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Prostate Cancer Progression to Androgen Independent Disease: The Role of the Wnt/β-Catenin Pathway

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1. Introduction

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 (Feldman & Feldman, 2001). 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 (Niu et al, 2010). 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 (Feldman & Feldman, 2001). 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 its 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 (Nui et al, 2010).

The AR is required for the development of prostate carcinogenesis from early prostate intraepithelial neoplasia (PIN) to organ-confined or locally invasive primary tumors

(Koochekpour, 2010). 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) (He et al., 1999; He et al, 2000; Loy et al, 2003; Bennett et al., 2010) and components of the cytoskeleton (Veldscholte et al., 1992; Bennett et al., 2010), 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 (Nazarteh & Weigel, 1996; Feldman & Feldman, 2001). 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 (Feldman & Feldman, 2001) such as prostate specific antigen (PSA), a routine biomarker for prostate cancer diagnosis and progression (Bennett et al, 2010, Whitaker et al, 2008), and, probasin, a prostate-specific gene that has been exploited as a marker of prostate differentiation (Johnson et al., 2000). The AR has both a cytoplasmic and nuclear distribution, and shows a certain degree of trafficking either to or from the nucleus (Mulholland et al., 2002). 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 (Mulholland et al., 2002).

1.1 AR structure and function

The AR gene is located on the X chromosome (q11-12), and contains eight exons that produces a protein of approximately 920 amino acids (Bennett et al., 2010). 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 (Feldman & Feldman, 2001), 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 (Bennett et al, 2010). There are three regions of tri-nucleotide repeats, which include poly-glutamine (Q) and poly-glycine tracts (Choong & Wilson 1998; Bennet et al., 2010). The poly-Q tract is encoded by a polymorphic CAG repeat (Southwell, et al., 2008). The length of the repeats inversely affects the stability of the AR-NTD and C-terminal LBD interaction, and, AR expression and activity (Chamberlain et al., 1994; Ding et al., 2004; Bennett et al, 2010). 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 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 (He et al., 1999; He et al., 2000; Hong et al., 1999; Xu & Li, 2003; Bennett et al, 2010). 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 (Lemon & Tijian,

2000; Bennett et al., 2010) in preparation for DNA transcription (Shen et al., 2005; Bennett et al., 2010).

The LBD folds into 12 helices. 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 (Bennett et al., 2010). The NTD interacts with the LBD through its sequence motifs ^{23}FQNLF^{27} and ^{433}WHTLF^{437} (He et al., 2000; Simental et al., 1991; Bennett et al., 2010), while co-activators/co-regulators (E.g. SRC/p160 family of co-activators) bind to the LBD by a highly conserved consensus sequence, the LXXLL (L is Leucine and X is any amino acid) motif (also known as the NR box) (Bennett et al., 2010). 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 (Bennett et al., 2010). 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) (Yang et al, 2002).

The LBD AF2 domain is comprised of helices 3, 4, 5 and 12 (Gelmann, 2002). 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 (Masiello et al., 2004). 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 (Miyamoto et al., 1998; Miyamoto & Chang, 2000; Rahman et al., 2004; Bennett et al., 2010). However, it was shown that the AR NTD and CTD interaction was not 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 (Kemppainnen, et al., 1999; Chang & McDonnell, 2002; Masiello et al., 2004).

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 (Bennett et al., 2010). 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 (Liao et al., 2003; Bennett et al., 2010). 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 (Cheng et al., 2002; Heinlein & Chang, 2002; Liao et al., 2003; Bennett et al., 2010). 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) (Bennett et al, 2010). 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 (Cutress et al., 1998; He et al., 2002; Loy et al., 2003; Ozanne et al., 2000; Rahman et al., 2004; Schaufele et al., 2005; Heinlein & Chang, 2002; Bennett et al., 2010). 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 (He et al., 2000; Bennett et al., 2010).

The DBD (559-624) is comprised of two zinc fingers domains created by three α-helices and a 12 amino acid C-terminal extension (Feldman & Fledman, 2001). 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 (Bennett et al, 2010). It is thought that Lysine (Lys;K) 580 and Arginine (Arg;R) 585 in the first zinc finger bind to the second and fifth nucleotide pairs in the first ARE repeat: GGTACA, respectively to the second and fifth nucleotide pairs in the first ARE repeat: GGTACA (Gewirth & Sigler, 1995; Luisi et al., 1991; Schwabe et al., 1993; Rastinejad et al., 1995; Gelmann, 2002). 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 (Rastinejad et al., 1995; Gelmann et al, 2002). 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 (Haelens et al., 2003). The AR, glucocorticoid, mineralcorticoid and progesterone receptors recognize the 5′-TGTTCT-3′ core sequence (Haelens et al., 2003). However, it has been found that ARs can also recognize specific AREs that consist of two hexameric half-sites separated by 3 base pairs (Claessens et al., 1996; Rennie et al., 1993; Verrijdt et al., 1999; Verrijdt et al., 2000; Shaffer et al., 2004). 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 (Shaffer et al, 2004). It is thought that protein-protein interactions play a role in discriminating AR and other steroid mediated effects (Adler et al., 1993; Pawlowski et al., 2002) to enable ARE dependent gene transcription rather than the activation of other HREs.

1.2 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) (Feldman & Feldman, 2001). 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 (Bennett et al., 2010). Phosphorylation of the AR by MAPK, JNK, AKT, ERK, and p38, increases AR response to low level of androgens, estrogens, and anti-androgens as well as enhances the recruitment of co-activators (Bennett et al., 2010). 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 (Blok et al., 1998; Bennett et al., 2010). 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 (Lin et al., 2002; Koochekpour, 2010). Moreover, transactivation of the AR largely relies upon the phosphorylation of Ser213, Ser506, and Ser650 (Bennett et al., 2010). Phosphorylation of the AR is required for its effects within the nucleus and the AR should remain hyperphosphorylated to mediate its transcriptional role (Koochekpour, 2010). 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 (Grossmann et al., 2001; Gioeli et al., 2002; O-Mallet et al., 1991; Koochekpour, 2010). Specifically, Yang et al., (2005)

demonstrated that 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 (Fu et al., 2004; Koochekpour, 2010). 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 (Poukka et al., 2000; Koochekpour, 2010).

1.3 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) (Bennett et al, 2010). 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 (Koochekpour, 2010). 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% (Marcellie et al., 2000; Koivisto et al., 1997; Taplin et al., 1995; Tilley et al., 1996; Taplin et al., 1999; Feldman & Feldman, 2001). 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 (Koochekpour, 2010). Genetic susceptibility seems to be more significant in patients <55 years old (Koochekpour, 2010). Recently, a R726L mutation was reported in only Finnish patients with sporadic or familial CaP (Gruber et al., 2003; Mononen, et al., 2000; Koochekpour, 2010). 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 (Ingles et al., 1997; Gruber et al., 2003; Crocitto et al., 1997; Koochekpour, 2010). Koochekpour, (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 (Koochepour, 2010). 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 (Veldscholte et al., 1992; Feldman & Feldman, 2001). 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 (Feldman & Feldman, 2001). Other mutations such as the H874Y (Histidine 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 (Darimont et al., 1998; McInerney et al., 1998; Nolte et al., 1998; Shiau et al., 1998; Westin et al., 1998; Song et al., 2003). 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) (Taplin et al., 1995; Thrompson et al., 2001; Song et al., 2003). 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 (Thomspon et al., 2001; Song et al., 2003). Additionally, a L701H mutation was also identified in conjunction with the T877A mutation in MDA PCa 2a cell lines (Zhao et al., 1999; Feldman & Feldman, 2001). 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 (Zhao et al., 2000; Feldman & Feldman, 2001). 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 (Feldman & Feldman, 2001). Novel truncated AR mutants, mRNA splice variants and mutant AR lacking exon 3 tandem duplication (coding for C-terminal portion of the DBD) have also been found in the CWR22R derived cell line 22RV1 (AI-CaP) (Marcias et al., 2009; Koochekpour, 2010). 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.

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 (Palmberg et al., 2000; Feldman & Feldman, 2001). 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 (Gregory et al., 2001; Feldman & Feldman, 2001).

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 (Koochekpour, 2010). 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% (Labrie et al., 1986; Feldman & Feldman, 2001). 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 (Trucia et al, 2000). After castration, adrenal androgens could account for as much as 40% of the total DHT in the prostate (Labrie et al., 1993; Trucia et al., 2000).

Hormone receptors that are activated by ligand independent mechanisms are known as 'outlaw' receptors (Feldman & Feldman, 2001). Certain growth factors such as Insulin Growth Factor (IGF)-1, Keratinocyte Growth Factor (KGF), and Epidermal Growth Factor (EGF) have been demonstrated to activate the 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) (Feldman & Feldman, 2001). 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 (Feldman & Feldman, 2001).

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 Wnt signaling pathway plays a significant role in CaP tumor progression. Although it is rare to find genetic mutations in components of the Wnt pathway such as APC or β-catenin in CaP, the deregulation of this pathway is thought to be an early event in tumorigenesis. Moreover, β-catenin, the key regulator of the Wnt pathway, is a direct co-activator of the AR.

2. Wnt signaling pathway: An overview

The Wnt signaling pathway, responsible for a vast array of biological functions, is activated by 19 Wnt isoforms (For a complete list of Wnt isoforms, refer to Chien et al., 2009). The Wnts are a family of secreted glycolipoproteins, which are conserved in all metazoan animals. Wnt ligands activate the Wnt pathway by binding to a seven-pass transmembrane frizzled (Fzd) receptor in conjunction with its co-receptors, LDL receptor related proteins 5 and 6 (LRP5/6). Other factors such as R-spondin, Norrin and Wise may also facilitate Fzd stimulation (Kharaishvili et al., 2011). Signaling by these powerful morphogens functions to direct cell proliferation, cell adhesion, tissue development, oncogenesis, tumor suppression, and cell-fate determination (MacDonald et al., 2009). As a result, defective Wnt signal transduction plays a critical role in a range of hereditary diseases and cancers such as polycystic kidney disease (Wuebken & Schmidt-Ott, 2011), Alzheimer's disease (De Ferrari et al., 2007), hepatocellular carcinoma (Ji et al., 2011), colorectal cancer (Fearon, 1995) and other malignancies. Here, we will focus on the role of Wnt signaling in the development of prostate carcinogenesis.

Wnt signaling can be divided into two categories: the canonical Wnt pathway and the non-canonical Wnt pathway. The former is activated by a certain subset of Wnt proteins that affects a potent oncoprotein, β-catenin, while the latter operates independently of β-catenin signaling. The canonical Wnt pathway is the most well understood and has been implicated in regulating cell-cell adhesion as well as cell cycle control. The planar cell polarity (PCP) pathway and the calcium (Ca^{2+})-dependent pathway have been identified as non-canonical,

however evidence has yet to show whether these two pathways are truly distinct or simply part of a larger signaling network.

2.1 The non-canonical (β-catenin independent) Wnt pathway

In β-catenin independent signaling, Wnt pathways are activated via Fzd receptors and do not involve LRP5/LRP6. Binding of non-canonical Wnts, namely Wnt4, Wnt5a, and Wnt11, to Fzd can influence cell polarization, and embryonic processes such as convergent extension and cochlea development (Veeman et al., 2003; Shih et al., 2007). The asymmetrical distribution of transmembrane receptors and intracellular proteins such as Fzd and the scaffold phosphoprotein Dishevelled (Dvl) respectively, regulate the activation of Rho-family GTPases. The end-stop of the planar cell polarity (PCP) pathway is signaling via Rho-associate kinase (ROCK) and c-Jun NH-terminal kinase (JNK) (Macheda & Stacker, 2008). The second β-catenin independent signaling pathway is characterized by the release of intracellular calcium ions by the stimulation of G-proteins. Increased calcium levels are sufficient to elicit a response from two calcium-sensitive enzymes: 1) calcium/calmodulin-dependent kinase II, (CaMKII), 2) protein kinase C (PKC) (Kuhl, 2004; Kohn & Moon, 2005). The Wnt/Ca^{2+} pathway has been postulated to play a role in tumor progression as the upregulation of Wnt5a in melanoma cells furthered invasiveness by restructuring the actin cytoskeleton (Weerartna et al., 2002).

There is emerging evidence that the non-canonical pathway competes with canonical Wnt signaling. Certain non-canonical Wnts such as Wnt11 and Wnt5a have been shown to antagonize Wnt/β-catenin signaling, although the mode of action is still unclear (Railo et al., 2008). Proposed mechanisms include competitions for Dvl molecules and alternative degradation pathways involving Siah-APC instead of GSK3β-β-TrCP (Veeman et al., 2003; Topol et al., 2003; Kharaishvili et al., 2011).

2.2 The canonical Wnt (β-catenin dependent) pathway

In the absence of Wnt, β-catenin is targeted for degradation by the 'destruction complex' comprising adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β) and casein kinase (CK1), all of which are anchored to a scaffold protein, Axin. Phosphorylation of β-catenin by CK1 at Ser45 primes the sequential phosphorylation at Thr41, Ser37, and Ser33 by GSK3β. Phosphorylation at Ser33 and Ser37 allows recognition of β-catenin by an E3 ubiquitin ligase subunit, β-TrCP (β-transducin repeat-containing protein), resulting in ubiquitination and subsequent proteasomal degradation (MacDonald et al., 2009). Strict regulation of cytosolic β-catenin levels via the destruction complex ensures to some extent the nuclear availability of β-catenin. However, in the presence of a Wnt signal, β-catenin is stablized to increase in cellular levels and subsequently translocate to the nucleus where it becomes transcriptionally active—the hallmark of the canonical Wnt pathway.

The canonical Wnts, primarily Wnt3, Wnt3a, and Wnt6, bind to Wnt-Fzd-LRP 5/6 complexes to activate Dvl, which then disables GSK3β activity and stimulates LRP5/6 phosphorylation. Phosphorylation of LRP5/6 on its cytoplasmic tail leads to Axin docking at the plasma membrane, thus preventing the constitutive destruction of β-catenin (Davidson et al., 2005; Shih et al., 2007). Consequently, β-catenin accumulates in the cytoplasm and ultimately translocates to the nucleus, where it acts as a co-activator of T-Cell Factor/Lymphoid Enhancer Cell (TCF/LEF) family of DNA-binding proteins to mediate Wnt target gene transcription. Interestingly, the mechanism underlying the nuclear localization of β-catenin remains unclear as β-catenin does not contain a Nuclear

Localization Sequence (NLS) nor does it utilize the conventional importin nuclear transport system (Clevers, 2006). It is likely that NLS containing chaperones such as APC, Axin and RanBP3 (Ran binding protein 3) shuttle β-catenin into the nucleus (Clevers et al., 2006). To date there has been no mandatory chaperone identified as an essential carrier for the nuclear transport of β-catenin. Furthermore, TCF-pygopus complexes have also been implicated in β-catenin's nuclear retention (Stadeli, et al., 2006).

In the absence of β-catenin, the TCF/Groucho complex represses gene expression. The interaction between β-catenin and TCF results in the physical displacement of repressor Groucho, leading to the transactivation of downstream target genes often overexpressed in cancer. (Clevers, 2006; Macdonald et al., 2009) For a comprehensive, updated overview of Wnt target genes, refer to http://www.stanford.edu/~rnusse/wntwindow.html.

2.3 Canonical Wnt pathway and CaP progression

CaP development has been linked to Wnt signaling abnormalities and the stabilization of β-catenin (Chesire et al., 2002; Yardy & Brewster, 2005). The canonical Wnts are secreted during early prostate development but are thought to rapidly diminish in the adult prostate (Yu et al., 2009). Expression of Wnts in mature prostate is therefore unfavorable. Studies have shown constitutive activation of the canonical Wnt pathway due to the deletion of exon 3 on β-catenin which caused hyperplasia, squamous cell transdifferentiation (Bierie et al., 2003), and high-grade prostatic intraepithelial neoplasia (HGPIN) in the adult prostate and sustained growth even after androgen ablation (Yu et al., 2009).

Although exon 3 mutations of β-catenin only occur in 5% of primary CaP, 20% of advanced CaP showed an overall increase in β-catenin levels (Chesire et al., 2000; Gerstein et al., 2002). This suggests that aberrant expression of β-catenin is likely responsible for late CaP tumorigenesis. For instance, in mouse prostate expressing SV40-large T-antigen (LPB-Tag), a powerful deactivator of p53 and retinoblastoma (Rb) family of tumor suppressors, the integration of a non-degradable β-catenin gene provided additional morphological changes by transforming areas of benign HGPIN into invasive adenocarcinoma, along with an elevated expression of matrix metalloproteinase (MMP)-7 (Yu et al., 2011). MMPs are proteases known to facilitate membrane invasion by catalyzing the breakdown of the extracellular matrix (Bonfil et al., 2007). In this sense, gain of cell transformation and cell aggression via β-catenin/Wnt signaling may be attributed to the upregulation of MMPs, which are known Wnt target genes. Further, cell motility is endowed by epithelial-mesenchymal transition (EMT), a process by which epithelial cells acquire mesenchymal cell phenotypes such as enhanced invasiveness and greater migratory capacity (Kalluri & Weinberg, 2009). Accumulation of free β-catenin due to a loss of its cytoplasmic binding partner, E-cadherin, contributed to EMT in colon epithelial cells (Novak et al., 1998) while nuclear localization of β-catenin promoted and maintained EMT induced by c-FosER fusion protein in mammary epithelial cells (Eger et al., 2000; Eger et al., 2004). Likewise, GSK3β, a negative regulator of β-catenin, was downregulated in LNCaP/HIF-1α and IA8 EMT positive CaP cell lines, suggesting that β-catenin stabilization correlated with EMT characteristics in prostate tumorigenesis (Jiang et al., 2007). Additionally, Zhao et al., (2011) showed that shRNA knockdown of β-catenin expression in LNCaP/HIF-1α cells caused a reversal of mesenchymal properties and metastatic potential. This repression of β-catenin also attenuated invasive potency, increased E-cadherin expression, retained cytoplasmic β-catenin, and downregulated mesenchymal markers such as vimentin, N-cadherin and

MMP-7 (Zhao et al., 2011). Last but not least, the activation of Wnt signals in LNCaP cells resulted in expression of neuroendocrine (NE) markers, NSE and Chr.A, signifying Wnt/β-catenin in the development of neuroendocrine differentiation (NED) (Yang et al., 2005). This is confirmed by *in vivo* studies, which have revealed areas of NED in mouse prostates expressing dominant active β-catenin and T-antigen (Yu et al., 2011).

While β-catenin is the point of interest in terms of Wnt signaling and cancer progression, dysfunction of other components within the Wnt pathway can be equally detrimental. A classic example is the APC truncation that occurs in over 80% of colorectal cancer (Quyn et al., 2008). APC is an integral part of the 'destruction complex' that prevents β-catenin from exhibiting its oncogenic properties. Accordingly, APC loss-of-function fosters cell proliferation and differentiation, specifically the growth of adenomatous polyps in the colon. Although there are relatively rare incidences of APC mutations in human CaP, the *APC gene* has been shown to be modified in primary and metastatic CaP, through processes such as promoter hypermethylation and somatic alterations (Jeronimo et al., 2004; Brewster et al., 1994; Bruxvoort et al., 2007). Moreover, deletion of the *APC gene* in mouse CaP models stimulated the rapid development of AI-CaP (Bruxvoort et al., 2007). Despite these data, the role of APC in CaP remains controversial as a recent study found APC variants in several clinical specimens of CaP to be non-functional (Yardy et al., 2009). In the same study, the scaffold protein, Axin, was modified in 6% of advanced CaP cell lines with four Axin polymorphisms identified (Yardy et al., 2009). Furthermore, a strong correlation was shown between Axin2 and CaP progression (Pinnarbasi et al., 2010). Finally, the knockdown of Wnt receptors, Fz2 and Ror2, and the removal of the co-receptor, LRP5, significantly reduced DU145 cells' invasive capacity and new bone formation in MDA CaP 2b – a bone-derived CaP cell line, respectively (Li et al., 2008, Yamamoto et al., 2010). Evidently, Fz2 and Ror2 function to facilitate CaP aggression while LRP5 mediates CaP induced bone metastases.

Wnt antagonists are a family of secreted proteins capable of obstructing Wnt signaling. Common antagonists include certain members of the Dickkopf (DKK) family, the secreted Frizzled-related protein (sFRP) family and the Wnt inhibitory factor (WIF) family, all of which are frequently downregulated in human cancers (Kharaishvili et al., 2011). These inhibitors are categorized based on their binding preference. Members of the sFRP and WIF-1 class bind directly to Wnt ligands, which may either block Wnt-Fzd interaction or form nonfunctional Fzd complexes. The DKK protein family binds to co-receptors of the Wnt receptor complex, LRP5/6, to inhibit canonical Wnt signaling (Kawano & Kypta, 2003). In PC-3 cell lines, approximately 88% exhibited hypermethylation of the WIF-1 promoter region, which corresponded to a decrease in WIF-1 expression (Yee et al., 2010). This was observed in 64% of primary CaP tumors (Wissmann et al., 2003). On the other hand, the restoration of WIF-1 expression in PC-3 reverted EMT and enhanced paclitaxel-induced apoptosis, and, in xenograft mouse models decreased tumor size by approximately 63%; this was accompanied by an increase in epithelial markers, E-cadherin and Keratin-18, and a decrease in the mesenchymal marker, vimentin (Yee et al., 2010, Ohigashi et al., 2005). Similar results in PC-3 were obtained by the reintroduction of Frzb/sFRP-3, a potential tumor suppressor that prevented EMT, and decreased MMP-2, MMP-9 and AKT activation (Xi et al., 2005). Additional studies of PC-3 cell lines demonstrated that sFRP-1 negatively regulated AR function, however, by neither Wnt/β-catenin signaling nor the non-canonical pathways (Kawano et al., 2009). Instead, the sFRP-1/Fzd complex may have been responsible for another pathway, closely resembling that of Wnt5a signaling. The

therapeutic relevance of sFRP-1 remains elusive, as its attributes are largely dependent on certain cellular contexts. Examination of sFRP-1 treated prostate epithelial cells showed the downregulation of Wnt/β-catenin signaling, but unexpectedly, a reduction in apoptosis and stimulation of cell proliferation (Joesting et al., 2005). Unlike sFRP-1, sFRP-4 appeared to suppress anchorage independent growth, proliferation rate and mesenchymal expression in PC-3 cells, irrespective of AR functionality (Horvath et al., 2007).

The roles of DKK family of antagonists are becoming increasingly clear. Hall et al., (2008) reported that DKK-1 expression was elevated in early CaP development but became suppressed as CaP cells metastasized to the axial skeleton. Enforced DKK-1 expression in osteoblastic CaP cells was also shown to reduce bone formation and induce osteolytic activity (Hall et al., 2008). In this regard, DKK-1 was required at a high level initially to inhibit osteoprotegerin, a suppressor of osteoclastogenesis, downstream of the Wnt pathway, which led to osteolytic lesions that facilitated tumor growth (Glass et al., 2005, Hall et al., 2008). Once CaP cells had invaded the bone, DKK-1 levels subsequently minimized as new bone formation required Wnt activation to propagate osteoblastic activity. This was in line with more recent experimental data showing that intercardiac injection of stably expressing DKK-1 Ace-1 cells (a CaP cell line that produces mixed osteoblastic and osteolytic lesions) into mice, increased the appearance of a subcutaneous tumor mass and decreased Ace-1-induced osteoblast activity (Thudi et al., 2011). Taken together, these results support the association between DKK-1 overexpression in CaP metastases and a decreased overall patient lifespan (Hall et al., 2008).

3. β-catenin in CaP progression

3.1 β-catenin

Despite the clear regulatory role of upstream Wnt factors such as the Wnt ligands, inhibitors, and receptors in CaP progression, the major mediator of Wnt signal activation is β-catenin. β-catenin is a 781 amino acid protein composed of three distinct regions: the central armadillo domain containing 12 imperfect repeats of 42 amino acids, the amino (N) – terminal containing phosphorylation sites vital for ubiquitin mediated proteosomal degradation and the carboxyl (C) – terminal housing the transactivation domain required for gene activation (Huber et al., 1997). β-catenin serves two major functions. At the adherens junctions, β-catenin links E-cadherin to the actin cytoskeleton via α-catenin. The β-catenin-E-cadherin interactions maintain efficient cell-cell adhesion and structural integrity of tissue architecture. This adhesive property of β-catenin juxtaposes against its oncogenic functions exerted within the nucleus, where TCF/LEF transcriptional factors complex with β-catenin to activate gene transcription. β-catenin's remarkable capability to partake in both cell signaling and adhesion can be explained by the existence of differing molecular forms of the same protein. Gottardi & Gumbiner, (2004) discovered that a TCF-specific form of β-catenin was generated after Wnt activation. This selective type was incompatible with cadherin's binding domain. The majority of E-cadherin-β-catenin dimers were found only when β-catenin was bound to α-catenin. Other organisms such as *C. elegans* utilize several β-catenins to differentially control cell adhesion and signaling, vertebrates transform β-catenin into distinct structural configurations to maintain the same degree of coordination and regulation. The failure to do so, as frequently occurs in cancer, is a common mechanism by which carcinogenesis is facilitated. Therefore, it is key to fully unravel the complex

machineries associated with this potent oncogene. This section of the review will discuss the structural basis of β-catenin's functionality, in terms of subcellular interactions, Wnt-mediated localization, and Wnt-independent signaling.

3.1.1 Armadillo repeat domain

The armadillo repeats (residues 141-664), each repeat consisting of 3 α-helices, helices 1 (H1), 2 (H2), and 3(H3), are densely packed, forming an overall cylindrical conformation. The armadillo tandem repeats form a superhelical molecule featuring a long, positively charged groove, which constitutes binding sites for the majority of β-catenin's interactors (Xing et al., 2008). The floor of this groove is made up of H3 helices. Although the groove shows high binding affinity for various molecular partners, the full-length protein interactions are rather weak, pointing to the significance of the terminal regions (Piedra et al., 2001; Castano et al., 2002). The proteolysis-resistant armadillo domain is also highly conserved and structurally rigid relative to the unstable terminal domains, which are sensitive to trypsin digestion (Xing et al., 2008; Huber, 1997). The inflexibility of the domain is caused by the extensive contacts between the 12 repeats, ensuring the stability of the continuous hydrophobic core (Huber et al., 1997). Deviations from the regular repetitions of residues create imperfect repeats, particularly on repeat 10, where an insertion of 20 amino acids between H1 and H2 surrounds the groove and affects ligand binding (Huber et al., 1997). The extra sequence hosts a binding surface for 14-3-3ζ, an important modulator of β-catenin transactivation by AKT (Fang et al., 2007; Xu et al., 2007). Other irregularities include a missing H1 in the seventh armadillo repeat and the kinked helices of the first repeat, but their functional roles are not clearly determined (Xu et al., 2007). Huber et al., (1997) hypothesized the seventh armadillo repeat as a site of potential hinge action since the lost helix would grant some local flexibility. Additional crystallographic analysis and mutational studies defined the importance of repeat 7 for TCF interaction and armadillo protein function (Graham et al., 2000; Tolwinski & Wieschaus, 2004).

3.1.2 Features of the armadillo groove

The long, positively charged groove is comprised of 12 armadillo repeats and forms β-catenin's ligand-recognition domain, which hosts mutually exclusive interactions with its numerous molecular partners. The positive charge of the groove and its negatively charged ligands assist β-catenin interaction. Superimposition of 3D crystal structures of a variety of β-catenin complexes, including TCF, the cadherins and APC, exposed a binding region (repeats 5-9) shared by the common ligands (Choi et al., 2006). Specifically, repeats 6-8 forms a special part of the groove containing a series of asparagine (Asn) residues that engage the polypeptide backbone of a diverse cohort of ligands (Gottardi & Gumbiner, 2001). Ligands recognizing β-catenin all contain a conserved consensus sequence, containing Aspartic Acid (Asp) and Glutamine (Glu) amino acids that form two disulfide bridges between Lys435 and Lys312 of β-catenin, respectively (Choi et al., 2006). Graham et al., (2000) dubbed these covalent bonds as "charged buttons" as they were required to affix the partners to β-catenin's armadillo domain. Despite the commonalities, each ligand interacts with the groove in a distinct manner: E-cadherin's cytoplasmic domain interacts with the entire span of the armadillo domain; TCF interacts with repeats 3-5 by its amphipathic helix C-terminal; ICAT, an inhibitor of TCF-β-catenin complex formation, is limited to only repeats 11 to 12 (Choi et al., 2006). In each case, the ligand appears to undergo conformational adjustments

to properly accommodate the rigid groove (Gottardi & Gumbiner, 2001). This is supported by the fact that most of β-catenin's partners are poorly structured in the absence of β-catenin or other ligands. Measurements of cadherin, TCF, APC and Axin by techniques such as NMR, circular dichroism and fluorescence anisotropy have confirmed the native instability of these proteins. For example, as independent entities, the entire β-catenin binding domain of E-cadherin was found to be completely unstructured and TCF failed to adopt its secondary structure (Huber & Weiss, 2001; Knapp et al., 2001; Choi et al., 2006).

3.1.3 N- and C- terminal domains

The unstructured terminal tails flanking the armadillo repeat domain are highly flexible, and are proposed to regulate ligand binding. For instance, the interaction between the C-terminal (residues 696- 781) and the armadillo repeats limit the binding of E-cadherin and other co-factors such as the TATA-binding protein (Piedra et al., 2001). The N-terminal (residues 1 – 134) can also interact with the central domain, however, with low affinity when the C-terminus is absent, while the deletion of the N-terminus resulted in a tighter binding of the C-terminal to the armadillo domain (Castano et al., 2002). These results indicate that the two termini are interdependent and interact with the armadillo domain in a fold-back fashion. The possibility of this mechanism, nonetheless, is challenged by recent quantitative analyses of ligand interaction from isothermal titration calorimetry (ITC). Choi et al., (2006) reasoned that competitive inhibition by the terminal tails does not occur, instead, the tails may directly influence the binding of ligands or other allosteric sites on the arm domain to facilitate β-catenin interaction. The possibility of weak transient interactions was negated by data obtained from Nuclear Magnetic Resonance (NMR) spectroscopy – The NMR spectrum of ^{15}N-tagged C-terminus of β-catenin was negligibly affected by the armadillo repeats (Xing et al., 2008; Gottardi & Peifer, 2008). The proximal regions of the C-terminal have also been shown to form an α-helix, designated as Helix C, which modulates Wnt-mediated transcription (Xing et al., 2008). The significance of this particular helix is well documented: truncated *Drosophila* armadillo lacking the Helix C failed to initiate transactivation, whereas truncation of the C-terminus up to the Helix C preserved signaling capacity (Gottardi & Peifer, 2008). Equally, Helix C was found on transcriptionally active forms of β-catenin in *C. elegans* but not on an adhesive form which preferred the cadherins (Schneider et al., 2003; Gottardi & Peifer, 2008). Moreover, experiments delivering truncated armadillo void of the N-terminus into the nucleus revealed an absence of β-catenin- TCF complexes, suggesting that the N-terminus influences, if not to a greater extent than the C-terminus, the gene transcription and chromatin remodeling functions possessed by β-catenin (Chan & Struhl 2002; Tolwinski & Wieschaus, 2004). Further crystallographic and NMR investigations suggested the dynamism of the unstructured tails distal to the Helix C: the negatively charged N- and C-tails respond to the positively charged groove in a highly variable manner and do not interact in a static conformation (Xing et al., 2008). Hence, the tails may "shield" the armadillo repeat domain from any non-specific interaction, or act as "intramolecular chaperones" of the armadillo repeat domain to facilitate ligand binding and to prevent self-aggregation of the repeats (Xing et al., 2008).

3.1.4 β-catenin and the destruction complex

The 'destruction complex' responsible for β-catenin turnover was described to encompass four major entities – the scaffold protein, Axin, the nuclear chaperone, APC and the

phosphorylation kinases, GSK3β and CK1. In the absence of Wnt stimulation, cytoplasmic β-catenin is phosphorylated at site Ser45 by CK1, priming the sequential phosphorylation at sites Ser33, Ser37, and Thr41 by GSK3β. In addition to the subsequent recognition by β-TrCP followed by proteosomal degradation, GSK3β and CK1 mediated phosphorylation has a major impact on the functionality of β-catenin. Unmodified β-catenin at GSKβ residues Ser33, Ser37 and Thr41 has been characterized as intrinsically more active than the pool of β-catenin that are phosphorylated (Staal et al., 2002; Maher et al., 2010). In other words, transactivation by β-catenin can be altered by phosphorylation. Using monoclonal antibodies detecting for β-catenin specifically unmodified at Ser37 and Thr41 (active β-catenin), Maher et al., (2010) indicated that active β-catenin exists in a monomeric form and was found in far fewer proportions relative to the total pool of β-catenin. That being said however, the low levels of active β-catenin were almost exclusively located in the nucleus. Furthermore, Maher et al., (2010) observed that β-catenin phosphorylated at Thr41/Ser45 was spatially uncoupled from β-catenin phosphorylated at Ser33/Ser37/Thr41. This suggested that phosphorylation at Ser45 by CK1 extended beyond a simple priming gesture (Maher et al., 2010). Since the majority of the Thr41/Ser45 phosphorylated β-catenin translocated to the nucleus, it is entirely possible that phosphorylation at Ser45 configures an active form of β-catenin (Maher et al., 2010). In contrast, β-catenin phosphorylated at Ser33/Ser37/Thr41 was generally cytoplasmic and was ultimately subjected to protein degradation. The F-box protein, β-TrCP, recognizes β-catenin at its doubly phosphorylated destruction motif, thereby causing ubiquitination at specific lysine residues by the larger $SCF^{\beta\text{-}TrCP}$ complex (Wu et al., 2003) The helical region just prior to the destruction motif (residues 20-31) is also required for successful β-TrCP interaction (Megy et al., 2004).

The scaffold protein Axin facilitates the phosphorylation-dependent degradation of β-catenin by anchoring β-catenin, APC, CK1 and GSK3β to specific binding sites. The β-catenin binding domain of Axin includes a highly conserved helical region that interacts with armadillo repeats 3 and 4 of β-catenin's positively charged groove (Xing et al., 2003). The helical region on Axin is C-terminal to the GSK3β binding site and runs roughly parallel to the superhelix formed by β-catenin's armadillo repeats. This places GSK3β at the N-terminus of β-catenin to augment the phosphorylation efficiency of GSK3β by **20,000 fold (Dajani et al., 2003). Alternatively, the anchored CK1 and GSK3β can phosphorylate Axin to increase its affinity for β-catenin (Mo et al., 2009). External factors, such as WTX, a tumor suppressor encoded by a gene mutated in Wilms tumors, may aid the degradation of β-catenin by binding directly to β-TrCP (Major et al., 2007). WTX antagonized Wnt signaling in mammalian cells, and this effect was abrogated by the siRNA knockdown of WTX expression (Major et al., 2007). Co-immunoprecipitation assays indicated that WTX can also interact with Axin, APC and β-catenin. In effect, WTX is likely to be another component of the destruction complex, exerting its influence on β-catenin perhaps just prior to ubiquitination (Kennell & Cadigan, 2009).

APC is of particular interest, as it has been proposed to participate in a range of roles in the destruction complex. Firstly, as a nuclear exporter of β-catenin, it is able to restrict TCF interaction and thus gene transcription (Henderson, 2000; Rosin-arbesfeld et al., 2000; van de Wetering et al., 1997; Xing et al., 2004) This model can be partly supported by the nuclear accumulation of β-catenin in colorectal cancer cells (SW480) expressing mutated APC, as well as elevated β-catenin levels due a complete loss of the *APC gene* observed in *Drosophila* (Kennell & Cadigan, 2009). More directly, transient transfection of wild type APC into

SW480 diminished nuclear levels of β-catenin and increased the degradation of β-catenin (Henderson, 2000). Treatment with leptomycin B (LMB), a nuclear export inhibitor of APC, or the mutagenesis of the NES on APC, abolished the reduction in transcriptional activity and total β-catenin levels (Neufeld et al., 2000). As well, the loss of functional NES resulted in increased levels of β-catenin within the nucleus. Taken together, these data support the role of APC as a nuclear chaperone of β-catenin.

Structurally, APC binds to β-catenin by either its three 15-amino acid repeats (15 aa) or seven 20-amino acid repeats (20 aa) at its central domain (Rubinfeld et al., 1993; Su et al., 1993). The 15 aa repeats are not modified by phosphorylation and bind to armadillo repeat 5-8 of β-catenin, overlapping the regions of TCF binding site (Spink et al., 2001). In spite of this physical arrangement, the 15 aa repeats cannot hinder TCF-β-catenin interaction (Spink et al., 2001). Likewise, truncated APC maintaining the intact 15 aa repeats retains the ability to bind to β-catenin but fails to down-regulate β-catenin expression (Munemitsu et al., 1995). The 20 aa repeats are thought to be more functionally important; though, peptide competition studies showed unphosphorylated 20 aa repeats adopt the same binding surface on β-catenin as the 15 aa repeats (Spink et al., 2001; Xing et al., 2004). Despite their sequence similarities, the different β-catenin-binding repeats have crucial differences. The 20 aa repeats are highly conserved and can be phosphorylated on the SXXSSLSXLS consensus motif (Xu et al., 2007). Phosphorylation at this motif by GSK3β and CK1 drastically increases APC interactions with β-catenin by 300- to 500- fold (Xing et al., 2004). In fact, phosphorylation of the third 20 aa repeat has by far, the tightest binding affinity for β-catenin (Liu et al., 2006). Interestingly, the deletion of this site accounts for the majority of APC mutations in colorectal cancer (Bienz & Clevers, 2000; Nathke, 2004; Polakis et al., 1995, Xu et al., 2007). Crystal structures of the complex between the 20 aa repeats and the armadillo groove of β-catenin has led to greater insight. Xing et al., (2004), determined that the phosphorylated 20 aa repeats of APC binds to the armadillo repeats 1-5, and a single 20 aa repeat with its flanking residues covers the entire span of β-catenin's structural groove. Consequently, the large binding area, along with the high affinity between the phosphorylated 20 aa repeats and the armadillo groove, may play critical roles in regulating β-catenin function. Indeed, binding competition assays have confirmed phosphorylated APC disrupts β-catenin-TCF interaction, in part, due to APC residues N-terminal to the 20 aa repeat which adopt a conformation identical to that of TCF and E-cadherin (Xing et al., 2004)

Consistent with results showing reduced β-catenin levels upon expression of APC, Axin interacts with APC through its regulator of G-protein signaling (RGS) domain to promote the destruction of β-catenin (Hinoi et al., 2000). On the other hand, APC requires the Ser-Ala-Met-Pro (SAMP) repeats, in conjunction with the 15 aa and 20 aa repeats, in the central domain to effectively interact with Axin (Behren et al., 1998, Hart et al., 1998, Kennell & Cadigan, 2009). Although APC cannot independently induce GSK3β-dependent phosphorylation of β-catenin, the synergy between APC and Axin considerably increased levels of GSK3β modified β-catenin (Hinoi et al., 2000; Kennell & Cadigan, 2009). This suggests APC, along with the kinases, are essential in forming complexes with Axin to mediate the degradation of β-catenin. The current models hold that APC sustains the efficiency of the 'destruction complex' by controlling the release and the recruitment of β-catenin. The basis of these models is that the phosphorylated 20 aa repeats on APC, not the 15 aa repeats nor the unmodified 20 aa repeats, competitively inhibits β-catenin-Axin interaction (Xing et al., 2003). In one system, Axin recruits β-catenin bound to the 15 aa repeats, which then allows the efficient phosphorylation of the N-terminal serine and threonine residues by

GSK3β and CK1 (Xing et al., 2003). The latter kinases then phosphorylate the third 20 aa repeat on APC, dramatically increasing its affinity for β-catenin so that the Axin-β-catenin complex is derailed. Axin's β-catenin binding domain becomes free for the next available substrate while APC is dephosphorylated as it moves way from GSK3β's active site. Subsequently, β-catenin dissociates from APC and β-TrCP targets the released β-catenin for ubiquitination and degradation. An alternative model suggests phosphorylated APC first transports β-catenin to the 'destruction complex', where dephosphorylation of APC reduces its binding affinity, causing β-catenin to bind preferentially to Axin (Kennell & Cadigan, 2009). While β-catenin is recognized by β-TrCP, GSK3β and CK1 phosphorylate APC to renew the cycle once again. In both scenarios, a candidate dephosphorylation agent is protein phosphatase 2A (PP2A). This multimeric phosphatase promotes β-catenin turnover and causes β-catenin stabilization when inhibited (Xing et al., 2003). In *in vitro* studies, PP2A was shown to directly dephosphorylate APC (Xing et al., 2003).

3.2 β-catenin and cell adhesion

The progression of many cancers, including prostate metastasis, involves the loss of cell adhesion and contact inhibition. This can be attributed to the aberrant regulation of cell adhesion molecules (CAMs), which comprise the cadherins, integrins, selectins and immunoglobulin. E-cadherin is a calcium-dependent transmembrane glycoprotein responsible for mediating intercellular adhesion as well as structural integrity. The cytoplasmic domain of E-cadherin interacts with the entire span of β-catenin's armadillo repeat domain, and features multiple, quasi-independent binding regions (Huber & Weis, 2001). However, only the last 100 residues of the E-cadherin cytoplasmic domain make contact with the large binding interface on β-catenin – and this extensiveness may render the interaction resistant, in most cases, to single point mutations on β-catenin's E-cadherin binding sites (Huber & Weis, 2001; Gottardi & Gumbiner, 2001). The cytoplasmic domain can be subdivided into five regions, I-V, based on their distinct interaction with β-catenin's armadillo repeats. The most functionally relevant are region II and IV, where certain phosphorylation events occur to affect binding affinity. An α-helix in region II is packed against β-catenin Tyr654 on armadillo repeats 11-12. $pp60^{c\text{-}src}$ -induced phosphorylation of Tyr654 reduced the affinity of E-cadherin for β-catenin by 6-fold as the *in vitro* transfection of $pp60^{c\text{-}src}$ led to junctional instability and gain of invasive phenotype associated with metastasis (Roura et al., 1999; Behrens et al., 1993; Huber & Weis, 2001). In general, overexpression and constitutive activation of tyrosine kinases contributes to abnormal growth, in situ carcinogenesis and metastasis (Lilien & Balsamo, 2005). Region IV hosts consensus sequences for casein kinase II (CK-2) and GSK3β-mediated serine phosphorylation. The residues, Ser 684, 686 and 692, enhance E-cadherin binding to β-catenin by either salt bridges or hydrogen bonds, but only when they are phosphorylated. These sites are part of the extended PEST (Pro-Glu-Ser-Thr) sequence responsible for cadherin degradation; masking of the PEST domain when β-catenin binds consequently prevents degradation. Furthermore, site-directed mutagenesis of these key residues resulted in the loss of cell-cell adhesion and the attenuated β-catenin/E-cadherin interaction (Huber & Weiss, 2001).

Another key component of the adhesion complex is α-catenin, a protein linking the actin filaments to the E-cadherin bound β-catenin. The amphiphathic helix (residues 118-141) N-terminal to the first armadillo repeat of β-catenin forms the major binding surface for α-

catenin (Huber &Weis, 2001). Residues 146-149 adopt a helix in a direction different from that of residues 118-141. The conserved residue Tyr142 is a critical regulator this region as it affects α-catenin-β-catenin interaction: phosphorylation of Tyr142 dissociated β-catenin from α-catenin with the simultaneous loss of cell adhesion (Piedra et al., 2003; Ozawa et al., 1998; Lilien & Balsamo, 2005). Crystal structure of chimeric protein, α-β-cat, a fusion complex between the binding domains of β-catenin and α-catenin, revealed that the amphiphathic helix structure collapses past residue 142, as the firm helix would introduce steric clash (Huber & Weis, 2001). Thus, the non-helical region between residues 142-144 creates a hinged region, which can accommodate both β-catenin and α-catenin simultaneously (Wu et al., 2007). While this supports the notion that α-catenin can directly interacts with the actin cytoskeleton while bound to the E-cadherin/β-catenin complex to facilitate structural integrity, recent evidence have led to an alternate mechanism that more accurately describe the mode of interaction. A prerequisite for actin interaction is for α-catenin to be in its homodimeric form; however, the homodimerization interface impedes β-catenin binding sites located on α-catenin (Drees et al., 2005). The monomeric form of α-catenin primarily binds to β-catenin but exhibits low affinity for actin. Thus, α-catenin cannot interact with both β-catenin and actin concomitantly. It seems, overall, α-catenin modulates actin dynamics in the presence of E-cadherin (Drees et al., 2005; Wu et al., 2007).

In CaP, both the deregulation of the E-cadherin/α-catenin complex and the down-regulation of E-cadherin/β-catenin complex were correlated with a high Gleason score and in some cases, low patient survival (Richmond et al., 1997). Furthermore, activated AR was found to repress E-cadherin gene expression and contribute to mesenchymal-like appearance and tumor metastasis (Liu et al., 2008). DHT was essential both *in vitro* and *in vivo* to induce the down-regulation of E-cadherin (Liu et al., 2008). Androgen-mediated EMT, characterized by a loss of E-cadherin, was inversely correlated with levels of AR expression in prostate tumor epithelial cells (Zhu et al., 2010). Thus, minimal AR activation was needed for maintenance of EMT (Zhu et al., 2010). The loss of α-catenin expression has also been observed in PC-3 cell line which was pivotal to the maintenance of cell-cell adhesion (Ewing et al, 1995; Verras & Sun, 2006). In addition, Sasaki et al., (2000) and Yang et al., (2002) demonstrated that the reintroduction of E-cadherin to E-cadherin negative cell line, TSU-Pr1, shifted the localization of β-catenin from the cytoplasm to the cell membrane. Furthermore, there was a reduction of nuclear levels of β-catenin and a corresponding decrease in AR mediated transcription by β-catenin (Yang et al, 2002). Conversely, the loss or reduction of E-cadherin expression from TSU-Pr1 cell lines enhanced AR mediated transcription due to the increased cytoplasmic and nuclear levels of β-catenin (Verras & Sun 2006). Hence, in CaP progression the integrity and presence of E-cadherin affects the redistribution of β-catenin and functionally affects AR induced cell growth and survival.

3.3 β-catenin and post translational modifications

A plethora of post-translational modifications occur on β-catenin to tightly regulate its cellular activity. This includes Ser/Thr phosphorylation, tyrosine phosphorylation, ubiquitylation, acetylation and O-glycosylation. The aforementioned modifications can occur on the N-terminus, the C- terminus, or the surface of the armadillo repeat domain, suggesting they do not alter β-catenin's 3-dimensional conformations on a large scale. Notably, tyrosine phosphorylation, in addition to its role of disassembling adherence junctions as discussed earlier (Section 3.2), has long been implicated to affect the

transcriptional activity of β-catenin. For example, phosphorylation of Tyr654 can increase β-catenin's interaction with the basal transcriptional machinery TATA-binding protein by disassociating the C-terminal from the armadillo repeat domain. Mutation of Tyr654 to glutamate released β-catenin from cadherins and enhanced its activity as a co-activator of transcription, although there is no data suggesting that nuclear β-catenin is phosphorylated at this site (Piedra et al., 2001; Lilien & Balsamo, 2005) Interestingly, phosphorylation of Tyr142 by c-Met acts as a molecular switch that transforms the adhesive form of β-catenin into one that preferentially binds to BCL9-2, effectively increasing the transcription of Wnt target genes. β-catenin containing a mutated Tyr142 did not efficiently bind to BCL9-2, resulting in a dramatic decrease in Wnt target gene transcription (Brembeck et al., 2004). Similar outcomes were mimicked by the CK2-mediated phosphorylation of Thr393, which potentiated Wnt signaling by instilling β-catenin with resistance to proteosomal degradation and an elevated co-transcriptional function (Song et al., 2003).

β-catenin was recently reported to be post-translationally modified by O-linked N-acetylglucosamine (O-GlcNAc). While there was minimal O-GlcNAcylation of β-catenin in CaP, normal primary prostate cells exhibited significantly higher levels of O-GlcNAcylated β-catenin (Sayat et al., 2008). O-GlyNAcylation refers to a covalent modification of serine and threonine residues of mammalian glycoproteins (Brockhausen et al., 2009). This involves the attachment of a single monosaccharide of O-GlcNAc to the hydroxyl of serine or threonine amino acid residues by an O-glycosidic bond (Brockhausen et al., 2009). The addition and removal of O-GlcNAc groups is a reversible process that utilizes two *nucleocytoplasmic* enzymes, O-linked β-N-acetylglucosamine transferase (OGT) and β-D-*N*-acetylglucosaminidase (O-GlcNAcase), respectively (Guinez et al., 2004). The functional aspect of the O-GlcNAc modification of β-catenin was first reported by Sayat et al., (2008) and demonstrated that increasing cellular levels of O-GlcNAc-β-catenin resulted in diminished levels of nuclear β-catenin and a corresponding increase in cytoplasmic β-catenin. Moreover, TOPFlash-luciferase activity showed that the transcriptional function of β-catenin was inversely correlated to its O-GlcNAcylated levels (Sayat et al., 2008). Taken together, these data suggests that O-GlcNAcylation of endogenous β-catenin negatively regulated its nuclear localization and transcriptional activity in CaP and primary prostate cell lines. Such results have clear implications on the nuclear availability of β-catenin and to its transcriptional function including AR transactivation. However, the question of how O-GlcNAc modification may affect AR-β-catenin transcriptional activation, interaction and TCF-AR competition remains to be answered.

Levy et al., (2004) showed that lysine acetylation positively modulates β-catenin's transcriptional activity. Specifically, acetylation at residue Lys345 located in armadillo repeat 6, increased binding affinity of β-catenin for Tcf-4, and required the acetyltransferase activity of coactivator p300. Mutation on Lys345 severed the coopertivity between p300 and β-catenin, which served to reduce β-catenin's co-activation function. Interestingly, competition assays revealed that the acetylated form of β-catenin had lower affinity for the AR while the non-acetylated form better competed for the AR. This suggests a reciprocal relationship between the β-catenin-TCF interaction and the β-catenin-AR axis.

3.4 β-catenin and transactivation

The nuclear localization of β-catenin is the hallmark of the canonical Wnt pathway. Nuclear β-catenin activates gene transcription by forming a complex with TCF/LEF family of DNA-

binding proteins to mediate the transcription of Wnt target genes such as cyclin D1 and c-myc. Although the exact sequence of events that occur once β-catenin has translocated into the nucleus remains elusive, there are several models explaining the role of β-catenin in gene activation. The simplest explains β-catenin as a co-activator by providing a transcriptional activation domain to TCF/LEF (Sokol, 2011). Another model proposes that β-catenin heterodimerizes with TCF/LEF to supplant repressor proteins, Groucho/TLE, CtBP or HDACs, and thereby switching TCF/LEF from a quiescent state into one that is transcriptionally active (Stadeli et al., 2006; Sokol, 2011). Since TCF is DNA bound, changes in chromatin structure are also necessary to lift the transcriptional blockade imparted by the repressor proteins (Narlikar et al., 2002; Daniels &Weis 2005). β-catenin was found to interact with numerous other chromatin modifying proteins such as histone acetyltransferases (HATs), cyclic AMP response element-binding protein (CBP) or its close relative p300, TATA binding protein (TBP) and Brg-1 to assemble a multimeric complex in conjunction with TCF/LEF (Daniel & Weis, 2005).

The TCF/LEF family consists of Tcf-1, Tcf-3, Tcf-4 and Lef-1, and may function to either activate or repress the transcription of a plethora of genes depending on the availability of β-catenin in the nucleus (Ravindranath & Connell 2008). While Tcf-1 and Tcf-4 may play dual roles as both an activator and repressor, Lef-1 exists predominately as an activator whereas Tcf-3 is often a repressor (MacDonald et al., 2009). Upon Wnt activation, approximately 50 residues of Tcf-4 within the N-terminal interact with β-catenin in two distinct binding surfaces (Wu et al., 2007): an extended region (residues 13-25) that interacts with armadillo repeats 4-9 and an α-helix formed by residues 40-50 that binds to armadillo repeats 3-5. In the former interaction, Asp16 and Glu17 form salt bridges with armadillo residues Lys435 and Lys508, respectively, which fastens Tcf-4 to the positively charged groove (Poy et al., 2001). Asp16 is particularly important as it accounts for high affinity binding to β-catenin. In addition, hydrophobic interactions between the Tcf-4 side chains are critical for effective binding. Compared to wild type Tcf-4, the truncated proteins lacking these side chains showed almost a 60% reduction in transcriptional activity (Poy et al., 2001). The second binding interface involves the N-terminal to the DNA-binding high motility group (HMG) domain and overlaps the binding interface for transcriptional repressors Groucho/TLE (Wu et al., 2007). Thus, these proteins are displaced when β-catenin binds with a higher affinity.

Along with β-catenin, many other co-activators of TCF have been identified. BCL9 is an adaptor protein proposed to aid transactivation by providing docking sites for other transcriptional machinery such as pygopus (Sampietro et al., 2006). BCL9 has not only been found to interact with β-catenin/TCF complex to activate transcription, but also been found to sequester β-catenin in the nucleus (Kireghoff et al., 2006). The crystal structure of a β-catenin/BCL9/Tcf-4 complex revealed that BCL9 interacted with β-catenin at a region N-terminal to the structural groove of the armadillo repeat domain. The β-catenin binding domain on BCL9 forms an α-helix, but unlike other co-activators, the helix does not overlap the binding sites of other β-catenin partners and can be mutated to prevent proper β-catenin-BCL9 binding without compromising the integrity of other indispensible interactions (Sampietro et al., 2006). Sampietro et al., (2006) demonstrated that simultaneous mutations within hydrophobic pockets of the first armadillo repeat, especially on residues L156A and L159A, effectively abolished BCL9 binding but not that of E-cadherin and α-catenin, the only two known proteins that bind to the same region on β-catenin. This

suggests that BCL9 interacts with β-catenin through unique, hydrophobic contacts and underscores the therapeutic potential of small molecule inhibitors to prevent the transcription of Wnt target genes via precise interferences of the BCL9-β-catenin complex.
A variety of antagonists and agonists functions to further regulate β-catenin/TCF-mediated transcription. A devoted nuclear antagonist, ICAT (inhibitor of β-catenin and TCF), inhibits binding of β-catenin to Tcf-4 *in vitro* and has been shown to decrease Tcf-4-induced reporter activity (Tago et al., 2000; Tutter et al., 2001; Daniel & Weis 2002). This inhibitory attribute is due to its high affinity for the armadillo repeats 5-10, which are shared between TCFs, APC, and cadherins. Moreover, the helical domain of ICAT can inhibit co-activators, namely p300; in fact, ICAT exhibits bipartite inhibition: its helical domain disrupts CBP/p300 binding, while its extended region prevents β-catenin-TCF interaction (Daniel & Weis, 2002). Positive modulators of β-catenin/TCF-mediated transcription include Galectin-3 (gal-3) and Daxx. Interestingly, the overexpression of gal-3 in the nucleus is associated with tumorigenesis and metastasis in colon, prostate, and tongue squamous carcinoma cells (Danguy et al., 2000; Honjo et al., 2000; Van de Brule et al., 2000). Shimura et al., (2004) demonstrated that gal-3 promotes transcription of Wnt target genes, cyclin D1 and c-myc, and colocalizes with β-catenin to induce transcriptional activity of Tcf-4 up to 13 fold. The binding region of gal-3 (residues 1-131) overlaps with that of α-catenin and may cause displacement of β-catenin from the plasma membrane (Shimura et al., 2004). Another positive co-regulator is Daxx, which potentiates β-catenin/Tcf-4-mediated transcription possibly by removing binding of repressors Groucho/TLE (Huang et al., 2009). Surprisingly, Daxx can also down-regulate DNA binding capacity of nuclear hormone receptors including the AR (Shih et al., 2007).

4. Wnt/β-catenin-AR mediated cross-talk

Alterations in β-catenin distribution and expression have been reported in patient CaP samples with a general trend of increased β-catenin levels in AI-CaP and a greater nuclear presence of β-catenin with increased Gleason grade (Whitaker et al., 2008). As mentioned previously, recent evidence has demonstrated that the Wnt signaling pathway partakes in mediating CaP progression. Activating mutations of β-catenin, the major regulator of this pathway, are found in 5% of prostate cancers (Song et al., 2003). Such mutations directly contribute to altered growth of the CaP, but also increase AR activity (Cronauer et al., 2004). However, since mutations of β-catenin occur focally, it is still a subject of debate whether such alterations to β-catenin represent a late event in prostate cancer progression (Verras & Sun, 2006). It is unlikely that mutational activation of β-catenin is the primary cause. Evidence is clear that increased AR expression results in sensitivity to androgen and that increased AR expression alone is sufficient to transform primary CaP into a more aggressive AI phenotype. The recent identification of a physical interaction between AR and β-catenin and AR and TCF was an exciting new development for understanding the mechanism underlying CaP progression. In effect, the observed accumulation of β-catenin and increase in AR activation are likely in interplay to arbitrate selective gene expression programs that potentiate prostate carcinogenesis.

4.1 The AR-β-catenin Interaction: Structure

β-catenin contains five LXXLL motifs situated within the armadillo region. The LXXLL motifs are found on the second alpha-helix of armadillo repeats 1, 3 7, 10, and 12 (Pai et al., 1996; Huber et al., 1997; Mulholland et al., 2005; Song et al., 2003). However, deletion

mutants of repeats 7, 10 and 12 indicated that these regions were not necessary for AR/β-catenin binding (Yang et al., 2002; Song et al., 2003; Mulholland et al., 2005). A possible explanation for this observation was that the leucine residues of the armadillo repeats may be buried within hydrophobic cores, thus inaccessible to binding the AR LBD (Mulholland et al., 2005). On the other hand, Yang et al., 2002 showed that the central armadillo repeats 1-6 of β-catenin were responsible for the LBD interaction. Using a yeast two hybrid system, Yang et al., 2002 showed that deletion of the AR N-terminal activation domain alone (134-671) or deletions that combined the central armadillo domain (671-781; repeat 1-7) of β-catenin and the N-terminal of the AR resulted in no interaction. This indicated that the primary binding region of the AR encompassed the N-terminus and the first seven armadillo repeats of β-catenin (Yang et al., 2002). However, when deleting repeat 6, the interaction was essentially abolished. These results were confirmed using site directed mutagenesis protocols to generate internal deletions mutants of β-catenin that either lacked repeats 7, 10, 12, 6, or 5. Again, deletions of repeat 7, 10 or 12 had no effect in LBD-β-catenin interaction; deletion of repeat 6 or 5 abolished this interaction. In addition, analysis of AR transcriptional activity (measured by luciferase reporter construct, MMTVpA3-Luc), using β-catenin mutants lacking repeat 6 together with an AR expression vector, showed no enhancement of AR transcriptional activity in CV-1 (AR null) cells. The study by Yang et al., (2002) clearly described that armadillo repeats 1-6 were required for β-catenin AR interaction and AR directed transcriptional activity. However, the LXXLL motifs within β-catenin may not directly contribute to AR binding (Yang et al., 2002).

The LBD is thought to be sufficient for AR-β-catenin interactions. As mentioned previously, β-catenin alone can increase androgen dependent transcription. Using an androgen responsive MMTV LTR luciferase promoter assay, Song et al., (2003) demonstrated that when cells were co-transfected with a GAL4-AR LBD fusion protein and herpes simplex virus VP16-β-catenin fusion protein, there was a rescue of agonist dependent AF2 activity in the AR LBD. Moreover, co-transfection of the NTD and VP16-β-catenin had a synergistic effect on reporter expression (Song et al., 2003). This indicated that the LBD can change its conformation to form a binding area that accommodates co-activator binding. Accordingly, Song et al., (2003) reported that binding of β-catenin to the AR modulated the NTD through its interaction with the AF2 region and this interaction was adjacent but not identical to the AF2 binding site for TIF2. Residues K720 and Valine (V) 716 located on Helix 3 were necessary for AR- β-catenin and AR-TIF2 interaction, respectively (He et al., 1999; Song et al., 2003). Mutation to the AR LBD specifically at K720A reduced AR NTD interactions by 50% and completely abolished β-catenin binding. AR LBDs with either V716R or K720A were both able to maintain DHT ligand binding. Thus, the synergistic effects of β-catenin and NTD were mediated by the independent binding of each to the AR LBD (Song et al., 2003). In addition, two-hybrid interactions with GAL4-AR LBD and AR mutants that lost either the NTD ^{23}FQNLF^{27} or the ^{433}WHTLF^{437} motifs were able to reduce binding to the GAL4-AR LBD (Song et al., 2003). However, in the presence of VP16-β-catenin, there was still a mild interaction of the AR mutants with β-catenin. This suggested that the NTD/CTD interaction of the AR was required for the efficient interaction of the AR and β-catenin. Although, Song et al., (2003) reported that the effects of β-catenin on AR dependent transcription in the presence of TIF2 and/or NTD were small, β-catenin could modulate TIF2 activation of AR mediated MMTV-Luc reporter activity and enhanced the effects of AR NTD. In this case, β-catenin may be facilitating the stabilization and recruitment of

additional transcriptional machinery to enhance transcriptional activation as it too, can directly form complexes with TIF2-AR complexes.
Bicalutamide, flutamide and cyproterone acetate (CPA) are the AR antagonists routinely used for CaP treatment. Hydroxyflutamide, the active metabolite of flutamide, are potent antagonists *in vivo*. Although these compounds can antagonize wildtype AR activation, these anti-androgens were also found to activate mutant ARs that were identified in patients. Moreover, β-catenin seemed to play a role in enhancing AR mutant transcriptional activity. The T877A mutation within the LBD is prevalent in hormone refractory CaP and *in vivo* studies have shown that T877A AR mutants could be stimulated by hydroxyflutamide but not by bicalutamide. Interestingly, Masiello et al., (2004) demonstrated that hydroxyflutamide liganded T877A AR was strongly activated by β-catenin and also stimulated interaction between the AR NTD and T877A LBD. In contrast, CPA liganded T877A mutant AR was not activated by β-catenin and neither was the CPA bound wildtype AR. While β-catenin mediated co-activation of the T877A AR was enhanced in the presence of hydroxyflutamide, T877A had no effect on β-catenin recruitment by CPA. Recently, a novel AR W741C (Tryptophan to Cysteine) mutation was isolated from bicalutamide treated LNCaP cells (Masiello et al, 2004). The significance of this mutant was that it was also identified in a patient receiving bicalutamide therapy. Interestingly, this mutation enable bicalutamide liganded W741C mutant AR to be activated by β-catenin. Such evidence shows that β-catenin is a key modulator AR structure, function and ligand sensitivity, all of which are contributing factors in prostate tumorigenesis to AI disease.

4.2 WNT/β-catenin signaling and AR

The activation of the Wnt signaling pathway results in the stabilization of β-catenin and its cytoplasmic accumulation. This requires deactivation of the destruction complex as well as β-catenin's phosphorylation at Tyrosine 142 (Tyr142). Phosphorylation at this site decreases β-catenin's interaction with α-catenin. Wang et al., (2008) used castration resistant mouse models to demonstrate consistently greater levels of Tyr142 phosphorylation of β-catenin together with increased AR expression in mouse samples. Gene expression studies also indicated that there was a decrease in Wnt transcription factors, Tcf-3 and LEF, as well as the target genes, MYC and CCND1, (Wang et al, 2008). Furthermore, β-catenin inhibitors including CSNK2B, CSK1E, GSK3B, TP53, WNT5A and PLCB4 were also decreased. Therefore, in AI disease progression, the cytoplasmic pool of β-catenin is increased while the downstream effects of β-catenin-TCF transcriptional activity are suppressed (Wang et al., 2008).
The notion that there was a direct interaction between the AR and β-catenin was first established by Truica et al., (2000), who demonstrated that β-catenin was able to enhance AR transactivation, alter the sensitivity of AR to ligands and relieve the repression of anti-androgens on AR mediated transcription. Using coimmunoprecipitation studies, Trucia et al., (2000) determined that β-catenin interacted with the AR in the absence of hormone in LNCaP cell lines which expressed the T877A mutant AR; however, upon administration of DHT, this interaction was increased. In addition, constitutive expression of a stabilized β-catenin (S33F), a mutant that increases β-catenin's half-life, potentiated luciferase reporter activity by 2.5 fold in the presence of androgen (similar results were also observed using the probasin promoter); β-catenin had no effect on reporter activity in the absence of androgen, signifying an androgen dependent mechanism. Similarly, expression of wild type AR in AR-

negative cells, TSU-Pr1 and PC-3, increased AR transcriptional activity of a luciferase reporter 2-4 fold in the presence of androgen and β-catenin relative to baseline.

The modulatory role of β-catenin on AR ligand binding specificity was further confirmed by luciferase reporter assays that measured AR mediated transcription by β-catenin in the presence or absence of adrenal steroids, androstenedione and DHEA. Androstenedione and DHEA are weak androgens that mimic actions of testosterone. Transfection of S33F β-catenin and wild type AR in TSU-Pr1 cell lines showed increased AR-directed transcription with 1nM androstenedione which was comparable to the AR activation caused by 1nM testosterone. Alongside β-catenin's ability to increase AR sensitivity to ligands, β-catenin was also able to alter AR's specificity to ligand activation. Administration of R1881, an agonist of the AR, together with increasing concentrations of bicalutamide, diminished the antagonistic effects of bicalutamide in a dose dependent manner in the presence of β-catenin. Similar results were observed using estradiol. The above study by Trucia et al., (2000) was a novel development into prostate tumorigenesis as its etiology was no longer limited to AR directed transcription but now encompassed β-catenin, another oncogenic activator. β-catenin had the ability to structurally alter the AR LBD so that it may accommodate other steroids and ligands to enhance AR directed transcriptional activation. Hence, it seems as though β-catenin's role has moved beyond its functions as a co-regulator of TCF/LEF transcriptional activation to now include a greater purpose in modulating AR and/or Wnt directed prostate tumorigenesis.

The fact that β-catenin does not have a NLS make its function as a co-activator of transcriptional activity dependent on chaperones for nuclear import. β-catenin's ability to bind the AR provides β-catenin a means to enter the nucleus. Mulholland et al., (2002) provided novel evidence for ligand mediated AR-β-catenin nuclear translocation which was also accompanied by an increase in the expression of AR genes. Confocal microscopy data of LNCaP cells demonstrated that in the absence of ligand, AR was diffusely spread throughout the cells, while β-catenin was localized at the cell membrane, cytoplasm and nucleus. Upon administration of ligand, the AR and β-catenin both became strongly nuclear as observed by greater nuclear staining. There was a moderate decrease in cytoplasmic levels of β-catenin with no significant change at the cell borders (similar results were obtained from transient transfection of the AR using the AR-null PC-3 cell line). More importantly, co-localization of β-catenin and AR was present. Such evidence demonstrates that the AR mediated translocation of β-catenin was distinct from that of APC-β-catenin nuclear-cytoplasmic shuttling (Mulholland et al., 2002).

Mulholland et al., (2002) were also able to show an AR dependent binding of β-catenin to the probasin promoter and confirmed this specificity by antisense or shRNA knock down of β-catenin, which resulted in decreased PSA gene expression. This was also shown by Li et al., (2004), who demonstrated that β-catenin could be recruited to the PSA promoter. Such studies brought mechanistic insight into the co-regulatory functions of β-catenin and its role in differentially regulating AR responsive genes and downstream Wnt/AR transcription factors such as c-myc and the cyclins (Mulholland et al., 2002). Cyclin D1 is a regulator of cell cycle progression and was found to promote mitogenesis and antimitogeneic effects through activation of the cyclin dependent kinases dictated by the AF-1 domain of AR (Petre et al., 2002; Petre-Draviam et al., 2003; Mulholland et al., 2005). Interestingly, stabilization of β-catenin induced little change in cyclin D1 expression, although greatly increased the levels of c-myc (Gounari et al., 2002; Petre-Draviam et al., 2003; Mulholland et al., 2005). Currently,

the relationship between cyclin D1 and β-catenin signaling activity is poorly correlated and literature agrees that increased cyclin D1 levels in prostate adenocarcinomas is a rare event and is not a clinical predictor of prognosis (Mulholland et al., 2005). On the other hand, cyclin D1 has been shown to bind the AR NTD in both ligand dependent and independent conditions, to mediate the repression of AR transcriptional activity. This interaction was also arbitrated without the requirement of an LXXLL motif (Reutens et al., 2001; Petre et al., 2002; Petre-Draviam et al., 2003; Mulholland et al., 2005). To date, cyclin D1 is well recognized as a co-repressor of the AR, however, the significance of this negative regulation conferred by cyclin D1 remains to be elucidated.

The crosstalk observed between the AR and Wnt targets, such as cyclin D1, questions whether other components of the Wnt/β-catenin pathway influence the oncogenicity of the AR. For example, Verras et al., (2004) demonstrated that cultured CaP cell lines activated by Wnt3a ligand increased AR transcriptional effects even without androgenic ligands; however, this was only observed in AR positive CaP cells. Using a PSA driven promoter luciferase assay, LNCaP cells treated with Wnt3a culture medium increased endogenous AR mediated transcription from the PSA promoter. Yang et al., (2006) further demonstrated that Wnt signaling could also increase AR mRNA expression. The AR gene is a target for Wnt signaling as TCF promoter binding elements are present within the AR promoter region. Surprisingly, even with greater levels of AR mRNA, the expression of AR protein was much reduced. Yang et al., (2006) suggested that the decrease in AR protein was likely associated with ubiquitin proteosomal degradation mediated by increased phosphorylation of MDM2 by phosphorylated AKT. Alternatively, Schweizer et al., (2008) showed that overexpression of AR in the presence of Wnt1 activation in PC-3, CWR22Rv1 and LNCaP led to an increase in luciferase reporter activity driven by a LEF-dependent promoter relative to Wnt1 stimulation alone. Based on these results, the AR seemed to have the ability to augment Wnt transcriptional activity in CaP cells. Treatment with agonists and antagonists of the AR, however, inhibited LEF reporter activity even in the presence of Wnt stimulation. Schweizer et al., (2008) reasoned that ligand bound AR may lead to interactions with other cofactors within the AR pathway thus, reducing the ability of the AR to signal through the Wnt/β-catenin pathway. Such cross regulation of the AR and TCF/LEF indicates that the Wnt and AR pathway can differentially regulate gene expression programs that can feedback onto each other. Moreover, AR signals can be potentiated under androgen ablation by Wnt or AR signal activation alone. This raises questions then, to how β-catenin, the common regulator between both pathways, contributes and divides its functions between AR and Wnt signaling.

4.3 β-catenin-TCF-AR axis

The evidence so far clearly indicates that β-catenin can interact with the AR to shuttle to the nucleus and modulate AR ligand specificity and transcriptional function (Truica et al, 2000, Chesire et al, 2002, Yang et al, 2002; Mulholland et al, 2002, Pawlowksi et al, 2002). Chesire & Isaacs, (2002) went on further to show that AR activity also had consequences for β-catenin/TCF target gene expression. Co-transfection of a luciferase reporter containing a PSA enhancer and probasin promoter (pBK-PSE-PB), and a β-catenin/TCF dependent reporter (pOT), demonstrated that AR positive cell lines (CWR22-Rv1 and LAPC-4) suppressed β-catenin/TCF signaling (CRT) in the presence of ligand. This was determined using a stabilized mutant β-catenin (identified in a hormone refractory patient) that had an interstitial deletion (Δ24-27) encompassing the entire GSK3β phosphorylation domain.

However, androgen induced suppression of CRT did not necessarily correlate with increased AR transcriptional activity. In AR dose response assays, CRT decreased as a function of total amount of liganded AR and not on AR transcriptional output. For example, overexpression of AR in CWR22-Rv1 cells reduced AR transcriptional activity; although the interference observed for ligand dependent CRT was more prominent (Chesire & Isaacs, 2002). Inhibition of CRT was also evident in cells with greater intrinsic CRT activity. Upon transient expression of AR and R1881 treatment, CRT in SW480 and HCT-116 (with loss of APC and APC mutations, respectively) was still inhibited. Alternatively, inhibition of the AR by anti-androgens such as CPA and bicalcutamide alleviated androgen induced CRT repression. This suggested that AR mediated suppression of CRT was not dependent on cell specific factors (Chesire & Isaacs, 2002). Androgen dependent repression of CRT was also observed using a cyclin D1 promoter based luciferase reporter. Treatment of CWR22-Rv1 cells with AR ligand reduced the induction of cyclin D1 promoter by β-catenin. AR-negative cells did not have the same response, suggesting that AR expression was required for androgen induced CRT suppression (Chesire & Isaacs, 2002). Together with the fact that there is AR mediated repression of CRT as well as reduced CRT target gene expression of cyclin D1, it is unlikely that concentrations of cyclin D1 required to repress the AR during CaP progression would be achieved (Mulholland et al., 2005). Hence, negative regulation of cyclin D1 by the AR via the interference of CRT is likely a mechanism to counter regulate its co-repressor.

Co-factors bind liganded AR through the AR LBD, suggesting that β-catenin's modulatory function for AR and CRT activity may occur by the AR's restriction on TCF's access to β-catenin (Chesire & Isaacs, 2002). Using an AR expression construct deleted in its DBD (Δ538-614), Chesire & Isaacs, (2002) postulated that a mutant AR limited in its target gene expression capacity could retain its ability to bind to β-catenin through the LBD and inhibit CRT independent of AR target gene transcription. Despite the fact that previous studies demonstrated AR LBD alone was sufficient for β-catenin binding, Chesire & Isaacs, (2002) found that only the wildtype AR reduced CRT when compared to the mutant AR (Δ538-614). This was consistent with the fact that AR gene expression was not required for CRT interference. Further investigation showed that androgen dependent suppression of CRT was abolished with the overexpression of Tcf-4. Removing the N-terminal (ΔN) and HMG DNA binding domain (ΔHMG) of Tcf-4 (β-catenin and DNA binding sites, respectively) reduced the inhibition of CRT much less than wildtype Tcf-4. The Tcf-4 ΔNΔHMG double mutant was also unable to inhibit ligand dependent repression of CRT by the AR. Ectopic expression of Lef-1 conciliated AR signaling and potentiated CRT activity. This ultimately suggested that disruption of the AR-CRT equilibrium in CaP was likely due to a competition between Tcf-4 and AR for β-catenin.

In order to establish the mechanism by which this competition was occurring, Chesire & Isaacs, (2002) further evaluated the effects of Tcf-4 on AR transcription. Full length, ΔN, and/or ΔHMG constructs of Tcf-4 (which all fail to bind β-catenin) were able to impede AR activation of the pBK-PSE-PB promoter and block R1881 induced AR activity. Chesire & Isaacs, (2002) suggested that AR transcription by Tcf-4 likely did not involve decreased β-catenin access by TCF itself. It was more likely that CRT suppression was rather a consequence of the competition for β-catenin rather than AR target gene expression. Mulholland et al., (2003) lends further support to this hypothesis through the use of transcriptional reporter assays which demonstrated that wildtype TCF reduced the activity of AR (ARR3-Luc)-responsive reporter, while the ΔN TCF mutant did not have such an

effect. Alternatively, when PC-3 cells were co-transfected with NTD/DBD or LBD/DBD AR deletion mutants and a TCF promoter luciferase reporter construct, TOPflash, the LBD/DBD mutants were capable of repressing TOPflash luciferase activity in the presence of DHT, while the NT/DBD mutant was not able to do so. TOPflash activity was repressed in a dose dependent manner and alternatively, Casodex was able to alleviate this repression. Thus, the LBD but not the NTD is required for TCF repression. Mulholland et al., (2003) went further to show that there was co-localization of TCF and AR within the nucleus. Using deconvolution microscopy, co-transfection of HcRed-Tcf and AR-EGFP constructs in LNCaP, SW480, and PC3, resulted in partial colocalization of Tcf and AR in the presence of DHT. In addition, co-expression of β-catenin-EGFP constructs with HcRed-AR or HcRed-TCF in LNCaP cells demonstrated a reduced colocalization of β-catenin with TCF upon treatment of DHT and correspondingly, increased colocalization of β-catenin and the AR; in the absence of DHT there was an increased colocalization of β-catenin with TCF. This suggested that β-catenin had the ability to shuttle between the AR and TCF androgen dependently (Mulholland et al., 2003). Treatment with Casodex reduced AR mediated depletion of TCF-β-catenin interaction and diminished androgen sensitive co-immunoprecipitation of endogenous AR and β-catenin (Mulholland et al., 2003).

It has been previously shown that steroid hormone receptor binding can be enhanced by an HMG DBD (Amir et al., 2003). For example, Yuan et al., (2001) determined that the AR can interact with sequence specific HMG box transcription factor SRY, a member of the SOX family of HMG proteins. Likewise, Tcf-4 is a sequence specific HMG transcription factor. Amir et al., (2003) demonstrated a direct interaction between Tcf-4 and AR via the AR DBD, independent of β-catenin. Using a glutathione-S-transferase (GST) fusion protein pull-down experiments, Amir et al., (2003) showed that ^{35}S-labeled Tcf-4 bound specifically to the GST-AR-DBD (aa 556-628) fusion protein (deleted in both the AR NTD and LBD). Tcf-4 did not bind the the AR hinge region (aa 634-668). Alternatively, Amir et al., (2003) confirmed that Tcf-4 repression of AR transcriptional activity was independent of β-catentin. They hypothesized that Tcf-4 repression of AR signaling may be due to the sequestration of β-catenin rather than a direct AR-Tcf-4 interaction. However, co-transfection of CV-1 (AR null) with AR expression vector, pSVARo, ARE_4-Luciferase reporter, and, β-catenin and Tcf-4 expression vectors only resulted in a partial reversal of Tcf-4 mediated repression of AR transcriptional activity relative to Tcf-4 expression alone. This was despite the fact that quantities of β-catenin transfected into the CV-1 cells could readily enhance AR activity. In order to confirm that the results observed were mediated by direct Tcf-4 and AR DBD interaction and not due to AR-β-catenin interactions, CV-1 cells were co-transfected with a VP16-AR DBD (aa 501-660) fusion protein and Tcf-4. Similar to wildtype AR the VP16-AR-DBD (aa 501-660) could be repressed by Tcf-4. This supported that AR transcriptional activity was not due to any negative effects of AR-β-catenin binding and was directly a result of an interaction between Tcf-4 and the AR. The fact that β-catenin could only partially reverse Tcf-4 mediated AR transcriptional activity suggested that β-catenin may lack the ability to displace a co-repressor. Co-repressor activity of Grouch/TLE proteins have been well recognized to repress Tcf-4 mediated signaling. Previously, it has been demonstrated that Groucho/TLE could bind the AR N-terminus and decrease AR transcriptional activity (Shroder, 1993; Amir et al., 2003). The limited capacity of β-catenin to alleviate Tcf-4 repression may possibly be due to its inability to compete with AR-Tcf-4-Groucho/TLE complexes (Amir et al., 2003).

Alterntively, Amir et al., (2003) went on to show that TCF could recruit β-catenin to the AR in the absence of the AR LBD. Tcf-4 binds β-catenin at its N-terminus leaving the C-terminal HMG domain free for AR binding. Thus, Amir et al., (2003) reasoned that Tcf-4 may serve to recruit β-catenin to the Tcf-4-AR complex. First, Amir et al., (2003) confirmed that β-catenin alone did not bind GST-AR-DBD. This was not surprising as it has consistently been shown that β-catenin binds to the AR LBD. Interestingly, upon co-incubation of both β-catenin and Tcf-4 with GST-AR DBD (aa 556-628), co-immunoprecipitation studies demonstrated an increase in β-catenin interaction with Tcf-4-GST-AR DBD (aa 556-628) complexes. Thus, the two β-catenin binding sites within AR-Tcf-4 complex serves as a sensitive target for β-catenin mediated transcriptional activation and also provides co-operative regulatory control over both AR and Wnt target genes (Amir et al., 2003).

The evidence for the TCF-β-catenin-AR axis is still at its infancy. However, studies strongly support the cross regulatory mechanisms that are in play between β-catenin, TCF, and AR during CaP progression. The abovementioned reports suggest that β-catenin can shift its attention between AR and TCF in an androgen dependent manner in addition to modulating AR ligand specificity, sensitivity, and transcriptional activity. Additionally, the fact that there is direct competition for β-catenin by TCF and AR transcriptional machinery further adds to the complexity of the AR-β-catenin axis and introduces another contributing factor for β-catenin mediated regulation of Wnt and AR signaling in CaP progression.

4.4 β-catenin-PI3K-AR axis

The Phosphatidylinositol-3 Kinase (PI3K) and Wnt pathway have both been implicated in the progression of CaP. Specifically, there is interplay between these two pathways through the common factor, GSK3β. Moreover, the loss of the tumor suppressor and negative regulator of the PI3K pathway, PTEN is a common occurrance in CaP causing the constitutive activation of the PI3K pathway. Consequently, there is an increased activation of the end effector protein, AKT, through its phosphorylation at key serine and threonine residues. Excessive activation of AKT results in increased cell growth, cell survival and inhibition to apoptosis. AKT has many substrates, however, for the purposes of this review we will focus on GSK3β. Activation of AKT leads to the inhibition of GSK3β through its phosphorylation at Ser9 resulting in the subsequent accumulation of β-catenin. In effect, the association between AKT-GSK3β and GSK3β-β-catenin bring the PI3K and Wnt pathways, respectively, at a junction where β-catenin's stability and nuclear availability for AR transactivation may be regulated.

Many cell lines of metastatic (LNCaP, PC-3) or AI (22RV-1) CaP have highly active PI3K/AKT activity which has also been correlated with a increased Gleason grade (Mulholland et al., 2006). To further elucidate the role of the PI3K pathway in CaP progression, Sharma et al., (2002) demonstrated that the inhibition of the PI3K pathway by LY294002 inhibited AR transactivation of the PSA gene in LNCaP cells. As expected, phosphorylation of GSK3β was reduced and nuclear levels of β-catenin correspondingly decreased 2-3 fold upon LY294002 treatment. Co-expression of a dominantly active AKT reversed this inhibition of AR activity. This suggested that repression of AR activity by LY294002 was through the inhibition of PI3K and the subsequent inactivation of AKT activity (Sharma et al, 2002). To confirm that LY294002 mediated repression was through GSK3β, Sharma et al., (2002) used a wildtype β-catenin or a β-catenin mutant containing a point mutation within the N-terminal GSK3β phosphorylation site, to demonstrate that AR

transcriptional expression was only reduced by LY294002 for wildtype β-catenin and not for the β-catenin mutant. Since this mutant was void of a GSK3β binding site, the results from this study suggested that GSK3β was involved in β-catenin regulation of AR activity through the PI3K pathway (Sharma et al., 2002).

GSK3β is ubiquitously expressed within CaP cells, including LNCaP, PC-3 and DU145 (Wang et al., 2004). Wang et al., (2004) showed that GSK3β could regulate the AR through its phosphorylation. Using purified GST tagged AR N-terminal (aa 38-560), GST-AR DBD-LBD (aa 551-918) and His_6-AR LBD (aa 666-918), Wang et al., (2004) demonstrated that GSK3β significantly phosphorylated the AR N-terminal (aa 38-560), while only slightly phosphorylating the DBD-LBD and LBD fragments. Furthermore, the presence of GSK3β inhibited GAL4-AR-N-terminal transcriptional response to a luciferase reporter (pG5-Luc) but did not do so for the GAL4-AR LBD which contained the AF2 domain. This suggested that the inhibition of AR transactivation by GSK3β was likely mediated by the NTD AF1 domain.

In order to confirm GSK3β's regulatory role on the AR, Wang et al., (2004) examined AR activity in LNCaP cells transfected with an androgen responsive luciferase reporter (MMTV-Luc) and wildtype GSK3β. The addition of GSK3β reduced AR activity in a dose dependent manner which was then alleviated by lithium chloride (LiCl) treatment (an inhibitor of GSK3β) (Wang et al., 2004). The physical association of GSK3β to the AR was shown through GST pull down assays which demonstrated that the GSK3β interacting domain within the AR was on both the AR NTD and CTD (Wang et al., 2004, Wang et al., 2006). Thus, these results suggested that GSK3β mediates its inhibitory effects by phosphorylating the AR to diminish the interaction between the NTD and CTD, which is necessary for AR transcriptional activity (Salas et al., 2004; Wang et al, 2004; Mulholland et al., 2001).

Tyrosine 216 (Tyr216) phosphorylation of GSK3β is an activating modification that was found to be increased upon androgen stimulation (Liao et al., 2003). Phosphorylation of Tyr216 was inhibited by bicalutamide or by LY294002 suggesting that the PI3K pathway was required for androgen induced GSK3β Tyr216 phosphorylation (Salas et al., 2003). Moreover, the distribution of GSK3β was also dependent on its phosphorylation status. Using GSK3β mutants, Y216F and a GSK3β deleted at its first nine amino acids (GSK3βΔ9), the Y216F mutant was predominantly found in the cytoplasm while the GSK3βΔ9 was more dominant in the nucleus and able to co-localize with the AR in the presence of the androgens. The accumulation of the GSK3βΔ9 was also associated with the suppression of AR mediated transcription which was thought to be due to the elevated phosphorylation of the AR by GSK3β (Salas et al., 2003). Salas et al., (2003) went further to show that the AR and GSK3β were capable of co-localizing in the nucleus using immunohistochemical analysis which supported the physical interaction between these two molecules.

In contrast, some studies have reported that GSK3β was necessary for AR mediated gene expression rather than its inhibition (Liao et al., 2003; Mazor et al., 2004). Using a PSA- SEAP reporter (androgen responsive secreted alkaline phosphatase reporter; described in Ref.24 of Liao et al., 2003) transfected into LNCaP cells (known to have inactivated GSK3β due to Ser9 phosphorylation) treated with LiCl in the presence or absence of R1881 agonist, Liao et al., (2003) demonstrated that LiCl abolished androgen dependent PSA-SEAP activity; this was also evident for cells treated with PI3K inhibitor LY294002. Furthermore, LiCl treatment on LAPC-4 cell line (containing wildtype AR and PTEN) dramatically suppressed PSA expression in the presence of R1881 (Liao et al., 2003). This effect was further confirmed with siRNA knockdown of GSK3β gene. Mazor et al., (2004) also supports this hypothesis by

demonstrating that overexpression of GSK3β in LNCaP cells increased AR transcriptional activity. Interestingly, there was a decrease in AR protein levels upon GSK3β inhibition (Mazor et al., 2004). Mazor et al., (2004) suggested that GSK3β's ability to phosphorylate AR may also increase AR stability as GSK3β has been shown to regulate the stability of many proteins such as Axin, and β-catenin.

Clearly there is evidence supporting PI3K/AKT/GSK3β role in AR transactivation, however, the mechanism of GSK3β activity through this pathway in CaP progression still remains elusive and conflicting. Mulholland et al., (2006) comments on this paradoxical effect and postulates that it is likely that basal activity of GSK3β is required for AR function and any increase in GSK3β activity such as the case for phosphorylated Tyr216, may result in decreased AR function directly through AR phosphorylation or indirectly by influencing β-catenin stabilization.

5. Current therapy, implications and future directions

The reciprocal interactions and interplay between the AR/Wnt-β-catenin 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 Wnt pathway occuring at various levels: a) Wnt ligands transactivate the AR, b) β-catenin interacts with the AR to increase AR mediated transcriptional activity, c) GSK3β negatively regulates AR transcription through the PI3K pathway, d) cyclin D1 (TCF/LEF target gene) can interact with AR to inhibit AR transcriptional activity, and e) competition for β-catenin occurs between AR and TCF/LEF (summarized in Wang et al., 2008). The integration of these oncogenic pathways potentiates the progression from AD-CaP to AI-CaP whereby cell growth and survival, in part, hinges on the availability of β-catenin. Furthermore, the modulatory role of β-catenin on AR expression and transactivation, and ligand specificity and sensitivity, suggests that β-catenin works through a range of intensities. Accordingly, the design of future therapeutic strategies will require the dynamic interplay between AR and β-catenin to be addressed.

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 only increasing patient survival for 2 months (Shen & Shen, 2010). The recent developments on β-catenin's regulatory function in altering the structural intergrity of the AR poses a dilemma for anti-androgens (Eg. bicalutamide, flutamide and CPA) as these agents lose their efficacy in the presence of β-catenin's modulatory effects. Moreover, the ability of β-catenin/Wnt pathway to synergistically heighten AR signaling together with non-genomic cross talk between other pro-survival factors make targetable areas for therapy difficult. Future therapies will have to be evaluated according to tumour type and be individualized to specific alterations that occur during CaP progression (Ewan & Dale, 2008). Specifically, putative chemotherapeutic agents that inhibit the shuttling of β-catenin into the nucleus (Yardy & Brewster, 2005) or those that abolish potential oncogenic AR/β-catenin interactions such as inhibitors that target AR LBD or the first six armadillo repeats may be effective (Mulholland et al, 2005). Furthermore, inhibition of upstream Wnt or PI3K signaling may pose a viable option (Mulholland et al, 2005). The caveat for such therapeutic designs is that the Wnt pathway is

important for normal cell renewal; therefore, the goal is to balance therapeutic effects with minimal harm to cellular homeostasis (Ewan & Dale, 2008).

The oncogenic role of the Wnt/β-catenin pathway in CaP progression is clearly evident. However, the mechanisms underlying the interplay between Wnt and AR signaling still remains unclear. Therefore, understanding how β-catenin-AR-TCF interaction and Wnt-AR crosstalk are regulated in CaP progression will provide a means to elucidate the complexities and contexts of AI disease that are necessary for successful therapeutic intervention.

6. References

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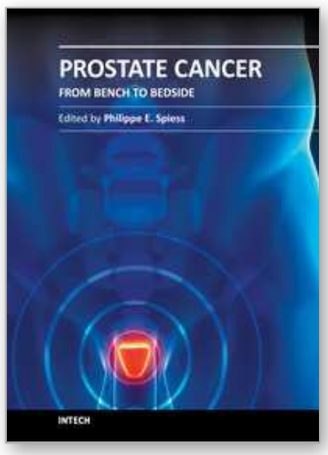

Prostate Cancer - From Bench to Bedside

Edited by Dr. Philippe E. Spiess

ISBN 978-953-307-331-6
Hard cover, 528 pages
Publisher InTech
Published online 25, November, 2011
Published in print edition November, 2011

The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Jacqueline R. Ha, Yu Hao D. Huang and Sujata Persad (2011). Prostate Cancer Progression to Androgen Independent Disease: The Role of the Wnt/β-Catenin Pathway, Prostate Cancer - From Bench to Bedside, Dr. Philippe E. Spiess (Ed.), ISBN: 978-953-307-331-6, InTech, Available from: http://www.intechopen.com/books/prostate-cancer-from-bench-to-bedside/prostate-cancer-progression-to-androgen-independent-disease-the-role-of-the-wnt-catenin-pathway

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