogenic phenotype. Conversely, mutational inactivation of the ability of p110α to interact with RAS has the opposite effect by decreasing the oncogenicity of helical domain mutants and minimizing downstream signaling [83]. On the other hand, kinase domain mutants become independent of RAS binding, and its oncogenicity is preserved [83]. PIK3R1 mutations occur within a stretch of six residues (560-565) located in the inter-SH2 domain of p85 [83]. This area is the contact point for p85 with the C2 domain of p110α whereby mutation leads to improper binding to p110α and relieve the inhibitory interaction of p85 [83]. Enhanced AKT signalling, stimulation of cell replication, and oncogenic transformation were some of the observed effects [83, 104, 105]. As such, p85 mutations in the inter-SH2 domain can be thought to be equivalent to activation mutations of the p110α C2 domain.

2.2. Phosphatase and tensin homolog deleted on chromosome TEN (PTEN)

The tumor suppressor, PTEN, is a dual phosphatase that has activity for both lipid and protein substrates. It is a gene that is lost in both heritable and spontaneous cancers where germline mutations cause autosomal dominant hamartoma tumor syndromes and where spontaneous missense mutations occur frequently in the central nervous system (20%), endometrial (39%), colorectal (9%), skin (17%), prostate (14%), and breast (6%) cancers [95]. Its role within the PI3K pathway serves to negatively regulate PI3K signalling. PTEN functions to remove phosphates in position 3' from phosphoinositides [93, 106, 107], therefore, returning PIP_3 to PIP_2 and terminating the PI3K signal. Monoallelic loss (loss of heterozygosity) and/or mutation of PTEN thus, leads to a hyperactive PI3K pathway to drastically impact tumor growth and disease severity. PTEN mutants that retain protein tyrosine phosphatase activity but lose the ability

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to dephosphorylate PIP_3 are found in many tumours indicating that PTEN lipid phosphatase activity is required for tumour suppression.

PTEN is tightly regulated at the transcriptional level as well as by post translational modification, primarily through ubiquitylation. Incidentally, the levels of PTEN are controlled by PI3K itself, through the regulation of the transcription factor NF-κB, while, PPARβ/δ agonists and TNFα repress PTEN expression [93, 108]. Furthermore, the activity of PTEN is also controlled by the PI3K pathway. In p85 conditional knockout mice, the loss of p85 resulted in PTEN activity, while loss of p110δ isoform regulated PTEN activity through a RhoA-ROCK-depedent signaling [93, 109]. Currently, NEDD4-1 is the first and only identified E3 ligase for PTEN [93, 110]. Similar to PTEN, NEDD4-1 is also regulated by the PI3K pathway, thus representing a positive feedback for PTEN degradation and PI3K activation [93, 111]. More often than not, heterozygous alterations of PTEN are most common in the initial steps of tumorigenesis. Surprisingly, complete PTEN deletion does not have pro-tumorigenic effect. For example actute PTEN loss within prostate cells leads to a strong p53 dependent senescence response that opposes cancer progression. Hence, it can be suggested that tumors may not select for a complete loss of function of PTEN during the initial states of tumorigenesis. For example, in CaP patients, approximately 70% of tumors have heterozygous alteration in PTEN at presentaiton and then lose the other allele at later stages [93].

The co-existence of both PIK3CA mutations and PTEN loss has been observed in various cancers. This suggests that these two genetic aberrations are not completely redundant and may have additional selective advantage [95]. Yuan and Cantley, (2008) postulate that PTEN and p110α exist in a negative feedback loop to regulate pathway activity, such that any alterations to these enzymes results in heightened oncogenic potency of the PI3K pathway.

2.3. AKT/PKB

The formation of PIP_3 is the central initiating event which functions to recruit plekstrin homology (PH) domain containing proteins to the plasma membrane. Of relevance here, is AKT/PKB, as it is the critical mediator of signal tranduction events downstream the PI3K cascade. There are three members of the AKT family (AKT1, AKT2, and AKT3) and they are broadly expressed to have some isoform specific features [87]. AKT1 is the major isoform implicated in cancers, whereas AKT2 is more so involved in insulin signaling and glucose transport. AKT3 on the other hand has well known features and functions, however is thought to play a specific role in brain tissue [86, 112].

The AKT gene encodes a serine/threonine kinase with an amino-terminal PH domain, a central catalytic domain, and a carboxyl-terminal regulatory domain. The regulation of AKT function is two- fold, requiring its translocation to the plasma membrane and its sequential phosphorylation at Threonine 308 (T308) and Serine 473 (S473). Within unstimulated cells, AKT is constitutively phosphorylated at S124 and T450. Upon PIP_3 formation, there is direct interaction of AKT to PIP_3 via is PH domain. Here, PDK1 phosphorylates AKT on T308. The phosphorylation of T308 is a priming event to mediate the phosphorylation of S473 by PDK2, now thought to be the mammalian target of rapamycin complex 2 (mTORC2). This secondary event is necessary for maximal activation of the kinase, increasing AKT activity 10-fold [86, 113,

114]. Once activated AKT has many substrates within the cytoplasm and nucleus, including those that regulate apoptosis, proliferation, and protein translation. Although the activation of AKT has been well established, there is little known regarding the dephosphorylation of AKT as no AKT specific phosphatase has been identified. However heat-shock protein 90 (HSP90) has been demonstrated to protect AKT from dephosphorylation by the ubiquitous phosphatase, PP2A.

The activation of AKT regulates many cellular processes including cell proliferation and survival, cell size and glucose homeostasis, metabolism, angiogenesis, and tissue invasion [86, 93]. Amplification and mutations of AKT have been reported for pancreas, ovarian, head and neck and breast cancers. This includes a recently identified missense mutation to the PH domain of AKT1 (E17K) [95]. Such a mutation resulted in constitutive association of AKT with the plasma membrane and its prolonged activation. The biological effects of AKT activation relevant to cancer is primarily associated with cell survival, proliferation and growth. First, AKT functions as an anti-apoptotic response to various stimuli. This is through a series of phosphorylation and inhibition events of key pro-apoptotic proteins including, BAD, MDM2 and members of the Forkhead family of proteins.

BAD is a member Bcl-2 family of pro-apoptotic protein where these members form non-function hetero-dimer complexes with the survival factor BCL-X_L [87]. Once AKT phosphorylates BAD on S136, it prevents the interaction of BAD with BLC-X_L to restore the anti-apoptotic function of BCL-X_L [86, 115]. AKT also phosphorylates the pro-death enzyme, caspase 9, and inhibits its catalytic activity; this is in addition to preventing the nuclear localization of the Forkhead family of transcription factor, FKHR which transcriptionally inhibits the expression of pro-apoptotic proteins, BIM and FAS ligand. Alternatively, an indirect mechanism of AKT regulation of apoptosis is mediated by the NF-κB pathway and p53. Specifically, phosphorylation of and hence, the activation of IκB kinase (IκK) results in the degradation of NF-κB inhibitor, IκB, causing the nuclear translocation of NF-κB and the expression of anti-apoptotic genes. The pro-apoptotic effects of p53 tumour suppressor protein are mediated by AKT phosphorylation of the p53 binding protein MDM2. MDM2 is a negative regulator of p53 function as it targets p53 for ubiquitin mediated proteosomal degradation through its E3 ubiquitin ligase activity. The phosphorylation of MDM2 increases the efficiency by which MDM2 translocates to the nucleus thereby enhancing p53 degradation.

The proliferative effects of AKT activation can be attributed to its role by inactivating the cell cycle inhibitor p27 and p21, and, by inhibiting the enzyme, glycogen synthase kinase (GSK) 3β at its Serine 9 phosphoryaltion site. The regulation of cell cycle progression is through cyclin-cyclin-dependent kinase (CDK) complexes and CDK inhibitors (CKI). p27and p21 are CKIs that become phosphorylated by AKT and through indirect mechanisms, AKT phosphorylation can modulate the expression of CKIs as well as their activities. Phosphorylation of p27 renders it inactive and promotes cell cycle entry. Additionally, phosphorylation of the transcription factor, FOXO3A, by AKT causes the nuclear expulsion of the transcription factor, and therefore decreases the expression of p27 [116]. Alongside CKIs, cyclin D1 levels are important for G1/S phase transition through the cell cycle. AKT has an important role in preventing cyclin D1 degradation by inhibiting the cyclin D1 kinase, GSK3β. This prevents the phosphorylation

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of cyclin D1 thereby increasing its levels to enable cell cycle progression. Interestingly, cyclin D1 expression is also tightly controlled by FOXO3A. Upon AKT phosphorylation of FOXO, its exclusion from the nucleus increases cyclin D1 expression. In effect, FOXO3A is considered a transcriptional repressor for this gene.

The significance of AKT in cancer progression is further heightened by its role in cell growth and metabolism. In highly proliferating tumor cells, there is rapid synthesis of macromolecules to meet the biosynthetic demands required by the cell. Incidently, AKT is one of the main regulators of protein translation and ribosome biogenesis [93], facilitating the means for cell growth. This is primarily achieved through the serine/threonine kinase, mammalian target of rapamycin (mTOR or FRAP1) Complex 1, which is composed of the protein kinase mTOR and a series of interactors. This complex serves as a molecular sensor of nutrient availability and in effect, modulates protein synthesis. It is unlikely that the PI3K/AKT pathway is the sole simulator of mTOR activity. Nonetheless, AKT's phosphorylation of two independent substrates of this complex contributes to the oncogenic phenotype. Specifically, AKT phosphorylates and inactivates the GTPase-activating protein (GAP) Tuberous Sclerosis Complex (TSC) 2 which forms a complex with TSC1 to inhibit the GTPase, Ras-homolog enriched in brain (Rheb). Rheb then directly interacts with mTOR and activates mTORC1 through the inhibition of FKBP38, the negative regulator of mTORC1. Alternatively, the phosphorylation and inhibition of another negative regulator of mTORC1, PRAS40 (proline-rich AKT substrate of 40kDa), enhances the activity of mTORC1 through its competition with GTPase Rheb. Altogether then, AKT promotes the activation of mTORC1 which initiates the translational machinery to produce ribosomes and increase the rate of protein synthesis. REFS

2.4. Mammalian Target of Rapamycin (mTOR)

mTOR is a member of two distinct complexes, mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) [83, 117]. It is thought that the mTORC1 complex plays a more dominant role in tumor progression while the mTORC2 complex is more significant to mediating signals to the cytoskeleton [116] and now identified as the factor responsible for the phosphorylation of AKT at S473. This phosphorylation event maximizes the activity of AKT and opens its targets to include PRAS40 and FOXO [83]. Moreover, it is another mechanism through which to provide positive feedback on the PI3K pathway [86,113].

The mTORC1 pathway is a central point of signal integration for growth factor signalling, energy state (AMP levels), and, nutrient and oxygen availability [118] which are fundamental for regulating tumor cell growth. The particular interest for this pathway has been largely determined by the discovery of the specific inhibitor, rapamycin, which blocks mTORC1 activity through yet unknown mechanisms. mTORC1 is comprised of Regulatory Associated Protein of TOR (RAPTOR), LST8 and PRAS40 [83]. The complex itself has many substrates, which upon its activation phosphorylates and activates S6 kinases (S6K) 1 and 2 (activation of protein translation and ribosome biogenesis), as well as inactivates 4E binding protein (4EBP) 1, 4EBP2, 4EBP3, which releases the inhibition of eukaryotic intiation factor 4E (eIF4E). mTOR dependent protein synthesis affects 5' untranslated polypyrimidine tracts of complex secondary mRNA structures. Such mRNA structures require eIF4A helicase activity together with

eukaryotic initiation factors eIF4E and eIF4G to from the EIF4F initation complex. Altogether, the main effect is to upregulated protein synthesis.

Although the PI3K/AKT pathway serves to activate the mTORC1 pathway, mTORC1 itself negatively regulated the PI3K pathway. Over the years, studies have shown that mTORC1 inhibition can lead to PI3K activation. Moreover, mTOR activity can be suppressed by PI3K inhibitors such as wortmannin and LY294002 [87]. However, it is unclear whether the mechanism of activation of mTOR by AKT can completely drive tumorigenesis. As rapamycin can inhibit AKT dependent cancers, it is presumed that in some part mTORC1 does have tumorigenic effects. Although there is correlation between increased translation and tumorigeneisis, whether this is sufficient for increased cancer susceptibility is yet to be determined.

3. The PI3K/Akt signaling pathway and prostate cancer

In the recent years, emerging evidence has strongly linked the deregulation of the PI3K pathway to prostate carcinogenesis and castrate resistance, although its precise role remains elusive. Two components of this pathway, PTEN and PI3K, are currently the focus of intense investigation and this section aims to address their role in the pathology of prostate cancer.

3.1. The incidence of genetic *PTEN* alteration

The deletions involving the chromosome 10q, which hosts the *PTEN* locus, 10q23, in CaP is a frequently observed phenomenon. Modifications to *PTEN* in various stages of CaP have been characterized to include both homozygous and hemizygous deletions, as well as inactivating mutations. Although the incidence and the modes of these alterations have been inconsistent across studies, the severity of *PTEN* loss seems to correlate with disease progression [119]. Whereas locally confined CaP presents homozygous deletions of *PTEN* ranging from 0% to 15%, the incidence within metastases can increase up to 30%. Likewise, heterozygosity loss occurs in 13% of the locally confined cases and up to 39% in metastatic phenotypes [120]. Further support has comefrom interphase fluorescence in situ hybridization (FISH) analysis of histologic sections, which reported genomic deletion of *PTEN* in 23% of high-grade intraepithelial neoplasia (HGPIN) and 68% of prostate tumors [121]. Recently, Han et al., (2009) demonstrated that *PTEN* deletion occurs in 9% of premalignant prostate, a proportion which increases to 17% in localized CaP and to 54% when metastasized. Functional loss of *PTEN* can also be generated through point mutations, which are seen in upwards of 16% of primary tumor and 20% to 30% in advanced stages [120]. Taken together, these studies suggest the deletion of *PTEN* is likely a late genetic occurrence in CaP progression.

3.1.1. Mechanism of PTEN loss

Although the incidence *PTEN* alterations in CaP have been extensively characterized in the past ten years, the mechanism by which genomic *PTEN* deletions occur remains to be elucidated. The high frequency of large-scale chromosomal events leading to the loss of *PTEN* locus suggests unique features that may enhance DNA rearrangements at 10q23. Yoshimoto et al.,

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(2012) identified recombination hotspots known as segmental duplications (SD) 17 and 18 to be located between *PTEN* and *BMPR1A*. The SDs are typically part of a 1-400 kB genomic region exhibiting over 90% homology [123, 124] responsible for improving the likelihood of constitutional microdeletion events [125]. Utilizing meta-analysis of published prostate cancer genomes to map 10q23 deletion sites and FISH for confirmation, Yoshimoto et al., (2012) demonstrates SD17-SD18 colocalizes with a deletion breakpoint hotspot occurring in 69% of PTEN losses, which suggests SD17 and SD18 facilitate homology-dependent rearrangements of DNA that lead to a *PTEN* deletion breakpoint. The presence of these SDs thus destabilizes the genome, predisposing CaP progenitors to genomic microdeletions that ultimately result in *PTEN* loss. Subsequent attenuated *PTEN* expression has been shown to further diminish genomic stability [126], leading to the acquirement of other chromosomal abnormalities [127]. Cells bearing homozygous *PTEN* deletion would have significant stronger growth advantage and predominate due a constitutively activation of the PI3K pathway. This sequence of events may explain the progressive loss of *PTEN* as prostate turmorigenesis continues.

3.1.2. The clinical and cellular impact of PTEN loss

The functional loss of *PTEN* in CaP has been shown by numerous studies to confer poor clinical prognosis and predict disease progression. Genomic *PTEN* deletions studied through either immunohistochemistry or FISH have been correlated with increased Akt phosphorylation, higher Gleason grade, biochemical relapse, angiogenesis, and larger tumor sizes [123, 128-132]. Specifically, Yoshimoto et al., (2007) demonstrated that haploinsufficiency of *PTEN* is associated with an earlier onset of biochemical relapse after prostatectomy while bi-allelic deletion of *PTEN* is associated with an even shorter time to relapse. Additionally, loss of *PTEN* near the time of prostatectomy correlated strongly with extraprostatic extension and seminal vesicle invasion.

Decreased expression of PTEN profiled by high-density tissue microarray was shown to also increase the risk of tumor recurrence after radical prostatectomy [129]. Similar findings were reached in immunohistochemical evaluation of PTEN expression in CaP glands. Using a nested case-control study, the group Chaux et al., (2012) found patients with reduced PTEN expression was at a higher risk of relapse, independent of identified clinicopathological covariates. Their previous study also linked attenuated PTEN levels to faster onset of metastasis in CaP patients [134]. Additionally, the use of transgenic mouse models have served to recapitulate features of PTEN loss in humans and concomitantly fostered a greater understanding of the PI3K pathway alongside clinical studies. Prostate specific $PTEN^{-/-}$ null knockout mice proceeds linearly from acquiring prostatic intraepithelial neoplasia (PIN) to adenocarcinoma to metastasis, mimicking the disease progression in human CaP [135]. The prostate tumors also exhibited temporary regression following androgen ablation, but eventually proliferated androgen independently. Further, mice with one deactivated *PTEN* allele combined with *p27KIP1* loss exhibit accelerated spontaneous neoplastic transformation and tumorigenesis [136]. These studies of mouse and human prostate cancers combine to emphasize haploinsufficiency of *PTEN* as a key predictor of disease states in prostate cancer.

3.2. The role of PI3K isoforms

The catalytic isoform p110β and its regulatory complex p85*α* have been shown to mediate AR transactivation in the presence of androgens [137]. Overexpression of wild type p110β led to androgen-independent AR transactivation while the overexpression of p110*α* gene showed no effects. Interestingly, short interference RNA (siRNA) disruption of p110β gene in prostate cancer cells abrogated tumor progression *in vivo*. Moreover, clinical analysis of tumor samples linked high p110β and p85*α* expression at the mRNA and protein level to malignant prostate tumors, metastasis and poor differentiation.

Conditional knockout mouse models of p110β have further provided insight into the oncogenic potential of the catalytic subunit. Prostate epithelium remained normal in the absence p110β alone while PTEN loss alone resulted in tumor growth in the anterior lobe by 12 weeks; subsequent ablation of the *p110β* gene rescued PTEN null anterior prostate from tumorigenesis [138]. Increased phosphorylation of Akt on Ser473 was achieved through PTEN loss while additional ablation of *p110β* attenuated Akt activation. These results are not attributable to changes in the p110*α* subunit as minimal changes in tumor growth and Akt phosphorylation were observed upon *p110α* ablation. One study ascribed the differential functions of the p110*α* and p110β catalytic subunits to the distinct pools of PIP3 they generate [139]. The p110*α*, in response to growth factor stimuli, will cause an immediate flux of PIP3 coupled with efficient Akt phosphorylation, whereas p110β will maintain a basal level of PIP3 with minimal effects on Akt phosphorylation. Together with the observation that p110β-specific inhibitors effectively reduce Akt phosphorylation in the absence of *PTEN in vitro* [139], oncogenic transformation of prostate cancer cells upon PTEN loss is likely derived from the p110β-catalyzed pool of PIP3 [138]. These data collectively support distinct functionalities of the p110*α* and p110β catalytic subunits in PI3K/AKT signaling.

Recent studies have also shed light onto the third isoform of PI3K catalytic subunit, p110δ. Tzenaki et al., (2012) reported CaP cells that contain high levels of p110δ activity have dampened PTEN functionality. Treatment with p110δ -specific inhibitor in DU145 cells promoted PTEN activation, reduced Akt phosphorylation and inhibited cell proliferation. In another cell line (22Rv1) with wild-type PTEN and low p110δ expression, measured basal PTEN activity was comparatively higher than that in DU145 cells. Inhibition of p110δ in 22Rv1 likewise did not affect Akt phosphorylation status or cell proliferative abilities. Hence, the development of p110δ -selective inhibitors may hold promise since blocking p110δ activity will also indirectly inhibit other catalytic isoforms through PTEN activation.

4. PI3K/AKT and AR Signalling axis

The role of the PI3K/AKT pathway in CaP cell proliferation, survival and progression from AD to AI disease has been linked to androgen receptor (AR) transcriptional activity, stability and expression. This section of the review will discuss the various modes of crosstalk between the PI3K-Akt and AR axis.

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4.1. PI3K/PTEN and AR

The activation of Akt has provided a mechanistic link between PI3K and AR transactivation. However, other modes of interaction have been shown. AR can directly interact with the p85 regulatory subunit of PI3K. Upon their binding, the AR enhances PI3K enzyme activity to ultimately upregulate Akt phosphorylation [141]. Conversely, EGFR stimulated PI3K activity was decreased in PC-3 cells transfected with wildtype AR relative to AR null PC-3 cell line [141-142]. PI3K activity was further reduced upon R1881 treatment, suggesting AR activation as a negative regulator of PI3K stimulation. The role of androgens within the AR-PI3K axis has also shown their significance in modulating cell proliferation and growth. Multiple reports have demonstrated that androgens enhance PI3K activity and increase downstream Akt phosphorylation. In NIH3T3 fibroblasts, there was rapid activation of the PI3K/AKT pathway upon androgen stimulation. While this required the presence of the AR, AR transactivation was not essential [141, 143]. Low concentration of androgen further stimulated the association of the AR with Src and PI3K, which triggered the cells into S-phase entry [141, 143].

The negative regulator of the PI3K pathway, the PTEN tumor suppressor gene, as discussed previous is frequently inactivated in CaP as well as in CaP cell lines which include PC-3 and LNCaP. Functional loss of PTEN is associated with increased AKT phosphorylation, higher Gleason score, and poor prognosis [120, 144]. The development of conditional mouse models of PTEN -/- has shown that PTEN alone can drive the progression of CaP through invasion, metastasis and AI proliferation [82]. Although there was heightened PI3K activity, there was continued evidence of AR gain-of-function despite reduced steroid ligand levels [45, 82]. For example, prostatic epithelium in PTEN-/- mice was still sensitive to androgen withdrawal. As such, Mullholland et al., (2006) suggest that while the AR remains functional and sensitive to androgens, the PI3K/AKT and AR oncogenic signaling may complement and compensate for one another during the time of androgen ablation therapy. This is supported by studies demonstrating cells lacking PTEN had elevated PI3K/AKT axis activity upon androgen withdrawal [45, 46, 82]. Hence, PTEN loss may allow for epithelium with sufficient PI3K/AKT signaling to maintain cell proliferation and promote AR gain of function [82].

Currently, there is no direct method that supports PTEN loss for promoting AR-specific gene activation. However, CaP xenograft studies have shown that amplification of AR does occur [82, 147] under a PTEN null background, which can be correlated to increased AR stabilization [82]. Li et al, (2001) demonstrated that PTEN itself can negatively regulate AR gene targets such as PSA, reduce the nuclear localization of the AR and promote receptor degradation through caspase 3 or the proteosome [82]. Alternative to AR amplification and stabilization, the loss of PTEN may also contribute to AR activation through the various coregulators of the AR, this includes ARA70 [149] and ARA54 [150]. Such regulation by PTEN would allow for heightened AR response to low androgen concentrations or responsiveness to non-androgen ligands. On the other hand, at low androgen levels, AR expression alone can also stimulate cell proliferation (Denmeade et al., 1996) while PTEN restoration induced apoptosis and growth arrest [151]. However, in the presence of androgens, PTEN expression was sufficient to reduce cell proliferation but not induce apoptotic response [82, 148, 152]. PTEN loss then,

results in an indirect AR gain-of-function phenotype by establishing an environment that may increase AR oncogenicity and CaP metastatic potential.

4.2. AKT and AR cross-talk

The regulation of the AR by Akt through direct and indirect modes of interactions has been demonstrated in literature. These include, but not limited to 1) direct phosphorylation of the AR by AKT, 2) AKT/mTOR dependent regulation of the AR, 3) AR interaction with FOXO family of transcription factors downstream AKT, and 4) AR regulation by AKT via the Wnt/GSK3β/β-catenin cross-talk.

4.2.1. AKT and direct AR phosphorylation

AKT binds to the AR to directly phosphorylate at AR consensus sites, S213 and S791. Upon phosphorylation by AKT, the AR becomes transcriptionally active under physiological androgen concentrations. However, a study Xin et al, (2006) demonstrated that AR phosphorylations at S213 and S791 were not critical for tumor progression, which indicated that AR phosphorylation may not be the sole regulatory event inducing AR transcriptional activity [153, 141]. Alternatively, at high androgen concentrations, AKT can protect CaP cells from apoptosis and suppress AR transcriptional activity by phosphorylation at S210 and S790 [49]. Lin et al, (2002) also demonstrated that AR phosphorylation by AKT resulted in MDM2 mediated ubiquitlyation of the AR, leading to its proteosomal degradation. Taken together, these data indicate AKT-mediated regulation of AR activity is dependent on the external environment.

4.2.2. PI-3K/AKT/mTOR and AR

A potent nutrient and growth/survival pathway kinase, mTOR has been implicated in the progression of several types of cancer. Interestingly, it has been identified as a regulator of AI-CaP growth, but not CaP growth, by Ghosh et al. (2005). Further, it has been implicated in regulating the progression to androgen-independent disease [155-156] alongside AR signaling. There are three key routes through which mTOR could potentially interact with AR signaling: through its kinase activity as mTORC1 or mTORC2, post-transcriptional regulation via regulation of translation factors, or indirectly through a signaling cascade. Several broad studies have implicated a connection between the two molecules. Sircar et al. (2009) showed that AKT activation, mTOR activity and AR nuclear expression concurrently occur in several PTEN-null patient samples. Additionally, Müller et al. (2012) demonstrated that loss of phosphorylation of Ser2448 of mTOR, an inhibitory alteration, resulted in decreased levels of cellular AR in ERG-fusion-positive prostate cancer cells. Kaarbø et al. (2010) also show a reduction of AR by mTOR in LNCaP cells. Furthermore, they demonstrate that mTOR inhibtion increases ligand-dependent AR activity.

Work by Wang et al. (2008) further demonstrated evidence of a connection between mTORC1 signaling and AR by demonstrating an induction of AR activity by rapamycin. The application of rapamycin to LNCaP and C4-2 cells resulted in inhibition of the mTORC1 pathway, but an

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increase in AR transcriptional activity. They further determined that this relationship was through AKT. Inhibition of mTORC1 by rapamycin was shown to activate a parallel AR-mediated survival pathway putatively downstream of mTORC2. This potentially matches results by Müller et al. (2012), who showed that loss of mTOR deactivation resulted in AR reduction, suggesting an inverse relationship between the two molecules. Upon dual inhibition using rapamycin and bicalutamide, apoptosis occurred [159], signifying that there is a parallel compensatory effect for mTORC1 signaling through AR. This relationship was expanded on by Wu et al., (2010) who determined that the relationship between AR and mTORC1 is dependent upon testosterone availability, and generates a self perpetuating cycle that promotes cell survival. In low testosterone conditions, application of bicalutamide repressed mTOR activity, as did siRNA against AR in both low and high testosterone conditions. This was shown to be through AR repression of TSC2, a negative regulator of mTORC1. Conversely, rapamycin treatment induced AR activity, as was previously shown by Wang et al. (2008). Furthermore, in low testosterone conditions, where AR activity is low and thus TSC2 is not repressed, bicalutamide leads to apoptosis [160].

These data support the compensatory AR-mediated growth pathway found by Wang et al. (2008), as the lack of TSC2 repression would inhibit mTOR signaling while bicalutimide inhibits AR, leading to a similar dual inhibition of mTORC1 and AR which Wang et al. (2008) reported to induce apoptosis. The interactions between mTOR and AR have been shown to play a key role in progression to AICaP. The mechanism elucidated by Wu et al. (2010) demonstrated that LNCaP cells can become attuned to low testoterone by mTOR/AR crosstalk. When testosterone is low, AR activity drops, but so then does AR-mediated inhibition of TSC2. This represses mTOR activity, leading to induction of AR activity once more. If this cycle is perpetuated, the cells will become able to self-induce AR signaling, and thus also reestablish mTORC1 activity, leading to the development of an androgen-resistant and highly proliferative tumor. Additionally, LNCaP cells acclimatized to low-testosterone conditions showed resistance to glucose deprivation, another way of repressing mTOR. The mTORC1 repression leads to higher levels of AR transactivity, activating a postulated second survival pathway that can compensate for the canonical PI-3K/AKT/mTOR axis. Thus, inhibition of one pathway would sensitize to inhibition of the other. Only by inhibition of both mTOR and AR was apoptosis achieved in both studies [159, 160]. Squillace et al. (2012) show similar results using bicalutamide and ridaforolimus, an mTOR inhibitor. In their study, they [161] also demonstrate that the combination therapy does not induce apoptosis in healthy RWPE-1 PTEN-expressing prostate cells. This is important, as it indicates that the targeted AR/mTOR system is an aberrancy in the tumour.

AR/mTOR dual inhibition was shown to be key to regulation of CaP progression by Schayowitz et al. (2010), who demonstrated that usage thereof can prolong androgen sensitivity of a tumour. Using mouse models, Schayowitz et al. (2010) showed that combination therapy had significant effect on both xenografted androgen-dependent (LNCaP) and androgen-independent (HP-LNCaP) tumours. In both LNCaP and HP-LNCaP xenografts, treatment with a single inhibitor caused no significant decrease in tumour volume, while combination therapy reduced tumour volume significantly. Bicalutamide or everolimus (an mTOR inhibitor) did

not decrease tumour volume in LNCaP xenografts, while combination treatment offered significant reduction after 15 days [162]. Likewise, in HP-LNCaP xenografts bicalutamide, everolimus or VN124-1 (a novel androgen/AR inhibitor) did not significantly decrease tumour volume, while dual inhibition did. Interestingly, combination everolimus/VN124-1 treatment reduced tumour volume far more than did everolimus/bicalutamide treatment. Everolimus/VN124-1 treatment also resulted in significant decreases in AR, p-mTOR, p-S6K and p-S6 levels as visualized by western blotting [162], signifying an inhibition of the AR/mTOR pathways. Single inhibition often led to increased pathway activation, further evidencing compensatory crosstalk. This study is notable because treatment of xenografts with the combination treatment was not overcome, nor was its efficacy as compared to tumour volume decreased over a 45-day period. This indicates that sensitivity to the dual inhibition was maintained, and compensatory crosstalk did not rescue the xenograft. Suppression of CaP progression to AI disease in this manner was also shown by Friedrichs et al. (2011), albeit through a different treatment. Omega-3 polyunsaturated fatty acids (PUFAs) were shown to inhibit CaP progression through suppression of mTOR and AR signalling. Application of DHA, an omega-3 PUFA, inhibited AKT signaling and decreased cell growth in AI clones of LNCaP. Friedrichs et al. (2011) also observed the effect of omega-3 PUFAs on suppression of CaP progression using an assay that mimics progression to androgen-independent disease with androgen ablation. In the control group, ~35% of the cells initally underwent growth arrest and then recovered, while ~42.5% stayed arrested. In the omega-3 PUFA groups (+DHA, +EPA), a majority of cells stayed arrested, as did cells treated with an AKT inhibitor. Treatment with DHA was also accompanied by suppression of AR and p-mTOR expression, along with downregulation of p-S6 and of p-TSC2, an AKT target. This data taken together with data by Wang et al. (2008), Wu et al. (2010), Schayowitz et al. (2011) and Squillace et al. (2012) suggest that suppression of both mTOR and AR signaling is key to inhibition of CaP progression, and that single inhibition leads to activation of the other in a compensatory mechanism.

Such a mechanism was postulated by Wang et al. (2008) to be downstream of mTORC2. Facompre et al., (2012) report that that mTORC2 is involved in an AR-mediated growth pathway. Addition of 5μM p-XSC, or 1,4-phenylenebis(methylene)selenocyanate, a known AKT and AR inhibitor, was shown by Facompre et al. (2012) to inhibit mTORC2 kinase activity *in vitro,* supporting Wang et al. (2008) in proposing that mTORC2 plays a role in AR-mediated crosstalk. This dual inhibition of AR and mTORC2 could indicate that either p-XSC is an mTORC2 inhibitor and inhibits AR downstream of TORC2, or that AR is upstream of mTORC2 and thus suppression of AR inhibits mTORC2 signaling. Treatment with p-XSC resulted in decreased growth of both androgen-dependent LNCaP and androgen-independent C4-2 CaP cell lines by ~25%, with rapamycin showing similar results [163]. As indicated by several other sources, dual inhibition had a far more marked effect. Addition of 1nM rapamycin in combination with the mTORC2/AR inhibitor p-XSC heavily decreased cell viability by ~50% in LNCaP calls, and by ~60% in C4-2 cells. The dual inhibitor treatment resulted in extremely efficient repression of phosphorylation downstream of both mTOR complexes, further supporting the postulation that an AR survival pathway is related to mTORC2. The role of mTORC2 in AR signaling has been investigated by Fang et al. (2011), who report that an mTORC2-mediated growth pathway is downstream of AR. Treatment of CWR22R3 cells with

DHT led to proteosome-mediated degradation of p27, a protein that induces cell cycle arrest through CDK inhibition. Such an action would contribute to the inhibited growth noted in CaP progression. This degradation was shown to be through mTORC2-mediated phosphorylation of AKT at Ser473, but not Thr308. AKT has previously been shown to be phosphorylated at Thr308 by PDK1 [165], so the modification of AKT in this context seems to be purely mediated by mTORC2 without influence from PDK1 molecules, leading to selective activation of only certain downstream substrates, such as SGK and PKCa. Fang et al. (2011) went on to demonstrate that DHT stimulation of AR is inducing nuclear accumulation of SIN1, a factor required for complexing of mTORC2, which signals for the assembly of mTORC2 and subsequent partial phophorylation of AKT. The actual phosphorylation of p27, required for its degradation, could be mediated by AKT or one of its selectively activated substrates. Growth pathways downstream of AKT, such as the SGK pathway, would also lead to increased viability and proliferation of CaP. Xu et al. (2006) implicated mTOR in a similar manner, showing that AR induces cyclin proteins, especially cyclin D1, D2 and D3. RT-PCR results did not indicate a similar increase at an mRNA level. This was because AR regulation of cyclin D was at a post-transcriptional level, through mTOR. Co-activation of cyclin proteins together with degradation of p27, a CDK inhibitor, could lead to potent activation of a CDK-Cyclin growth pathway.

AR has also been shown to be post-transcriptionally modified by mTOR [167] in an EGF/PI-3K/AKT-dependent manner. This study elucidates the manner in which mTORC1 regulates and rapamycin induces AR. Through modulation of the interactions between translation initiation factor eIF4E and scaffolding protein eIF4G, mTOR putatively regulates the rate of translation of AR. Thus, rapamycin inhibition of mTORC1 would lead to an increased rate of translation of AR, leading to increased expression and revitalization of mTORC1 signaling. This combined pathway contains a failsafe in the form of the mTOR-mediated repression of AR and the AR regulation of TSC2, leading to cyclic and self-perpetuating support of two growth pathways. This pathway has consistently been implicated as important to CaP survival, growth and progression to AICaP. The data suggests that this crosstalk leads to maintainance of parallel mTORC1 and mTORC2 survival pathways. The pathway is protected from itself: induction of mTOR signaling decreases AR activity, which would enhance TSC2 and thus return mTORC1 to normal levels, rescuing AR. Repression of mTORC1 induces AR through attenuation of its post-transcriptional inhibition, leading to downstream mTORC2/CDK-Cyclin signaling. Additionally, AR represses TSC2, revitalizing the mTORC1 pathway. Repression of AR increases TSC2 activity, leading to inhibited mTORC1 and thus increased AR, hypersensitizing the cell and possibly leading to progression towards AI disease.

4.2.3. PI-3K/AKT/FOXO and AR

FOXO, a family of apoptosis-promtoing transcription factor, has shown relevance to AR and prostate cancer progression. AR has previously been shown to be a positive regulator of the PI-3K pathway, which represses FOXO family transactivity. Additionally, tissue microarray data from TM3-AR CaP cells treated with testosterone display marked downregulation of 65 FOXO-family proteins [168]. Sixteen of these have been shown to be important in development,

including FOXO1. These data suggest that AR and FOXO are antagonistic towards one another. Li et al. (2001) first indicated an antagonistic nature of AR and PTEN/FOXO.

Ma et al. (2008) characterized one side of this antagonism, showing that FOXO1 mediates PTEN inhibition of AR. By expressing fragments of FOXO1 and determining their ability to repress AR activity, they discerned that the FOXO1 inhibition of AR required its AD and forkhead box. Further, the inhibition of AR was found to be through disruption of its NTD/CTD interaction. This disruption was found to be mediated by FOXO1 binding the AR NTD and repressing interaction with SRC1, a promoter of AR activity [169]. Completing the dichotomy, Li et al. (2003) showed that AR can also disrupt the activity of FOXO-family transcription factors, including FKHR. Androgen treatment and subsequent AR activation in PTEN-null cells was shown to repress FKHR and related FOXO-family protein activity in a manner independent of transcriptional coactivators. This repression was found to be by complexing of activated AR and FKHR, leading to an inability of FKHR to bind DNA. Two points of interaction were found for each molecule: AR binds to the FKHR C-terminus and binds weakly to the forkhead domain, while FKHR binds to the AR NTD and weakly to the LBD. Through flow cytometry, Li et al. (2003) demonstrated that FKHR when bound by AR can no longer induce cell cycle arrest, thereby leading to an attenuation of its role in growth control. This suggests that androgen ablation therapy might reintroduce FKHR and related FOXO activity, leading to arrest of cell growth. Thus, the progression to androgen independence would require compensation for androgen deprivation, such as those discussed earlier. In particular, mTOR and FOXO seem to have related roles. AR inhibition of FOXO combined with mTOR-mediated growth signaling could lead to potent CaP proliferation. Additionally, mTOR-related crosstalk could rescue FOXO-mediated AR inhibition.

Another similarity between FOXO and mTOR crosstalk with AR is the role of p27. Unlike FOXO1 and FKHR, FOXO3a has been shown to transcriptionally upregulate AR by binding its promoter [171], while AR deactivates FOXO3a [172]. FOXO3a also promotes transcription of p27 via its promoter [173]. These interactions lead to a system where AR deactivates FOXO3a, leading to inhibition of p27. If AR were reduced, FOXO3a would become active, promote AR expression and thus reinstate the same state. Disruption of this cycle was shown by Li et al. (2007), using a DIM compound called B-DIM. Treatment with B-DIM repressed FOXO3a binding to the AR promoter while maintaining its binding to the p27 promoter, leading to cell cycle arrest. In CaP, redundant repression by mTOR might prevent this method of rescue. Additionally, AR disruption of FOXO proteins in conjunction with AR/mTOR crosstalk would lead to a deadly regulatory loop whereby cell cycle regulation is suppressed and growth is promoted. Zhang et al. (2010) also showed potential for FOXO-based therapy in response to AR signaling. Methylseleninic acid (MSA) can be metabolized to methylselenol, which has been shown to have anticancer effects and to induce apoptosis [175]. Zhang et al (2010) found that treatment of LNCaP cells with MSA leads to induction of FOXO1 expression and transactivity. Additionally, knock down of FOXO1 after MSA treatment was found to nullify its apoptotic effects. The critical role of FOXO1 in this context was found to be repression of AR activity, though the mechanism for this inhibition remains unknown. Based on results from Ma et al. (2008), FOXO1 could be attenuating NTD/CTD interactions of AR. Results from

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treatments with FOXO-associated drugs such as B-DIM and MSA implicate it as an important molecule in AR-mediated CaP growth and its role as an antagonist of AR could potentially implicate it in CaP progression. Since AR/FOXO crosstalk is seemingly similar but opposite to AR/mTOR crosstalk, investigation of both AKT-dependent and AKT-independent crosstalk between the two pathways could elucidate important mechanisms of CaP progression.

4.2.4. PI-3K/AKT/GSK3β and AR

The evidence thus far clearly shows that increased activation of the PI-3K/AKT signaling pathway and transcriptional activity of AR are closely intertwined. The role that AKT plays in modulating AR activity, however, remains obscure. One of the many downstream substrates of AKT, GSK3β has also been shown to play a role in AR regulation [176-179], and is ubiquitously expressed in CaP cell lines, including COS-1, PC-3, LNCaP and DU145 (Wang et al., 2004). It has increased expression in AICaP cell lines [176, 179], and appears to be a key player in the progression of CaP to androgen-independent disease. However, the nature of this role is at present ambiguous. In varied contexts, GSK3β has been shown to both promote and antagonize AR transactivaton independently of its interactors and other substrates in both a ligand-dependent and ligand-independent manner.

A repressor of several EMT pathways, GSK3β has been shown by Salas et al. (2004) to be capable of repressing ligand-dependent AR activity by phosphorylation. Transfection of wild type GSK3β or constitutively active GSK-3B$^{\Delta 9}$, a mutant of GSK3β devoid of its first 9 amino acids, into AR-expressing LNCaP, A103 and V28 cells significantly increased phosphorylated AR compared to transfection of empty pCMV$_4$ or inactivated tyrosine 216 mutated GSK3β (GSK3β^{Y216F}). Furthermore, treatment of cells with LiCl, a GSK3β inhibitor, significantly decreased phosphorylation of AR (Salas et al., 2004), indicating that altered modification of AR is indeed due to the activity of GSK3β. In AR- COS-1 prostate cancer cells, co-transfection of wtGSK3β or GSK3β$^{\Delta 9}$ with AR lead to increased phosphorylation of AR, as opposed to cotransfection of AR with empty pCMV$_4$ or GSK3β^{Y216F} which did not elevate phosphorylation of AR. This suggests that the activity of GSK3β is essential to the phosphorylation of AR, as the active forms of GSK3β were the only ones to display an effect. Upon treatment of AR-expressing COS-1 cells with LY294002, phosphorylation of AR increased [176]. This is due to reduced deactivation of endogenous GSK3β through indirect inhibition of AKT via the PI3K pathway. When these cells were treated with LiCl, AR phosphorylation decreased in a dose-dependent manner, indicating that these results are related to increased GSK3β activity. In following with the increased phosphorylation of AR, Salas et al. (2004) also reported that AR-mediated transactivation in the presence of R1881 (metribolone), a synthetic nonmetabolizable androgen, was decreased with increased GSK3β activity. This was shown through luciferase reporter assays both with an ARE-driven ARE$_2$LUC construct as well as with a PB-LUC (a promoter from an endogenous AR target). By using C-terminal and N-terminal domain mutants of AR, Salas et al. (2004) were able to determine that GSK3β preferentially phosphorylates AR on its CTD. Furthermore, usage of a GST-tagged ARLBD revealed that GSK3β could phosphorylate AR on its LBD. This may provide a mechanism by which AR-driven transcription is decreased by GSK-3β. The effect of GSK3β being through the AR LBD is further

evidenced by the fact that Salas et al. (2004) did not note any suppression of the ligand-independent, constitutively active AR5 and AR104 constructs.

Wang et al. (2004) also showed a reductive effect of GSK3β on AR transcription. Co-transfection of AR, wtGSK3β and two reporter constructs in a dual luciferase system with an ARE-driven promoter (ARE_4) revealed that increased GSK3β decreased AR transactivation. Further, usage of a constitutively active GSK3β mutant ($GSK3\beta^{S9A}$) further restricted AR-driven transcription. These data taken together suggest that GSK3β kinase activity regulates the level of AR transactivation. GSK3β was also shown to decrease AR transcription in LNCaP cells, which express endogenous AR. These effects were shown to be reversible by LiCl treatment. However, in contrast to the work by Salas et al. (2004), Wang et al. (2004) demonstrated that GSK3β phosphorylates AR on its NTD more significantly than its LBD or a DBD-LBD fragment by using GST-tagged fragments. Moreover, they showed that GSK3β repressed transcription by GAL4-AR-N-terminal in the presence of a pG5-Luc reporter, which contains the ligand-independent AF-1 domain, while failing to repress activity of the AF-2 domain-containing GAL4-AR-LBD. These data suggest that GSK3β inhibits ligand-independent activity of AR. Wang et al. (2004) also demonstrated that GSK3β binds to the CTD and NTD of AR in both transfected and endogenously expressing CaP cell lines, leading to the postulation that GSK3β-mediated suppression of AR transcription may be due to attenuated AR CTD-NTD interactions, which are required for transactivation. Supporting the interaction of the two molecules, Salas et al. (2004) noted that there was a physical co-distribution of the two molecules in CaP cell lines and in CaP tissue. Salas et al, (2004) reported that inhibition of GSK3β by Ser9 phosphorylation is elevated in the androgen-dependent LNCaP in comparison to the androgen-independent PC3 and DU145 CaP lines, which may signify an increased role for GSK3β in androgen-dependent tumours. Liao et al (2004) also show that GSK3β Tyr216 phosphorylation is elevated in AICaP cells, especially 22-RV1. Aberrant activity of the PI-3KAKT signaling system has been demonstrated in AI 22-RV1 CaP cells, and has been associated with an increased Gleason grade [82], which in turn has been shown to be an accurate predictor of progression to AICaP [182]. These data taken together suggest that the increased activity of PI-3K/AKT in AICaP, usually due to PTEN deficiency, may have the effect of disabling GSK3β and thus increasing activity of AR in a ligand-independent manner.

Work by Liao et al. (2004), Mazor et al. (2004) and Schütz et al. (2011) contradicts Salas et al. (2004) and Wang et al. (2004), reporting that inhibition of GSK3β actually represses AR-mediated transcription. Mazor et al. (2004) reported that GSK3β sequestration or knockdown inhibits AR signaling, while transfection of a constitutively active form ($GSK3\beta^{S9A}$) into LNCaP cells with majority Ser9-phosphorylated GSK3β increased AR transcriptional activity. Moreover, this effect was independent of its downstream substrate, the oncogenic β-catenin. Liao et al. (2004) demonstrate that GSK3β is necessary for ligand-dependent transcriptional activity to occur. In the presence of LiCl, two other GSK3β inhibitors RO318220 and GF109203X and siRNA against GSK3β, R1881-stimulated AR transcriptional activity as measured by a PSA-SEAP reporter was significantly reduced. This was not due to reduced nuclear translocation, as no inhibitors blocked AR nuclear localization with R1881 treatment. Knockdown of AKT and β-catenin, another substrate of GSK3β, did not yield any similar results, implicating that

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GSK3β activity is directly inducing the observed effect. Interestingly, in the presence of R1881 GSK3β Tyr216 phosphorylation was also increased, signifying a synergistic relationship between GSK3β and the androgen-dependent AR signaling cascade.

Schütz et al. (2011) further complicate the story, reporting that GSK3β is necessary for androgen-independent AR activity, though not by directly affecting the AF-1 or AF-2 activity domains. Instead, this inhibition is in a CRM1-dependent manner, as discerned in an earlier study [179]. CRM1 is an export receptor for substrates containing an L-rich NES, likely acting in a RanGTP-dependent manner [182]. CRM1 activity is inhibited by leptomycin B (LMB). Upon treatment of 22-RV1 cells with SB216763, a GSK3β inhibitor, AR was increasingly localized in the cytoplasm and experienced a two-fold drop in the nucleus. When LMB was added, the effects were reversed. AR transcriptional activity was also shown to drop with the inhibitor, and was rescued by LMB. Furthermore, AR association with CRM1 was shown to increase with SB216763, and a putative binding site was reported to be located within the C-terminal LBD. This was found using a mutant deleted of its LBD (Schütz et al., 2010). In their future work, Schütz et al. (2011) showed that unliganded AR in AI LNCaP lines, which was localized to the nucleus, is exported upon application of SB216763 in a CRM1-dependent manner, rescued by LMB. Decreased AR signaling with GSK3β was also shown in vivo using a tumor-engrafted chick choriallantoic membrane model. Of note, knockdown (shRNA) or long-term inhibition (SB216763) reduced the nuclear and cellular levels of AR respectively. Mazor et al (2004) showed similar data, suggesting that GSK3β may also play a role in maintaining stability of the AR protein.

The evidence clearly indicates that GSK3β plays a crucial role in regulation of AR; however the nature of that role is highly controversial. Upregulation of GSK3β has been shown to be associated with an elevated Gleason grade [180], which would suggest that support of AR signaling would be likely. Moreover, as Gleason grade often indicates increased risk for AICaP progression [182], however, GSK3β is also a target of AKT for Ser9 phsophorylation, which deactivates its kinase activity. Thus it would seem detrimental that GSK3β induction and reduced inhibition of the PI-3K pathway occur concurrently, as is the situation in several AICaP lines such as 22-RV1. It may be important to note that while Salas et al. (2004) and Wang et al. (2004) made liberal usage of overexpression models, while Liao et al. (2004), Schütz et al (2010) and Schütz et al. (2011) used mainly endogenous protein. This is largely due to the nature of the work, using GSK3β as a suppressant as opposed to studying the effects of repressed GSK3β activity, however ectopic expression can alter a system from the *in situ* function. From the presented results, it becomes apparent that endogenous GSK3β-AR interactions seem to be AR-promoting. Mullholland et al. (2006) suggests that a baseline level of GSK3β may be necessary for AR activity, and ectopic expression may alter the nature of the system, causing an inhibition and suggesting that GSK3β Tyr216 phosphorylation may ultimately be AR-inhibitory. However, the recent results by Schütz et al. (2011) contradict this, while results by Mazor et al. (2004) indicate that overexpression of GSK3β in a system with active endogenous GSK3β, such as 22-RV1 cells, has little effect. The mechanism outlined by Schütz et al. (2010) may be key to note: GSK3β could have a higher affinity for CRM1, thereby preferentially preventing AR export and thus promoting AR transactivation. Further, it would seem that

these interaction play a different role in ligand-dependent and AI disease, which may suggest a role in promoting the progression of CaP to an AI state. Alteration of GSK3β or alteration of its interactions *in situ* may play an important role in regulating GSK3β function with respect to CaP, as it has been shown to play a wide variety of tumour-suppressing and oncogenic roles when in different environments. Thus, a change in cellular context may be key to its role in CaP progression.

4.3. PI-3K/Wnt/AR Axis

The Wnt pathway and the PI-3K have both been implicated in CaP progression. Additionally, crosstalk has been evidenced between the two systems, usually downstream of AKT. In particular, GSK3β is a common intermediary between the Wnt and PI-3K pathways through which crosstalk is often implicated. In the Wnt pathway, GSK3β phosphorylates β-catenin, the central effector of the pathway, to mark it for ubiquitination and subsequent proteosomal degradation [184]. β -catenin is a multifunctional protein that both aids in the stabilization of the adherens junction with E-cadherin and activates transcription of Wnt target genes. Wnt ligands activate the Wnt pathway by binding to their seven-pass transmembrane receptor frizzled (Fzd) and its co-receptors LDL receptor related proteins 5 and 6 (LRP5/6). The Wnt pathway is divided into the canonical Wnt pathway, which signals through β-catenin, and the non-canonical Wnt pathway. The non-canonical Wnt pathway includes the calcium dependent pathway and the planar cell polarity pathway, both of which play vital roles in development. [185,186]. The canonical Wnt pathway is stimulated when a member of a subset of Wnt ligands binds Fzd. This transduces a signal through dishevelled (Dvl) to disrupt the β-catenin destruction complex, made up of adenomatous polyposis coli (APC), casein kinase 1 (CK1) and GSK3 β unified by the scaffolding protein Axin. By sequential phosphorylation, ubiquitination and degradation in the presence of an active destruction complex, β-catenin is maintained at reasonable levels. Upon stimulation by a Wnt ligand, GSK3βis deactivated by Dvl and Axin is seuestered to the membrane by the now-phosphorylated LRP5/6. This allows β-catenin to accumulate unchecked, and translocate to the nucleus. The mechanism for β-catenin translocation remains unclear. In order to play its role as a transcativator, β-catenin must bind its nuclear interactor T-cell factor (TCF). In a cell unstimulated by Wnt activation, TCF is bound to its repressor, Groucho. With Wnt activation, β-catenin displaces Groucho, and the β-catenin/TCF complex transcribes a plethora of Wnt target genes, many of which play oncogenic roles. In this way, β-catenin itself is a potent oncogene.

Wang et al. (2008) used castration resistant mouse models to demonstrate that AR expression seems to be concurrently expressed with increased levels of cytoplasmic β-catenin that is unattached to the adherens junction. This is significant because free β-catenin has the potential to shuttle to the nucleus and activate transcription of Wnt target genes. Trucia et al. (2000) establish a direct significance for this co-expression: β-catenin and AR can directly interact, leading to enhanced AR signaling and hypersensitivity to androgens. In LNCaP cells β-catenin and AR were shown to complex both in the presence and absence of androgen, but binding was markedly enhanced in the presence of DHT. However, using a stabilized mutant of β-catenin (β-catenin S33F) it was shown that AR activity is only enhanced in the presence of

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androgen, signifying a ligand-dependent activation. This was measured using a luciferase reporter assay. Trucia et al. (2000) went on to show that β-catenin binds AR on its LBD, and reduces the effects of bicalutamide on AR. This was shown to be through alteration of the AR LBD, broadening the scope of AR-ligand interactions to include other ligands. In this way β-catenin was show by Trucia et al. (2000) to be a co-activator of AR, providing it with increased significance beyond its role as a coactivator of Wnt target genes with TCF. β-catenin's interaction with AR were shown to be increasingly important by Mullholland et al. (2002), who showed that the AR/β-catenin complex can serve as a vehicle for β-catenin translocation in a ligand-dependent manner. Treatment with androgen in LNCaP cells led to colocalization of AR and β-catenin to the nucleus. Mullholland et al. (2002) went on to show that there are several points of overlap between β-catenin-driven and AR-driven transcription by noting several common targets, including cell cycle proteins such as cyclin D1. Others since have showed interaction between the two molecules, both in support [67] and in contention [47, 189]. In fact, data by Chesire et al. (2002) indicates that ligand-dependent AR/ β-catenin interactions inhibit β-catenn/TCF activity.

The most obvious point of crosstalk between PI-3K and Wnt is their common intermediary, GSK3. Sharma et al. (2002) investigated the crosstalk between these two molecules and AR. Treatment of LNCaP cells with LY294002 resulted in inhibition of AR-driven PSA expression, demonstrating a regulation of AR activity similar to that seen by Li et al. (2001). Upon application of LY294002, phosphorylation of AKT decreased, as did inhibitory phosphorylation of GSK3 β. In conjunction with the lack of deactivation of GSK3 β, nuclear accumulation of β-catenin was significantly reduced. Usage of a mutant β-catenin mutated at its GSK3 βphosphorylation site attenuated the results, showing that the modulation of AR transactivation by the PI-3K pathway occurs through β-catenin. This finding is contradicted by Liao et al. (2004) and Mazor et al. (2003), who demonstrate that GSK3 βis required for AR transactivation. Liao et al (2004) showed that knockdown of β-catenin by pooled siRNA does not affect the levels of R1881-stimulated AR transactivation as measured using a PSA-SEAP reporter construct. Mazor et al. (2003) also show that depletion of β-catenin levels by siRNA treatment does not inhibit transactivation by endogenous AR in 22-RV1, LNCaP and CWR-R1 cells. In fact, they demonstrate that knockdown of β-catenin leads to increased levels of AR activity. It is worthy to note that both Liao et al. (2004) and Mazor et al. (2003) worked with primarily endogenous proteins, using knockdown models, and demonstrating that endogenous β-catenin is not a co-activator of AR. Mazor et al. (2003) notes the importance of confirming results obtained using ectopic expression with studies of endogenous protein.

For the most part, this section focuses on crosstalk through GSK3β. However, it is important to note that AKT modulates a variety of substrates downstream of PI-3K, and a number of these could be means for crosstalk. Hoogeboom et al. (2008) noted that FOXO interrupts β-catenin/TCF transcription by binding and sequestering β-catenin. This type of interference could play a role in inhibiting β-catenin/AR transcription as well, should that interaction take place. If β-catenin Is truly a coactivator of AR, or if AR does act as a shuttle for β-catenin, a great depth of understanding could be arrived at. In order to understand CaP progression and the roles of the Wnt, PI-3K and AR pathways therein, these interactions must be studied and

understood. Should β-catenin coactivate with AR, the question arises as to whether it might do the same with FOXO. Many other proteins adapt functions based on their interactors and β-catenin is no different, being responsible for maintaining anchorage dependence when interacting with E-cadherin at the adherens junction. Mazor et al. (2003) had justification in commenting that the endogenous interactions of a protein should be understood. Until the relationships underlying β-catenin and its interactors are characterized, its role in CaP progression will remain elusive.

5. Current therapy, implications and future directions

The reciprocal interactions and interplay between the AR and PI3K/AKT axis suggests that the underlying mechanism potentiating CaP progression is complex and impacts the very balance of these prosurvival pathways. Current literature shows that there is indeed crosstalk between the AR and PI3K/AKT pathway occuring at various levels. The integration of these oncogenic pathways potentiates CaP tumorigenesis and this is further complicated by the levels of androgens and stage of CaP progression. In effect, the transition from AD-CaP to AI-CaP in prostate carcinogenesis provides major clinical challenges. Androgen ablation and/or anti-androgen therapies are only temporarily effective. Such therapies yield a hormone refractory tumor that is essentially untreatable with the most effective standard chemotherapeutic regimens which only increase patient survival for 2 months [191]. In this case, the pharmacological challenge then, will be to consider the contributions from both PI3K/AKT and AR signalling pathways throughout CaP progression [82].

The mTORC1 pathway has been a primary focus for drug development due to the discovery of rapamycin [93]. However, selective inhibitors from this family of compounds have not proven to be effective. Although, it seems promising to use drug combinations for the inhibition of the main survival pathways (mTORC1, PI3K, AKT) this may incidently result in high toxicity. The concept of intercepting signaling cross-talk with drug combinations to target multiple nodes of integration and/or multiple kinases may be useful in controlling upstream and downstream the PI3K pathway. In addition, the ability of the PI3K/AKT pathway to synergistically heighten AR signaling together with non-genomic cross talk between other pro-survival factors make targetable areas for therapy difficult. Now, with the integration of the Wnt/β-catenin signalling pathway in AR regulation the interplay between PI3K, Wnt and AR signaling becomes further complicated. As such, putative chemotherapeutic agents that inhibit upstream the Wnt or PI3K signaling may pose a viable option [194].

The oncogenic role of the PI3K/AKT pathway in CaP progression is clearly evident. However, the mechanisms underlying the interplay between PI3K and AR signaling still remains unclear. Therefore, understanding how crosstalks are regulated in CaP progression will provide a means by which to elucidate the complexities and contexts of AI disease that are necessary for successful therapeutic intervention.

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