Src Kinase and Androgen Receptor in Prostate Cancer

A Thesis

Submitted to the Faculty of

Worcester Polytechnic Institute, Worcester, MA

In partial fulfillment of requirements of the

Degree of Master of Science

in

Biology and Biotechnology

by

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May 2010

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ABSTRACT

Src signaling plays an important role in prostate cancer (PrCa) progression. It has previously been shown that Src interacts with androgen receptor (AR) and enhances AR transactivation. Although it has been shown that Src promotes AR activity, the underlying pathway has not been defined.

To help characterize the Src-AR pathway, the cellular localizations of Src, p-Src, AR, pAR, and Prostate Specific Antigen (PSA, an AR target gene) were analyzed in androgen-dependent (AD) LNCaP cells and in androgen-independent (AI) castration-resistant C4-2B cells. Using sub-cellular fractionation, the data showed that treatment of AD cells with synthetic androgen R1881 increased p-Src, AR, pAR, and PSA in the nucleus, while the levels of c-Src remained unchanged. Treatment of AI cells with R1881 increased pSrc and AR in the nucleus, while the levels of c-Src and PSA remained unchanged. When using Immunofluorescence microscopy, R1881 did not appear to increase the nuclear levels of p-Src or c-Src, so perhaps this technique is not as sensitive or quantitative as sub-cellular fractionation/immunoblots.

The presence of PSA in the nucleus was unexpected given its well-proven role as a secreted protein. Nuclear PSA was observed upon androgen stimulation in AD and AI cells, and in the nucleus of AI cells upon androgen deprivation. Given PSA's ability to induce cell division and decrease apoptosis when transfected into cells, its presence in the nucleus may imply that PSA acts there to help induce tumorigenesis.

The effect of Src on AR activity was further studied by transfection of a dominant negative src (SrcK298M) in AD and AI cells. Transfection with SrcK298M did not affect PSA expression in LNCaP cells, but strongly inhibited PSA levels in AI cells.

Integrin signaling through Src was investigated in PrCa by ligand binding assay in AD and AI cells. The data showed that $\alpha_V\beta_3$ integrin (but not $\alpha_V\beta_6$) upon attachment to fibronectin or TGFβ-latency associated peptide (TGFβ-LAP) increases p-Src levels in AD and AI cells, while the levels of c-Src, PSA, and AKT remain unchanged. Thus, $\alpha_V\beta_3$ integrin facilitates Src signaling, but the activation does not appear to affect AR transactivation.

In conclusion, these data show that Src is required for AR activity and, consequently, PSA expression in AI prostate cancer cells, but not in AD cells. These data also suggest that the nuclear co-localization of p-Src, AR and PSA might allow macromolecular interactions, which can further enhance AR transactivation and promote disease progression. With respect to the switch in tumor progression from an AD to AI state, the data indicate that the integrin-Src pathway does not include AKT or PSA (and not AR by deduction), so perhaps other non-AR pathways help facilitate tumor growth at the AI state.

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ACKNOWLEDGEMENTS

I take this opportunity to thank my advisor Dr. Lucia R. Languino (UMass Medical School) for all her support and guidance throughout the research process. It was her continuous motivation, and her ability to bring out the best of me, that led to the successful completion of this thesis.

I would also like to extend my gratitude to my committee members Dr.Samuel Politz and Dr. Dave Adams, and the Biology & Biotechnology Department, WPI for their approval to work with Dr. Languino, and for their valuable advice.

I would like to thank Dr. Tao Wang for his constant effort, interest in my project and for providing unique insights into the development of this project. He has gone above and beyond in helping me complete and learn from this work, and I greatly appreciate the time and effort he has put in to make this experience possible for me.

I would like to thank Dr. Hira Lal Goel, Dr. Jing Li, and Dr. Marco Trerotola for their constant encouragement, technical help, and for making this experience both enjoyable and educational for me.

I would also like to thank Dr. Swarna Mehrotra and Dr.Dario Alteri lab for their generous donation of time and materials related to the project.

I would also like to extend my gratitude to Heather Ehlers and Alana Calpai administrative assistants in the Cancer Biology department (UMass), and Carol Butler administrative assistant in the Biology and Biotechnology department (WPI) for their help in arranging committee meetings.

Lastly, I would like to thank my family and friends for their continuous support and encouragement, which kept me going during difficult times.

THESIS PURPOSE

It has previously been shown that Src phosphorylates and activates AR in AI PrCa cells (Guo et al., 2006). Hence, it can be hypothesized that Src plays an important role in PrCa disease progression. Src activation promotes AR nuclear translocation and promotes AR transactivation. However, the mechanism of Src-regulated AR activity in PrCa remains to be explored. It is also known that cytoplasmic domains of integrins interact with Src and results in Src activation. Thus, this integrin-Src pathway may help regulate AR activity.

The first and second part of this thesis focused on the role of Src in the regulation of AR activity in PrCa. Src activity is known to be important in PrCa since tumors in which Src activity has been blocked show reduced tumor volume (Recchia et al., 2003). Since AR is found to be localized in the nucleus upon ligand binding, and increased Src kinase activity is found in PrCa tumor xenografts, I studied AR, Src, and PSA localization in PrCa cells upon stimulation with synthetic androgen R1881. Then, I studied the effect of Src expression and phosphorylation on AR activity in LNCaP (AD) and C4-2B (AI) PrCa cell lines. And finally, I analyzed the role of an integrin-Src-AR pathway on the localization of Src, p-Src, AR, p-AR, and PSA.

Therefore, this thesis investigated novel mechanisms, which modulate AR activity by studying Src localization, expression and phosphorylation in androgen-dependent and independent cell lines, and the potential ability of integrins to activate this pathway.

BACKGROUND

Prostate cancer is the second leading cause of cancer death among men in Western countries. In 2010, 192,280 new cases, and 27,360 estimated deaths are predicted to occur due to PrCa in United States (Jemal et al., 2009) (**Table 1**). Patients with advanced PrCa initially benefit from androgen-ablation therapy, which leads to temporary tumor remission due to apoptosis of androgen-sensitive tumor cells. However, the recurrence of AI tumors is inevitable for most patients, and renders the conventional hormone ablation therapy ineffective (Feldman and Feldman, 2001; Debes and Tindall, 2004). It has, therefore, become a focus of intensive study to understand the mechanisms underlying the development of hormone-refractory PrCa.

Estimated New Cases*

Males			Females		
Prostate	192,280	25%	Breast	192,370	27%
Lung & bronchus	116,090	15%	Lung & bronchus	103,350	14%
Colon & rectum	75,590	10%	Colon & rectum	71,380	10%
Urinary bladder	52,810	7%	Uterine corpus	42,160	6%
Melanoma of the skin	39,080	5%	Non-Hodgkin lymphoma	29,990	4%
Non-Hodgkin lymphoma	35,990	5%	Melanoma of the skin	29,640	4%
Kidney & renal pelvis	35,430	5%	Thyroid	27,200	4%
Leukemia	25,630	3%	Kidney & renal pelvis	22,330	3%
Oral cavity & pharynx	25,240	3%	Ovary	21,550	3%
Pancreas	21,050	3%	Pancreas	21,420	3%
All Sites	**766,130**	**100%**	**All Sites**	**713,220**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	88,900	30%	Lung & bronchus	70,490	26%
Prostate	27,360	9%	Breast	40,170	15%
Colon & rectum	25,240	9%	Colon & rectum	24,680	9%
Pancreas	18,030	6%	Pancreas	17,210	6%
Leukemia	12,590	4%	Ovary	14,600	5%
Liver & intrahepatic bile duct	12,090	4%	Non-Hodgkin lymphoma	9,670	4%
Esophagus	11,490	4%	Leukemia	9,280	3%
Urinary bladder	10,180	3%	Uterine Corpus	7,780	3%
Non-Hodgkin lymphoma	9,830	3%	Liver & intrahepatic bile duct	6,070	2%
Kidney & renal pelvis	8,160	3%	Brain & other nervous system	5,590	2%
All Sites	**292,540**	**100%**	**All Sites**	**269,800**	**100%**

Table 1: Cancer Statistics 2009 (Jemal et al., 2009).

Androgen-Independent Prostate Cancer

Prostate cancer develops in the prostate gland, and the tumor cells can metastasize from prostate to other parts of the body, such as lymph nodes and bones. Disease diagnosis involves screening prostate specific antigen (PSA) levels present in serum. PSA is a member of the human kallikrein family of serine proteases (Heinlein and Chang, 2004). PSA is secreted by the prostate gland, and is found in small quantities in normal patients. It is a 33kDa, human kallikrein gene family member, and a serine protease that inhibits coagulation of the seminal fluid (Balk et al., 2003). The major substrates of PSA are seminogelin I and II that mediate gel formation in the semen (Balk et al, 2003). Other PSA substrates include fibronectin, TGFβ, PTH-related peptide, and plasminogen (Yousef et al., 2001). PSA was initially detected in the cytoplasmic vesicles and secretory granules of epithelial cells (Sinha et al., 1987). Serum PSA levels are often elevated in PrCa disease. PSA is a one of the major AR-dependent target genes, and is clinically monitored to detect early stage disease and the emergence of recurrent tumors post-therapy (Nash and Melezinek, 2000; Ryan et al., 2006). Thus, readouts of AR activity are critical for the assessment of disease progression and therapeutic outcome. PrCa initially presents as an AD disease, therefore hormone ablation therapy is often used. However, the disease progresses to a state where it becomes AI. In these cases the AR becomes hypersensitive and responds to very low levels of androgens. There are five different proposed pathways, hypersensitive pathway, promiscuous pathway, outlaw receptor pathway, bypass pathway, and lurker pathway, which can lead to AI PrCa (Feldman and Feldman, 2001). In the hypersensitive pathway, AR becomes hypersensitive to low levels of androgens (**Fig 1**). The factors that might contribute to hypersensitivity include mutations in the AR gene, or increased levels of co-activators. In the promiscuous pathway, the specificity of AR broadens due to the production of

other steroid hormones or even AR antagonists that can activate AR. In the outlaw pathway, there is production of outlaw receptors by crosstalk with growth factor signaling pathways, which can phosphorylate and activate AR. Unlike the three pathways discussed above, the bypass pathway is independent of AR. In this pathway, the pro-apoptotic mechanisms that are normally blocked by androgen are, instead, inhibited by a parallel mechanism such as the up-regulation of Bcl2. In the lurker cell pathway, AI PrCa cells are lurking in the normal prostate and become selected for by therapy (**Fig 1**).

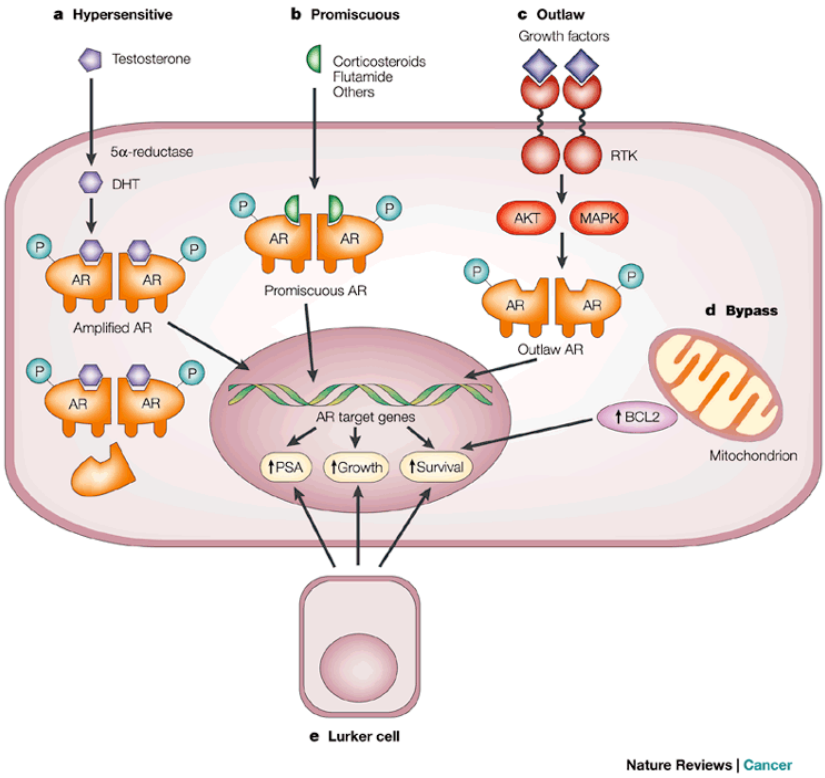


Figure 1: **Androgen-Independent Pathways in Prostate Cancer.** Five different pathways have been proposed to result in androgen-independent prostate cancer: hypersensitive pathway, promiscuous pathway, outlaw receptor pathway, bypass pathway, and lurker pathway (Feldman and Feldman, 2001).

By understanding the mechanism behind AI PrCa, specific therapies could be designed to target the cause behind this disease.

Androgen Receptor

AR plays a central role in PrCa development and disease progression. AR, is a member of the steroid hormone receptor family which is primarily responsible for mediating the physiological effects of androgens by binding to specific DNA sequences, known as androgen response elements (AREs) (Heinlein and Chang, 2004). AR, also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4), is a type of nuclear receptor, which is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone (Roy et al., 2001). The AR gene is located on the X chromosome at Xq11-12 (Roy et al., 2001). The main function of the AR is as a DNA-binding transcription factor, which regulates gene expression. AR appears to be involved in various aspects of the disease, from its commencement to the development of treatment resistance.

In the absence of androgens, AR usually stays in the cytoplasm in an inactive form. Upon androgen binding the AR, the AR forms a dimer, which moves from the cytoplasm to the nucleus (Roy et al., 2001; Marcelli et al., 2006) (**Fig 2**). The nuclear translocation of AR results in the binding of AR to ARE's in the regulatory region of target genes as a transcription factor (Heinlein and Chang, 2004).

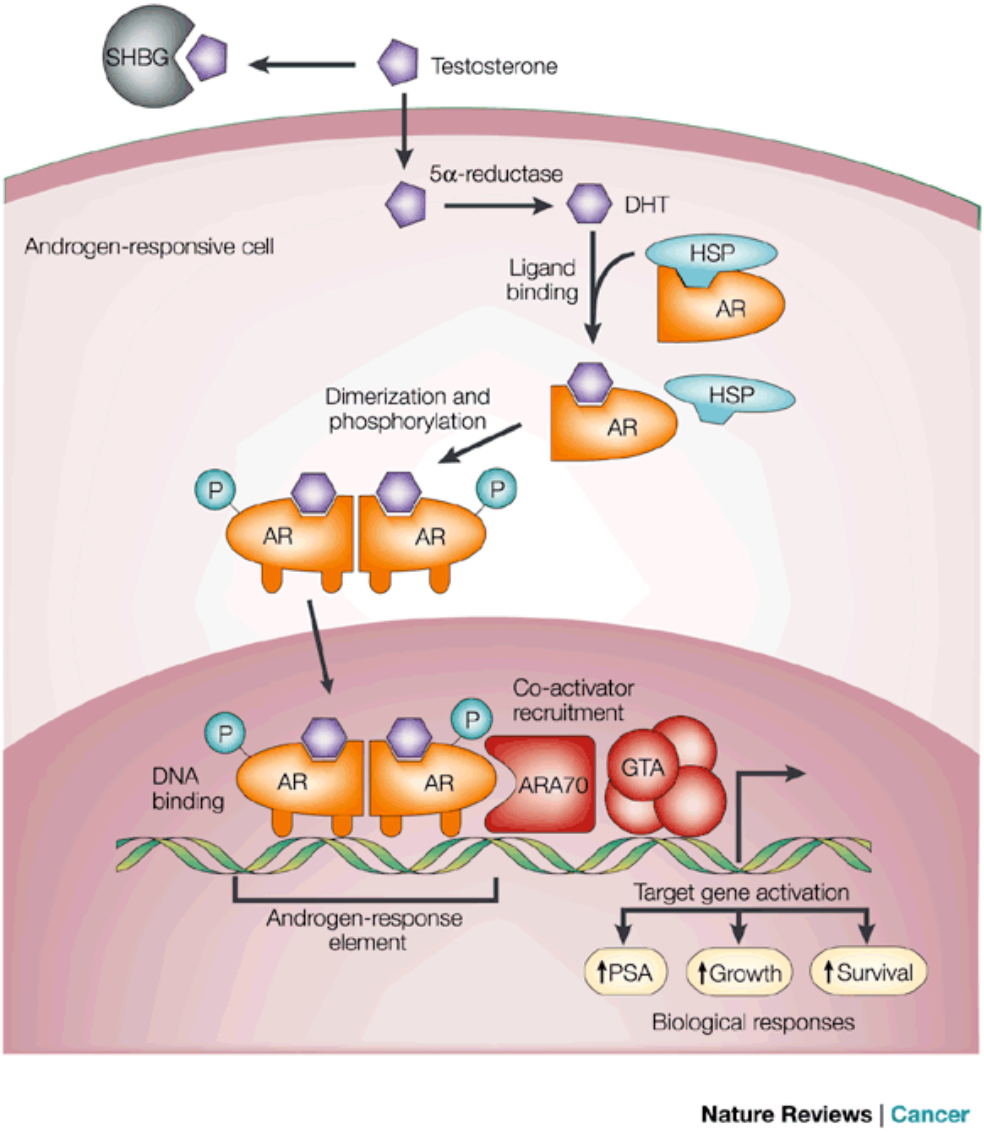


Figure 2: **Androgen Receptor Activation.** The unliganded AR localizes in the cytoplasm as a heteromeric complex with heat shock proteins (Hsp). Upon androgen binding, the AR undergoes conformational changes, gets dimerized and moves from the cytoplasm to the nucleus. The nuclear translocation of AR results in the binding of AR to androgen response elements in the regulatory region of target genes as a transcription factor (Feldman and Feldman, 2001).

The AR gene is composed of three domains: N terminal domain (NTD), DNA binding domain (DBD) and a C-terminal domain or ligand-binding domain (LBD) (**Fig 3**).

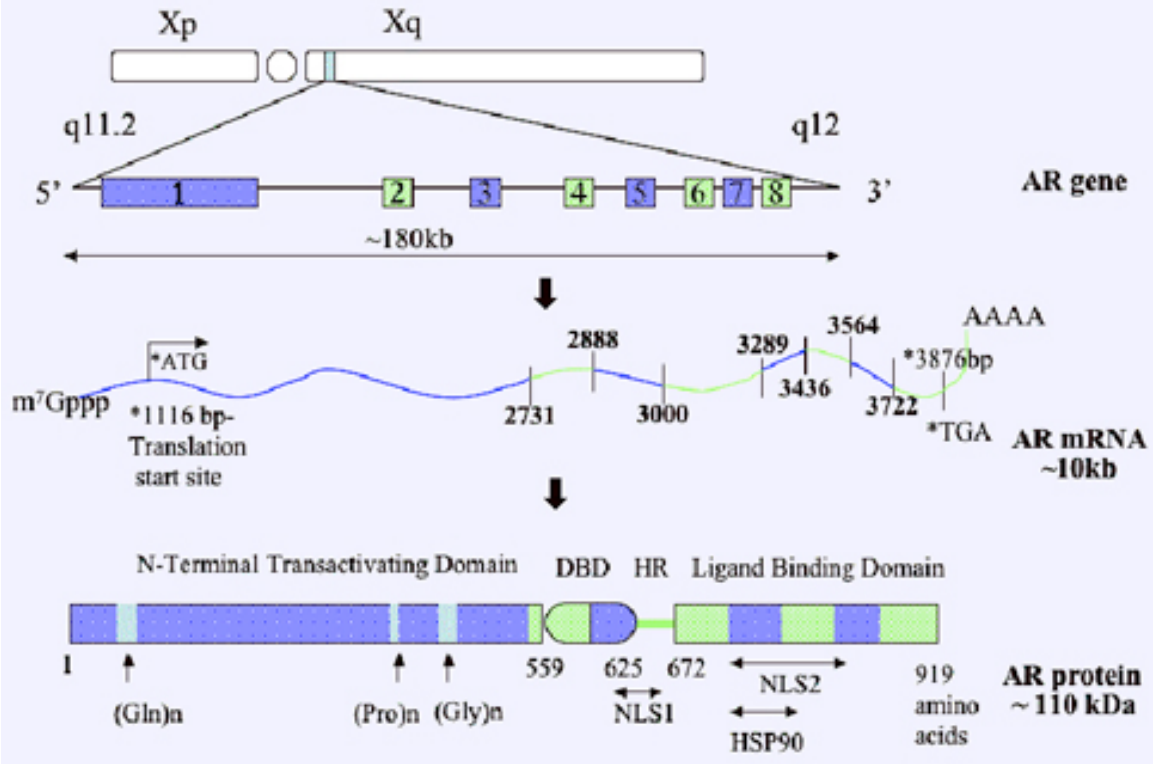


Figure 3: **Androgen Receptor Gene.** The AR gene is composed of three domains: N terminal domain (NTD), DNA binding domain (DBD) and a C-terminal domain or ligand-binding domain (LBD) (Litvinov et al., 2003).

The NTD and LBD domains contain the sub-domains called activation function-1 (AF-1), and activation function-2 (AF-2), respectively (Litvinov et al., 2003). AF-1 and AF-2 interact with several other co-factors that lead to transactivation of AR. AF-1 and AF-2 docking sites specifically recognize motifs like LXXLL and FXXLF which are present in different co-factors (van de Wijngaart et al., 2006). The FXXLF motif is also present in the NTD that interacts with the LBD of AR, although the function of this interaction is not clear. FXXMF is the common motif found in PAK6 and Supervillin. FXXLF is frequently found in ARA50 and ARA70 (Miyamoto et al., 1998), while the FXXFF motif is frequently found in Gelsolin, cdc37 (van de Wijngaart et al., 2006). These interactions of the AR with co-factors result in either

phosphorylation or dephosphorylation of AR that may cause either activation or inactivation, respectively, of AR in PrCa.

AR and Androgen-Independent PrCa

Activation of the AR plays a role in AI PrCa. Multiple mechanisms including stimulation by tyrosine kinases have been postulated. Several phosphorylation sites exist in the AR protein. Three tyrosine sites have been studied so far: tyr-267, tyr-363 and tyr-534 (Guo et al., 2006; Mahajan et al., 2007). It was found that the tyr-534 site was evolutionally conserved, which suggested that tyr-534 might be of functional importance in hormone refractory cancer (Guo et al, 2006). A mutation at tyr-534 diminished Src-induced tyrosine phosphorylation of AR (Guo et al., 2006). Hormone-refractory tumor lysates were blotted with anti-pAR (Y534) antibody, and an increase in pAR expression levels was found in AI tumors as compared to hormone naïve tumors. It was also reported that tyrosine phosphorylation regulates AR nuclear translocation and transcriptional activity (Guo et al., 2006). Hence, it can be hypothesized that AR tyrosine phosphorylation has a role in the progression of PrCa to the AI state.

Integrins and Prostate Cancer

Integrins have been found to play a major role in PrCa progression (Goel et al., 2008). Integrins play an important role in cell signaling. They are crucial regulators of differentiation, growth, survival, migration and invasion. The adhesive contacts between cells and the extra cellular matrix (ECM) are mediated by integrins (Fornaro et al., 2003). They are also involved in tumor growth and metastasis. Integrins are dimers of alpha and beta subunits (**Fig 4)**. Twenty four unique integrin dimers can form through different combinations of alpha and beta subunits.

α_{IIb}, α_6, β_1, β_3 and β_6 integrin subunits are up-regulated in PrCa, while α_v, α_7, α_3 β_{1C}, β_4 integrins are down-regulated (Goel et al., 2008).

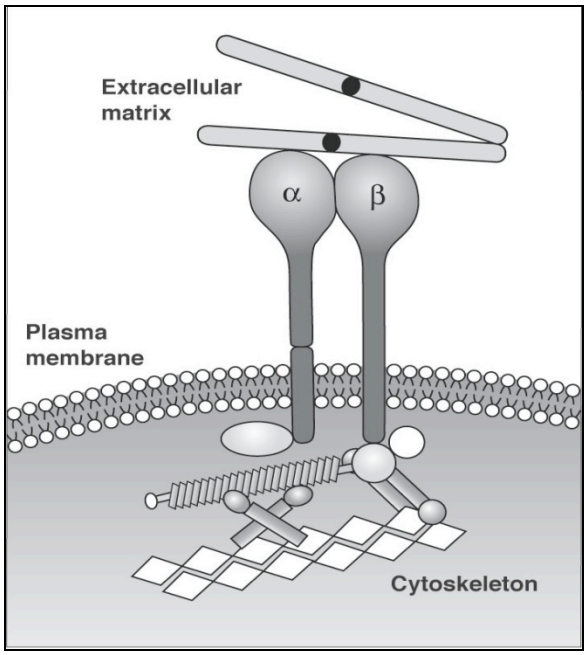


Figure 4: Integrins link extracellular matrix proteins to the cytoskeleton (Goel and Languino, 2004).

The integrin $\alpha_v\beta_6$ is neo-expressed in many carcinomas, such as colon (Agrez et al., 1994), breast (Arihiro et al., 2000), oral squamous carcinoma (Jones et al., 1997), ovarian cancer (Ahmed et al., 2002), and *in vitro* studies suggest that $\alpha_v\beta_6$ promotes epithelial cell spreading, migration, and proliferation (Breuss et al., 1995). siRNA-mediated down-regulation of AR in $\alpha_v\beta_6$ integrin expressing LNCaP cells shows a strong inhibition of PSA expression levels (Wang et al., unpublished data), suggesting that $\alpha_v\beta_6$ integrin mediates specific pathways that are AR dependent, and contributes to increased AR transcriptional activity. Immunofluorescence studies in LNCaP cells expressing $\alpha_v\beta_6$ integrin showed that AR was localized in the nucleus, even under starvation conditions (Wang et al., unpublished data). This suggests that $\alpha_v\beta_6$ integrin

promotes AR translocation and transcriptional activity via an AI mechanism.

Integrins promote a wide range of adhesion-dependent effects, including proliferation, survival, migration or invasion. In particular $\alpha_V\beta_3$ integrin is associated with the progression of a variety of human tumors, including melanomas and carcinomas of the prostate, breast, cervix and pancreas. It enhances tumor cell migration, survival and increased growth factor release. It has been previously shown that $\alpha_V\beta_3$ integrin strongly supports Src-mediated oncogenic transformation through an interaction at the β_3 cytoplasmic tail (Huvneers et al., 2007). Src phosphorylates $\alpha_V\beta_3$ integrin, but this phosphorylation is not required for $\alpha_V\beta_3$ integrin-mediated support for tumor formation (Huvneers et al., 2007).

Thus, integrins regulate Src-mediated oncogenic transformation and various aspects of morphological transformation through different pathways. It has also been reported that $\alpha_V\beta_3$ integrin promotes anchorage-independence through Src mediated pathways (Desgrosellier et al., 2009). Hence, to define the integrin-Src pathway involved in PrCa disease progression will be of utmost importance.

Src Regulation of AR

Src and its function in cellular mechanisms

Src is a non-receptor tyrosine kinase originally discovered in the 1970s (Martin, 2001). It is a 60 kDa protein that consists of three domains, an N-terminal SH3 domain, a central SH2 domain and a tyrosine kinase domain (Martin, 2001) (**Fig 5**). The Src kinase family is comprised of nine members: Src, Lyn, Fyn, Yes, Lck, Hck, Blk, Fgr and Yrk (Fizazi, 2007).

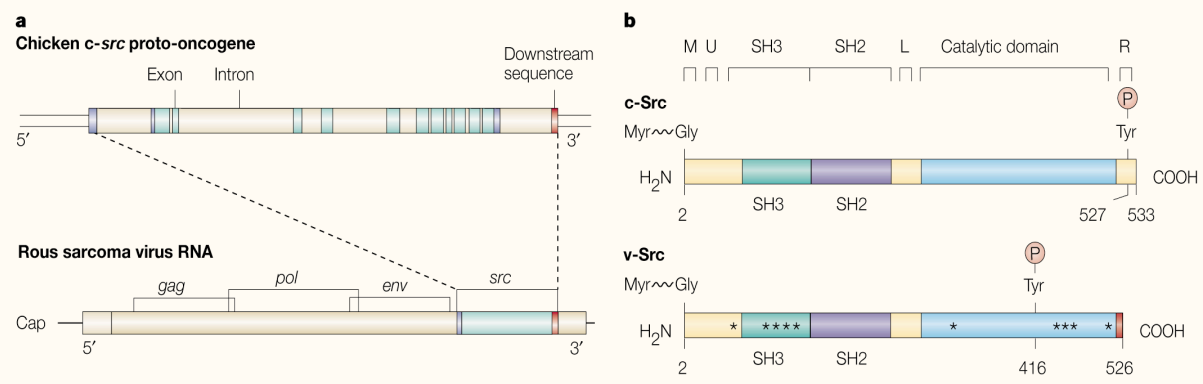


Figure 5: **Src Gene and Structure. a)** The v-*Src* and c-*Src* genes. The v-*Src* gene (bottom) lacks the introns present in c-*Src* (top), and in v-*src* the 3'-terminal sequence of c-*Src* is replaced by a downstream sequence. In addition, several activating point mutations are present within v-*Src* (not shown). A non-defective Rous sarcoma virus strain, such as that shown, contains the viral structural genes (*gag*, *pol* and *env*), plus the viral-transforming gene, v-*Src*. The Bryan strain is replication-defective and lacks a functional *env* gene; some variants of the Bryan strain are also defective in *pol*. **b**) Structural features of c-Src and v-Src proteins. Residue numbers and other details are specific to chicken c-Src and the v-Src protein of the Schmidt–Ruppin strain of RSV. The amino terminus of the protein is myristoylated at Gly2. Positions of the membrane-binding (M), unique (U), SH2, SH3, linker (L), catalytic and regulatory (R) domains of c-Src are shown; the substituted carboxy-terminal sequences in v-Src are shown in red. Asterisks represent amino acid substitutions in v-Src (Martin, 2001).

Auto-inhibition of the Src kinase domain results from the interactions between SH2 (Src Homology domain 2) and SH3 (Src Homology domain 3) domains. Auto-phosphorylation at tyr-416, and de-phosphorylation at tyr-527, are required for activation of c-Src (Cooper et al., 1986). Csk (Carboxy-terminal Src kinase) phosphorylates at site tyr527, which results in inactivation of c-Src (Okada and Nakawaga, 1989). Phosphorylation at site tyr-527 was found to be the key event in regulating c-Src kinase activity. Deletion of this site could account for the oncogenic activation of Src, and this variant is referred to as v-Src (Martin, 2001) (**Fig 6**).

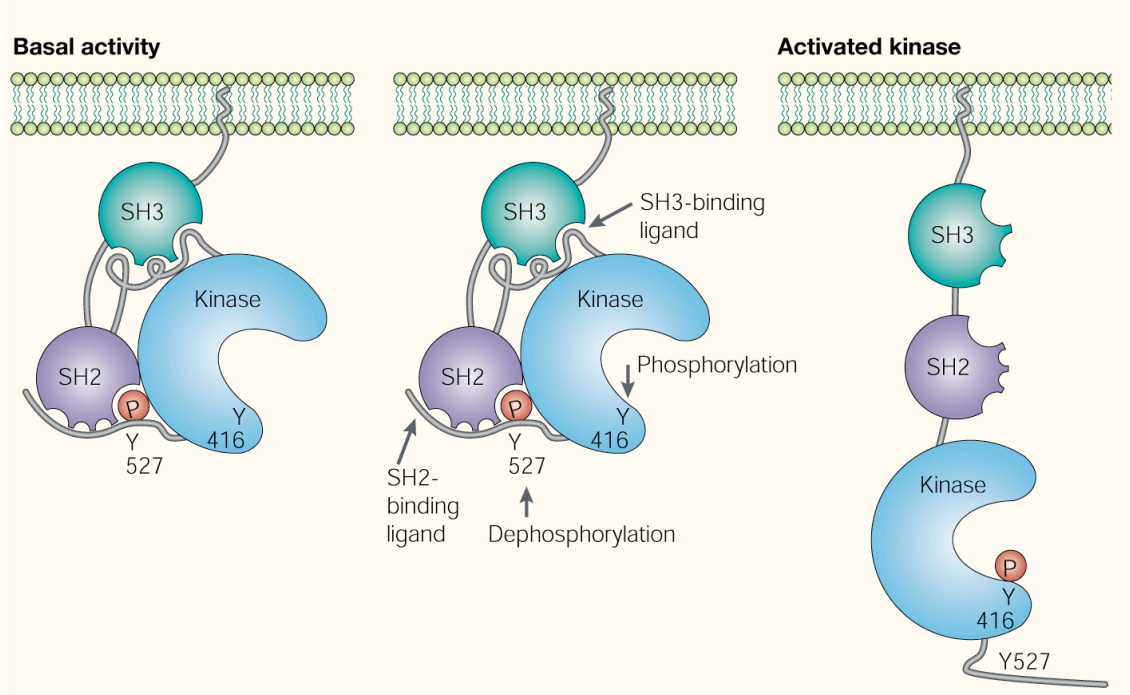


Figure 6: **Src Activation.** Src protein consists of three domains, an N-terminal SH3 domain, a central SH2 domain and a tyrosine kinase domain. Auto-inhibition of the kinase domain results from the interactions between SH2 and SH3 domains. Auto-phosphorylation at tyrosine 416 and dephosphorylation at tyrosine 527 are required for activation of c-Src. Deletion of Tyr527 accounts for the oncogenic activation of Src and the variant is referred to as v-Src (Martin, 2001).

Src has been found to be involved in the control of many functions, including cell adhesion, growth, movement and differentiation (Dehm and Bonham 2004). Src can be activated by different surface receptors, like receptor tyrosine kinase, G-protein coupled receptor, and integrins (Martin, 2001). Growth factors like PDGF (platelet derived growth factor) activate c-Src by recruiting it to surface receptors like PDGFR which results in further downstream intracellular signaling (Ralston and Bishop, 1985; Kypta et al., 1990) (**Fig 7**).

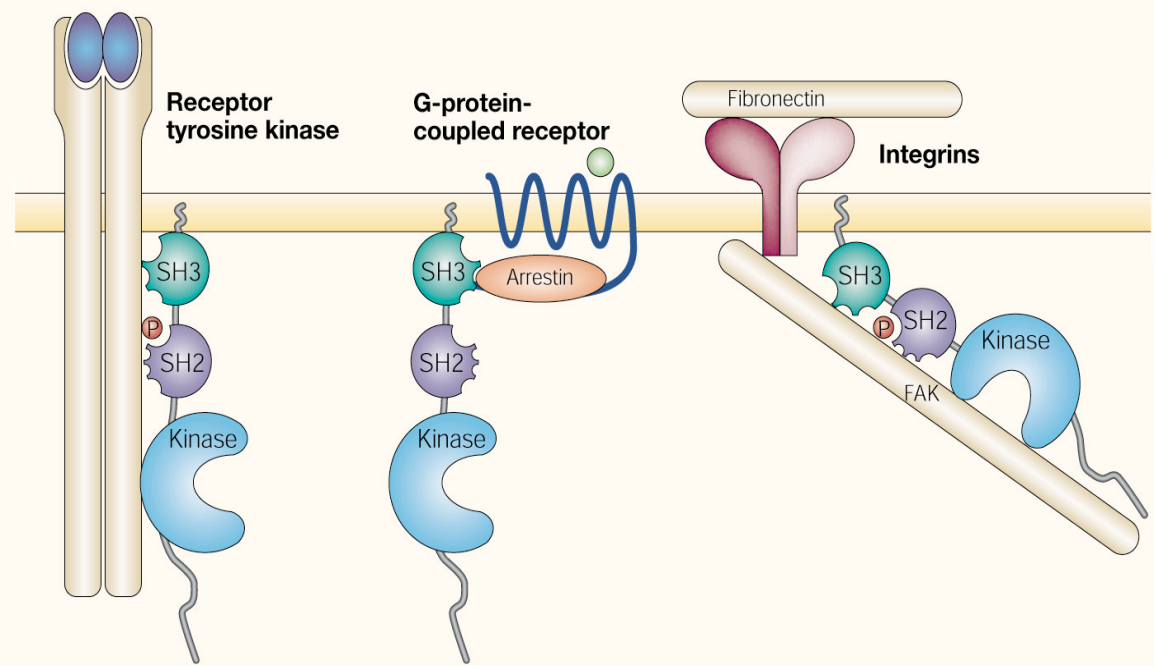


Figure 7: **Src Activation and Function.** Src can be activated by different surface receptors, like receptor tyrosine kinase, G-protein coupled receptor, and integrins. Src signaling is found to be involved in the control of many functions including cell adhesion, growth, movement, and differentiation (Martin, 2001).

Many substrates of src have been characterized. Several of these Src substrates, including FAK (focal adhesion kinase) (Lipfert et al., 1992), Cas (Crk and Src associated substrate) (Sakai et al., 1994), and Stat3 (Turkson et al., 1998) were found to be important for integrin signaling and growth regulation. Src was also found to play a role in mitogenesis, as it is found to be involved in the MAPK and PI3K-mTOR pathways (Penuel and Martin, 1999). Src activity is up-regulated in lung, colon, breast, prostate and pancreatic cancer (Dehm and Bonham, 2004). Hence, Src can play an important role in PrCa disease progression.

Src and Prostate Cancer

Multiple studies have supported a role for Src tyrosine kinase and its family members in

PrCa (reviewed in Fizazi, 2007). Src may contribute to AI-PrCa by mediating signals from various factors, including growth factors and chemokines (Lee et al., 2004). It has been reported that Src may be involved in the initial transition from an AD to AI state (Lee et al., 2001; Unni et al., 2004). Several studies show that inhibition of Src signaling decreases proliferation, invasion and migration of PrCa cells that have detached from the primary site (Recchia et al., 2003), suggesting involvement of Src in PrCa progression. It was found that Src kinase activity gets up-regulated in androgen refractory PrCa (Guo et al., 2006). Tumor lysates from hormone refractory prostate xenograft models were studied, and it was found that there were high expression levels of p-Src in hormone-refractory prostate xenograft tumors compared to their hormone sensitive counterparts (Guo et al., 2006). Studies were also done in human prostate tissue samples from hormone-naïve and hormone-refractory patients. This may suggest a possible role of Src in AI-PrCa. Increased Src expression was also found in TRAMP mouse models of PrCa (Asim et al., 2008). Together, these studies confirm a crucial role of Src in PrCa disease and its progression.

Src and its potential role in integrin-mediated signaling

A signaling partnership is reported among integrins, Src, and FAK, which subsequently can result in recruitment of proteins containing SH2 domains to trigger adhesion-induced cellular responses (Hanks and Polte, 1996). FAK phosphorylation at tyr-397 mediates a direct interaction with the Src SH2 domain, which results in activation of Src (Hanks and Polte, 1996). Recently it has been shown that $\alpha_V\beta_3$ integrin mediates anchorage-independence and tumor cell malignancy in a Src-dependent manner (Desgrosellie et al., 2009). In breast cancer, $\alpha_6\beta_4$ integrin was found to promote cancer cell-motility and invasion through a mechanism involving

Src, AKT and NFAT (Kim et al., 2009). Hence, Src tyrosine kinase is found to play a significant role in integrin-mediated signaling through indirect association with integrins in cancer progression and metastasis.

Src phosphorylation activates AR

Src interacts and phosphorylates AR at its tyr-534 site (Guo et al., 2006; Kraus et al., 2006), which is responsible for AR activation. By transfecting PrCa cell lines with kinase active (SrcY527F) and dominant-negative (SrcK295M) constructs, luciferase activity was studied using a PSA-luc promoter. It was found that SrcY527F induced the activity of the PSA promoter, suggesting that Src may directly modulate AR transcriptional activity (Guo et al., 2006). Specific Src inhibitor SU6656 reduced the AR-driven luciferase activity (Guo et al., 2006). Hence, this suggests that Src interaction with AR results in AR activation.

Though it has been shown that Src-induced phosphorylation regulates the translocation of AR from the cytoplasm to the nucleus, it is still unclear how tyrosine kinase phosphorylation of AR induces its translocation (Guo et al., 2006). It is possible that phosphorylation may induce conformational changes in the ligand-binding domain of AR, and therefore, even in the presence of very low levels of androgen (as in androgen refractory cancer), the androgen responsive genes are activated and AR transcriptional activity takes place.

Understanding the mechanism behind AI PrCa will help in building a novel and effective strategy to inhibit PrCa growth and metastasis.

MATERIALS AND METHODS

Reagents

Synthetic androgen R1881 was obtained from Perkin-Elmer. The following rabbit polyclonal antibodies (pAbs) were used for immunoblotting: pSer81-AR (Dr. Daniel Gioeli), p-Src (Y416, Cell Signaling), AKT1/2/3 (H-136), ERK1 (C16, Santa Cruz), PSA (DAKO Cytomation), and goat polyclonal RCC1 (Santa Cruz). The following mouse monoclonal antibodies (mAbs) were used against androgen receptor (AR) (AR441, Santa Cruz), c-Src (Santa Cruz and Cell Signaling), and α-tubulin (DM1A, Sigma) for immunoblotting. Purified non-immune mouse IgGs (mIgG) and rabbit IgGs (rIgG) (Pierce) were used as negative controls. For immunofluorescence, rabbit pAb AR (N20, Santa Cruz), p-Src (stimulatory site Y416, Cell Signaling), and mAb for c-Src (Santa Cruz) were used. HRP-conjugated secondary antibodies to mouse, rabbit (Cell Signaling) and goat (Santa Cruz) were used for immunoblotting. Rhodamine and fluorescein-conjugated secondary antibodies used for immunofluorescence were purchased from Jackson Laboratories. 4', 6-diamidino-2-phenylindole (DAPI) used for immunofluorescence was obtained from Molecular Probes, Invitrogen.

Androgen Deprivation and Androgen Stimulation

Androgen Deprivation: Cells were cultured in media containing 2% charcoal stripped serum (CSS) for 24 hrs. This serum was prepared by treating fetal bovine serum (FBS) with HCl-treated charcoal. This medium does not have detectable levels of androgens or steroids that might activate AR (based on our laboratory's internal standard data).

Androgen Stimulation: Cells were cultured in media containing 2% CSS for 24 hrs, and then stimulated with R1881 (1 nM) for 2 hrs or 24 hrs depending upon the experimental conditions.

Cells and Culture Conditions

LNCaP (AD) and C4-2B (AI) cells were obtained from ATCC. Cells stably transfected with either $\alpha_V\beta_3$ or $\alpha_V\beta_6$ integrin genes were obtained from Zheng et al. (1999) and Manes et al. (2003). Cells were cultured in RPMI (GIBCO, Invitrogen) supplemented with 5% FBS (Gemini Bioproducts Inc.), 2 mmol/L glutamine, 100 μg/ml streptomycin-100U/ml penicillin, 0.1 mmol/L nonessential amino acids and 1 mmol/L sodium pyruvate (GIBCO, Invitrogen). RPMI supplemented with 2% CSS was used for starvation of the cells. For transient transfection experiments, cells were transfected with wild-type Src (wt-src) or dominant negative Src (SrcK298M) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Sub-Cellular Fractionation

A total of $3x10^6$ cells were trypsinized, and the pellet was washed once with 1XPBS. The pellet was resuspended in Buffer A (10 mM HEPES at pH 7.9, 10 mM KCL, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_2VO_3, 0.1% Triton X-100, protease inhibitors, 0.2 mM PMSF), vortexed and kept on ice for 30 min. Cell homogenate was spun at 3,300 RPM for 15 min at 4^oC using a microcentrifuge. The supernatant obtained from the above step was removed and stored as cytosolic extract.

The cytosolic extract can be further clarified by centrifuging at 16,000 RPM, to remove any contaminants. The pellet obtained (nuclear fraction) was incubated in an equivalent volume of buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and protease inhibitors) for 30 min, and centrifuged at 13,000 RPM for 15 mins to remove contaminants such as chromatin and nuclei. The supernatant obtained from the above centrifugation was stored as the "nuclear fraction". Note: All steps were performed under ice-cold conditions i.e. at 4^{o}C (Zou et al., 2002).

Immunoblotting

The protein concentration for each cell lysate was quantified with a BCA protein assay kit (Pierce) to allow equal loading on the gel. Proteins were separated by denaturing SDS-Polyacrylamide Gel Electrophoresis, then transferred to PVDF membrane (Millipore). Membranes were blocked for 3 hrs with 5% nonfat dry milk-TBST or 5% bovine serum albumin (BSA)-TBST buffer. Primary antibodies were diluted in blocking buffer according to the manufacturer's protocols, and subsequently incubated with the blots for 2 hr at room temperature, or overnight at 4°C. Primary antibodies against pSer81-AR, p-Src, and c-Src were diluted in 5% BSA-TBST buffer. The membranes were washed three times with TBST and incubated with a 1:2000 dilution of the appropriate horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG in the blocking buffer for 2 hr at room temperature. After washing, the blots were developed with enhanced chemiluminescence (Boston Bioproducts) and exposed to BioMax X-ray film (ISC Bioexpress) (Fornaro et al., 1998; Goel et al., 2004).

Immunofluorescence

Cells were grown on cover-slips coated with poly-L-lysine (1 mg/ml) in RPMI supplemented with 5% FBS. After starvation for 24 hrs in RPMI containing 2% CSS medium, cells were grown in the presence of ethanol (EtOH) or R1881 (1 nM or 0.1 nM) for 2 hrs. Cells were fixed in 4% paraformaldehyde for 5 min, and washed twice with phosphate buffer saline (1X PBS). The cells were permeabilized with 1X PBS containing 0.2% Triton-X100, and blocked with 5% BSA in 1X PBS for 1 hr at room temperature. Cells were incubated with primary antibodies diluted in 5% BSA-PBS buffer for 1 hr at room temperature. After washing with 1X PBS, the cover-slips were incubated with goat anti-rabbit rhodamine or anti-mouse fluorescein-conjugated secondary antibody for 1 hr at room temperature. Cells were washed twice for 5 min each with 1X PBS. Finally, the cells were counterstained with DAPI to visualize nuclei before mounting. The slides were visualized using an Olympus IX71 inverted microscope with IPLab V3.55 (Scanalytics, Inc, Rockville, MD), magnification 400X.

Cell Adhesion Assay

Cell adhesion assays have been described previously (Zheng et al., 1999). Stable transfected $\alpha_V\beta_6$-LNCaP and $\alpha_V\beta_3$-LNCaP cells were starved overnight in a serum free RPMI culture medium. Cells were seeded on the culture plates coated with FN (3 mg/ml), LAP-TGF-β (0.5 mg/ml) or BSA. Cells (10^6) were plated on the substrate for 1 hr in serum-free RPMI medium before collecting cell lysates.

Small Interfering RNA Transfection

LNCaP cells were transfected with β_{1A} and β_{1C} integrin siRNA duplexes (IDT Inc.). The sequences of β_{1A} siRNA were: sense strand, 5'-AUGGGACACGGGUGAAAAUTT-3'; antisense strand, 5'-AUUUUCACCCGUGUCCCAUTT-3'. The sequences of β_{1C} siRNA were: sense strand, 5'-CCUCUGACUUCCAGAUUCCTT-3'; antisense strand, 5'-GGAAUCUGGAAGUCAGAGGTT-3'. siRNA duplexes were transfected using Oligofectamine (Invitrogen) at a final concentration of 20 nM. After two rounds of transfection, cells were harvested 48 hrs post-transfection.

Statistical Analysis

For immunofluorescence experiments detecting AR, p-Src, c-Src, or PSA, a total of 700 cells were counted for each time point under different experimental conditions. Each experiment was repeated three times. The average and standard deviation values were calculated for every experiment. Statistical analysis was performed pairwise using the Student's t-test. All p-values were based on two-tailed tests.

RESULTS

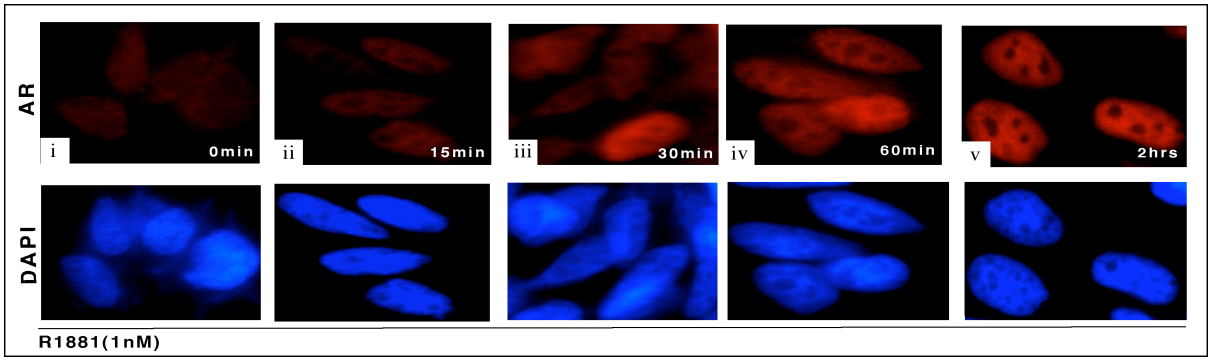
Androgen Promotes Nuclear Localization of AR

Steroid hormone receptors are ligand–activated transcription factors. The intracellular movement and nucleo-cytoplasmic trafficking of steroid hormone receptors has been well researched, and the data indicate that unliganded receptor localizes in the cytoplasm as a heteromeric complex with heat shock proteins (Roy et al., 1999; Roy et al., 2001). Upon ligand binding, the receptor undergoes conformational changes, homodimerization, nuclear translocation, and initiation of target gene expression. AR is localized in the nucleus upon stimulation with dihydroxy-testosterone (DHT), and is exported back into the cytoplasm upon DHT withdrawal (Roy et al., 1999; Roy et al., 2001).

In the first set of experiments of this thesis, the dynamic trafficking of AR in the LNCaP (AD) cell line was studied over a period of time under starvation and stimulation conditions. AR translocation was evident as soon as 15 minutes after stimulation with synthetic androgen R1881 (**Fig 8A**), and accumulated in the nucleus after 2 hrs of stimulation with R1881. Under starvation conditions, AR was predominantly localized in the cytoplasm (**Fig 8B**). These results confirm the previously published data (Roy et al., 2001; Marcelli et al., 2006), and validate the androgen sensitivity of our LNCaP/R1881 system.

Fig 8

(A)



(B)

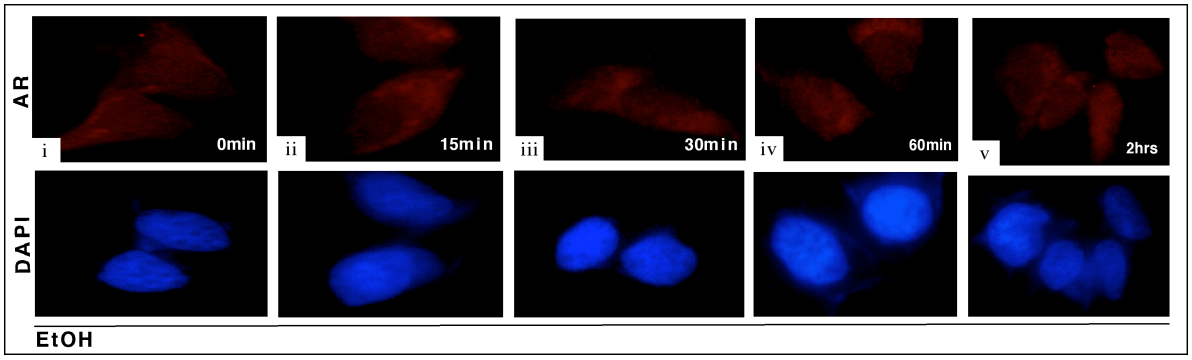


Figure 8: AR Nuclear Localization After R1881 Stimulation. LNCaP (AD) cells were seeded on poly-L-Lysine (1 mg/ml)-coated cover-slips for two days. Cells were starved for 24 hrs, then stimulated with R1881 (1 nM) (upper panel), or ethanol (EtOH) (vehicle) (lower panel) for different time points. Immunofluorescence was performed by staining the cells with anti-AR (N20). Nuclear staining was performed using DAPI. Pictures were taken at different time points as indicated.

The AR nuclear export mechanism was also studied over a period of time. Cells were stimulated for 2 hrs with R1881, then R1881 was removed. Pictures were visualized using IPLab V3.55 at different time points (**Fig 9**). It was found that AR localized in the nucleus after 2 hrs of stimulation with R1881, but gradually started to move out from the nucleus after withdrawal of R1881. At 2 hrs after the withdrawal of R1881, weak diffused cytoplasmic and nuclear AR staining patterns were observed. Hence, I conclude that AR shuttles to and from the

nucleus, and its movement depends upon androgen stimulation or withdrawal. AR activity is known to be high in PrCa, which is a result of AR nuclear translocation. In recurrent PrCa, AR activity is found high among patients exhibiting an androgen-independent phenotype (Feldman and Feldman 2001). This androgen-independent AR activity can be the result of unmonitored AR activation through several co-factors, growth factors, integrins, or other signaling mechanisms.

Fig 9

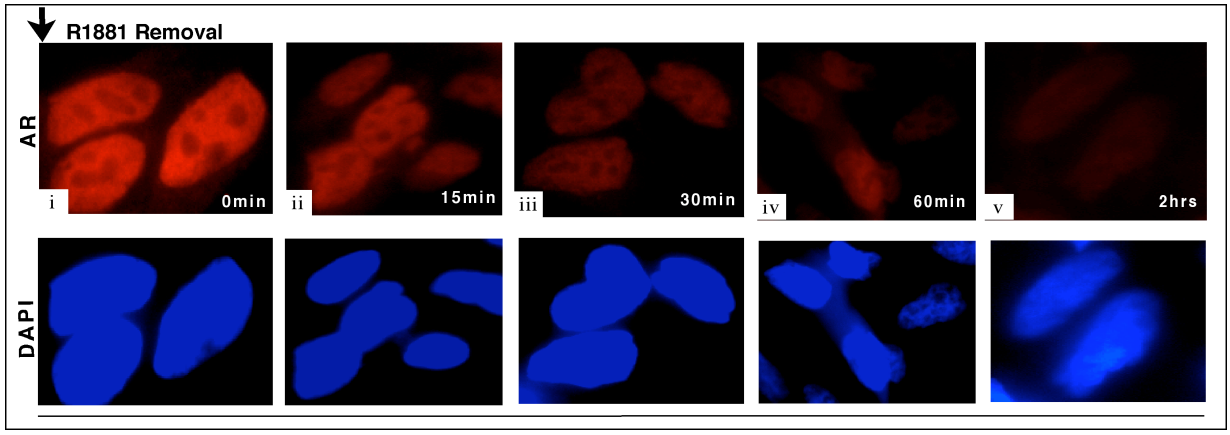


Figure 9: AR Dynamic Trafficking. LNCaP (AD) cells were seeded on poly-L-Lysine (1 mg/ml)-coated cover-slips for two days. Cells were starved for 24 hrs, then stimulated with R1881 (1 nM) for 2 hrs. R1881 was removed and immunofluorescence was performed by staining cells with anti-AR (N20) at different time points as indicated. Nuclear staining was performed using DAPI. Pictures were taken at different time points.

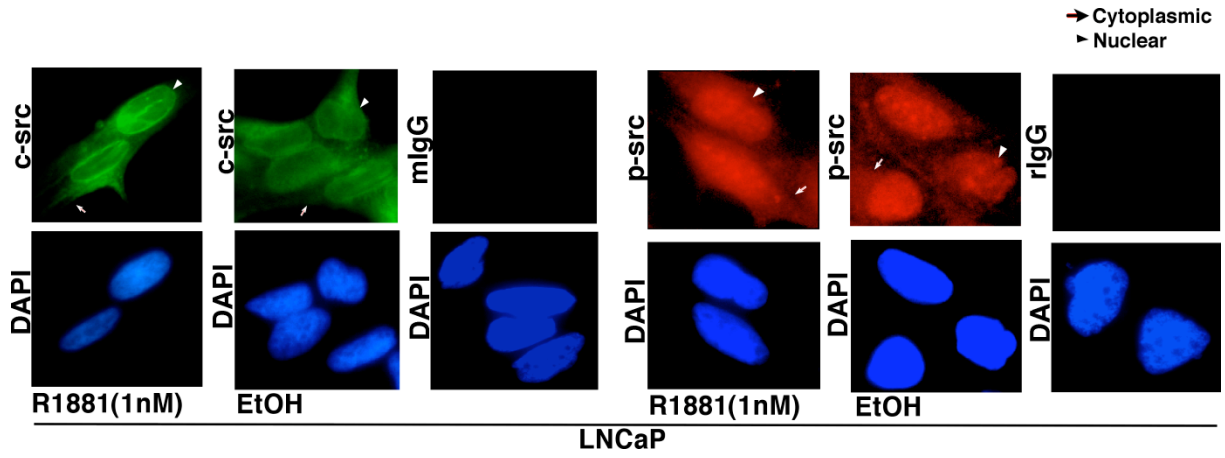
Androgen Does Not Affect p-Src or c-Src Localization Based on Immunofluorescence

AR cooperates with many co-factors, and recently Src was found to be an important co-factor that affects AR activity in PrCa (Guo et al., 2006). Src, which is known to be involved in tumor growth and metastasis in PrCa, affects AR translocation and activity *in vitro* (Gioeli et al., 2002). During *in vivo* studies, hormone-refractory PrCa tumor xenografts exhibited increased Src phosphorylation when compared with hormone-sensitive xenografts. To confirm a definite

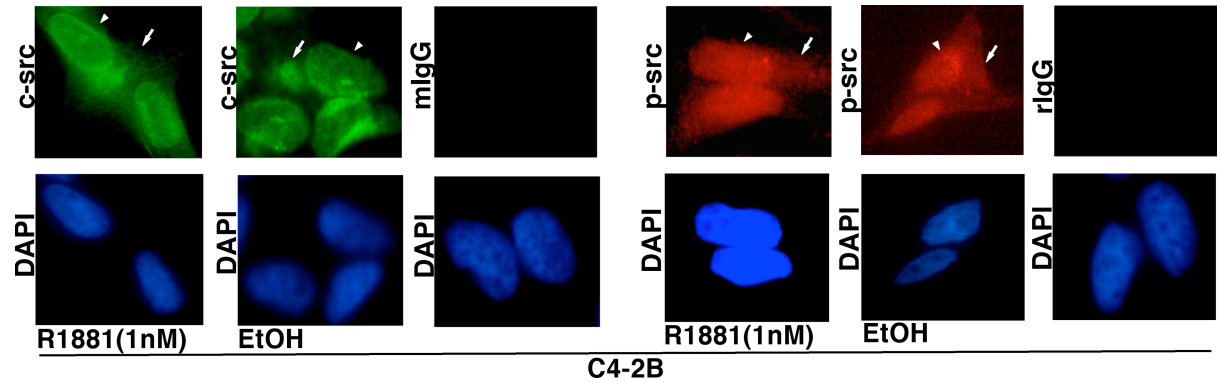
role of Src in the regulation of AR activity in PrCa, I investigated the effect of R1881 AR ligand on Src phosphorylation at stimulatory site Y416 (p-Src), and total cellular scr (c-Src) localization and expression in LNCaP (AD) and C4-2B (AI) PrCa cells in the presence and absence of R1881. p-Src and c-Src were found to be localized in the cytoplasm as well in the nucleus irrespective of R1881 stimulation (**Fig 10**). No statistically significant differences ($p>0.05$) were observed (**Fig 10C**). Hence, I conclude that based on immunofluorescence, androgen stimulation does not affect p-Src or c-Src localization in PrCa cells.

Fig 10

(A)



(B)



(C)

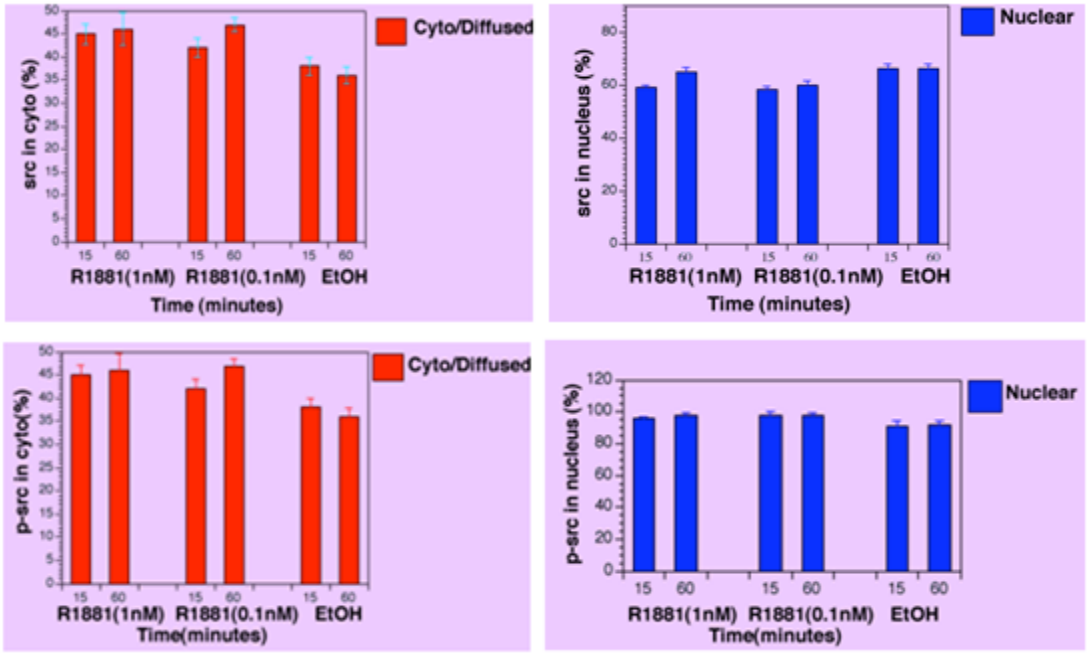


Figure 10: Based on Immunofluorescence Microscopy p-Src and c-Src Are Localized in the Cytoplasm and Nucleus Irrespective of Androgen Status. LNCaP (AD) **(Panel A)** and C4-2B (AI) **(Panel B)** cells were seeded on poly-L-Lysine (1 mg/ml) coated cover-slips for 2 days. Cells were starved for 24 hrs, then stimulated with R1881 (1 nM) or ethanol (vehicle) for 15 min or 60 min. Immunofluorescence was performed by staining cells with antibodies against c-Src or p-Src. Nuclear staining was performed using DAPI. **(Panel C)** The graphs represent the mean of triplicates ± SD, while p values are based on three different experiments. Error bars denote standard error. There were no statistically significant differences, $p>0.05$.

Androgen Affects Src and AR Activation Based on Cell Fractionation

Using sub-cellular fractionation, c-Src and p-Src expression levels were analyzed in the presence and absence of R1881. R1881 stimulation increased p-Src levels in both LNCaP (AD) and C4-2B (AI) cells (**Fig 11**). The localization of c-Src and p-Src were also studied under these conditions. In agreement with the immunofluorescence results, R1881 stimulation did not affect the localization of total cellular c-Src when analyzed by cell fractionation. c-Src was found in both cytoplasmic and nuclear fractions, independent of androgen status, in either AD or AI cell

lines. In contrast to c-Src, the levels of p-Src clearly increased in the nucleus (and lesser so in the cytoplasm) after androgen stimulation, for either LNCaP cells (left panel, third row) or C4-2B cells (right panel, top row), indicating that androgen affects Src *activation* in both cell lines.

Fig 11

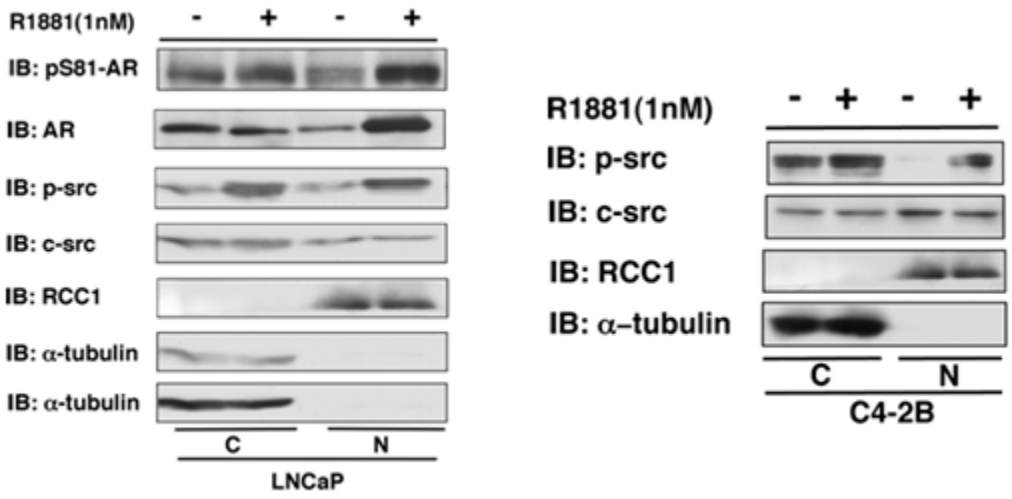


Figure 11: Nuclear Localization of Src and AR. LNCaP (AD) and C4-2B (AI) cells were starved for 24 hrs using RPMI containing 2% CSS followed by stimulation with R1881 (1 nM) or ethanol for 24hrs. Cell lysates were subjected to fractionation, cytoplasmic and nuclear fractions were collected. The collected fractions were subjected to immunoblotting (IB) using mAbs against AR (AR441), c-Src and α-tubulin (DM1A). pAbs were used against p-Src (Y416), pS81-AR and RCC1 (C-20). α-tubulin was used as a loading control for the cytoplasmic fractions. RCC1 was used as a loading control for the nuclear fractions. C, cytoplasmic fraction; N, nuclear fraction.

The cell fraction technique was also applied to AR. AR was found to localize in the nucleus of LNCaP (AD) (**Fig 11**, left panel, second row; **Fig 12A**, top row) and C4-2B (AI) cells (**Fig 12A**, top row) upon stimulation with R1881 for 2 hrs, as compared with non-stimulated (vehicle treated) cells. I also found that upon stimulation with R1881, pS81-AR (an AR site indirectly phosphorylated by Src) (Yang et al., 2009) also localizes in the nucleus of LNCaP cells (**Fig 11**, left panel, top row). In addition, increased expression levels of AR were found in

the cytoplasmic fractions from vehicle treated cells as compared to cytoplasmic fractions from R1881 treated cells. Therefore, it can be suggested that upon ligand binding AR becomes activated (p-AR) and localizes to the nucleus.

The cell fractionation technique was also applied to PSA. Translocation of AR from the cytoplasm into the nucleus is required for AR to exert its transcriptional activity (Heinlein and Chang 2004). PSA is a gene strongly regulated by AR activity, therefore, I examined PSA levels in cytoplasmic and nuclear fractions in R1881 treated and untreated cells. High PSA levels were found in nuclear and cytoplasmic fractions of R1881-treated LNCaP (AD) cells, and no expression of PSA was found in vehicle treatment (**Fig 12A**, left panel, second row). In C4-2B (AI) cells, PSA was found in approximately equal amounts in nuclear and cytoplasmic fractions, in either androgen-stimulation or deprivation conditions (**Fig 12A**, right panel, second row). Immunofluorescence studies done for PSA localization in LNCaP (AD) and C4-2B (AI) cells upon androgen stimulation and deprivation revealed similar results as obtained by fractionation experiments (**Fig 12B**). Overall, these data show that androgen stimulation activates Src (p-Src), increases nuclear levels of AR, and increases cellular levels of PSA. The nuclear localization of PSA previously thought only to be a secretory protein is novel. The nuclear co-localization of AR, Src, and PSA might play an important role in disease progression.

Fig 12A

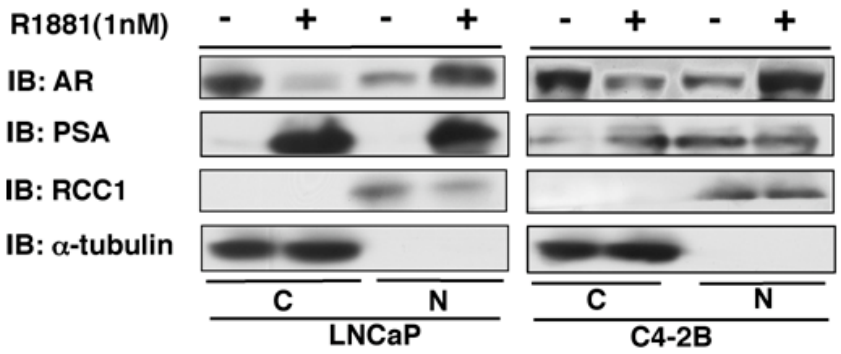


Fig 12B

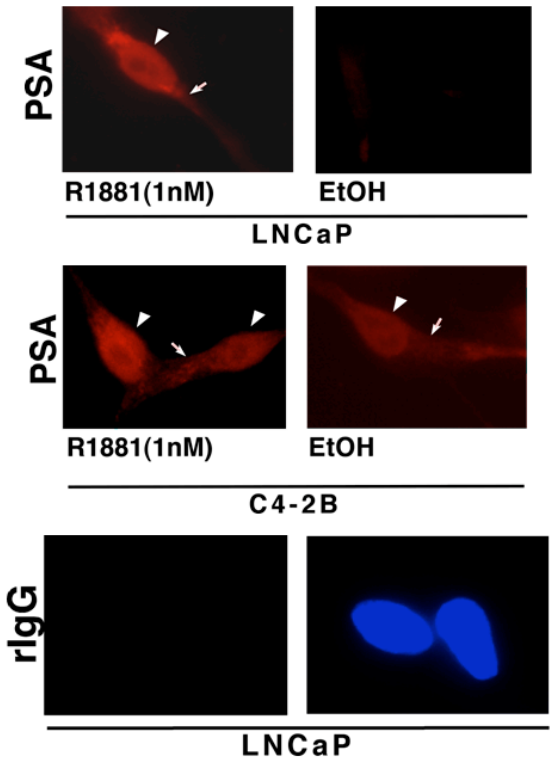


Figure 12: AR and PSA Nuclear Translocation. **(A)** LNCaP (AD) and C4-2B (AI) cells were starved for 24 hrs using RPMI containing 2% CSS, followed by stimulation with R1881 (1 nM) or ethanol for 24 hrs. Cell lysates were subjected to fractionation, then cytoplasmic and nuclear fractions were collected. The collected fractions were subjected to IB)using mAbs against AR (AR441) and α-tubulin (DM1A). **(B)** LNCaP

(AD) and C4-2B (AI) cells were seeded on poly-L-Lysine (1 mg/ml)-coated cover-slips for two days. Cells were starved for 24 hrs and stimulated with R1881(1 nM) or ethanol (EtOH). Immunofluorescence was carried out by staining cells with pAb against PSA. rIgG was used as a negative control for staining. Nuclear staining was performed using DAPI.

Src Differentially Affects AR Activity in Androgen-Dependent and Independent PrCa Cell Lines

To study the effect of Src kinase on AR transactivation, transfection studies were performed in the LNCaP (AD) cell line using plasmids encoding wt-src or SrcK298M. The plasmids were transiently transfected into LNCaP (AD) cells, and AR, PSA, and AKT levels were studied 72 hrs post-transfection. I observed that there was no difference in expression levels of AR in cells transfected with SrcK298M plasmid compared to cells transfected with wt-src (**Fig 13,** left panel).

Fig 13

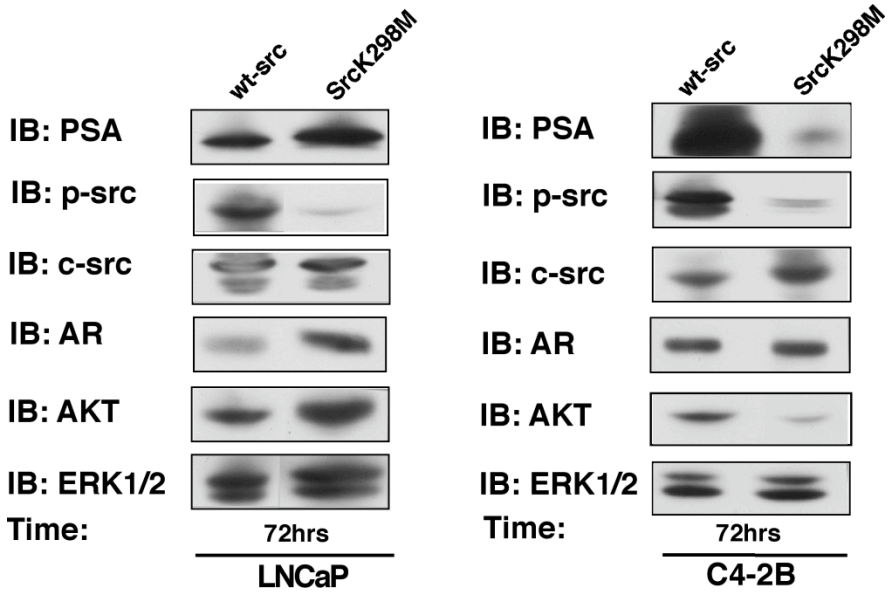


Figure 13: Src Differentially Affects AR Activity in AD and AI PrCa Cells. LNCaP (AD) and C4-2B (AI) cells were transiently transfected with plasmids encoding wt-src or SrcK298M using Lipofectamine 2000 (Invitrogen). Cell lysates were collected after 72 hrs of transfection, and were subjected to IB using mAbs specific for AR (AR441) or c-Src. Primary Abs were used against p-Src (Y416), PSA, AKT1/2/3, or ERK1/2 (C16) (the latter was used as a loading control). Dominant negative src inhibits AR activity (PSA levels) in AI PrCa cells, but has no effect on AR activity in AD PrCa cells.

In addition to androgen signaling, which plays an important role in PrCa, the phosphotidylinositol 3-kinase (PI3K) pathway represents another important mechanism with roles in PrCa. Kinase AKT modulates AR activity, which is cell-type and passage number-dependent (Lin et al., 2003). Therefore, AKT and PSA levels were analyzed. In AD cells (**Fig 13**, left panel), AKT levels were comparable in cells transfected with SrcK298M and wt-src, although a slight decrease in AKT was sometimes observed in SrcK298M samples. Thus, in AD cells, dominant-negative src expression appears to have no strong effect on PSA or AKT levels. However, in C4-2B (AI) cells (**Fig 13**, right panel), SrcK298M transient transfection inhibited AR activity (as measured by PSA and AKT levels) compared to cells transfected with wt-src. Thus, dominant-negative src appears to down-regulate AR activity in the AI PrCa cell line, but not in the AD cell line. Thus, we conclude that Src differentially affects AR activity in AD and AI PrCa cell lines.

$\alpha_V\beta_3$ Integrin Increases Src Activation in a Ligand-Dependent Manner

Integrins regulate Src-mediated oncogenic transformation and various aspects of morphological transformation through different pathways (Goel et al., 2008). It has also been reported that $\alpha_V\beta_3$ integrin promotes anchorage-independence through Src-mediated pathways (Desgrosellier et al., 2009). The interaction of Src with $\alpha_V\beta_3$ integrin is required for tumor formation. With respect to PrCa, integrin signalling may play a key role in the switch from early

stage AD tumors to later stage AI tumors (Goel et al., 2008). Hence, I studied the integrin-Src pathway in PrCa cells using an integrin ligand-binding assay with LNCaP (AD) and C4-2B (AI) cell lines stably expressing $\alpha_V\beta_3$ integrin (**Fig 14**). I found that transfection with $\alpha_V\beta_3$ integrin increases p-Src levels upon attachment to ligands fibronectin or TGFβ-LAP, as compared to control cell lines expressing $\alpha_V\beta_6$ integrin, in either AD (Fig 14 A) or AI (Fig 14B) cell lines. Therefore, I concluded that $\alpha_V\beta_3$ integrin increases p-Src levels in a ligand-dependent manner.

Fig 14

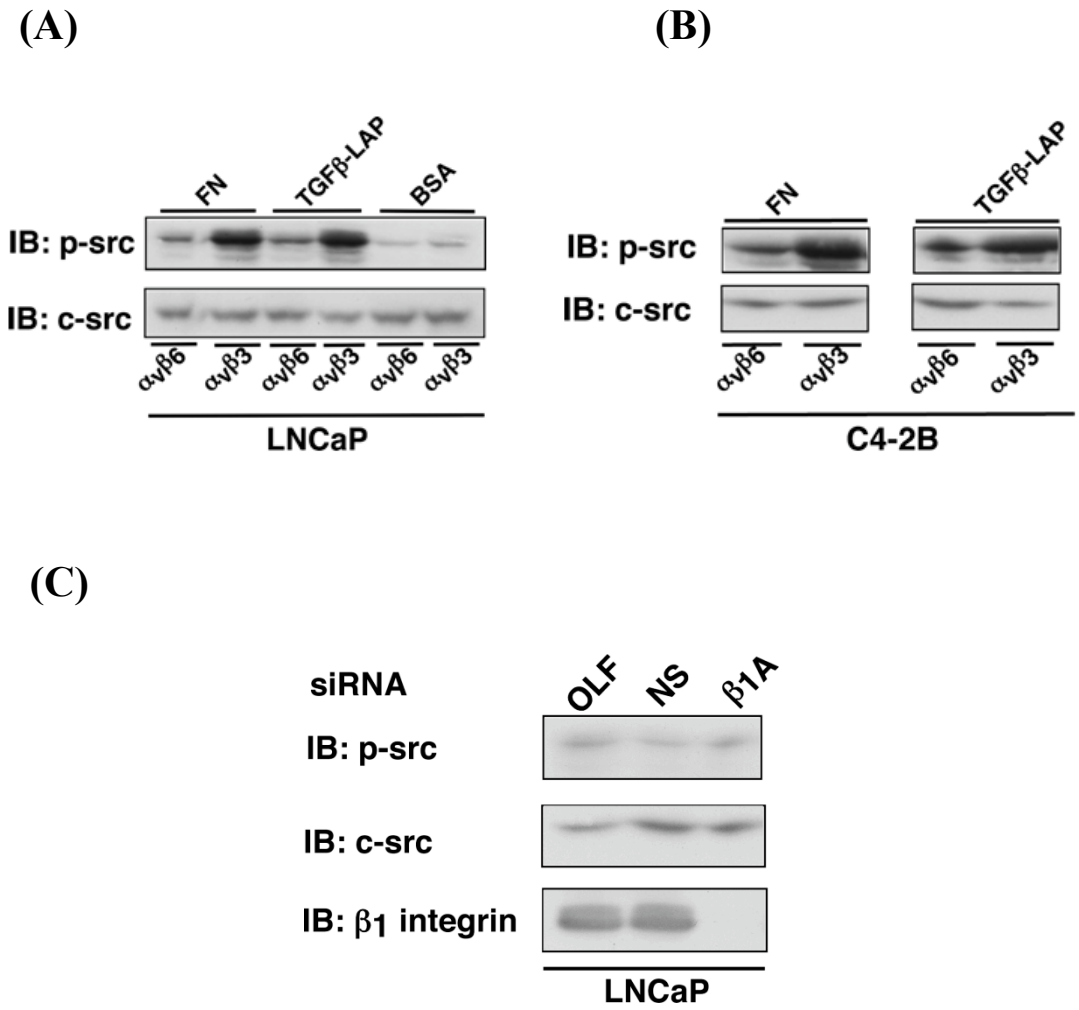


Figure 14: $\alpha_V\beta_3$ Integrin Increases Src Activation. LNCaP (AD) **(A)** and C4-2B (AI) **(B)** cells were starved in serum-free culture medium for 24 hrs before incubation with ligands fibronectin (FN) (3 mg/ml), LAP-TGF-β (0.5 mg/ml), or BSA for 1 hr. Cell lysates were collected and

subjected to IB using mAbs specific for c-Src. Primary Abs were for c-Src or p-Src (Y416). **(C)** LNCaP cells were transfected with β_{1A} siRNA or NS siRNA using Oligofectamine (Invitrogen). Cell lysates were collected 48 hrs post transfection and subjected to to IB using Abs specific for c-Src, p-Src (Y416), or β_{1A} integrin.

To prove integrin specificity of the observed effect, I also studied whether β_{1A} integrin, which is endogenously expressed in many cell lines affects Src activation. Therefore, I down-regulated β_{1A} integrin by siRNA in LNCaP (AD) cells, and found that inhibition of β_{1A} integrin has no effect on Src activation as compared to the non-silencing control (**Fig 14C**). Hence, we concluded that Src activation is integrin-specific and ligand-dependent.

I also studied the effect of $\alpha_V\beta_3$ integrin on AR activity in both LNCaP (AD) and C4-2B (AI) cell lines (**Fig 15**). I found that there was no apparent change in the total cellular levels of PSA or AKT as compared to mock transfected cells, although small changes in sub-cellular levels (i.e. in the nucleus) can not be ruled out. In conclusion, although $\alpha_V\beta_3$ integrin when bound to ligand strongly activates p-Src, it does not appear to affect AR activity.

Fig 15

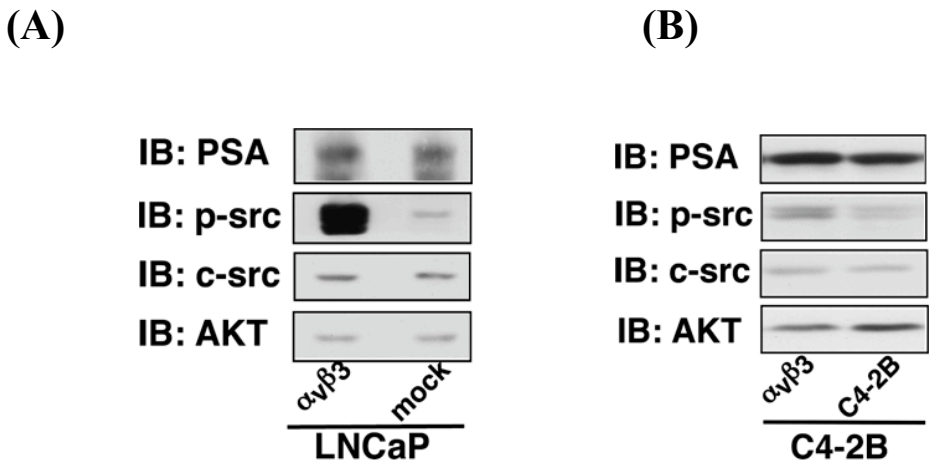


Figure 15: $\alpha_V\beta_3$ Integrin Does Not Affect PSA Expression Levels. LNCaP **(A)** or C4-2B **(B)** cells were tranfected with a plasmid encoding $\alpha_V\beta_3$ integrin, or were mock transfected. Cell lysates were collected, and subjected to IB using Ab for PSA, p-Src (Y416), c-Src, or AKT (loading control).

DISCUSSION

The purpose of this thesis was to analyze the role of Src in the regulation of AR activity, and to investigate a potential effect of $\alpha_V\beta_3$ integrin, a surface receptor known to be upregulated in PrCa (Goel et al., 2008), on AR activity through Src. Src signaling plays an important role in PrCa progression. Its inhibition has previously been shown to decrease tumor volume (Recchia et al., 2003). It has also been shown that Src can interacts with AR to enhance AR transactivation. Although it has been shown that Src can promote AR activity, the underlying pathway has not been defined, nor has it been shown whether Src activation of AR plays a role in integrin-Src signalling. The novel findings described in this thesis show that the cytoplasmic tyrosine kinase Src (Martin, 2001) differentially affects AR activity, evaluated by measuring PSA levels, in AD and AI PrCa cells. Specifically, Src was found to stimulate AR activity in AI cells but not in AD cells. The results also show that Src, AR and PSA localize in the nucleus, suggesting a macromolecular interaction which could further enhance AR transactivation. Finally, I demonstrate that although $\alpha_V\beta_3$ integrin increases Src phosphorylation, its activation does not affect PSA levels mediated by AR.

Src, AR, and PSA Localization

The first set of investigations focused on studying the localization of Src, p-Src, AR, pAR, and PSA, in AD and AI cells, in the presence and absence of androgen. Using sub-cellular fractionation, the data showed that treatment of AD cells with synthetic androgen R1881 increased p-Src, AR, pAR, and PSA in the nucleus, while the levels of c-Src remained

unchanged. Treatment of AI cells with R1881 increased pSrc and AR in the nucleus, while the levels of c-Src and PSA remained unchanged. However, when using immunofluorescence microscopy, R1881 did not appear to increase the nuclear levels of p-Src or c-Src, so perhaps this technique is not as sensitive or quantitative as the sub-cellular fractionation/immunoblots.

The increase in p-Src levels in PrCa cells treated with R1881 supports previously published work (Kraus et al., 2006). The findings with the R1881-induced increase in nuclear AR make sense, since Src has previously been shown to promote the nuclear localization of various transcription factors, including AR and RUNX3, and AR nuclear import is crucial for its function as a DNA transcription factor (Guo et al., 2006; Cutress et al., 2008; Goh et al., 2010).

PSA in the Nucleus

While performing these studies, I unexpectedly found the AR target gene, PSA, in the nucleus of AD and AI PrCa cells, suggesting a possible role of PSA in gene transactivation. PSA has long been known to be a secreted protein that functions to prevent seminal fluid coagulation, but it is also known to promote cell growth (Niu et al., 2008). Although its nuclear localization has not been reported, it was previously shown that PSA increases ARA70/AR-mediated transactivation (Niu et al., 2008). Thus, the nuclear localization of PSA may help define its specific role in PrCa progression. With respect to cancer therapeutics, targeting PSA itself might be needed to stop PSA-induced PrCa progression. Initial clinical studies with PSA-encoding recombinant vaccinia vectors (rV-PSA) demonstrated safety and immunogenicity (Sanda et al., 1999; Gulley et al., 2002). Phase I and II clinical trials with a PSA vaccine have shown a 44% reduction in the death rate, and an 8.5-month improvement in median overall survival in men with castration-resistant PrCa (Lubaroff et al., 2009; Kantoff et al., 2010). Phase

III trials with PROSTVA are currently in progress**.** These data suggest that targeting PSA will be a novel therapeutic strategy to treat patients with recurrent PrCa.

Dominant-Negative Src Experiments

Src signaling is involved in androgen-induced proliferation of PrCa cells (Migliaccio et al., 2000) and may also participate in the transition to AI growth (Lee et al., 2001; Lee et al., 2004; Unni et al., 2004). Src promotes AI growth and metastasis (Lee et al., 2004; Chang et al., 2007). Previous reports on Src kinase activity in PrCa cells show that constitutively active src increases AR transactivation, while dominant-negative src (in agreement with this thesis data) decreases AR transactivation in AI PrCa cells (Guo et al., 2006). The Guo et al. study also showed that Src phosphorylates AR in AI-prostate cancer cells (Guo et al., 2006). Consistent with these published data, I have shown that a dominant-negative variant of src (SrcK298M) prevents AR activity in C4-2B AI cells. However, this effect is specific for AI cells, but is not observed in AD LNCaP cells. In these latter cells, the Src dominant-negative variant does not prevent AR activity. These results, may have profound implications in the strategy currently used in several clinical trials which are targeting Src in PrCa (Guarino, 2010). It has been reported that dominant-negative src destroys the interaction of RACK1 (receptor for activated C kinase 1), AR, and Src (Kraus et al., 2006) in PrCa cells. Therefore, the effect of Src in AI PrCa cells may be due to a differential expression of scaffolding proteins such as RACK1 that help bring kinase and substrate together (Kraus et al., 2006).

Integrins and Src Activity

The third and final purpose of this thesis was to investigate the potential effects of $\alpha_V\beta_3$

integrin on AR activity. $\alpha_V\beta_3$ is a surface receptor previously shown to be upregulated in prostate cancer, through Src. Integrins promote a wide-range of adhesion-dependent effects in tumor cells. Most integrins activate FAK and thereby also other members of the Src Family Kinaes (SFK), which causes activation of several downstream pathways (Guo and Giancotti, 2004). Integrins bind ECM ligands, and act to couple cells to their microenvironments.

Here I show that $\alpha_V\beta_3$ integrin increases Src activation in AD and AI PrCa cells. Previous reports show that $\alpha_V\beta_3$ integrin alters TGF-β signaling in mesenchymal epithelial cells (MECs); the ability of TGF-β to promote invasion is mediated by phosphorylation of TGFβ-receptor II by Src kinase (Galliher and Schiemann, 2006). I confirm that $\alpha_V\beta_3$ integrin, but not $\alpha_V\beta_6$ integrin, increases Src activation upon attachment to ligands fibronectin or TGFβ-LAP (Desgrosellier et al., 2009). This shows that in addition to Src activation by androgens in AD cells, Src can also be activated by integrins in a ligand-dependent and integrin-specific manner. Since Src activation increases AR activity, I hypothesized that $\alpha_V\beta_3$ integrin can promote AR transactivation through Src. Thus, I investigated the effect of $\alpha_V\beta_3$ integrin–mediated Src activation on PSA expression levels. The findings show surprisingly that the $\alpha_V\beta_3$ integrin–Src pathway does not affect PSA expression levels. Therefore, I concluded that the $\alpha_V\beta_3$ integrin–Src signaling pathway is AR-independent, or that this pathway upregulates a different AR target gene. Indeed, PSA is not the only AR target gene; other AR responsive genes include TMPRSS2, PRKCD (Protein kinase C delta), PYCR1 (Pyrroline-5-carboxylate reductase 1), and many others (Jariwala et al., 2007; Bolton et al., 2007). Thus, potential increases in these other AR-responsive proteins should be analyzed in integrin-transfected cells to help determine whether AR participates in the integrin-Src pathway. Studying the integrin-mediated changes in the expression levels of these genes, and other genes known to be activated by Src, will likely

bring new insights in the mechanisms underlying the integrin-Src pathway.

OVERALL CONCLUSIONS

From the present study I conclude that Src is required for AR activity and, consequently, PSA expression in androgen-independent prostate cancer cells, but not in androgen-dependent cells. The findings in this thesis of the nuclear co-localization of p-Src, AR, and PSA appears to be consistent with their roles in disease progression. Given PSA's ability to induce cell division and decrease apoptosis when transfected into cells, its presence in the nucleus as first shown in this thesis may imply that PSA acts there to help induce tumorigenesis. With respect to integrins, cells transfected with $\alpha_V\beta_3$ but not $\alpha_V\beta_6$ increase Src activity, although the increased Src activity does not appear to affect AR transactivation.

Therefore, during integrin-Src signaling, which likely increases in the AI state, perhaps other AR-genes are involved, or other pathways besides AR helps maintain the tumorigenesis.

FUTURE EXPERIMENTS

The present investigation can be extended for future studies as follows:

1. *Analyze a potential role of PSA in promoting PrCa progression*. The experimental design could include: inhibiting or over-expressing PSA in LNCaP (AD) and C4-2B (AI) cell lines, and studying changes in gene expression, cell adhesion, cell migration, and cell viability. Analyzing the type of integrin expression at the cell surface by down-regulating or over-expressing PSA

could further help define the integrins that affect the PSA-AR pathway.

2. *Analyze a potential interaction between Src kinase and PSA in PrCa cells*. To study a possible interaction of Src-AR-PSA in PrCa cells, immunoprecipitation of PSA followed by immunoblotting with Src, and vice versa, will be required to confirm the interaction. This potential interaction can further unfold the mechanism behind nuclear localization of Src, AR and PSA. This interaction could also be investigated in an integrin-dependent environment to help define the specific integrin-Src-AR-PSA pathway in PrCa.

3. *Study the downstream effects resulting from $\alpha_V\beta_3$ integrin-Src activation*. To analyze the $\alpha_V\beta_3$ integrin-Src pathway in PrCa, hybridization arrays could be used to help identify specific genes up- or down-regulated by this pathway. RNA could be isolated from cells transfected with various integrins, with or without co-transfection with the Src dominant-negative plasmid, to hybridize to commercial arrays. It could be suggested that the $\alpha_V\beta_3$ integrin–Src signaling pathway is AR-independent, so this approach could help identify genes important to PrCa progression in an androgen-independent and AR-independent state. Candidates identified by arrays could be confirmed by RT-PCR. Further studies at RNA (RT-PCR) and DNA level (CHIP analysis) could determine the requirement of AR and its target gene to regulate gene transcription.

4. All of the above studies could be extended to PrCa mouse models *in vivo* to confirm the *in vitro* findings.

BIBLIOGRAPHY

Agrez, M., Chen, A., Cone, R. I., Pytela, R., and Sheppard, D. (1994). The αvβ6 integrin promotes proliferation of colon carcinoma cells through a unique region of the β6 cytoplasmic domain. J Cell Biol *127*, 547-556.

Ahmed, N., Pansino, F., Clyde, R., Murthi, P., Quinn, M. A., Rice, G. E., Agrez, M. V., Mok, S., and Baker, M. S. (2002). Overexpression of αvβ6 integrin in serous epithelial ovarian cancer regulates extracellular matrix degradation via the plasminogen activation cascade. Carcinogenesis *23*, 237-244.

Arihiro, K., Kaneko, M., Fujii, S., Inai, K., and Yokosaki, Y. (2000). Significance of α9β1 and αvβ6 integrin expression in breast carcinoma. Breast Cancer *7*, 19-26.

Asim, M., Siddiqui, I. A., Hafeez, B. B., Baniahmad, A., and Mukhtar, H. (2008). Src kinase potentiates androgen receptor transactivation function and invasion of androgen-independent prostate cancer C4-2 cells. Oncogene *27*, 3596-3604.

Balk, S. P., Ko, Y. J., and Bubley, G. J. (2003). Biology of prostate-specific antigen. J Clin Oncol *21*, 383-391.

Bolton, E. C., So, A. Y., Chaivorapol, C., Haqq, C. M., Li, H., and Yamamoto, K. R. (2007). Cell- and gene-specific regulation of primary target genes by the androgen receptor. Genes Dev *21*, 2005-2017.

Breuss, J. M., Gallo, J., DeLisser, H. M., Klimanskaya, I. V., Folkesson, H. G., Pittet, J. F., Nishimura, S. L., Aldape, K., Landers, D. V., Carpenter, W., and et al. (1995). Expression of the β6 integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. J Cell Sci *108 (Pt 6)*, 2241-2251.

Chang, Y. M., Kung, H. J., and Evans, C. P. (2007). Nonreceptor tyrosine kinases in prostate cancer. Neoplasia *9*, 90-100.

Cooper, J. A., Gould, K. L., Cartwright, C. A., and Hunter, T. (1986). Tyr527 is phosphorylated in pp60c-src: implications for regulation. Science *231*, 1431-1434.

Cutress, M. L., Whitaker, H. C., Mills, I. G., Stewart, M., and Neal, D. E. (2008). Structural basis for the nuclear import of the human androgen receptor. J Cell Sci *121*, 957-968.

Debes, J. D., and Tindall, D. J. (2004). Mechanisms of androgen-refractory prostate cancer. N Engl J Med *351*, 1488-1490.

Dehm, S. M., and Bonham, K. (2004). SRC gene expression in human cancer: the role of transcriptional activation. Biochem Cell Biol *82*, 263-274.

Desgrosellier, J. S., Barnes, L. A., Shields, D. J., Huang, M., Lau, S. K., Prevost, N., Tarin, D., Shattil, S. J., and Cheresh, D. A. (2009). An integrin α(v)β(3)-c-Src oncogenic unit promotes anchorage-independence and tumor progression. Nat Med *10*, 1163-9.

Feldman, B. J., and Feldman, D. (2001). The development of androgen-independent prostate cancer. Nat Rev Cancer *1*, 34-45.

Fizazi, K. (2007). The role of Src in prostate cancer. Ann Oncol *18*, 1765-1773.

Fornaro, M., Manzotti, M., Tallini, G., Slear, A. E., Bosari, S., Ruoslahti, E., and Languino, L. R. (1998). β1C integrin in epithelial cells correlates with a nonproliferative phenotype: forced expression of β1C inhibits prostate epithelial cell proliferation. Am J Pathol *153*, 1079-1087.

Fornaro, M., Plescia, J., Chheang, S., Tallini, G., Zhu, Y. M., King, M., Altieri, D. C., and Languino, L. R. (2003). Fibronectin protects prostate cancer cells from tumor necrosis factor α-induced apoptosis via the AKT/Survivin pathway. J Biol Chem *278*, 50402-50411.

Galliher, A. J., and Schiemann, W. P. (2006). β3 integrin and Src facilitate transforming growth factor-β mediated induction of epithelial-mesenchymal transition in mammary epithelial cells. Breast Cancer Res *8*, R42.

Gioeli, D., Ficarro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, A. D., White, F. M., Christian, R. E., Settlage, R. E., Shabanowitz, J.*, et al.* (2002). Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem *277*, 29304-29314.

Goel, H. L., and Languino, L. R. (2004). Integrin signaling in cancer. In Molecular targeting and signal transduction, R. Kumar, ed. (Norwell: Kluwer Academic Publishers), pp. 15-31.

Goel, H. L., Li, J., Kogan, S., and Languino, L. R. (2008). Integrins in prostate cancer progression. Endocr Relat Cancer *3*, 657-664.

Goh, Y. M., Cinghu, S., Hong, E. T., Lee, Y. S., Kim, J. H., Jang, J. W., Li, Y. H., Chi, X. Z., Lee, K. S., Wee, H.*, et al.* (2010). Src kinase phosphorylates RUNX3 at tyrosine residues and localizes the protein in the cytoplasm. J Biol Chem *285*, 10122-10129.

Gulley, J., Chen, A. P., Dahut, W., Arlen, P. M., Bastian, A., Steinberg, S. M., Tsang, K., Panicali, D., Poole, D., Schlom, J., and Michael Hamilton, J. (2002). Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer. Prostate *53*, 109-117.

Guarino, M. (2010). Src signaling in cancer invasion. J Cell Physiol *1*, 14-26.

Guo, W., and Giancotti, F. G. (2004). Integrin signalling during tumour progression. Nat Rev Mol Cell Biol *5*, 816-826.

Guo, Z., Dai, B., Jiang, T., Xu, K., Xie, Y., Kim, O., Nesheiwat, I., Kong, X., Melamed, J., Handratta, V. D.*, et al.* (2006). Regulation of androgen receptor activity by tyrosine phosphorylation. Cancer Cell *10*, 309-319.

Hanks, S. K., and Polte, T. R. (1996). Signaling through focal adhesion kinase. Bioessays *19*, 137-146.

Heinlein, C. A., and Chang, C. (2004). Androgen receptor in prostate cancer. Endocr Rev *25*, 276-308.

Huveneers, S., van den Bout, I., Sonneveld, P., Sancho, A., Sonnenberg, A., and Danen, E. H. (2007). Integrin αvβ3 controls activity and oncogenic potential of primed c-Src. Cancer Res *67*, 2693-2700.

Jariwala, U., Prescott, J., Jia, L., Barski, A., Pregizer, S., Cogan, J., P., Arasheben, A., Tilley W., D., Scher, H., I., Gerald., W., L., Buchanan, G., Coetzee, G., A., Frenkel, B. (2007). Identification of novel androgen receptor target genes in prostate cancer. Mol Cancer *6*, 6-39.

Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., and Thun, M. J. (2009). Cancer statistics, 2009. CA Cancer J Clin *59*, 225-249.

Jones, J., Watt, F. M., and Speight, P. M. (1997). Changes in the expression of αv integrins in oral squamous cell carcinomas. J Oral Pathol Med *26*, 63-68.

Kantoff, P. W., Schuetz, T. J., Blumenstein, B. A., Glode, L. M., Bilhartz, D. L., Wyand, M., Manson, K., Panicali, D. L., Laus, R., Schlom, J.*, et al.* (2010). Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. J Clin Oncol *28*, 1099-1105.

Kim, T. H., Kim, H. I., Soung, Y. H., Shaw, L. A., and Chung, J. (2009). Integrin (α6β4) signals through Src to increase expression of S100A4, a metastasis-promoting factor: implications for cancer cell invasion. Mol Cancer Res *7*, 1605-1612.

Kraus, S., Gioeli, D., Vomastek, T., Gordon, V., and Weber, M. J. (2006). Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor. Cancer Res *66*, 11047-11054.

Kypta, R. M., Goldberg, Y., Ulug, E. T., and Courtneidge, S. A. (1990). Association between the PDGF receptor and members of the src family of tyrosine kinases. Cell *62*, 481-492.

Lee, L. F., Guan, J., Qiu, Y., and Kung, H. J. (2001). Neuropeptide-induced androgen independence in prostate cancer cells: roles of nonreceptor tyrosine kinases Etk/Bmx, Src, and focal adhesion kinase. Mol Cell Biol *21*, 8385-8397.

Lee, M. S., Igawa, T., and Lin, M. F. (2004). Tyrosine-317 of p52(Shc) mediates androgen-stimulated proliferation signals in human prostate cancer cells. Oncogene *23*, 3048-3058.

Lin, H. K., Hu, Y. C., Yang, L., Altuwaijri, S., Chen, Y. T., Kang, H. Y., and Chang, C. (2003). Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J Biol Chem *278*, 50902-50907.

Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. J Cell Biol *119*, 905-912.

Litvinov, I. V., De Marzo, A. M., and Isaacs, J. T. (2003). Is the Achilles' heel for prostate cancer therapy a gain of function in androgen receptor signaling? J Clin Endocrinol Metab *88*, 2972-2982.

Lubaroff, D. M., Konety, B. R., Link, B., Gerstbrein, J., Madsen, T., Shannon, M., Howard, J., Paisley, J., Boeglin, D., Ratliff, T. L., and Williams, R. D. (2009). Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic results. Clin Cancer Res *15*, 7375-7380.

Mahajan, N. P., Liu, Y., Majumder, S., Warren, M. R., Parker, C. E., Mohler, J. L., Earp, H. S., and Whang, Y. E. (2007). Activated Cdc42-associated kinase Ack1 promotes prostate cancer progression via androgen receptor tyrosine phosphorylation. Proc Natl Acad Sci USA *104*, 8438-8443.

Manes, T., Zheng, D. Q., Tognin, S., Woodard, A. S., Marchisio, P. C., and Languino, L. R. (2003). $\alpha_V\beta_3$ integrin expression up-regulates cdc2, which modulates cell migration. J Cell Biol *161*, 817-826.

Marcelli, M., Stenoien, D. L., Szafran, A. T., Simeoni, S., Agoulnik, I. U., Weigel, N. L., Moran, T., Mikic, I., Price, J. H., and Mancini, M. A. (2006). Quantifying effects of ligands on androgen receptor nuclear translocation, intranuclear dynamics, and solubility. J Cell Biochem *98*, 770-788.

Martin, G. S. (2001). The hunting of the Src. Nat Rev Mol Cell Biol *2*, 467-475.

Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000). Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation. EMBO J *19*, 5406-5417.

Miyamoto, H., Yeh, S., Wilding, G., and Chang, C. (1998). Promotion of agonist activity of antiandrogens by the androgen receptor coactivator, ARA70, in human prostate cancer DU145 cells. Proc Natl Acad Sci USA *95*, 7379-7384.

Nash, A. F., and Melezinek, I. (2000). The role of prostate specific antigen measurement in the detection and management of prostate cancer. Endocr Relat Cancer *7*, 37-51.

Niu, Y., Yeh, S., Miyamoto, H., Li, G., Altuwaijri, S., Yuan, J., Han, R., Ma, T., Kuo, H. C., and Chang, C. (2008). Tissue prostate-specific antigen facilitates refractory prostate tumor progression via enhancing ARA70-regulated androgen receptor transactivation. Cancer Res *68*, 7110-7119.

Okada, M., and Nakagawa, H. (1989). A protein tyrosine kinase involved in regulation of pp60c-src function. J Biol Chem *264*, 20886-20893.

Penuel, E., and Martin, G. S. (1999). Transformation by v-Src: Ras-MAPK and PI3K-mTOR mediate parallel pathways. Mol Biol Cell *10*, 1693-1703.

Ralston, R., and Bishop, J. M. (1985). The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor. Proc Natl Acad Sci USA *82*, 7845-7849.

Recchia, I., Rucci, N., Festuccia, C., Bologna, M., MacKay, A. R., Migliaccio, S., Longo, M., Susa, M., Fabbro, D., and Teti, A. (2003). Pyrrolopyrimidine c-Src inhibitors reduce growth, adhesion, motility and invasion of prostate cancer cells in vitro. Eur J Cancer *39*, 1927-1935.

Roy, A. K., Lavrovsky, Y., Song, C. S., Chen, S., Jung, M. H., Velu, N. K., Bi, B. Y., and Chatterjee, B. (1999). Regulation of androgen action. Vitam Horm *55*, 309-352.

Roy, A. K., Tyagi, R. K., Song, C. S., Lavrovsky, Y., Ahn, S. C., Oh, T. S., and Chatterjee, B. (2001). Androgen receptor: structural domains and functional dynamics after ligand-receptor interaction. Ann NY Acad Sci *949*, 44-57.

Ryan, C. J., Smith, A., Lal, P., Satagopan, J., Reuter, V., Scardino, P., Gerald, W., and Scher, H. I. (2006). Persistent prostate-specific antigen expression after neoadjuvant androgen depletion: an early predictor of relapse or incomplete androgen suppression. Urology *68*, 834-839.

Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994). A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J *13*, 3748-3756.

Sanda, M. G., Smith, D. C., Charles, L. G., Hwang, C., Pienta, K. J., Schlom, J., Milenic, D., Panicali, D., and Montie, J. E. (1999). Recombinant vaccinia-PSA (PROSTVAC) can

induce a prostate-specific immune response in androgen-modulated human prostate cancer. Urology *53*, 260-266.

Sinha, A. A., Wilson, M. J., Gleason, D. F. (1987). Immunoelectron microscopic localization of prostatic-specific antigen in human prostate by the protein A-gold complex. Cancer *6*, 1288-93.

Turkson, J., Bowman, T., Garcia, R., Caldenhoven, E., De Groot, R. P., and Jove, R. (1998). Stat3 activation by Src induces specific gene regulation and is required for cell transformation. Mol Cell Biol *18*, 2545-2552.

Unni, E., Sun, S., Nan, B., McPhaul, M. J., Cheskis, B., Mancini, M. A., and Marcelli, M. (2004). Changes in androgen receptor nongenotropic signaling correlate with transition of LNCaP cells to androgen independence. Cancer Res *64*, 7156-7168.

van de Wijngaart, D. J., van Royen, M. E., Hersmus, R., Pike, A. C., Houtsmuller, A. B., Jenster, G., Trapman, J., and Dubbink, H. J. (2006). Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. J Biol Chem *281*, 19407-19416.

Yang, W. J., Chang, C. J., Yeh, S. H., Lin, W. H., Wang, S. H., Tsai, T. F., Chen, D. S., and Chen, P. J. (2009). Hepatitis B virus X protein enhances the transcriptional activity of the androgen receptor through c-Src and glycogen synthase kinase-3β kinase pathways. Hepatology *49*, 1515-1524.

Yousef, G. M., and Diamandis, E. P. (2001). The New Human Tissue Kallikrein Gene Family: Structure, Function, and Association to Disease. Endo Rev *2*, 184-204.

Zheng, D. Q., Woodard, A. S., Fornaro, M., Tallini, G., and Languino, L. R. (1999). Prostatic carcinoma cell migration via αvβ3 integrin is modulated by a focal adhesion kinase pathway. Cancer Res *59*, 1655-1664.

Zou, L., Cortez, D., and Elledge, S. J. (2002). Regulation of ATR substrate selection by Rad17-dependent loading of Rad9 complexes onto chromatin. Genes Dev *16*, 198-208.