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The Role Of IL-8-Mediated Src Family Kinase Activation In Tumor-Tumor And Tumor-Stromal Interactions In Metastasis Of Prostate Cancer

Lynnelle Thorpe

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THE ROLE OF IL-8-MEDIATED SRC FAMILY KINASE ACTIVATION IN TUMOR-TUMOR AND TUMOR-STROMAL INTERACTIONS IN METASTASIS OF PROSTATE CANCER

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THE ROLE OF IL-8-MEDIATED SRC FAMILY KINASE ACTIVATION IN
TUMOR-TUMOR AND TUMOR-STROMAL INTERACTIONS IN
METASTASIS OF PROSTATE CANCER

A

DISSERTATION

Presented to the Faculty of

The University of Texas
Health Science Center at Houston

And

The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, TX

May 2013
DEDICATION

To God, for giving me the ability and strength to pursue and finish this degree.

To my mother and father, Sheila L. and Willie H. Thorpe, and my sister, Alexia C. Lee,

for always believing in me and supporting me in all of my endeavors.
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I would like to thank all the members of Dr. Gallick’s laboratory, Nila U. Parikh, Jung-Kang Jin, Tanushree Chatterji, Sanchaika Gaur, Andreas Varkaris, Rachel Harris and Jian H. Song for their help, for always being ears to listen and for giving me encouraging words.
Men with localized prostate cancer (PCa) have a 100% five-year survival rate, but this rate drops to 33% for men with metastatic disease. A better understanding of the metastatic process is needed to develop better therapies for PCa. Aberrant activation of protein tyrosine kinases, including Src Family Kinases (SFKs) contribute to metastasis through numerous functions, one of which leads to increased expression of cytokines, such as IL-8. However, the relationship between Src activity and IL-8 regulation is not completely understood. In cell line models, I determined that IL-8 activates Src and in turn Src activates IL-8 demonstrating a feed forward loop contributing to the migration and invasion of PCa cells.

However, IL-8 is also produced by tumor-associated stromal cells. In bone marrow derived stromal cells (HS5), I demonstrated a feed forward loop occurs as was observed in tumor cells. HS5 conditioned media increased Src activity in PCa cells. By silencing IL-8 in HS5 cells, Src activity was decreased to control levels in PCa cells as was migration and invasion. Thus, stromal cells producing IL-8 contribute to metastatic properties of PCa by a paracrine mechanism.

To examine the effect of stromal cells on tumor growth and metastatic potential of PCa in vivo, I mixed HS5 and PCa cells and co-injected them intraprostatically. I
determined that tumor growth and metastases were increased. By silencing IL-8 in HS5 cells and co-injecting them with PCa cells intraprostatically, tumor growth and metastases were still increased relative to injection of PCa cells alone, but decreased relative to co-injections with PCa cells and HS5 cells.

These studies demonstrated: (1) a feed forward loop in both tumor and stromal cells, whereby IL-8 activates Src, derepressing IL-8 expression in PCa cells \textit{in vitro}; (2) stromal produced IL-8 activates Src and contributes to the migration and invasion of PCa cells \textit{in vitro}; and (3) stromal produced IL-8 is responsible, in part, for increases in PCa tumor growth and metastatic potential.

Together, these studies demonstrated that IL-8-mediated Src activity increases the metastatic potential of PCa and therapeutic agents interfering with the IL-8/SFK signaling axis may be useful for prevention and treatment of metastases.
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CHAPTER 1

INTRODUCTION
OVERVIEW OF PROSTATE CANCER

Prostate cancer (PCa) is the most common cancer in men in the United States and is the second leading cause of cancer mortality in western civilization. It is estimated that that there will be 241,740 new cases and 28,170 deaths in 2012 (1). The incidence of disease increases with age and as many as 35% of men ages 60-69 years old and 46% of men ages 70-81 years old will develop PCa (2,3). When diagnosed early, the 5-year relative survival rate for men with localized disease is nearly 100%, but when disease is no longer organ confined, the 5-year survival rate for men dramatically drops from nearly 100% to 29% (4). For localized disease, patients initially respond to the front-line androgen ablation or surgical castration therapies, but many patients progress to castration resistant prostate cancer (CRPC) where mortality increases. The increased mortality is due, in part, to sustained androgen receptor signaling.

Although recent therapies, discussed in detail in the current therapies section, have been developed that more effectively block androgen synthesis or inhibit androgen binding to AR, there are patients that still progress and eventually die (5-7). This suggests that other mechanisms, in conjunction with AR signaling, must be explored in CRPC. This also suggests that there are differences, either morphologically, mechanistically or biologically, associated with stages of PCa development. In light of this, we must first understand the current theories of how PCa develops and progresses to CRPC which will be discussed in the next sections.
DEVELOPMENT OF THE PROSTATE

To better understand the development of PCa, we must understand the morphology and organization of the prostate. The prostate is a small walnut size organ sitting below the bladder, posterior to the pubic bone and anterior to the rectum. Comprised of ~70% glandular tissue and 30% muscular tissue, the prostate houses an epithelial and stromal compartment separated by a basement membrane (8). The normal prostate gland comprises three cell types: luminal, basal and neuroendocrine cells. (Figure 1) The origin of each cell type is thought to develop from presumed prostate stem cells, located in the basal layer and proximal ducts of the prostate gland, which become differentiated and can be identified by their position, cell-type specific markers and function (9).

The luminal cell layer, composed of columnar epithelial cells and located in the lumen of the prostate, expresses the AR and prostate specific antigen (PSA), while, functionally, it secretes seminal fluids. The basal cells, located in the basal layer of the prostate gland, express low levels of AR, cytokeratins and cell adhesion molecules (CAMs), while, functionally, separating the stromal compartment from the luminal cells (10). The neuroendocrine cells, which express various basal cell markers, are unevenly distributed within the basal layer representing a post-mitotic population of cells. In addition, there is a stromal compartment that represents a cellular niche containing mesenchymal and fibroblastic cells as well as lymphocytes, endothelial cells, myofibroblasts and pericytes required to support the prostate for survival.

Androgen expression is a critical characteristic of prostate development. Androgen signaling occurs through the AR supporting survival of the prostate. AR
The normal epithelium consists of a luminal cells enveloped by a basal cell layer. The stromal cell compartment is the outermost portion of the prostate epithelium. Neuroendocrine cells are a post-mitotic cell type that can result transform into prostate cancer.
serves as a ligand-dependent transcription factor that, in the absence of ligand binding, is distributed diffusely throughout the cytoplasm bound to heat shock proteins in an inactive state. Ligand binding to the AR facilitates dimerization and nuclear translocation where AR dimers bind to androgen response elements (AREs) stimulating transcription of androgen regulated genes, such as KLK3, the gene encoding PSA protein (11-12). As a result, PSA, serine protease protein secreted by prostate, as a marker of AR function is widely used to assess prostate-specific AR activity (13-14). Although controversial, PSA screening methods are utilized to monitor disease progression and response to therapeutic intervention.

Thus far, we have described prostate development in terms of organization, anatomy and androgen regulation. McNeal provides a well-accepted theory assessing the anatomy of the prostate as a means to understand the regions of PCa development. McNeal describes two zones of the prostate: the peripheral and the transition zone. The peripheral zone, localized in the posterior portion of the gland, is considered the primary site where PCa develops and accounts for ~75% of the region where PCa originates. The transition zone, localized near the urethra, accounts for origination of ~20% of cancers, a less frequent site of cancer development (8,15).

**Benign Prostate Hyperplasia**

Benign prostate hyperplasia (BPH) represents a clinically significant pathologic process that the prostate undergoes, but is not considered a precursor of PCa. BPH is characterized by cellular proliferation and gland enlargement mainly in the periurethral and transition zones of the prostate. Considered to be a normal process for
aging men, only 10% of men with BPH require medicinal treatment making this condition treatable and curable (16).

**DEVELOPMENT OF PROSTATE CANCER**

**Prostate Stem Cells**

PCa arises from cancer stem cells (CSCs), but there is controversy as to whether the stem cell of origin is basal or luminal (17,18). The evidence that PCa arises from luminal cells is due to the presence of non-terminally differentiated secretory luminal cells along with minimal presence of basal cells and basal cell-markers (19). This theory is also supported by the expression of luminal cell markers, such as cytokeratins 8 and 18 (CK8 and 18), PSA, stem cell antigen-1 (Sca1) and prostate stem cell antigen (PSCA) in cancer cells (9,18,20-23). In addition, Liao et al. report that cancer stem cell differentiation to a luminal cell lineage also requires the presence of the microenvironment suggesting that determining the stem cell origin is further confounded by interactions with the microenvironment (24).

There is also evidence supporting the theory that PCa arises from a basal cell origin (25). Studies report that a small population of cells in the prostate tumor express basal markers, such as p63, hTERT and Bcl-2 (21,26). However, it is not clear whether there is a conversion between basal and luminal stem cells leading to the development of PCa. Wang et al. discuss two newer theories where, 1) there may be overlap in stem cell activities such that the basal and luminal cells have autonomous progenitors or 2) PCa arises from a basal cell progenitor that differentiates into luminal progenitor cells (27). These studies highlight that there are still confounding questions about the true
stem cell origin of PCa and what role the microenvironment plays, but it does shed light on possible mechanisms of tumor initiation (19).

**Prostatic Intraepithelial Neoplasia**

Prostatic intraepithelial neoplasia (PIN) is suggested to occur under two models - the stem cell hypothesis where transforming events in stem cells occur or by the stochastic model where PIN develops from any cell harboring specific gene mutations (28). The stem cell model is considered to be the most accepted model of PIN initiation. PIN, first identified by Bostwick and Brawer, is defined as highly proliferative epithelial cells, usually non-invasive, located within the normal prostatic acinar ducts exhibiting large acini (29). There are two types of PIN - low grade (LGPIN) and high grade (HGPIN) with different clinical outcomes. While LGPIN, usually, does not progress to PCa and is less reported, HGPIN represents the earliest recognized stage of carcinogenesis (30,31). Multiple studies report an increased frequency of HGPIN in prostates with PCa and, amongst other highly significant predictors of cancer, HGPIN ranks as having the highest risk ratio making it a marker with high predictive value for PCa development (31-33).

There are multiple progressive morphological, genetic and phenotypic changes associated with HGPIN in the progression to PCa, but some are also observed in PCa. Some morphological changes involve the presence of dysplastic epithelial cells with an enlarged nucleus and the disappearance of a defined basal cell membrane, although the presence of basal cells still exist (31). Some progressive genetic and phenotypic alterations associated with HGPIN, also observed in PCa, are decreased expression of genes, such as the NKX3.1 and p27 genes, while there are also decreases associated
with some genes involved in secretory differentiation, such as PSA and prostatic acid phosphatase. There is also an increase in other genes, such as b-cell lymphoma 2 (bcl-2), p53 and c-met (34-35). Along with morphological, phenotypic and genotypic changes, multiple studies demonstrate that there is an observance of PIN lesions within close proximity of malignant disease suggesting, at least morphologically, that PIN is a pre-neoplastic condition to PCa (36). PIN is best distinguished from PCa by its organ-confined phenotype and the inability of cells to become invasive.

**Progression to Prostate Cancer**

The transition from PIN to PCa involves progressive phenotypic and genotypic changes, mentioned above, but there is also evidence suggesting that androgen signaling is involved in the initiation of PIN and development of PCa (37). The initial survival and dependence of PCa is due in large part to the presence of androgens. One of the most predominant androgens found in the sera of males, testosterone, is converted by the steroid 5α-reductase type 2 (SRD5A2) into a higher affinity androgen, 5α-dihydrotestosterone (5α-DHT) in men with PCa (38). The subsequent binding of androgen to AR, as described earlier, results in the transcription of androgen-regulated genes that facilitate survival and proliferation. Huggins and Hodges provided some of the earliest studies demonstrating that androgens increase PCa cell growth leading to the development of multiple endocrine therapies (39). Androgen ablation therapies, such as bicalutamide, flutamide and leuprolide, were introduced to control disease (40). Patients respond to these therapies and a small subset of patients are cured, but many progress suggesting that AR is sustained and signaling still occurs (40-42). When patients
become resistant to this treatment, this disease is termed castration resistant prostate cancer (CRPC) and life expectancy drops.

Sustained signaling through AR, despite androgen ablation, is a major consequence of CRPC. In an effort to understand the mechanisms underlying androgen activity and the AR in CRPC, multiple *in vitro* and *in vivo* models have shown that, even in the absence of androgen regulation, cells still express androgen-responsive genes as well as AR (43-47). In fact, many cases of CRPC display aberrant AR activity due to changes, such as amplifications, splice variants and/or altered AR target genes (45,48,49). This suggests that CRPC utilizes a mechanism that bypasses the need for androgen without deregulation of AR. There are additional mechanisms, outlined below, that have been postulated describing reasons for aberrant activity of AR in CRPC.

One mechanism suggests that AR is active due to alternate sources of androgen, such as circulating dehydroepiandrosterone (DHEA) by the adrenal glands (50-52). Other mechanisms suggest that increased AR sensitivity to low androgen levels creates a hypersensitive mechanism, while increased levels of coactivators can also enhance AR sensitivity, but to androgen and non-androgen ligands (53,54). Non-androgen factors and non-androgen steroid hormones, such as estrogens and cortisol, are also thought to be responsible for sustained AR activity through promiscuous ligand binding (55). In conjunction with promiscuous ligand binding, studies demonstrate that AR activation is also achieved through growth factor and/or receptor tyrosine kinase pathways, such as IGF, HER-2/neu and Src, creating, what is commonly termed as an outlaw mechanism (56-57). Studies suggest that steroid-induced Src, a non-receptor
tyrosine kinase, activation results in association with AR stimulating signaling cascades that regulate metastatic events, such as proliferation and migration (53,59). This further suggests that, in CRPC where there is aberrant AR activity, Src activation may be a contributing factor, which will be discussed in detail later (59). Although all the mechanisms, albeit important in understanding the role of the AR in CRPC, are not mutually exclusive and may work together to sustain CRPC, more studies are needed addressing pathways that may lead to metastatic disease.

**Metastatic Disease**

While PCa, when detected at an early stage in patients, results in curable disease, its progression to CRPC can lead to aggressive and metastatic disease. Metastatic disease comprises distant metastases confined to the spleen, liver and, most frequently, the bone and lymph nodes. Bubendorf et al. report that 80% of men dying from PCa incurred bone metastases suggesting that metastatic sites offer a host of factors that support the growth and survival of PCa (60). Paget’s ‘seed and soil’ hypothesis, explaining the cross-talk between cancer cells and organ-specific microenvironments, is widely accepted as a model for understanding the interdependency of tumor cells and cells in their metastatic microenvironment and strongly suggests that organ-specific metastases require the metastatic ‘soil’ for continued tumor growth (61,62). Thus, understanding the metastatic process involved in cancer progression is important and we now understand that the biological aggressiveness of metastatic disease is vastly a result of interactions between PCa cells and their microenvironmental changes, increasing interest in studies that interrogate organ-specific metastases.
In an effort to understand the nature of how the microenvironment affects PCa, Kaplan et al. describe site-specific niches that exist to support tumor cells in their passage to distant sites and upon their arrival at the distant site, such as the primary tumor, vascular, osteoblastic and pre-metastatic niches (63). It has been further suggested that the preferential metastasis of PCa to specific organs is due, in part, to heterotypic cell interactions, but there may be modifications to distant tissue sites which prime the “soil” for cancer cells yielding a “pre-metastatic” niche (64). The pre-metastatic niche consists of immigrant and resident cells that “prime” the soil through signaling mechanisms conducive to tumor seeding (63,65,66). An example of a resident cell that may prime the “soil” are stromal cells. The bidirectional activities between stromal cells and PCa cells provides a host of factors which contribute to progression of metastatic disease (67,68).

Although there is ample evidence suggesting that bidirectional activities at the primary site contribute to PCa growth and survival, there has been increased interest in understanding the stromal-epithelial crosstalk at the metastatic site (69-71). Part of the reasoning is that, even though the genetic and molecular changes in tumor cells are required during tumor progression, they are not sufficient to fully drive tumors to be metastatic. As a result, studies suggest that the metastatic potential of PCa is a stroma-dependent event and the balance of stromal factors within the metastatic tissue determines metastatic behavior (43,72,73). This aspect is integral to the studies of my thesis.

The role of the stroma in metastatic disease involves factors, such as interleukin-6 (IL-6), CXCL-12, transforming growth factor beta (TGF-β), vascular
endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), that initiate signaling cascades increasing metastatic properties of PCa (74,75). This reciprocal signaling mechanism is thought to permit the promotion of metastasis to the host environment. More recent, in vivo and in vitro, studies support the hypothesis that organ-specific PCa cell and stromal interactions contribute to a “vicious cycle” whereby the stroma modifies the PCa cells and the PCa cells modify the stroma (76-78).

Increasing evidence suggests that there are many vicious cycles, not only, cultivating and maintaining an environment for cancer progression, but also transforming the stroma as PCa progresses to aggressive disease (59). By interrogating pathways integral to the tumor-stromal interactions at the metastatic sites, we may be able to develop strategies that effectively disrupt the stroma-dependent tumor interaction. An integral part of this thesis is to delineate factors expressed and secreted by the tumor and stroma contributing to metastatic disease. As a result, we must first understand the current molecular biology of PCa and factors that contribute to the initiation and progression of PCa.

**MOLECULAR BIOLOGY OF PROSTATE CANCER**

Both genetic and epigenetic events have been shown to play a role in the development and progression of PCa. Epigenetic changes, including methylation and acetylation, represent some of the most common changes seen in early stage PCa. In particular, DNA hypermethylation is a common event involving increased methylation of DNA in multiple genes. The most hypermethylated genes contributing to PCa initiation are glutathione S-transferase (GST) P1 gene, RASSF1A, RAB2B, MDR1 and APC. Of these hypermethylated genes, GSTP1 is cited heavily as being a major
contributor to PCa initiation. GSTP1 is an enzyme catalyzing the addition of glutathione to toxic compounds which in turn decreases cellular oxidative damage. Silencing and promoter hypermethylation of GSTP1 is observed in 90% of PCa and these changes may contribute to increases in cellular oxidative damage. Since GSTP1 expression is decreased during the differentiation of basal cells to luminal cells, there may be a predisposition to increased oxidative damage. Although important, these epigenetic changes are only one event contributing to PCa initiation and progression. There are also genetic changes, such as chromosomal losses, gains and rearrangements, contributing to PCa progression. The loss and gain of chromosomal regions, specifically regions of chromosomes 8, 10 and 21, represent some of the most frequently observed changes in PCa progression and will be discussed in detail below.

One of the most frequently altered chromosomes is chromosome 8 with frequent loss of 8p21 (79). Loss of the 8p21 region is an early and frequent event in PCa, seen in 85% of carcinoma. One gene located in this region is the prostate-specific homeobox transcription factor, Nkx3.1. Studies demonstrate that Nkx3.1 expression is decreased in PCa, while deletion of Nkx3.1 results in PIN. This is supported by mouse model studies demonstrating that Nkx3.1 -/- results in PIN lesions that do not progress to PCa, but that cooperation of Nkx3.1 loss and PTEN loss results in progression to PCa and metastatic disease (80,81). As a result, loss of Nkx3.1 expression is alone insufficient for progression to advanced PCa and involves other genetic events (82).

Another altered chromosome is chromosome 10 with frequent loss of 10q23. One gene located in this region is the phosphatase and tensin tumor suppressor, PTEN, which is also frequently lost in PCa (83). The loss of PTEN expression is mostly
observed in advanced stage disease and accounts for >50% of PTEN gene alterations in metastatic disease, while only a small fraction of primary tumors exhibit PTEN deletion or mutation (84,85). This evidence is supported by genetically engineered mouse model studies where PTEN inactivation in normal prostate epithelium drives the development of advanced PCa (84).

*Pten* deletion studies demonstrate that PCa progression to metastatic disease is a result of cooperation with multiple genes, such as simultaneous loss of *Nkx3.1* and *Pten* or inactivation of *Pten* and up-regulation of the proto-oncogene *c-Myc* (84,86-88). These studies provide strong evidence that PTEN loss plays an important role in early stage PCa and metastatic disease. There are also gains of chromosomal regions resulting in changes that contribute to PCa progression.

One of the most frequently gained chromosomal regions is 8q24. One gene frequently amplified in this region is *c-Myc*, a proto-oncogene known to regulate genes involved in cell proliferation. It is overexpressed in ~40% of primary tumors and ~90% of metastatic PCa (89,90). Mouse model studies report that prostate epithelium overexpressing c-MYC develop PCa, but there is no observance of metastatic lesions (91). More recent evidence suggests that progression to metastatic disease is thought to require additional genetic alterations (92,93). These results demonstrate that changes to chromosome 8, encompassing the Myc gene, play an important role in PCa, but Myc is not a major contributor in the progression to metastatic disease. While chromosomal losses and gains represent changes integral to PCa progression, chromosomal rearrangements are a more recent area of interest in PCa progression.
Chromosomal rearrangements were considered a rare event in PCa, but recent evidence suggests that a specific chromosomal rearrangement at 21q plays an important role in the progression of PCa. Studies demonstrate that the chromosomal rearrangement of 21q, results in the fusion of the transmembrane protease serine 2 gene (TMPRSS2) and the ETS gene, ERG, yielding a fusion protein found in the majority of PCa (94,95). Specifically, the androgen-responsive TMPRSS2 promoter fuses with select coding regions of the ERG family transcription factors, but other ETS-family genes are also involved in recurrent fusions (96). The overexpression of the ETS family of transcription factors accounts for ~60% of gene rearrangements in PCa and is considered the most frequent event in human PCa (97). The rearrangement between TMPRSS2 and ERG is the most frequent of all ETS-family genes. Although the functional significance is not fully understood, in vitro and in vivo studies suggest that activation of ETS genes enhances tumor-invasive properties (98,99). In addition, mouse model studies demonstrate that ERG expression along with PTEN loss results in HGPIN transition to PCa (99,100). These studies demonstrate that, although important to PCa progression, these gene rearrangements are alone insufficient in driving malignant disease.

The previous sections have discussed that epigenetic alterations and genetic changes play a role in PCa progression. Signal transduction changes which may or may not result from mutations comprise other changes that contribute to PCa progression. In particular, the overexpression of kinases has been an area extensively studied and there is more recent evidence implicating their role in PCa progression to metastatic disease. Many kinases have been implicated in PCa progression, such as Her2/Neu, EGFR,
PDGFR, FAK, Raf, VEGFR, PI3K/Akt, MAPK, Axl, MET and Src (101-106). In particular, our laboratory has provided evidence that the oncogenic activity of Src plays an integral role in PCa progression, especially, metastatic CRPC (107). The next section will discuss the role of SFK activity in PCa and will provide information on factors that may be associated with increased SFK activity in metastatic PCa.

THE ROLE OF SRC FAMILY KINASES IN PROSTATE CANCER

Of the nine family members, Src, Yes, Fyn, Lck, Lyn, Blk, Fgr, Yrk and Hck. Of these nine members, Src, Yes and Fyn are the most ubiquitously expressed. In normal tissues, SFKs act as key signal transduction molecules facilitating cellular signals elicited by receptor protein tyrosine kinases, cytokine receptors, integrins, ion channels and G-protein coupled receptors. Upon activation, SFKs regulate biological processes, such as adhesion, migration, invasion, cell cycle and cytoskeletal changes. These processes are regulated by mechanisms of SFK activity and involve changes in the structural features of SFKs, which are outlined below.

Src Family Kinase: General Structure and Mechanism of Activation

Peyton Rous was the first to isolate the virus named Rous Sarcoma virus from chickens. Years later, the identification of Rous Sarcoma virus as a type of retrovirus that harbored transforming protein, v-Src, was recognized (108). These findings contributed to the discovery, by Bishop and Varmus, of Src as the cellular form of v-Src which was determined to be the product of the transforming gene harbored by avian Rous Sarcoma Virus (109). A major finding regarding Src function demonstrated that the Src protein possessed intrinsic protein tyrosine kinase activity making it the first
identified gene product of this kind (110). Peyton Rous was awarded the Nobel Prize in Medicine for his efforts in the discovery of the virus (111,112).

The structure of SFKs is composed of the following domains important to the complete activation of the protein shown in **Figure 2a and b.** The Src homology domain-4 (SH-4) is important for membrane localization by either palmitoylation or myristoylation of which Src is the only member that does not undergo palmitoylation. The SH-3 domain is a domain that recognizes prolines in the Pro-xx-Pro motif of other substrates important for protein-protein interactions. The SH-2 domain recognizes phosphorylated tyrosine residue-containing substrates and also serves to stimulate protein-protein interactions. The SH1 domain serves as the catalytic kinase domain comprising Y^{419} which is autophosphorylated upon Src activation. The C-terminal also contains a negative regulatory phosphorylation residue at Y^{530}. In the inactive state, Y^{530} is phosphorylated forming an intramolecular interaction with the SH2 domain that also associates with the SH3 domain and the linking region between the SH2 and kinase domain. In particular, C-terminal kinase (Csk) and Csk homologous kinase are two kinases that inactivate SFKs and the intramolecular interactions limit the accessibility of substrates to be phosphorylated further maintaining an inactive Src state. In terms of regulation of Src activation, there are several mechanisms outlined below.

One mechanism involves dephosphorylation of Y^{530} which increases the dissociation of the C-terminal region from the SH2 domain resulting in an open confirmation. Multiple phosphatases dephosphorylate the Y^{530}, such as SH2-containing phosphatase 1, protein tyrosine phosphatase (PTP)1B and PTP1α (112,113-115).
Figure 2
Structure and Activation of Src Family Kinases

A. Structure of Src Family Kinase
Src family kinases consist of four domains and two regulatory regions that facilitate intra and intermolecular interactions. These domains represent Src homology domains. The regulatory regions are the negative regulatory (NR) domain and the unique region.

B. Regulation of Src activation
Src contains multiple domains serving different functions. SH4 domain contains a 15 amino acid sequence required for lipid modification along with a glycine needed for myristoylation of Src. This site is critical for membrane localization. The unique domain consists of multiple serine and threonine residues needed to facilitate interactions with proteins, such as PKA, PKC and cdk2/cyclin. The SH3 domain consists of the proline-rich (PXXP) motif needed for intra and intermolecular interactions. The SH2 domain binds proteins containing phospho-tyrosine sites. The SH1 domain is a highly conserved protein kinase domain containing Y419. This domain facilitates autophosphorylation of Y419 upon Src phosphorylation. Lastly, the negative regulator (NR) domain consists of Y530 which interacts with the SH2 domain upon Csk (C-terminal Src kinase)-mediated phosphorylation.
Other mechanisms involve deregulation of regulatory proteins, such as Csk, or increased activity of cytoplasmic proteins. However, for full SFK activation, autophosphorylation of Y^{419} must occur within the catalytic kinase domain. SFK activation is also activated by cellular signals, such as RTKs, integrins and cytokine receptors which modulate normal cellular functions, such as, survival, migration, proliferation and invasion. SFK activation has been demonstrated to be aberrantly active in multiple cancers, including PCa. However, studies have failed to provide evidence of mutations as a mechanism for this aberrant SFK activation along with previous studies suggesting that SFKs are not capable of cellular transformation. These findings raised questions about how SFKs contribute to cancer progression. This has prompted an increase in research investigating how SFK activity plays a role in cancer progression.

**Significance of SFK activity in cancer and PCa progression**

Clinical studies report that SFK activity is increased in patients with advanced disease correlating with shorter overall survival and increased metastatic incidence (116-118). *In vitro and in vivo* studies supporting these reports come from our laboratory and others demonstrating that SFK inhibition decreases tumor growth and metastatic potential in multiple cancers, including PCa (107,119-123). A pinnacle study, providing strong evidence that Src promotes tumor progression, by Agarwal et al. demonstrated that increased Src activity is an independent prognostic indicator at multiple stages of colon carcinoma (124). This provided foundational evidence suggesting that Src, of the many SFKs, may play a role in many stages of multiple cancers, including PCa.
Earlier reports demonstrate that Src is important in the development of PCa and progression (125-129). In contrast to earlier reports, recent evidence suggests that Src, along with Fyn and Lyn, result in transformation of prostate epithelium suggesting a more functional role in the early events of PCa progression (37). This study further reports that among Src, Lyn and Fyn, Src demonstrates the strongest transforming capacity and oncogenic phenotype. As a result, increased Src activity has been implicated as a central signaling component of PCa progression that contributes to changes in multiple stages of PCa progression. One thought regarding Src’s increased activity is that Src’s activity has also been demonstrated to be regulated by upstream signaling mechanisms which may contribute to changes in PCa progression. Upstream signaling is thought to be one mechanism by which Src activity is increased in cancer and, as such, the investigation of signaling mechanisms mediating Src activity in PCa has been of interest. Rather than provide all of the signaling mechanisms involved, this thesis aims to focus on signaling families that mediate Src activity in PCa progression, which will be discussed in the next section.

**Significance of Src signaling mechanisms in PCa**

Multiple studies demonstrate that Src activation can occur through activation of integrins, RTKs and cytokine receptors in many cancers show in Figure 3 (130-132).
Figure 3
Src-Mediated Pathways in Cancer
This figure displays pathways that Src regulates in multiple cancers. Src regulates pathways that modulate survival, angiogenesis, proliferation and motility/migration events.
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In PCa, our laboratory reports that integrin clustering regulates the recruitment of Src to activated FAK stimulating the activation of related signaling pathways, such as PI3K, Rho, Ras, paxillin, p130Cas, ERK and MAPK leading to changes in motility/migration, invasion and cell spreading (131). In addition, there are multiple RTKs with intrinsic protein kinase activity that have been demonstrated to modulate Src activation in PCa, outlined in multiple reports (103,133-136). Our laboratory offers additional reports demonstrating that tyrosine kinases, receptor and non-receptor, contribute to metastatic properties of PCa cells (107, 137,138). This work has provided a great deal of evidence showing that signaling through integrins, RTKs and other tyrosine kinases, not only regulates Src activation, but facilitates changes in metastatic properties of PCa. More recently, another upstream signaling mechanism has received more attention for its role in tumor progression, GPCRs, but whether it modulates Src activation and facilitates changes to metastatic properties of PCa progression is unclear and is the focus of this thesis.

GPCRs are considered to be the largest family of cell surface molecules facilitating signal transduction pathways regulating biological functions through stimuli, including peptide growth factors, hormones and cytokines. Studies demonstrate that the expression of GPCRs and GPCR ligands are increased in multiple cancer cell lines and tumors, including PCa (138-141). This has been outlined in a comprehensive review of the role of GPCRs in cancer by Dorsam and Gutkind (142). There is emerging evidence demonstrating that GPCRs and GPCR ligands, such as interleukins, initiate intracellular signaling cascades involving many factors, including protein kinases and cytokines, regulating many metastatic properties of PCa (143-145). This information,
provided in the next section, is minimal and limited in scope, but provides foundational support for the role of GPCR ligands and GPCRs in PCa progression.

There is evidence demonstrating that Src-mediated changes may be influenced by GPCR ligands, such as IL-8, in PCa, the focus of this thesis (146,147). One study, from our laboratory, demonstrates that Src inhibition decreases interleukin 8 (IL-8) cytokine production in PCa cells suggesting that Src-mediation of PCa progression may be regulated by, not only integrins and RTKs, but also by GPCRs and/or GPCR ligand upstream signaling (148-149). However, there is limited information about what GPCR-initiated signaling cascades are involved in regulating Src activation and what metastatic properties it modulates in PCa. As a result, it is of interest to this thesis to identify GPCR-regulated mechanisms that facilitate Src-mediated changes in metastatic properties of PCa. However, signaling mechanisms facilitating Src activation in PCa provide only one possibility of how Src regulates PCa progression. There may also be Src signaling mechanisms present in the tumor microenvironment which contribute to PCa progression, an emerging field of study and another focus of this thesis. The following section will discuss Src’s role in the tumor microenvironment.

**Significance of Src in the tumor-stromal microenvironment**

The role of Src in the tumor microenvironment has been mostly observed in bone metastases and in osteoclast and osteoblast functions. Initial studies demonstrate that Src -/- mice undergo osteopetrosis, a bone-remodeling disorder, causing defective bone resorption by osteoclasts (150). This study led to multiple studies dedicated to Src’s role in bone based upon the fact that Src inhibition may decrease bone turnover. Studies supporting this theory demonstrate that inhibition of Src increases osteoblast
differentiation and bone formation while decreasing osteoblast proliferation. Src also regulates the bone remodeling process by negative regulation of osteoblasts and positive regulation of osteoclasts (151). The use of Src inhibitors in *in vitro* studies, such as dasatanib and saracatanib, demonstrated decreases in bone regulatory mechanisms, such as osteoclast activity and PCa-cell induced osteoclast activity (152-154). As projected by the initial src -/- mouse study and the ensuing in vitro studies, multiple Phase I and II clinical trials have been conducted with SFK inhibitors demonstrating decreases of bone remodeling markers, such as N-telopeptie of type 1 collagen (urinary NTX) and C-telopeptide of type 1 collagen (serum CTX) in healthy men. This led to Phase I/II trials with saracatinib and dasatanib which showed promising results, in the dasatanib trial, in a small cohort of men with metastatic CRPC.

Although these studies suggest that Src modulates biological functions of the tumor and possibly the bone microenvironment, there are still some patients that will not respond to these therapeutic strategies. As a result, we must investigate other cell types in the tumor microenvironment contributing to tumor progression that contain Src-mediated signaling cascades. Earlier reports show that increased Src expression in bone marrow stromal cultures correlates with increased growth factor and cytokine stimulation (155). Another study demonstrates that Src activity mediates prostate epithelial cell migration to prostate stromal cells in the presence of growth factors (156). These studies suggest that Src regulates signaling pathways in cells supporting and associated with PCa progression, but the mechanism of Src activation and signaling pathways associated with Src activation in the stromal microenvironment are unclear. However, there is information to suggest that, not only Src activation, but factors
produced and secreted by the stromal microenvironment contribute to PCa progression. Recent studies suggest that one of those stromal produced factors, IL-8, modulates tyrosine kinase activation, but it is unclear if or how tumor and stromal produced IL-8 modulates the tyrosine kinase activation of Src to promote the metastatic potential of PCa. As a result, an integral part of my PhD thesis will be to determine how IL-8 contributes to Src activation in metastatic properties of PCa as well as explore how stromal produced IL-8 contributes to Src activation in PCa. The next section will introduce and discuss the function and roles of IL-8 in PCa.

THE ROLE OF INTERLEUKIN-8 IN PROSTATE CANCER

Of the many cytokines and chemokines discovered, IL-8 represents one that modulates a variety of biological processes, not observed, by other cytokines. Much of the work addressing the roles of cytokines in PCa has been focused on IL-8, IL-12 and IL-6. IL-6 has been associated with tumor burden and metastatic CRPC, but in vitro studies have identified IL-6 more as a facilitator of growth and survival while IL-12 has been shown to modulate, primarily, invasion of PCa cells in vitro (158-161). It is evident that these cytokines play important roles in modulating biological functions of PCa progression, but it is unclear what role IL-8 signaling plays in PCa progression to metastatic disease and the mechanism of IL-8 signaling in biological functions of PCa. This thesis will discuss these topics in upcoming sections. The following section will, first, discuss IL-8 structure and regulation.

Interleukin 8: General Structure and Regulation

IL-8 (also known as CXCL8, GCP-1 and NAP-1) is an 8 kDa member of the CXC chemokine family. The IL-8 protein belongs to a family of CXC chemokines
denoted by two cysteines surrounding any amino acid. IL-8 is also classified according to the absence or presence of the ELR (Glu-Leu-Arg) motif in the N-terminus. IL-8 is a 99-amino acid precursor which is processed as a mature protein by cleavage of the leader sequence. Two forms of the mature protein exist, a 72-amino acid protein and a 77-amino acid protein with the 72-amino acid protein representing the most predominate form. (Figure 4a and b) It was first discovered as a pro-inflammatory cytokine on chromosome 4 secreted by human blood monocytes and later redefined as a “chemokine” for its chemotactic capabilities.

Since then, IL-8 has been shown to be expressed and secreted by multiple cell types including stromal cells and tumor cells (162). Binding studies indicate that IL-8 binds to two G protein coupled receptors, CXCR1 and CXCR2. CXCR1 exhibits high affinity for IL-8 while CXCR2 exhibits affinity for three ligands, IL-8, GROα and NAP-2 (163,164). Although the role of CXCR1 and 2 are important to the function of IL-8, there is still controversy over how the receptors contribute to biological functions and how they initiate changes in cancer progression which will not be a highlight of this thesis. This thesis will address the importance of IL-8 in cancer progression with an emphasis on its significance to PCa progression outlined in the next section.

**Significance of IL-8 in cancer progression**

Clinical evidence demonstrates that increased IL-8 expression and serum levels correlates with poor prognosis and recurrent disease in many cancers, including melanoma, ovarian, colon, lung, breast, lymphoma, head and neck small cell carcinoma, liver and prostate (165-171). Therapies targeting IL-8 have been investigated, such as
Figure 4
Structure and Regulation of Interleukin 8

A. Illustration of IL-8 and sites required for activity. The ELR sequence is essential to the binding of the IL-8 receptors and for its activity. The tyrosine and lysine residues and the alpha helical region located near the C-terminus are required for binding of the IL-8 receptors as well.

B. Transcriptional regulation of IL-8. The picture shows transcription factors important in IL-8 transcription. The basal levels of transcription are regulated by binding of the NF-kB repressing factor (NFR) to the negative regulatory element obstructing NFk-B binding. Binding of octamer-1 (OCT-1) occupies the C/EBP site and deacetylation of histone proteins by histone deacetylase 1 (HDAC-1) modulates basal transcription as well. Upon either induced or constitutive activity, NFk-B translocates to the nucleus binding near NRF. C/EBP occupies the OCT-1 site while CREB binding protein (CBP)/p300 initiates chromatin remodeling through histone acetylation and activator protein-1 (AP-1) transcription factor becomes phosphorylated as well. The culmination of these events results in transcription of IL-8.
the administration of the humanized monoclonal antibody ABX-IL-8. ABX-IL-8 administration demonstrated decreased metastatic potential of melanoma in xenograft models and decreased tumor growth in bladder cancer (172-173). Although this treatment did not provide significant clinical benefit, it provided necessary information regarding how IL-8 signaling modulates tumor progression in a subset of cancers. Many of these studies prompted the investigation of a potential role for IL-8 in PCa progression.

**Significance of IL-8 in PCa progression**

Zabransky et al. demonstrate that lenalidomide treatments decreased IL-8 serum levels in patients with PCa, but, interestingly, IL-8 levels remained high in patients who progressed (169). A phase I/II trial with Dasatanib demonstrates that a cohort of responding patients display a decrease in serum IL-8 while non-responders showed either increased or stable IL-8 levels. Dayanni et al. report a correlation between dasatanib levels and decreased serum IL-8. This preliminary data strongly suggests that IL-8 may be an additional marker of response, but a larger study is warranted and has been completed with results pending. Further support comes from *in vitro* and *in vivo* studies demonstrating that IL-8 expression correlates with tumorigenecity and metastasis of PCa (172,173). However, there is controversy and unclear evidence concerning IL-8’s particular role in metastatic CRPC.

*In vitro* studies have demonstrated that IL-8 overexpression in androgen-positive cells desensitizes PCa cells to anti-androgens, such as bicalutamide and the DNA damage agent, oxaliplatin, while IL-8 also reduces growth inhibition (146,174,175). In contrast, another report demonstrates IL-8 increases the proliferation
of PCa cells, but this is not observed in the presence of bicalutamide (176). Other conflicting results from these and other studies are surrounded around how IL-8 regulates AR and PSA levels (146, 147, 176). These controversies have also led to an unclear understanding of how IL-8 signaling modulates PCa progression and the metastatic properties it involves. This thesis will identify IL-8 signaling pathways involved in regulating metastatic properties of PCa progression to metastatic CRPC. The next section will discuss studies prompting more investigation about the signaling mechanisms and biological processes surrounding the role of IL-8 in PCa progression.

**Significance of IL-8 in metastatic properties of PCa**

IL-8 induces classical biological functions including chemotaxis, migration, calcium mobilization, protein translation, inflammatory responses and cell proliferation, with chemotaxis and migration being the first identified biological effects in normal cells, illustrated in Figure 5. IL-8 was recognized as a regulator of chemotaxis through neutrophil-activation *in vitro* (163). Later studies demonstrated that IL-8 is involved in other biological functions of normal cellular functions and cancer cell functions. One of the first studies observing IL-8 in PCa demonstrated that neutralization of IL-8 decreases angiogenic activity and tumorigenecity of PC-3 cells (249). Since then, it has been well established that IL-8 plays a critical role in angiogenesis of cancer cells (177, 178). Kim et al. demonstrated that PCa cell lines express high levels of IL-8 contributing to increases in microvessel density increasing prostate tumor growth (179). *In vivo* studies by Inoue et al. also report increased VEGF and IL-8 expression demonstrating a correlation between neovascularization and IL-8 expression (173). More recent studies have been aimed at determining factors that potentially regulate IL-
8 or that IL-8 regulates modulating angiogenesis (146,147,175,176). There have been many advances made in understanding the role of IL-8 in the process of angiogenesis in PCa, but there has been and increasing effort put towards understanding how IL-8 contributes to other biological processes of PCa, such as migration and invasion and its suggested role in the progression to metastatic PCa.

Although the first studies demonstrate IL-8 as a novel chemoattractant, subsequent reports determined that IL-8 also regulates shape change and directional migration (180-182). As a result of these studies, IL-8 has been identified as a migratory regulator in multiple cancers, acute myeloid and lymphoid leukemia (AML and ALL) and melanoma being the first cancers to describe IL-8 as a relevant chemoattractant and migratory factor (183,184). Bar-Eli et al. demonstrates that IL-8 directly regulates matrix metalloproteinase (MMP) gene expression increasing invasion providing some of the foundational studies for examining the invasive role of IL-8 in numerous cancers, including PCa (185). Following these studies, Reiland et al. provided one of the first PCa studies demonstrating that PCa cells show significant increases in migration and invasion in response to exogenous IL-8 (186). Inoue et al. also demonstrates that overexpression of IL-8 in PCa cells significantly increases migration, while antisense transfection of IL-8 in high IL-8 expressing PCa cells decreases migration (173). More recent studies have demonstrated that IL-8 increases the migration of androgen-independent PCa cells suggesting that IL-8 production regulates PCa progression in the absence or in the place of androgen regulation (146,147). This evidence suggests that IL-8-mediated migration and invasion participates in PCa
progression to a metastatic phenotype, but there are questions about the signaling mechanisms involved.

**Significance of IL-8 signaling in metastatic properties of PCa**

Although information regarding IL-8 signaling cascades modulating biological functions of PCa is increasing, the mechanisms are still unclear. A comprehensive review by Waugh et al. provides a schematic of suggestive IL-8 signaling pathways that contribute to biological functions of cancer progression (175). There is information regarding specific canonical IL-8 signaling pathways regulating biological functions of cancer and it is suggested that protein tyrosine kinase activity is involved, where the clinical observation of increased IL-8 expression and SFK activity in PCa tissues, these reports provide interesting implications for a cooperative role of IL-8 and Src in PCa progression. This phenomenon had not been explored until recently where the results are interesting, but inconclusive, and are limited in scope.

In PCa, studies suggest that IL-8 signals through tyrosine kinases, such as Src and FAK, during the transition of androgen dependent PCa to androgen independent PCa (146,147). A recent study demonstrates that IL-8 participates in the transition of androgen dependent PCa to androgen independent PCa by increasing AR transcriptional activity (176). These reports suggest that, not only, do tyrosine kinases activate signaling mechanisms important for metastatic properties in PCa cells, but that this may occur through IL-8 cytokine-induced stimulation of tyrosine kinase activity in metastatic CRPC (146,147). As a result, this thesis will address if IL-8 and Src cooperate to induce changes in biological functions related to PCa progression to

~ 31 ~
**Figure 5**
**IL-8-Mediated Signaling Pathways in Cancer**

This is a simplified illustration of how IL-8 regulates multiple pathways that lead to changes in angiogenesis, proliferation, invasion and survival. The solid blue lines are known pathways that IL-8 regulates, such as the canonical IL-8 activation of PLC and PKC. The dashed blue lines represent less understood pathways of IL-8 regulation, such as the IL-8-mediated regulation of RhoGTPase. Lastly, the orange solid lines represent pathways that stimulate transcription factor activity. This illustration displays known and unknown pathways of IL-8 regulation and modulation of multiple biologic functions of cancer.

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metastatic disease. In addition to understanding the contributions of factors expressed and secreted by the tumor and their role in tumor progression, PCa is also influenced by the microenvironment. As a result a major thrust of this thesis will be to address how IL-8 signaling in the tumor microenvironment affects PCa progression. The next section will discuss the importance of understanding IL-8 in the tumor microenvironment and what role that plays in PCa progression.

**Significance of IL-8 in the tumor-stromal microenvironment**

It is well established that IL-8 is a potent cytokine secreted by cell types surrounding and interacting with cancer cells. The intratumoral production of IL-8 is associated with increased survival and growth, but IL-8 production also occurs by cells supporting the tumor. Initial studies demonstrate that IL-8 is expressed and secreted in abundance by bone marrow stromal cells (190,191). Other studies demonstrate that IL-8 is elevated in bone remodeling cells and fibroblastic stromal cells, two cell types directly interacting with PCa cells. Earlier studies supporting this demonstrate that the stromal compartment has a direct impact on epithelial cell behavior (192). However, initial studies reported the stromal compartment as a passive structure of support with very little aid to the biological function of cells. Over the past decade, there has been increasing evidence demonstrating that the stromal compartment provides a permissive and supportive environment of survival for cancer cells. As a result, it is suggestive that cancer progression, including PCa, is also a result of microenvironmental-mediated effects.

These microenvironmental-mediated effects have been studied in other cancers and have demonstrated that cancer-produced growth factors and cytokines modulate
bone formation (193,194). Interestingly, evidence also demonstrates that IL-8 can induce fibroblast or stromal derived cells to become reactive stroma. (196). As the information grows concerning how factors, such as IL-8, modulate the microenvironment, there is less information indicating the sources of IL-8 production, albeit tumor-produced and/or stroma-produced. There is also little evidence discussing how this IL-8 production affects PCa progression and what IL-8 signaling pathways are involved.

It is possible that a heterotypic crosstalk mechanism exists whereby IL-8 production by either the stromal or tumor compartments modulates IL-8-mediated signaling pathways in the tumor compartment to increase PCa progression. Since, there is evidence supporting a collaborative role of IL-8 and Src in PCa progression, there is a possibility that the crosstalk mechanism in the stromal compartment involves these factors. However, this has not been studied and will be addressed in this thesis.

We know that the tumor microenvironment provides a rich milieu of factors contributing to PCa cell survival, proliferation, migration and invasion. IL-8 production by fibroblasts and stromal cells is associated with survival of PCa cells, but we do not know if Src is involved in this mechanism and whether this is an IL-8 autocrine and/or paracrine mediated event. There have been preclinical mouse model studies addressing the role of IL-8 in the metastatic potential of PCa (173,196). These studies suggest that overexpression of IL-8 increases the metastatic potential of PCa in mouse models, but the microenvironment-mediated nature of PCa leads us to hypothesize that the tumor is not the only contributing cell type in PCa progression to metastatic disease.
Questions that are still unclear are how and why the tumor-stromal interaction affects the metastatic potential of PCa, which is integral to this thesis. These questions are capitalized by current efforts to assess therapeutic strategies that target factors expressed and/or secreted by both tumors and microenvironmental cells. The next section will discuss current therapeutic strategies in PCa and will highlight the efforts made in developing strategies that target the tumor and microenvironment.

**CURRENT STRATEGIES IN TREATING PROSTATE CANCER**

**Treatment strategies for localized and castration resistant prostate cancer**

Treatment of localized PCa usually involves radiation or surgery which eradicates nearly 100% of the cancer, but there are 30-50% of these patients that will incur either a local or distant recurrence. For recurring patients, first-line therapies involve androgen deprivation therapies (ADT), such as luteinizing hormone-releasing hormone (LHRH) and anti-androgens, but unfortunately in many of these patients, CRPC develops. Many of the earlier treatment options for CRPC, such as mitoxantrone-based treatments or bisphosphonates, provided palliative benefits, but did not provide overall survival benefits (197,198). In 2004, the randomized clinical trials, TAX 327 and SWOG 9916, reported the first evidence for increased survival benefit and reduced PSA in patients with metastatic CRPC using docetaxel-based chemotherapies and resulted in FDA approval for use of docetaxel as a treatment for metastatic CRPC (199,200). Docetaxel was the only FDA approved drug used to target metastatic CRPC. Now, multiple drugs have been approved due to the increased number of drugs entering into clinical development. The number of drugs in clinical
development has increased, in part, due to the advances in the understanding of the cellular and biological mechanisms of PCa progression.

The principal increase in understanding that has led to new drugs is that androgen ablation does not effectively block AR signaling. As a result, there has been increased development of anti-hormonal therapies, such as abiraterone acetate and MDV3100. Abiraterone acetate, an inhibitor of the key enzyme for hormonal synthesis (cytochrome P17), demonstrated a >50% decline in PSA in CRPC docetaxel-treated patients in a phase II trial with 38% of the patients showing partial response and also demonstrated an overall survival of ~4 months with abiraterone treatment. In April 2011, abiraterone became an FDA-approved anti-androgen therapy for use in metastatic CRPC (5,201). MDV3100, an AR antagonist, has also been used in a phase I/II study whereby patients treated with chemotherapy showed >50% decrease in PSA and non-chemotherapy treated patients displayed >60% decrease in PSA levels (202). MDV3100 (Enzalutimide) is now an FDA approved therapeutic agent that disrupts translocation of AR to the nucleus decreasing AR binding to DNA and preventing coactivator recruitment which is a proposed mechanism thought to contribute to tumor regression of CRPC (202). These results suggest that patients with androgen responsive metastatic CRPC may gain from therapeutics targeting continued AR signaling. However, targeting sustained androgen signaling is only one aspect of current therapeutic strategies addressing the cellular events in metastatic CRPC. Therapies targeted against signaling molecules, such as growth factor and tyrosine kinase signaling, upregulated in metastatic CRPC are needed as well.
The observed aberrant expression of kinase pathways, such as Her2/Neu, EGFR, PDGFR, FAK, Raf, VEGFR, PI3K/Akt, MAPK, Axl, MET and Src, in metastatic CRPC have prompted the development of many therapeutic agents (203-206). Detailed information regarding these pathways will not be discussed further, but are mentioned to highlight that targeting aberrantly active kinase pathways is an increased area for therapeutic strategy. However, I will discuss recent efforts in the development of therapeutic strategies for targeting the aberrant activity of the tyrosine kinase, Src, a major subject of this thesis. These recent efforts are, in part, due to studies from our laboratory and others demonstrating aberrant Src activity in the progression of PCa to metastatic CRPC and in the microenvironmental compartment as well.

Evidence suggests that the expression of Src in multiple microenvironment cell types contributes to the progression of PCa. Mouse model studies using Src-/- mice develop osteopetrosis (bone-building disease) suggesting that Src expression impairs osteoclast activity important for the balance of the bone remodeling process (207). The disruption of Src activity also increases bone-building activities by negative regulation of osteoblasts (151). Araujo et al. provides a comprehensive review demonstrating that SFK inhibition has anti-osteoclast activity preventing osteolytic metastases in breast cancer and decreased growth of tibially-injected prostate tumors (208). These studies have prompted the development of SFK inhibitors, such as AZD0530 (Saracatinib) and Dasatanib, that demonstrate inhibition of tumor growth and osteoclast activity. Saracatinib was introduced as a dual SFK/Abl inhibitor and has demonstrated promising
in vitro and in vivo results and has entered into phase I/II trials (209,210). The clinical importance of SFK inhibition by Saracatinib is still pending.

Dasatinib is another SFK inhibitor that is considered to be the most clinically studied Src inhibitor (212). Dasatinib was developed as a potent biochemical inhibitor of Src and Bcr/Abl kinase activity in multiple solid tumors, including PCa. Initial in vitro findings demonstrated that dasatinib, now known to be a multi-kinase inhibitor, decreases SFK activity in PCa cell lines (107,117). Preclinical studies from our laboratory demonstrate that dasatinib decreased lymph node metastases as well as migration and invasion of cells in vitro (107). In 2009, our institution completed a Phase 1/2 trial on dasatinib + docetaxel, with results so promising a worldwide Phase 3 trial has been completed with results pending (211). Some of the preliminary results demonstrate that combination therapy of docetaxel and dasatinib in metastatic CRPC (CA180-086) showed dramatic decreases in bone alkaline phosphatase, BAP, and urinary N-telopeptide, UNTx and 28% of patients displayed a reduction in size and number of lesions due to bone scan (213). The remaining endpoints of overall survival and progression free-survival are being analyzed. These studies suggest that dasatinib may serve therapeutic benefit for patients by targeting, not only, metastatic CRPC, but also the tumor microenvironment. As mentioned, osteoblast and osteoclasts are two cell types in the tumor microenvironment affected by Src, but cytokine-producing cell types, such as fibroblasts are also located in the tumor microenvironment. One of the cytokines produced by fibroblasts is IL-8 known to be important in PCa progression.

We are slowly gaining an understanding of the biological pathways and mechanisms over-expressed or deregulated in metastatic CRPC. The combination of
therapies targeting kinase-mediated signaling pathways in the tumor and the microenvironment may serve as one therapeutic strategy to disrupt the growth and progression of metastatic CRPC. As a result, this thesis will focus on understanding the contributions of Src signaling in metastatic disease and will identify signaling pathways associated with progression to metastatic disease.

**SUMMARY OF PROBLEM AND HYPOTHESIS**

Prostate cancer is a leading cause of cancer mortality in men in western civilization. While localized disease is almost always incurable, <40% of men will incur metastatic disease despite local therapies. As a result, therapies targeting factors involved in PCa progression will be beneficial in preventing metastatic spread. Two contributing factors to the metastatic spread of PCa are IL-8 and Src. These factors are also produced and expressed by microenvironmental cells, such as stromal cells, which take part in the bidirectional interactions increasing prostate carcinogenesis.

The goal of this Ph.D. dissertation is to investigate the role of IL-8-mediated Src family kinase activation in tumor-tumor and tumor-stromal interactions in metastasis of prostate cancer. Previous studies suggest that aberrant Src activity regulates functions that increase IL-8 secretion by PCa cells. In addition, IL-8 overexpression, by the tumor and stroma, and increased Src activity have, individually, been implicated as mediators of PCa progression. However, there is not a clear understanding of the relationship between IL-8 signaling and SFK activation. The questions surrounding IL-8 and SFK involvement in PCa are: 1) is there a feed forward loop whereby IL-8 induces Src activation further increasing IL-8 secretion which affects metastatic
properties of PCa? 2) is there a paracrine mechanism whereby IL-8 production by the stroma affects Src activation and metastatic properties of PCa? and 3) how does IL-8 production by the stroma affect tumor growth and metastatic potential?

The hypothesis tested in the work of this dissertation is that IL-8-mediated Src activation regulates increased metastatic potential of prostate cancer through tumor-tumor and tumor-stromal interactions. To test this hypothesis, I determined if IL-8 expression and secretion by PCa cells affects Src activity and migration in vitro. I next determined the effects of stroma-produced IL-8 on migration and invasion of PCa through Src activation in vitro. Lastly, I determined whether IL-8 production by the stroma affects tumor growth and metastatic potential in vivo. The work in this dissertation has led to a better understanding of how IL-8 signaling in PCa and the stroma modulates PCa progression as well as increased our understanding of the role of SFK activation in IL-8 signaling in PCa.
CHAPTER 2

MATERIALS AND METHODS
Cell Lines

The approach for this study was to use two well established cell culture models that mimic the progression of PCa from low metastatic to high metastatic potential with high metastatic potential representing advanced stage disease. The LNCaP human PCa progression model and the PC3 model contain isogenic variants of differing metastatic potential. The LNCaP cells are non-metastatic and androgen dependent while C4-2B4 cells are bone metastatic and androgen independent, as previously described (43). The LNCaP cell lines were provided by Dr. Mike Gray (MDACC). The PC3-MM2 cell line was created and kindly provided by the lab of Dr. Isaiah J. Fidler in the Department of Cancer Biology, the University of Texas MD Anderson Cancer Center. PC3 cells are low metastatic and androgen-dependent while PC3-MM2 cells are lymph node metastatic and androgen-independent, as previously described (214,215). The HS5 cell line is an immortalized fibroblastic stromal cell derived from human bone marrow and purchased from ATCC. This cell line was established as previously described (224).

Briefly, the LNCaP and C4-2B4 cell lines were maintained in RPMI 1640 medium (Sigma Aldrich) containing L-glutamine supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (HyClone), MEM Non-Essential Amino Acids (NEAA), Sodium Pyruvate and MEM Vitamin Solution. NEAA, Sodium Pyruvate and Vitamin Solution were purchased from Gibco. The PC3, PC3-MM2 and HS5 cell lines were maintained in Dulbecco’s Modified Eagle Media (DMEM)/ Ham’s F-12 (Sigma Aldrich) medium supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were also tested for mycoplasma using an enzymatic based method, MycoAlert™ Detection Kit, by Lonza and polymerase chain reaction
(PCR) method, Lookout® Plasma Elimination Kit by Sigma Aldrich. All cells were tested and found to be mycoplasma free.

**Reagents**

Recombinant IL-8 (72a.a.;rh-IL-8) was purchased from PeproTech. The thiazole-based dual Src/Abl kinase inhibitor, 2-acylamino-5-carboxamidothiazole (BMS-354825/dasatanib) was a gift from Bristol Myers Squibb.

**Construction of Lentiviral shRNA to IL-8**

For this study, PC3-MM2 and HS5 cells were infected with a GFP-labeled lentiviral-based IL-8 short hairpin RNA (shRNA). The lentiviral system is a well-established system developed by Didier Trono and the specific lentivirus system for IL-8 was made and kindly provided to us by Dr. Bar-Eli (215). Briefly, sense and antisense oligonucleotides from the IL-8 siRNA target sequence (5’-GCCAAGGA GUGCUAAAGAA-3’) were designed with a hairpin and sticky ends (ClaI and MluI), annealed to the green-fluorescent protein (GFP) labeled pLVTHM lentiviral gene transfer vector, used for DNA isolation and lentiviral production by the described method (216). A nontargeting sequence (5’-UUCUCCGAACGUGUCACGU-3’) was used as a control.

After plating of PC3-MM2 or HS5 cells, 500ul of viral supernatants were added to semi-confluent cells along with 6ug/ml of polybrene infection reagent (Millipore) and 500ul of growth media. After 48 hour incubation, GFP cells were visualized using fluorescence microscopy. Cells were then expanded to 150cm² tissue culture plates and GFP sorted by fluorescence activated cell sorting (FACS) using the Flow Cytometry and Cellular Imaging (FCCI) Core Facility at MD Anderson. qRT-
PCR and ELISA was performed to determine if endogenous and secretory IL-8 knockdown using methods in the previous mentioned sections on qRT-PCR and ELISA.

**Construction of IL-8 expression vector**

The work discussed in this section was done in collaboration with Lily Huang and Dr. Bar-Eli (Department of Cancer Biology, MDACC). The protein encoding region was amplified from human PC3-MM2 cDNA using the Touchdown PCR program. This method is useful in increasing the annealing specificity of PCR by using multiple cycles and multiple annealing temperatures enhancing formation of a more specific final PCR product. The sequences for the PCR primers were made with restriction enzyme sequences for Xho1 and Sal1 (Promega) - F- 5’ GGCTCGAG GCCACCATGACTTCCAAGCTGGCCGT-3’ and R- 5’ GAGTCGACTTATGATTCTCAGCCCTCT-3’. The program was as follows: 94°C for 2 min.; 10 cycles of 94°C for 15 sec., 68°C for 45 sec., -1.5 sec/cycle, 72°C for 2 min 30 sec.; 35 cycles of 94°C for 15 sec., 50°C for 45 sec., 72°C for 2 min 30 sec, + 3 sec/cycle; 72°C for 20 min. and holding at 4°C. PCR samples were purified using QIAquick PCR Purification Kit (Qiagen). Digestions were completed with purified PCR and pSI vector (Promega) according to manufacturer’s protocol (Promega). Digested vector and PCR were run on a crystal violet gel. Bands specific to IL-8 and digested pSI vector were extracted from the crystal violet gel using the gel extraction method and purified using the EZ Gel Extraction Kit (EZ Bioresarch) and Geneclean Turbo Kit (QBiogene) following the manufacturer’s protocol. The pSI vector and IL-8 purified DNA were ligated and transformations were performed using XL-10 Gold competent cells using the manufacturer’s protocol (Stratagene). After selection of 8-10 single colonies and
expansion, DNA was purified by performing the QIAPrep Spin Miniprep Kit according to the manufacturer’s protocol (Qiagen). Completion of cloning was observed by enzyme digestion and pSI/IL-8 DNA samples were confirmed by DNA sequencing using the DNA Analysis Core Facility (DAF) at MD Anderson Cancer Center.

**Construction of IL-8 mutagenesis vector**

We constructed a sequence that introduced point mutations in the shRNA target region without affecting the peptide sequences. The QuikChange II XL Site Directed Mutagenesis (Stratagene) method allowed us to make point mutations in the basic IL-8 expression vector through PCR-based methodology. Mutagenic primers were designed using the QuikChange Primer Design Program [www.agilent.com/genomics/qcpd](http://www.agilent.com/genomics/qcpd). The primers are as follows: F-5’

CTGTGTGAAGGTGCAGTTTTACCTAGAAGCGCAAAGGAGCTTAGATGTCAG
TGCTAAAG-3’ and R-5’ CTTTATGCACCTGACATCTAAGCTCCTTTGCGCTTCT
AGGTAAAACCTGCACCTCACCAGCAG-3’.

Briefly, synonymous mutations were introduced into the pSI/IL-8 DNA by performing PCR using PfuUltra Hotstart DNA polymerase. After Dpn1 digestion of amplified product, transformation into XL-10 Gold ultracompetent cells (Stratagene) was performed according to manufacturer’s protocol. After selection of 8-10 single colonies and expansion, DNA was purified by performing the QIAPrep Spin Miniprep Kit according to the manufacturer’s protocol (Qiagen). Completion of cloning was observed by enzyme digestion and pSI/IL-8 DNA samples were confirmed by DNA sequencing using the DNA Analysis Core Facility (DAF) at MD Anderson Cancer
pUC18 transformation and pWhitescript mutagenesis controls provided with the kit were used.

The resulting rescue vector, pSI/IL-8, was transfected into cells using Fugene 6 (Roche) according to manufacturer’s protocol and Quantikine IL-8 ELISA (R&D Systems) was performed to determine IL-8 levels.

**Immunoblot**

Cell lysates were extracted using previously described methods (107). Protein concentration was determined by RC DC BioRad Protein Assay Kit followed by spectrophotometric analysis by the Perkin Elmer Envision 2104 Multilabel Reader. Equal amounts of protein (50μg) were boiled at 100°C for five minutes, separated on 8% sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membrane at 100 volts at 4°C and blocked with either 5% BSA or 5% Milk dissolved in Tris-base with .05% Tween 20 (TBST).

Cell lysates were subjected to immunoblot analysis using the following antibodies: anti-pSRC<sup>Y416</sup>, anti-pAKT<sup>S473</sup>, anti-pAKT<sup>S308</sup>, anti-p38 MAPK, total AKT (Cell Signaling Technology, Inc.), CXCR1, CXCR2, pNF-κB/65 (Santa Cruz Biotechnology, Inc.), total SRC (Oncogene Science, Inc.), pFAK<sup>Y861</sup> (Biosource, Inc.), total FAK (BD Transduction, Inc.) and vinculin (Sigma, Inc.). Secondary antibodies used were as follows: anti-mouse IgG-HRP and anti-rabbit IgG-HRP (Bio-Rad, Inc.). The dilutions for primary antibodies used were 1:1000 with the exception of Vinculin 1:10,000. The dilutions for secondary antibodies used were 1:3000. The primary antibodies were applied overnight at 4°C, membranes were washed 5 times for 5 minutes at room temperature, secondary antibodies were applied to membranes for 1
hour at room temperature, membranes were washed again 5 times for 5 minutes at room temperature. After application of the enhanced chemiluminescence reagent ECL (Pierce, Inc.), blots were visualized on X-ray films. Experiments were completed at least twice.

Immunoprecipitation

Immunoprecipitation method was used to determine expression of the IL-8 receptors, CXCR1 and CXCR2. Cell lysates were extracted using previously described methods (107). To immunoprecipitate CXCR1, 4 ug of CXCR1 or CXCR2 antibody (Santa Cruz, Inc.) was added to 1mg of cell lysate and incubated overnight on a rotator at 4°C. Protein A-Agarose beads (Roche Applied Sciences) were used to form a complex between the CXCR1 receptor with high affinity for rabbit IgG and Protein G-Agarose beads (Thermo Scientific Pierce) were used to form a complex between the CXCR2 receptor with high affinity for mouse IgG. To prepare agarose beads, 50ul of Protein A or G beads were centrifuged at 12,000rpm for 20 seconds to remove supernatant. Beads were resuspended in 50ul RIPA buffer, lysate-antibody samples were added to the resuspended beads and samples were incubated for 2 hours on a rotator at 4°C. Samples were then centrifuged at 5,000rpm for 2 min at room temperature and supernatant was aspirated. To wash the lysate-antibody complex samples, ICKA wash buffer was added and samples were centrifuged for 5,000rpm for 2 min at room temperature. This step was repeated three times and 1X Lamelli sample buffer was added to samples and boiled for 5 minutes at 100°C. The supernatants were loaded onto a 6% SDS-PAGE gel and the remaining immunoblot was performed using the methods in the immunoblot section.
Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) and Quantitative Real Time-PCR (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) manufacturer’s protocol. Purified RNA was subjected to RT-PCR and qRT-PCR using the Peltier Thermal Cycler (MJ Research) and the MX3000™ Real-Time PCR System (Stratagene). The following primers were used: CXCR1F- 5’- GCTGTTGAGGTAC CTCCAG-3’, CXCR1R- 5’ AGTTCTTGGCACGTCATCG-3’; CXCR2F-5’-TCTG CTACGGATTCCACCCTG-3’, CXCR2R-5’ ACATTCTGTGCAAG GTGG-3’; IL-8F-5’ ATGACTTCCAGCTGGCCTG-3’, IL-8R- 5’ CCTCTTTCAAAAAACTTCTCCA CACC-3’. All PCR reactions were completed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control using the following primers: GAPDHF- 5’GAGGGGGGCAGGGGACCTG-3’, GAPDHR- 5’GGGA TGGG GGCTGGG AAG3’. For reverse transcriptase, 1ug of RNA was transcribed into cDNA using the Thermoscript™ RT-PCR System following the manufacturer’s protocol. PCR was completed with initial denaturation at 95°C for 5 minutes, 25 cycles of denaturation (95°C for 2 min), annealing (63°C (IL-8), 59°C (CXCR1/2), 62°C (GAPDH) for 45 seconds), extension (72°C for 2 minutes), and final extension (72°C for 10 minutes) after all cycles completed. PCR samples were analyzed on a 1.5% agarose gel containing ethidium bromide using diluted Biorad™ Tris/Acetic Acid/Ethlenediaminetetraacetic acid (EDTA) (TAE) buffer. Bands were visualized by Bio-Rad Gel Doc 2000 (BioRad, Inc.).

qRT-PCR was performed by extracting RNA using the aforementioned procedure. One microgram of RNA was transcribed into cDNA using TaqMan®
Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. The PCR reaction was made using TaqMan Gene Expression Assays (Applied Biosystems) for IL-8 (Hs00174103_m1) and GAPDH (HS02758991_g1) following the manufacturer’s protocol. Quantitative PCR was completed using the following cycling program: 60°C for 15 minutes, 95°C for 2 min., 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Gene expression was quantified relative to the GAPDH housekeeping gene. Threshold cycle (Ct) was determined for all cell lines and the relative level of transcript \(2^{\Delta\text{Ct}}\) was determined by the following formula: \(2^{(\text{Sample IL-8 Ct} - \text{GAPDH of Sample Ct})}\) and differences between cell lines were obtained using the formula, \(1/2^{\Delta\text{Ct}}\). Experiments were completed in triplicate and completed at least twice.

**Enzyme-linked immunosorbent assay (ELISA)**

To obtain conditioned media for ELISA, 50,000 viable cells were plated and the appropriate serum free medium was added for 24 or 48 hours. Conditioned media were subjected to centrifugation and stored at -20°C or used immediately. Remaining viable cells were detected by trypan blue exclusion and counted using the ViCell™ XR Cell Viability Analyzer (Beckman Coulter). For the ELISA, each conditioned medium was assayed for IL-8 using Quantikine® ELISA Human CXCL8/IL-8 Immunoassay (Research Diagnostics, Inc.: R&D Systems) according to the manufacturer’s protocol, and IL-8 levels were determined by comparison of absorbance of IL-8 protein to the standard curve. This system utilizes trypan blue exclusion to determine the population of viable cells and reports viable cells and total cell populations. These and subsequent experiments use viable cell counts for conducting experiments. The production of IL-8
was calculated to relative cell number. Experiments were completed in triplicate and completed at least twice.

**In vitro Cell Proliferation Assay**

To determine the rate of cell proliferation, cells were incubated for 12, 24, 48 or 72 hours with changes of medium every 48 hours. At each time point, cells were trypsinized and neutralized with growth medium, and viable cells were counted using the ViCell™ XR Cell Viability Analyzer. Cells were counted in triplicate and the experiments were repeated at least once.

To determine the effect of rhIL-8 on rate of cell proliferation, rhIL-8 was administered at concentrations of 10, 25, 50 or 100ng/ml in serum-free medium for 24 hours. Viable cells were determined by using the colorimetric CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega). Cells were counted in triplicate and the experiments repeated at least once.

**In Vitro Migration and Invasion Assays**

Migration and invasion assays were performed using boyden chamber inserts with 8.0µm-pore sizes. Invasion assays were performed using similar boyden chamber inserts with a BD Matrigel™ basement membrane matrix mimicking a physiological basement membrane. Briefly, 50,000 cells were plated in the top insert of a 24-well plate, and serum-free medium, growth medium or serum-free medium with recombinant IL-8 (rhIL-8) was added to the bottom chamber. After 18 hours, inserts were removed from the chamber, non-migratory cells were removed and migrated cells on the bottom of the insert were fixed and stained with HEMA 3® Stain Set (Fisher Scientific Company) according to the manufacturer’s protocol. After staining, migratory or
invasive cells were counted at 10x magnification in five random fields; counts were averaged for each insert and represented as cells migrated or invaded.

To determine the effects of dasatanib on migration and invasion, either 50nM or 100nM of dasatanib was added to serum-free medium in the bottom chamber, and inserts were placed atop wells for 18 hours. Migration and invasion were determined using the protocol already described. Experiments were completed in triplicate at least once.

**In vitro Co-culture migration and invasion assay of prostate cancer cells to immortalized human bone stromal cells**

To produce conditioned medium from stromal cells, 50,000 HS5 cells were plated in 24-well plates overnight, and serum-free medium was added to cells for 24 or 48 hours. Conditioned medium was used immediately or subjected to centrifugation and stored at -80°C.

To determine if stromal cell-conditioned medium affects migration and invasion of PCa cells, PC3 or PC3-MM2shIL-8 cells were plated in 24-well inserts in growth medium overnight. After the growth medium was removed and the cells washed, HS5 conditioned medium was placed in the bottom chambers of the plates and the inserts with PCa cells were placed on top of the bottom chambers for 12-24 hours. To determine the number of migrated or invaded cells, fixing, staining and counting were performed as described in the migration and invasion assay section. This assay was also completed with HS5 nontargeting control cells and shIL-8-cell conditioned medium. Each experiment was performed in triplicate.
**In vitro Co-culture of immortalized human bone stromal cell conditioned media with prostate cancer cells**

We tested whether co-culture of stromal cell conditioned medium with PCa cells affects Src activity. Briefly, HS5 or HS5shIL-8 cells were plated in 100 cm\(^2\) dishes, serum-free medium was added and conditioned medium was collected after 24 or 48 hours, subjected to centrifugation and stored at -80°C or used immediately. Each stromal cell-conditioned medium was placed on PC3 or PC3-MM2shIL-8 cells for 10, 15 or 60 minutes. Cell lysates were subjected to immunoblot analysis using the procedure already described.

**Orthotopic implantation of tumor cells**

Male athymic nude mice were purchased from the Department of Veterinary Medicine and Surgery Rodent Facility at MD Anderson Cancer Center. Animals were housed in pathogen-free conditions in the Research Animal Support Facility (RASF) at MDACC and used according to the institutional guidelines. In order to monitor PC3 cells *in vivo*, PC3 cells were plated in 12 well plates and infected with pre-made Renilla Luciferase-RFP fusion lentiviral particles (PC3RL) purchased from GenTarget, Inc (#LVP369). Briefly, 50μl of lentiviral particles were added to PC3 cells in fresh growth media and allowed to incubate for 48hrs. The transduction rate was determined by fluorescence imaging and sorted using MDACC Flow Cytometry and Cellular Imaging Core Facility.

Prior to orthotopic implantation, confluent PC3RL cells, HS5NT and HS5shIL-8 cells were detached and neutralized with growth media. A total of 5x10\(^6\) cells for the PC3RL group, 10x10\(^6\) cells for the PC3RL:HS5NT group and 10x10\(^6\) cells
for the PC3RL:HS5shIL-8 group were counted and subjected to centrifugation. Cells were resuspended in serum free DMEM in the following groups: .25x10^6 of PC3RL cells in 25ul, .25x10^6 of PC3RL cells plus .25x10^6 of HS5NT cells in 25ul and .25x10^6 of PC3RL cells plus .25x10^6 of HS5shIL-8 cells in 25ul.

For orthotopic implantations, male athymic nude mice (ages 3-5 wk) were anesthetized using Isoflurane. After which, mice were placed in a supine position and an incision was made in the lower abdomen. After prostate exteriorization, 25ul of SFM containing either PC3RL, PC3RL:HS5NT or PC3RL:HS5shIL-8 were injected into the lateral lobe of the prostate. Surgical clips were used to close the wound and removed one week post-surgery. To monitor tumor growth by luciferase activity, mice were injected with 100ul of Luciferin (GoldBio, Inc.) at 2.5 mg/ml and analyzed using the IVIS Imaging System (Caliper Life Sciences) in the Small Animal Imaging Facility at MDACC.

**Tissue Preparation**

After 20 days, mice were anesthetized with Isoflurane and mice were sacrificed. Primary tumors were excised, weighed and immediately portions were fixed in 10% formaldehyde in phosphate buffer. Another portion was embedded in OCT compound (Sakura Finetek) and flash frozen. The remaining portions were sealed in microcentrifuge tubes, flash frozen in liquid nitrogen and stored in -80°C. Iliac and renal metastases were determined by the enlarged size and opaque color observed and metastases were harvested.

**Phase I/II Dasatanib + Docetaxel Clinical Trial in Patients with Metastatic CRPC**
To determine the effects of dasatanib + docetaxel treatments in patients with metastatic CRPC, a Phase II clinical trial was performed and the parameters that were assessed were the maximum tolerated dose, toxicity profile and pharmacokinetics. The patient and eligibility criteria, study design and pharmacokinetics evaluation are outlined in detail in the study submitted by Araujo et al (211). Briefly, a small cohort of 46 patients were treated with intravenous administration of docetaxel and oral dasatanib administration in escalated dosages for 21 days. Blood samples were collected at days 1 (docetaxel alone), 14 (dasatanib alone) and 21 (docetaxel and dasatanib). Tumor progression was also measured using response evaluation criteria in solid tumors (RECIST) and bone radiographic measurements were taken pre- and post-treatments to indicate response. PSA and IL-8 levels were assessed using a cytokine platform.

Statistics

Statistical analyses were performed using GraphPad Prism 5 software (GraphPad, Inc.). Absorbance counts from proliferation assays and cell numbers from migration and invasion assays were compared using the Student t-test. All statistical tests were two-sided. Statistical values are as follows: nonsignificant (ns) $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$
CHAPTER 3
THE EFFECTS OF IL-8-MEDIATED SFK ACTIVITY IN PROSTATE CANCER IN VITRO
Although recent androgen deprivation therapies have demonstrated promising success in decreasing metastatic CRPC, there are still many patients that do not respond (5). Mechanisms involved in the development of tumor resistance to these AR-targeted therapies are unclear. As a result, there has been an increased effort in identifying signaling mechanisms in CRPC that may be targeted in addition to AR signaling.

Of the many signaling mechanisms increased in CRPC, one protein known to be increased in signaling mechanisms of CRPC is IL-8. Studies demonstrate that, upon androgen withdrawal, IL-8 production is increased and, upon unresponsiveness to anti-androgen therapy, circulating IL-8 levels are increased (146,173,218). Studies also demonstrate that increased IL-8 increases angiogenesis, migration and invasion (175,220). These results suggest that IL-8 production enhances metastatic disease, but it is unclear what IL-8-associated signaling molecules are involved in CRPC. Our laboratory demonstrates that SFKs regulate IL-8 secretory levels and recent in vitro evidence demonstrates that IL-8 stimulation of SFK activity is a partial contributor to the survival of IL-8 expressing cells against therapeutic effects (148). This suggests that SFKs regulate IL-8 and, in turn, IL-8 mediates SFK activity creating a feed forward loop. However, this has not been investigated in CRPC. As a result, this chapter will investigate if IL-8 affects Src activity in PCa cell lines and if this signaling axis affects biological functions of metastatic disease. To accomplish this, I used the non-metastatic LNCaP cell line and its metastatic variant, C4-2B4, and the metastatic PC3 cell line and its highly metastatic variant, PC3-MM2. Observations from this chapter will: (1) determine if IL-8 expression and Src activity are increased in metastatic disease, (2)
determine how IL-8 mediates Src activity in PCa and (3) determine if IL-8 mediation of SFK activity affects biological functions of metastatic disease.

**IL-8 receptors expressed in PCa cell lines**

IL-8 is known to signal through G-protein coupled receptors, CXCR1 and CXCR2. CXCR1 is the high affinity IL-8 receptor (14,159). Binding of IL-8 to CXCR1 is primarily responsible for mediating proliferative and motility functions of tumor cells (220). Shamaladevi et al. reports that CXCR1 contributes to changes in tumor growth and cell proliferation, while other reports demonstrate that CXCR2 does not contribute to tumorigenesis of PCa (186,221). These results suggest that the CXCR1 receptor is more relevant in PCa progression. As a result, I determined CXCR1 expression in the PCa cell lines, by examining mRNA and protein levels through RT-PCR and immunoblot analysis in LNCaP, C4-2B4, PC3 and PC3-MM2 cell lines. First, RT-PCR was performed to determine the level of CXCR1 mRNA expression in all PCa cell lines. **Figure 6a** shows that CXCR1 mRNA is expressed in all cell lines while displaying a decrease in mRNA expression in the PC3-MM2 cell line. Secondly, immunoprecipitations were performed using a CXCR1 antibody and immunoblot analysis was done to assess CXCR1 protein expression in all PCa cell lines. **Figure 6b** demonstrates that there is CXCR1 protein expression in all cell lines with a decrease in protein levels in PC3-MM2 cells. Studies, by other laboratories, demonstrate that CXCR1 undergoes receptor internalization and/or down-regulation upon IL-8 stimulation in cells with high IL-8 levels (166). This led me to ask the question of whether IL-8 levels are high or low in the cell models. As a result, I determined if IL-8 levels were increased in all PCa cell lines.
FIGURE 6 CXCR1 Expression in PCa Cell Lines
A. LNCaP, C4-2B4, PC3-PC3-MM2 cell lines were grown to sub-confluence, RNA was isolated and RT-PCR was performed using primer sequences targeting CXCR1.  B. Cells were grown to sub-confluence, immunoprecipitations were performed with the anti-CXCR1 antibody and cell lysates were subjected to immunoblot analysis.
IL-8 expression and protein production is increased in metastatic variants

I determined if there were differences in IL-8 mRNA expression and protein levels amongst the PCa cell lines and their metastatic variants. To determine the mRNA expression levels of IL-8 in PCa cell lines, qRT-PCR was performed. As shown in Figure 7a and c, qRT-PCR demonstrated that IL-8 mRNA expression levels were increased in the C4-2B4 cells relative to LNCaP cells and increased in the PC3-MM2 cells relative to PC3 cells (p≤0.001 and p≤0.01, respectively). To determine if IL-8 secreted levels correlated with mRNA levels, I performed an ELISA using the conditioned media of PCa cells and quantified the results according to cell number. The results demonstrate that IL-8 secretory levels were significantly increased in PC3-MM2 and C4-2B4 relative to their isogenic variants (p≤0.001 and p≤0.01, respectively). IL-8 levels were virtually undetectable in LNCaP cells, shown in Figure 7b and d. These results, first, demonstrate that IL-8 mRNA expression correlates with secretory levels. Second, the results demonstrate that there is a correlation between IL-8 levels and metastatic potential (LNCaP<C4-2B4<PC3<PC3-MM2).

Src activity is increased in metastatic variants

While the previous data show that IL-8 levels are increased in metastatic cell lines, our laboratory demonstrated that Src activity is increased in metastatic cells (PC3 and PC3-MM2) (107). As a result, I wanted to determine whether there was a correlation between IL-8 levels and Src activity in PCa cell lines and their metastatic variants. To accomplish this, immunoblot analysis was performed using phosphorylated SFK and total Src antibodies. Immunoblot analysis demonstrated that Src activity was significantly increased in C4-2B4 cells relative to LNCaP cells and
FIGURE 7  IL-8 mRNA Expression and Production in PCa Cell Lines

A. LNCaP, C4-2B4, PC3 and PC3-MM2 cells were grown to subconfluence and mRNA was isolated for qRT-PCR. IL-8 mRNA expression was assessed by qRT-PCR and reported as IL-8 mRNA expression relative to either LNCaP or PC3 IL-8 mRNA expression.

B. Cells were seeded, serum starved and conditioned media was collected from all cell lines. ELISA was performed to determine secretory levels of IL-8 in all cells and reported as pg/ml of IL-8 relative to total cell number. ** $p \leq 0.01$, *** $p \leq 0.001$
significantly increased in PC3-MM2 cells relative to PC3 cells (shown in Figure 8a and b). The results, first, demonstrate that Src activity is increased in the cell lines in the following order, LNCaP<C4-2B4<PC3<PC3-MM2. Second, the results demonstrate that although there is not a direct correlation between absolute IL-8 levels and Src activity the highly metastatic cells have the highest Src activity and IL-8 expression.

Next, to determine if exogenous IL-8 increases Src activity in low and high metastatic cells, I treated cell lines with 50ng/ml of recombinant IL-8 (rhIL-8) for 5, 10 and 30 minute timepoints. 50ng/ml of rhIL-8 was used as an optimal concentration demonstrated to increase downstream signaling in PCa cell lines as determined by previously published results (223). Addition of rhIL-8 significantly increased Src activity by 3.5 fold in LNCaP cells, 1.7 fold in C4-2B4 and 3.1 fold in PC3 cells, shown in Figures 9 and 10. However, there were no increases seen in the highly metastatic PC3-MM2 cell line. These results demonstrate that IL-8 increases Src activity in non- and low metastatic PCa cells, while demonstrating IL-8 does not increase Src activity in highly metastatic PCa cells.
FIGURE 8  Src Activity in Low and High Metastatic PCa Cell Lines
Cell lysates from (A) LNCaP and C4-2B4 cells, (B) PC3 and PC3-MM2 cells were subjected to immunoblot analysis. Src activity was determined by using anti-phospho Src Y419 and Total Src antibodies.
FIGURE 9  Effects of Exogenous IL-8 on Src Activity in the LNCaP Model

A and B. LNCaP and C4-2B4 cells were grown to subconfluence, serum starved and treated with rhIL-8 (50ng/ml). Lysates were analyzed by immunoblot and Src activity was determined by using anti-phospho Src Y419 and Total Src antibodies. Vinculin was used as a loading control. ** p ≤ 0.01, *** p ≤ 0.001
FIGURE 10  Effects of Exogenous IL-8 on Src Activity in the PC3 Model
A and B. PC3 and PC3-MM2 cells were grown to subconfluence, serum starved and treated with rhIL-8- (50ng/ml). Lysates were analyzed by immunoblot and Src activity was determined by using anti-phospho Src Y419 and Total Src antibodies. Vinculin was used as a loading control. *** $p \leq 0.001$
Exogenous IL-8 increases proliferation in low metastatic variants

Past studies demonstrated that overexpression of IL-8 in LNCaP cells increased proliferation (146, 223). However, I wanted to determine if exogenous IL-8 increases proliferation between low and high metastatic cells. Cells were plated and rhIL-8 was added to cells at 10, 50 and 250ng/ml concentrations for 72 hours and an MTS assay was performed. Figure 11a-c show that the LNCaP, C4-2B4 and PC3 cell lines demonstrate increased proliferation at 72hr ($p \leq 0.05$) with 250ng/ml of rhIL-8, while PC3-MM2 cells do not demonstrate changes in proliferation at any timepoint or rhIL-8 concentration, shown in Figure 11d. These results, first, confirm that IL-8 induces proliferation in the non-metastatic LNCaP cells and demonstrates that IL-8 induces proliferation in low metastatic C4-2B4 and metastatic PC3 cells. Second, these results demonstrate that IL-8 does not induce proliferation of the high metastatic cell line, PC3-MM2, likely because of high endogenous IL-8 expression and high Src activity, shown in Figure 7b and d Figure 8.

Exogenous IL-8 increases migration in low metastatic variants

Next, I determined if exogenous IL-8 affects migration in low and high metastatic cells. To accomplish this, cells were allowed to migrate for 18 hours in the absence or presence of rhIL-8 (50ng/ml). Media with 10% FBS was used as a control. As shown in Figure 12b, migration was significantly increased in LNCaP ($p \leq 0.01$), C4-2B4 ($p \leq 0.05$) and PC3 ($p \leq 0.001$) cells upon addition of rhIL-8, but this effect was not seen in the highly metastatic variant of PC3, PC3-MM2. These results demonstrate that IL-8 increases migration of low metastatic PCa and not the highly metastatic PCa cell, PC3-MM2.
FIGURE 11  Effects of Exogenous IL-8 on Proliferation of PCa Cells
A-D. LNCaP, C4-2B4, PC3 and PC3-MM2 cells were treated with 0, 10,50 and 250ng/ml of rhIL-8 in serum-free medium (SFM) or 10% FBS growth medium for 72 hours. The growth rate of viable cells was determined by MTS assay at 12, 24, 48 and 72 hour timpoints.* p ≤ 0.05, *** p ≤ 0.001
FIGURE 12  Effects of Exogenous IL-8 on Migration of PCa Cells
A. Migration assay of LNCaP, C4-2B4, PC3 and PC3-MM2 cells migrating toward 50ng/ml of rhIL-8 in serum free media (SFM) for 18 hours. Pictures were taken of migrated cells after fixation and staining. B. Graph of migrated cells to SFM, rhIL-8/SFM or growth medium. Numbers represent five random fields of cells counted at 10x magnification. Significance of differences within cell lines were calculated and statistics represent migration relative to SFM control. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$
SFK inhibition reverses IL-8-mediated increases in migration of low metastatic variants

While IL-8 increases the migration of low metastatic PCa cells, I next determined if IL-8 mediated increases in migration were SFK-dependent. PC3 cells were allowed to migrate in three conditions: (1) in the presence of the SFK inhibitor, dasatanib (50nM), (2) in the presence of rhIL-8 (50ng/ml) and (3) in the presence of dasatanib and rhIL-8 for 18 hours, shown in Figure 13a. Figure 13b shows that, as expected, rhIL-8 significantly increased migration (p≤0.05). In the presence of dasatanib, migration was significantly decreased (p≤0.01). However, the addition of IL-8 to dasatanib-treated PC3 cells did not overcome the dasatanib-mediated decreases in migration (p≤0.01). These results demonstrate that IL-8-mediated migration is an SFK-dependent event. Previous results from our laboratory demonstrate that SFK inhibition decreases IL-8 production. (138). Altogether, these results show IL-8 is regulated by SFKs and, in turn, SFKs regulate IL-8 suggesting that a feed forward mechanism is present. To determine if an IL-8-mediated feed forward loop exists, I knocked down IL-8 in highly metastatic PCa cells.

IL-8 shRNA decreases endogenous IL-8 expression and production

For these experiments, I chose the PC3-MM2 cells because they exhibit the highest IL-8 mRNA expression, secretory levels and Src activity amongst all PCa cells. Knockdown of IL-8 in PC3-MM2 cells was performed by using the IL-8 shRNA lentiviral vector kindly provided by Dr. Menashe Bar-Eli. Construction of the lentivirus is described in the Materials and Methods section. Cells were infected with a
FIGURE 13 Effects of SFK inhibition on IL-8-Mediated Migration
A. Migration assay of PC3 cells performed in the absence or presence of the SFK inhibitor, dasatanib, (50nM) with or without rhIL-8 (50ng/ml), for 18 hours. Pictures were taken of migrated cells after fixation and staining and cell were counted at 10x magnification. B. Graph illustrates cells that have migrated in the absence or presence of dasatanib and in the presence of rhIL-8 and dasatanib. Bars represent the average number of cells migrated from triplicate wells. * p ≤ 0.05, ** p ≤ 0.01
nontargeting control (GN18) or the shIL-8 vector and subjected to RT-PCR, qRT-PCR and ELISA to determine the efficiency of the IL-8 knockdown. The nomenclature for cells was as follows: PC3-MM2 (no vector control), PC3-MM2NT (PC3-MM2 nontargeting control) and PC3-MM2shIL-8 (PC3-MM2 IL-8 knockdown).

To determine if IL-8 knockdown was successful, first, I determined the mRNA expression level of IL-8 in PC3-MM2shIL-8 compared to PC3-MM2 cells by qRT-PCR. As shown in Figure 14a and b, IL-8 mRNA levels were significantly reduced in PC3-MM2shIL-8 cells relative to the PC3-MM2 cells (65% of PC3-MM2; \( p \leq 0.001 \)).

To determine if IL-8 secretory levels were inhibited, conditioned media was collected from all cell lines for ELISA detection as described in Materials and Methods. IL-8 secretory levels were also significantly decreased, by ~55% \((p \leq 0.001\)), shown in Figure 14c. Thus, the IL-8shRNA lentivirus vector could be used to examine the effects of IL-8 knockdown.

**IL-8 knockdown decreases Src activity and downstream targets in PCa cells**

Next, I determined if IL-8 knockdown decreases SFK activity. Lysates were subjected to immunoblot analysis and a Src\(^Y419\) specific antibody were used to detect activity shown in Figure 15. These results demonstrate that in the PC3-MM2shIL-8 cells, Src activity was decreased by 56% relative to PC3-MM2NT cells, shown in Figure 15a. This is the first evidence demonstrating that knockdown of IL-8 in PC3-MM2 cells results in decreased SFK activity.
FIGURE 14 Lentiviral Knockdown IL-8 in PC3-MM2
PC3-MM2 cells were infected with lentiviral IL-8 nontargeting vector (PC3-MM2 NT) and IL-8 shRNA vector (PC3-MM2 shIL-8). A and B. RNA was isolated from PC3-MM2, PC3-MM2NT and PC3-MM2shIL-8 cells and subjected to qRT-PCR using IL-8 specific primers. C. Cells were seeded, serum starved and conditioned media was collected from cells. Secretory IL-8 levels were assessed by ELISA in PC3-MM2, PC3-MM2NT and PC3-MM2shIL-8 cells. The results were normalized to total cell number. *** $p \leq 0.001$
FIGURE 15 Effects of IL-8 Knockdown on Src Activity and IL-8 Signaling
PC3-MM2, PC3-MM2NT and PC3-MM2shIL-8 cells were grown to subconfluency, lysates were extracted and analyzed by immunoblot. A. Immunoblot analysis using antiphospho- and anti-total SFK antibodies. B. Immunoblot analysis using antiphospho- and anti-total ERK and AKT antibodies. C. Immunoblot analysis using pNFk-B/65 antibody and vinculin as a loading control. Densitometry was done by quantititating the ratio of anti-phospho bands to anti-total bands or vinculin bands. Numbers represent arbritary units normalized to no vector control.
I also examined downstream targets important to Src and IL-8 function, such as AKT, ERK and NF-κB as seen in Figure 15b and c. The results demonstrate that IL-8 knockdown significantly decreases pAkt by 52%, pErk by 70% and NFκB levels by 90%. These observations indicate that IL-8 mediates SFK activity and downstream targets.

**IL-8 knockdown decreases biological functions of PC3-MM2 cells**

To determine if knockdown of IL-8 decreases migration and invasion in PCa cells, I performed migration and invasion assays by allowing cells to migrate for 18 hours. The results in Figure 16a and b demonstrate that knockdown of IL-8 significantly decreased migration ($p \leq 0.001$). Knockdown of IL-8 also decreased invasion ($p \leq 0.001$), Figure 16c and d. These results confirm that IL-8 mediates migration and invasion as shown by Figure 12.

**IL-8 rescue vector restores IL-8 expression and Src activity in PC3-MM2shIL-8 cells**

To determine if results of the IL-8 shRNA knockdown experiments were due to specific IL-8 targeting and not due to off-target effects, an IL-8 rescue vector (pSI/IL-8) was constructed using a PCR-based mutagenesis approach introducing point mutations in the shRNA target region without affecting the peptide sequences. The pSI/IL-8 rescue vector was described in Materials and Methods. For the rescue experiments, PC3-MM2shIL-8 cells were transfected with the pSI/IL-8 vector and an ELISA was performed to determine if IL-8 production was restored. Transfection of
FIGURE 16 Effects of IL-8 Knockdown on Migration and Invasion
A. PC3MM2NT and PC3-MM2shIL-8 cells were plated in boyden chambers and allowed to migrate for 18 hours. B. PC3MM2NT and PC3-MM2shIL-8 cells were plated in boyden chambers with Matrigel coating and allowed to invade for 18 hours. Pictures were taken of migrated (B) or invaded (D) cells after fixation, staining and numbers represent five random fields of cells counted at 10x magnification. Significance of differences within cell lines were calculated and statistics represent migration or invasion relative to nontargeting (NT) control. *** $p \leq 0.001$.
PC3-MM2shIL-8 cells with pSI/IL-8 rescue vector fully restored IL-8 production levels relative to PC3-MM2NT cells (Figure 17a). This result demonstrates the targeting vector does not have off-target effects.

Next, to determine if Src activity were restored, immunoblot analysis was performed with PC3-MM2NT, PC3-MM2shIL-8 and PC3-MM2shIL-8 + rescue vector. Figure 17b demonstrates restoration of Src activity in the PC3-MM2shIL-8 with the pSI/IL-8 rescue vector. This experiment demonstrates that Src activity is regulated by IL-8. These results demonstrate that there is a feed forward loop whereby IL-8 regulates Src activity and Src activity further regulates IL-8 production.

**IL-8 rescue vector restores migration and invasion in sh-knockdown**

To determine if rescue of IL-8 in PC3-MM2shIL-8 cells also restores migration and invasion, migration and invasion assays were performed with the pSI/IL-8 rescue vector. Figure 18 shows that, post-transfection with the IL-8 rescue vector, migration was restored relative to nontargeting control ($p \leq 0.001$). Invasion was also restored relative to nontargeting control ($p \leq 0.05$), shown in Figure 18. These results suggest that IL-8 is a regulator of the migratory and invasive events in the PC3-MM2 metastatic cells.
Mutagenesis experiments were performed introducing mutations that restore the expression of IL-8 without affecting the sh-mediated knockdown vector. PC3-MM2shIL-8 cells were transfected with the pSI/IL-8 rescue vector. A. ELISA was performed to determine the levels of IL-8 production in PC3-MM2shIL-8 and PC3-MM2shIL-8+IL-8 rescue vector. B. Lysates were extracted from PC3-MM2NT, PC3-MM2shIL-8 and PC3-MM2shIL-8 + IL-8 rescue vector cells and were analyzed by immunoblot using antiphospho- or anti-total Src antibodies. Densitometry was done by quantitating the ratio of pSrc\textsuperscript{Y419} bands to the ratio of Total Src bands and numbers represent arbitary units normalized to NT control.
FIGURE 18 Effects of Rescue of IL-8 in PC3-MM2shIL-8 on Migration and Invasion
A. PC3-MM2shIL-8 cells were transfected with the pSI/IL-8 rescue vector and cells were allowed to migrate or invade for 18 hours in serum free medium. Cells migrating or invading are reported as the average migrated or invaded cells. B. Cells were fixed, stained and counted at 10x in five random fields* $p \leq 0.05$, *** $p \leq 0.001$
CHAPTER 4

THE ROLE OF HS5 STROMAL-PRODUCED IL-8 ON SFK ACTIVITY AND PROSTATE CANCER PROGRESSION IN VITRO
PCa progresses due to changes not only in the tumor cells, but also in the microenvironment. *In vitro* studies demonstrate that stromal cells play a regulatory role in prostate carcinogenesis (77). The contributions of stromal cells in the progression of PCa can exist by physical contact, but also by factors secreted and/or expressed by the stromal cells, particularly cytokines. There are multiple studies, outlined in the introduction, demonstrating that stromal factors affect PCa progression, but there is unclear evidence regarding the role of stromal IL-8 production in metastatic disease.

Therefore, I have chosen to use a stromal IL-8 producing cell line as a model to investigate how IL-8 production by stromal cells affects biological functions of PCa cells *in vitro*. In this chapter, I used a stromal cell line, HS5, derived from bone marrow fibroblasts, known to produce IL-8 (224). The HS5 cell line is used in this chapter to investigate the role of stromal IL-8 production in the regulation of biological functions of PCa cells. Observations from this chapter will: (1) determine if stromal IL-8 affects migration and invasion of PCa cells and (2) determine if stromal IL-8 affects Src activity of PCa cells. Results are discussed in this chapter.

**HS5 stromal cells characterized by IL-8 expression and production and CXCR1 protein expression**

First, to determine if the high affinity CXCR1 receptor were expressed in the HS5 cells, CXCR1 was immunoprecipitated with an anti-CXCR1 specific antibody and immunoblot analysis was performed. Results are shown in Figure 19. *Figure 19c* shows that the CXCR1 receptor is expressed in the HS5 stromal cells. Along with CXCR1 expression, I examined IL-8 mRNA expression and IL-8 protein levels in HS5
FIGURE 19 IL-8 mRNA Expression and Production and CXCR1 Protein Expression in the HS5 Stromal Cell Line
A. HS5 cells were grown to subconfluence, serum starved for 24 hours and RNA was isolated for RT-PCR to determine IL-8 mRNA expression.  B. After seeding and serum starving HS5 cells, conditioned media from was collected and ELISA was performed to determine levels of IL-8.  C. Immunoprecipitation with anti-CXCR1 antibody and rabbit IgG (control) was completed and immunoblot analysis was performed to determine the expression of CXCR1.
stromal cells by performing RT-PCR and an ELISA. **Figure 19a** shows that the HS5 cells express IL-8 mRNA and **Figure 19b** shows that HS5 cells produce high levels of IL-8. Thus, these cells were a suitable model for studying stromal IL-8 production and its effects on PCa cells.

**Effects of HS5 stromal produced IL-8 on migration and invasion of PCa cells**

Next, I determined if conditioned media from HS5 stromal cells affects biological functions of PCa, such as migration and invasion. To accomplish this, I collected stromal conditioned media from the HS5 cells and allowed PC3 cells to migrate and/or invade for 18 hours. The results in **Figure 20a and b** demonstrate that HS5 stromal conditioned media significantly increased the migration of PC3 cells ($p \leq 0.01$). Results also demonstrate in **Figure 20c and d** that HS5 stromal conditioned media significantly increased the invasion of PC3 cells ($p \leq 0.01$).

**Knockdown of endogenous IL-8 in HS5 bone stromal cells**

After demonstrating in **Figure 20** that HS5 stromal conditioned media increases biological functions of PCa cells, I determined whether the HS5 stromal production of IL-8 contributes to these changes. To test this, I, first, knocked down IL-8 in the HS5 stromal cells using the same lentiviral system utilized for knock down of IL-8 in PC3-MM2 cells. HS5 cells were infected with the shIL-8 lentivirus (HS5shIL-8) as previously described. The nomenclature for infection with the nontargeting and shIL-8 vectors was HS5NT and HS5shIL-8, respectively. To determine if effective IL-8 knockdown was achieved, I assessed mRNA expression and secretory levels, by performing qRT-PCR and ELISA, respectively. As shown in **Figure 21a**, IL-8 mRNA expression was knocked down by 60% ($p \leq 0.01$). IL-8 secretory levels were knocked
FIGURE 20  Effects of HS5 Stromal Conditioned Media on Migration and Invasion of PC3 Cells
Conditioned media from HS5NT and HS5shIL-8 stromal cells was collected for use as a chemoattractant. PC3 cells were seeded in (A and B) migration or (C and D) invasion chambers, placed atop the HS5NT or HS5shIL-8 conditioned media and cells were allowed to migrate or invade for 18 hours. Cells were fixed, stained, counted at 10x in five random fields and reported as the average migrated or invaded cells. ** $p \leq 0.01$
FIGURE 21  Lentiviral Knockdown IL-8 in HS5 Stromal Cells
HS5 cells were infected with lentivirus particles containing the IL-8 nontargeting vector (HS5 NT) or IL-8 shRNA vector (HS5 shIL-8).  A. RNA was isolated from HS5, HS5NT and HS5shIL-8 cells and qRT-PCR analysis was performed.  B. Cells were seeded, serum starved and conditioned media was collected from cells. IL-8 production was measured by ELISA. The results were normalized to total cell number.  ** p ≤ 0.01
down to 55% in HS5shIL-8 cells \( (p \leq 0.01) \), shown in Figure 21b. These results demonstrate successful knock down of IL-8 in stromal cells.

**Knockdown of IL-8 in HS5 stromal cells decreases SFK activity and downstream targets**

I next determined if IL-8 knockdown decreases SFK activity in HS5 stromal cells. To do this, I compared SFK activity in NT and IL-8 knockdown cells by immunoblot analysis using antibodies specific to Src Y419 activity and total Src. Figure 22a shows that IL-8 knockdown decreased Src activity by 60% relative to HS5 cells. These results demonstrate for the first time that IL-8 regulates SFK activity in stromal cells.

I also determined if knockdown of IL-8 decreases downstream targets important to Src and IL-8 function, such as Akt, Erk and NFk-B. Figure 22b and c demonstrate that IL-8 knockdown significantly decreases pAkt by 75%, pErk by 70% and NFk-B levels by 80%. These results demonstrate that IL-8 mediates targets downstream of IL-8 and Src.

**SFK regulates IL-8 expression and Src activity in HS5 stromal cells**

Next, I determined if SFKs regulate IL-8 expression and secretory levels in HS5 stromal cells. To accomplish this, HS5 cells were treated with 10, 50, 100, 200 and 500nM of the SFK inhibitor, dasatanib. Cell lysates were subjected to immunoblot analysis and anti-phospho and total Src antibodies were used. I observed a 75% significant decrease in Src activity after treatment with 50nM dasatanib, while subsequent increasing concentrations abolished Src activity (Figure 23). These results demonstrate that SFK inhibition decreases Src activity in a dose-dependent manner in
FIGURE 22  Effects of IL-8 knockdown in HS5 cells on Src activity and IL-8 Signaling

HS5, HS5NT and HS5shIL-8 cells were grown to subconfluency, lysates were extracted and analyzed by immunoblot. A. Immunoblot analysis using antiphospho- and antitotal SFK antibodies. B. Immunoblot analysis using antiphospho- and anti-total ERK and AKT antibodies. C. Immunoblot analysis using pNFk-B/65 antibody and vinculin as a loading control. Densitometry was done by quantitating the ratio of anti-phospho bands to anti-total bands or vinculin bands. Numbers represent arbitrary units normalized to no vector control.
FIGURE 23  Effects of SFK Inhibition on Src Activity in HS5 Stromal Cells
SFK inhibition was performed by treating HS5 cells with dasatanib (10, 50, 100, 200 and 500nM) for 4 hours. Cell lysates were extracted and immunoblot analysis was performed by using antiphospho- or anti-total Src antibodies and by using a vinculin antibody as a loading control. Densitometry was done by quantitating the ratio of pSrc\textsuperscript{Y419} bands to the ratio of Total Src/Vinculin bands and numbers represent arbitrary units normalized to DMSO control.
HS5 stromal cells. It also demonstrates that maximal SFK inhibition occurs with 50nM dasatanib.

Next, to determine if Src regulates IL-8 mRNA expression in HS5 stromal cells, HS5 cells were treated with 10, 50 and 100nM of dasatanib and cells were collected for RNA isolation and RT-PCR was performed. Conditioned media was also collected to determine IL-8 secretory levels by ELISA. The results demonstrate that dose-dependent treatment of HS5 cells with dasatanib decreased IL-8 mRNA expression and secretory levels. IL-8 protein levels were significantly decreased by >60% with 50nM and 100nM dasatanib (Figure 24b), whereas IL-8 mRNA levels were significantly decreased by >50% at all dasatanib concentrations (Figure 24a). These results demonstrate that SFKs regulate IL-8 expression and secretory levels in HS5 stromal cells, similarly to how regulation occurred in tumor cells. This allowed me to test effects of stromal produced IL-8 on tumor cells.

**HS5 stromal production of IL-8 increases Src activity in PCa cells**

Next, I determined if stromal produced IL-8 regulates Src activity of PCa cells. To accomplish this, conditioned media from stromal cells (HS5NT and HS5shIL-8) was added to PCa cells with (PC3, PC3-MM2shIL-8 and PC3-MM2NT) for 10, 15 and 60 minutes. Cells were, then, subjected to immunoblot analysis and phosphorylated Src and total Src activity was assessed. First, I determined if HS5 stromal conditioned media increased SFK activity in low metastatic PC3 cells by adding HS5NT conditioned media. The results, shown in Figure 25, demonstrate that HS5 stromal conditioned media increased SFK activity in PC3 cells. I, next, determined if increased Src activity was a result of HS5 stromal produced IL-8 by adding HS5shIL-8-
FIGURE 24  Effects of SFK inhibition on IL-8 Expression in HS5 Stromal Cells
SFK inhibition was performed by treating HS5 cells with dasatanib. A. For IL-8 mRNA expression, dasatanib (10, 50 and 100nM) was added to HS5 cells for 4 hours and RT-PCR was performed with primers targeting the IL-8 sequence. B. For ELISA, cells were seeded, treated with dasatanib concentrations and conditioned media was collected from cells. The results were normalized to total cell number. ** p ≤ 0.01
FIGURE 25 Effects of HS5 Stromal Produced IL-8 on Src Activity in PC3 Cells
Conditioned media from HS5NT and HS5shIL-8 stromal cells was harvested. PC3 cells were grown until subconfluent and serum starved for 24 hours. Conditioned media from either HS5NT and HS5shIL-8 stromal cells was added to cells for 10, 15 and 60 minutes, cell lysates were extracted and immunoblot analysis was performed by using antiphospho- or anti-total Src antibodies and by using a vinculin antibody as a loading control. Densitometry was done by quantitating the ratio of pSrc bands to the ratio of Total Src/Vinculin bands and numbers represent arbritary units normalized to no treatment (0').
conditioned medium to PC3 cells. These results demonstrate that Src activity is decreased with addition of HS5shIL-8. These results demonstrate that IL-8 is one factor produced by HS5 stromal cells contributing to increases in Src activity. These results were also observed with PC3-MM2shIL-8 cells shown in Figure 26. As a control experiment, I determined if HS5NT or HS5shIL-8 conditioned medium affects SFK activity in cells with high IL-8 expression and Src activity. I added the conditioned medium to PC3-MM2NT cells using the same conditions as above. Results from this experiment demonstrate no change in Src activity with the addition of either HS5NT or HS5-shIL-8 conditioned medium (Figure 27). These results confirm results in Figure 9 demonstrating that exogenous IL-8 has no further effect on increasing Src activity on highly metastatic PC3-MM2 cells. Overall, these results demonstrate that stromal production of IL-8 increases SFK activity in PCa cells and IL-8 produced from stromal cells.

**Knockdown of IL-8 production in HS5 stromal cells decreases migration and invasion of PCa cells**

Next, I determined if stromal production of IL-8 affects migration and invasion. Conditioned medium from HS5NT and HS5shIL-8 cells was used as a chemoattractant and migration and invasion assays were performed. To determine if HS5 stromal conditioned medium increases migration of PC3 cells, HS5NT conditioned medium was used a chemoattractant. As shown in Figure 28a and b, HS5NT conditioned medium significantly increased migration of low-metastatic, PC3 cells ($p \leq 0.01$). Next, I determined if HS5 stromal cells producing less IL-8 decreases migration of PC3 cells by using HS5shIL-8 cell-conditioned medium as a
FIGURE 26  Effects of HS5 Stromal Produced IL-8 on Src Activity in PC3-MM2shIL-8 Cells
Conditioned media from HS5NT and HS5shIL-8 stromal cells was harvested. PC3-MM2shIL-8 cells were grown until subconfluency and serum starved for 24 hours. Conditioned media from either HS5NT and HS5shIL-8 stromal cells was added to cells for 10, 15 and 60 minutes, cell lysates were extracted and immunoblot analysis was performed by using antiphospho- or anti-total Src antibodies and by using a vinculin antibody as a loading control. Densitometry was done by quantitating the ratio of pSrc bands to the ratio of Total Src/Vinculin bands and numbers represent arbitrary units normalized to no treatment (0').
Conditioned media from HS5NT and HS5shIL-8 stromal cells was harvested. PC3-MM2NT cells were grown until subconfluency and serum starved for 24 hours. Conditioned media from either HS5NT and HS5shIL-8 stromal cells was added to cells for 10, 15 and 60 minutes, cell lysates were extracted and immunoblot analysis was performed by using antiphospho- or anti-total Src antibodies and by using a vinculin antibody as a loading control. Densitometry was done by quantitating the ratio of pSrc bands to the ratio of Total Src/Vinculin bands and numbers represent arbitrary units normalized to no treatment (0').
Conditioned media from HS5NT and HS5shIL-8 stromal cells was harvested. PC3 cells were seeded in (A and B) migration or (C and D) invasion chambers, chambers were placed atop the conditioned media (HS5NT or HS5shIL-8) and allowed to migrate or invade for 18 hours. Cells were fixed, stained, counted at 10x magnification in five random fields and reported as the average migrated or invaded cells. * $p \leq 0.05$, ** $p \leq 0.01$
chemoattractant. The results demonstrate that HS5 stromal cells producing less IL-8 significantly decreased migration relative to co-culture of PC3 cells with HS5NT cell-conditioned medium ($p \leq 0.05$). An invasion assay performed under the same conditions showed similar results, as seen in the right panel of Figure 28c and d. Figure 29a-d demonstrates that migration and invasion were also significantly decreased when PC3-MM2shIL-8 cells were co-cultured with HS5shIL-8 cell conditioned medium relative to HS5NT cell conditioned medium ($p \leq 0.01$). These results demonstrate that HS5 stromal production of IL-8 affects migration and invasion of PCa cells.
FIGURE 29 Effects of HS5 Stromal Conditioned Media on Migration and Invasion of PC3-MM2shIL-8 Cells
Conditioned media from HS5NT and HS5shIL-8 stromal cells was harvested. PC3-MM2shIL-8 cells were seeded in (A and B) migration or (C and D) invasion chambers, chambers were placed atop the conditioned media (HS5NT or HS5shIL-8) and allowed to migrate or invade for 18 hours. Cells were fixed, stained, counted at 10x in five random fields and reported as the average migrated or invaded cells. * $p \leq 0.05$, ** $p \leq 0.01$
CHAPTER 5

THE ROLE OF HS5 STROMAL-PRODUCED IL-8 IN TUMOR GROWTH AND METASTATIC POTENTIAL OF PROSTATE CANCER IN VIVO
PCa progression to metastatic disease is a result of multiple factors produced by the tumor and by the stromal microenvironment. There is *in vivo* evidence supporting the role of IL-8 tumor production in PCa progression whereby overexpression of IL-8 in LNCaP cells increases tumor growth and microvessel density (146). However, we know that stromal production of IL-8 contributes to PCa progression, as evidenced by chapter 4, where I demonstrate that stromal production of IL-8 is one factor increasing the migration and invasion of PCa cells *in vitro*. To date, there are no current *in vivo* models addressing if stromal production of IL-8 contributes to the metastatic potential of PCa. As a result, this chapter will assess if stromal IL-8 production affects PCa growth and metastatic incidence by performing co-inoculations with stromal cells and PCa cells. Observations from this chapter: (1) determines if the tumor-stromal interaction contributes to metastatic potential *in vivo* (2) determines if stromal IL-8 contributes to metastatic potential of PCa *in vivo*.

**Orthotopic co-inoculation of HS5 stromal cells and PCa cells**

This study was designed to determine if co-inoculation of stromal cells and PCa cells affects tumor growth and metastatic incidence. For co-inoculations, low metastatic PC3 cells and HS5 stromal cells (NT or shIL-8) were injected into the prostate of athymic nude mice in the following ratios: .25x10⁶ of PC3 cells for Group 1, .25x10⁶ of PC3 cells: .25x10⁶ of HS5NT cells for Group 2 and .25x10⁶ of PC3 cells: .25x10⁶ of HS5shIL-8 cells for Group 3 with an n=10 for each group. Tumors in mice were monitored by luciferase activity detected by bioluminescence imaging.

First, I determined the viability of the co-inoculation post-injection by culturing the stromal-prostate cancer cell combination *in vitro* and monitoring the
growth and viability for one week. Figure 30 shows a graph and representative pictures of the PC3:HS5 combination group from day 3 and 6 demonstrating that cells are viable.

**Effects of orthotopic co-inoculation of HS5 stromal cells and PCa cells on tumor growth and weight**

To assess tumor growth, mice were monitored every three days according to *in vivo* bioluminescence imaging using IVIS™-100 (Xenogen Co.) for changes in tumor growth and luciferase activity was used as a parameter of tumor growth. Figure 31 displays representative images of tumors in each mouse. After injection of luciferin, mice were anesthetized, placed in the bioluminescence imager and luciferase activity was measured. The red circles, in Figure 31a-c, represent the region of interest (ROI) encompassing the entire region of luciferase activity and the numbers represent luciferase activity within that region.

First, I determined if stromal cells increase tumor growth of PCa cells. As shown in Figure 32, there was a consistent increase in luciferase activity in all groups showing consistent tumor growth. The results also demonstrate that there were no significant differences in luciferase activity amongst different groups from day 0 until day 14. This result demonstrates that tumors grew similarly among all groups from day 0 to 14. At day 14, there was a significant increase in luciferase activity in the PC3:HS5NT group relative to PC3 alone group (p≤0.05), while there was no significant difference between PC3:HS5shIL-8 group relative to PC3 alone group. This demonstrates that stromal cells increase tumor growth of PCa cells as measured by luciferase activity.
FIGURE 30  Post-injection effects of Cell Viability and Growth with Stromal Cell and PCa Cells
Directly after co-inoculations of HS5 stromal cells and PC3/RFP luciferase labeled cells, cells were seeded together, counted and monitored for 6 days in culture. A. Cells were counted on days 3 and 6 and viable PC3 and HS5 cells were plotted in the graph. B. Pictures shown are representative of day 6 before sorting and counting cells. Pictures were taken at 4x magnification.
FIGURE 31 Determination of Luciferase Activity in Mice by in vivo Bioluminescence Imaging

Tumor growth was monitored by bioluminescence imaging mice every three days. Mice were injected with luciferin (20mg/ml) and placed under anesthesia. Mice were placed in the IVIS-100 Xenogen imager and luciferase activity was measured. Pictures were taken indicating luminescence value according to scale on right and region of interest (ROI) was indicated encompassing the entire region of luciferase activity. The luciferase values were calculated and used as a measurement of growth for each mice in each group.

The representative mice are as follows: (A) mouse in PC3 group, (B) mouse in PC3:HS5NT group and (C) mouse in PC3:HS5shIL-8.
FIGURE 32 Effects of HS5 Stromal Produced IL-8 on Tumor Growth
After intraprostatic injections of PCa cells and stromal cells in mice, tumor growth was monitored by imaging mice every two-three days, observing luciferase counts in each mouse by measuring the region of interest (ROI) of the tumor for each mouse.

, \( * p \leq 0.05 \), \( ** p \leq 0.01 \)
Next, I assessed whether stromal cells producing IL-8 affects tumor growth. The results demonstrate that, at day 14, there was a significant increase in luciferase activity in the PC3:HS5NT group relative to PC3:HS5shIL-8 group (p≤0.05). There was a larger increase seen at day 18 demonstrating that HS5 stromal produced IL-8 increases tumor growth.

**Co-inoculation of HS5 stromal cells and tumor cells increases tumor weight**

After termination of the experiment, tumor weights were measured. The average tumor weights were as follows: 126mg for PC3 group, 193mg for PC3:HS5NT group and 169 for PC3:HS5shIL-8 group. First, I determined if co-inoculation of PC3 cells and HS5 stromal cells increases tumor weight. The results demonstrate that there was a significant increase in tumor weight between the PC3:HS5NT group and PC3 alone (p≤0.0019). This result demonstrates that the tumor-stromal interaction contributes to tumor growth and suggests that HS5 stromal production of IL-8 contributes to increased tumor growth.

To determine if IL-8 production in stromal cells increases tumor weight, I compared the changes in tumor weights between the PC3:HS5NT and PC3:HS5shIL-8 groups. There was a significant decrease in the PC3:HS5shIL-8 tumor weights relative to PC3:HS5NT tumor weights (p≥0.05) demonstrating that stromal production of IL-8 contributes to tumor weight *in vivo*. Results also demonstrate that PC3:HS5shIL-8 tumor weights are significantly higher than PC3 alone (p≤0.0363), shown in Table 1. This result demonstrates that stromal cells producing less IL-8 increase tumor weights compared tumor cells alone suggesting that, expectantly, other factors contribute to
**TABLE 1 Effects of Stromal Production of IL-8 on Primary Tumor Weight of PC3 cells in an Orthotopic Mouse Model**

<table>
<thead>
<tr>
<th>Cell Line Combinations</th>
<th>Tumor Incidence</th>
<th>Median</th>
<th>IQR</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>10/10</td>
<td>128</td>
<td>(91.25-153.5)</td>
<td></td>
</tr>
<tr>
<td>PC3 + H55NT</td>
<td>8/10*</td>
<td>193.5</td>
<td>(182.5-225.3)</td>
<td>0.0019#</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0522^</td>
</tr>
<tr>
<td>PC3 + H5sshIL-8</td>
<td>10/10</td>
<td>165</td>
<td>(139.3-206.5)</td>
<td>0.0363#</td>
</tr>
</tbody>
</table>

*Abbreviations: NT, nontargeting; IQR, interquartile range; SD, standard deviation; LN, lymph node*

1. Interquartile range (IQR) is the range of the 75th and 25th percentiles of the tumor weight in each group.
2. * One mouse did not grow a tumor; another mouse was sacrificed on day 20 to examine possible metastases.
3. # PC3+H55NT and PC3+H5sshIL-8 groups had a statistically significant increase in tumor growth versus PC3 group.
4. ^ Moderate statistical significance in tumor growth between PC3+H55NT and PC3+H5sshIL-8 groups.
tumor weight. Overall, these results demonstrate that stromal production of IL-8 is one factor contributing to tumor weight.

Orthotopic co-inoculation of HS5 stromal cells and PCa cells increases metastatic incidence

To assess if metastatic incidence is affected by IL-8 producing stroma, the same groups were assessed for the presence of metastases. Mice were opened and thoroughly examined for iliac and renal lymph node metastases. Renal and iliac lymph nodes were observed and confirmed by H&E (107). All three mice groups had at least one metastasis either in the renal or iliac lymph nodes shown in Table 2. Mice injected with PC3 cells displayed on average 1.4 metastases. Mice injected with PC3:HS5NT cells displayed on average 3.5 metastases per mouse, while mice injected with PC3:HS5shIL-8 cells displayed on average 2.4 metastases per mouse. To determine if HS5 stromal cells increases metastatic incidence of PC3 cells, I compared metastases from PC3:HS5NT group and PC3 alone group. The results in Table 2 demonstrate that co-inoculation with PC3:HS5NT significantly increased metastatic incidence relative to PC3 alone ($p=.018$). These results demonstrate that co-inoculation of stromal cells with PCa cells increases metastatic incidence.

I, next, determined whether the increases seen in metastatic incidence were due to HS5 production of IL-8. The results demonstrate that PC3:HS5shIL-8 co-injections produced significantly less metastases in comparison to PC3: HS5NT ($p=.018$). These results demonstrate that stromal IL-8 production contributes to metastatic incidence. There was also a significant increase in metastatic incidence with co-inoculations of PC3:HS5shIL-8 relative to PC3 alone ($p=.0019$). This result demonstrates that stromal
TABLE 2 Effects of Stromal Production of IL-8 on Metastatic Incidence of PC3 Cells in an Orthotopic Mouse Model

<table>
<thead>
<tr>
<th>Cell Line Group</th>
<th>Iliac LN</th>
<th>Renal LN</th>
<th>Median</th>
<th>*IQR</th>
<th>p values for metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>14</td>
<td>0</td>
<td>1</td>
<td>(1-2)</td>
<td></td>
</tr>
<tr>
<td>PC3 + HS5NT</td>
<td>22</td>
<td>6</td>
<td>4</td>
<td>(2.25-4)</td>
<td>0.0003 #</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.018 ^</td>
</tr>
<tr>
<td>PC3 + HS5shIL-8</td>
<td>17</td>
<td>7</td>
<td>2.5</td>
<td>(2-3)</td>
<td>0.0019 #</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; IQR, interquartile range

*Interquartile range (IQR) is the range of the 75th and 25th percentiles of the metastatic incidence in each group.

# PC3+HS5NT and PC3+HS5shIL-8 groups had a statistically significant increase in metastatic incidence versus PC3 group.

^ PC3+HS5NT group had a statistically significant increase in metastatic incidence versus PC3+HS5shIL-8 group.
cells producing less IL-8 increase metastatic incidence compared to tumor cells alone suggesting that, expectantly, other factors contribute to metastatic incidence. Overall, results from this experiment demonstrates that stromal cells producing IL-8 increase metastatic incidence making IL-8 one factor involved in metastatic potential.

In addition, the co-inoculation groups (PC3:HS5NT and PC3:HS5shIL-8) were observed to have iliac and renal lymph node metastases, while inoculation with PC3 cells alone had only iliac lymph node metastases. This finding indicates that stromal IL-8 production alters the spectrum of lymph node metastases. Overall, this in vivo model demonstrates, for the first time, that stromal cells producing IL-8 increase the metastatic potential of PCa.
CHAPTER 6

CLINICAL SIGNIFICANCE OF IL-8 SECRETION AND SFK ACTIVITY IN METASTATIC CRPC
In clinical practice, PSA serves as the ‘gold’ standard for the detection of PCa. Although a frequent upward trend of PSA levels is evident in patients with advanced disease, there seems to be no definite, linear relationship between serum PSA levels and PCa stage or metastasis (212). This has led to controversies over its role as a biomarker for metastatic disease and suggests that it may instead better serve as a marker of predisposition to abnormalities of the prostate. As a result, the investigation of other markers, such as IL-8, are of great interest to this thesis. Several reports demonstrate detection of increased IL-8 in serum and tissue of patients with metastatic disease in comparison to disease-free patients or patients exhibiting benign prostatic hyperplasia (165,171). SFK activity is also increased in patients with metastatic PCa (129,225). Supporting in vitro and in vivo data, from our laboratory and others, demonstrates that IL-8 increases survival of PCa in the presence of docetaxel, while SFK inhibition by dasatanib, in preclinical models, demonstrates decreased tumor growth and metastasis and decreased growth of PCa in the bone (226,211). These results suggest that: (1) increased IL-8 levels contributes to disease progression and (2) SFK inhibition may decrease tumor progression and bone metastatic disease.

Results from my in vitro studies in Chapter 3 demonstrate that exogenous IL-8 increases PCa cell progression in an SFK-dependent mechanism. I also demonstrate in Chapter 4 and 5 that stromal IL-8 increases PCa progression in vitro and increases the metastatic potential of PCa in vivo. As a result, I investigated, clinically, if serum IL-8 levels in patients with metastatic CRPC are affected by dasatanib treatments. I was able to investigate this due to a Phase II trial with dasatanib+docetaxel in metastatic CRPC, conducted at MDACC by Dr. John Araujo. This clinical trial aimed to determine
if dasatanib alone or dasatanib+docetaxel treatments decreases metastatic CRPC.

Additional studies from this trial were to determine if PSA, bone turnover markers and IL-8 serum levels were changed. Observations from this study: (1) determined if patients with metastatic CRPC responding to SFK inhibition have decreased serum IL-8 levels and (2) determined if the changes in IL-8 levels correlates with PSA levels.

**Clinical administration of dasatanib decreases IL-8 levels in patients with metastatic CRPC**

This Phase I/II trial was designed to determine if dasatanib decreased metastatic CRPC by using bone radiographic measurements and PSA levels as a measure of response. I was interested in determining whether IL-8 levels were a better predictor of response in comparison to PSA levels. First, to determine if dasatanib decreases IL-8 levels in patients with metastatic CRPC, serum samples were collected at the following timepoints: before any treatments as a control and at day 21 constituting cycle 2/dasatanib+docetaxel. Samples were split into two groups- patients that responded to treatment and those that did not respond (responders vs. non-responders). ELISA was performed with all samples to determine the levels of serum IL-8 and PSA. I, first, determined if PSA levels predicted response. The results, in Figure 33b, demonstrate that PSA levels in responders were decreased and PSA levels in non-responding patients were also decreased. These results demonstrate that PSA levels of responding patients mimicked the PSA levels of those patients who did not respond. Next, I wanted to determine if IL-8 levels predicted response. Figure 33a demonstrates that IL-8 levels in responders were decreased, whereas IL-8 levels in non-responders remained elevated. These results demonstrate that IL-8 may be a better
FIGURE 33  Effects of Phase I/II | Dasatanib + Docetaxel Clinical Trial on IL-8 and PSA Production in Bone Metastatic Prostate CRPC

Serum samples collected from patients with bone metastatic PCa undergoing dasatanib + docetaxel treatments were assessed by radiographic measurements and ELISA was performed for PSA and IL-8 production. PSA and bone radiographic measurements were used as parameters of response. (A) IL-8 and (B) PSA serum levels in responders and non-responding patients after treatments. The waterfall plot illustrates responders (patients which do not progress on treatment) and non-responders (patients which progress on treatment). Blue bars represent responders and orange bars represent non-responders.
predictor of response for patients with metastatic CRPC. Although a wider study has been conducted and is pending, these results suggest that patients with metastatic disease exhibiting high IL-8 levels could be selected for dasatinib+docetaxel combination therapy.
CHAPTER 7

DISCUSSION
Protein tyrosine kinases signal through multiple pathways leading to subsequent changes in biological functions of PCa. One well-studied kinase family mediating these signaling pathways are SFKs, outlined in chapter 1. Through extensive studies, from our laboratory and others, we have demonstrated that increased SFK activation results in deregulation of migration, invasion, apoptosis, angiogenesis, proliferation and survival. Work by Park et al. demonstrates that SFKs play differential roles in PCa progression which has contributed to investigations providing a better understanding of the biological roles of SFK members and how they contribute to tumor progression.

As outlined in the Introduction, SFKs constitute nine family members of which three are ubiquitously expressed (Src, Yes and Fyn) and the remaining members are expressed in a tissue-specific manner. Src has been one of the most widely studied SFK members and has been shown to be increased in multiple cancers. In vitro evidence demonstrates that Src contributes to survival, migration and invasion in the progression of cancer. In vivo studies demonstrate that Src activation is a mediator of tumor progression in multiple cancers, but it was unclear whether Src activation was a contributor to progression of PCa to metastatic disease (127,131).

To better understand how Src activation contributes to metastatic disease, there has been an increased effort in the investigation of signaling factors regulating Src activity. We have learned that Src activity is not a result of direct activation by extracellular signals, but by activated PTKs, GFRs, GPCRs and integrins, outlined in the Introduction. However, there is less evidence describing a role for GPCRs and GPCR ligands in Src activation in PCa. This is important because there are studies
demonstrating that some GPCR ligands, which can also be classified as cytokines, contribute to PCa progression.

Of the many cytokines contributing to PCa progression, IL-8 is one factor that has been implicated in, not only, PCa progression, but in metastatic disease, due to its overexpression. In addition, similar to Src, we know that IL-8 mediates biological functions, such as angiogenesis, migration and invasion in PCa and, likewise, there is less information regarding signaling mechanisms regulating these biological functions. As a result, the investigation of pathways regulating IL-8 expression and Src activation are, currently, being studied as two separate signaling mediators of PCa progression. However, our laboratory provides evidence that Src regulates IL-8 production in PCa cells and, more recent studies, suggest that IL-8 activates PTKs, Src and FAK, to induce migration of PCa cells (138,147). These results suggest that the signaling mechanisms of Src and IL-8 are, not entirely exclusive, and that regulation of Src activation may be associated with IL-8 signaling in PCa. This becomes important because, although, we know that IL-8 signaling is increased in PCa, IL-8 signaling by cells in the microenvironment, such as stromal cells contributes to tumor progression.

So, it is possible that IL-8 signaling may also contribute to signaling mechanisms, such as SFK activity, and this mechanism may contribute to increases in PCa progression to metastatic disease. Delineation of whether IL-8 mediates SFK activity in PCa, either tumor-mediated or stromal-mediated, could also provide another mechanism of increased SFK activity in PCa progression.

The effects of IL-8-mediated SFK activity in metastatic prostate cancer in vitro
Previous studies demonstrate that IL-8 and Src, individually, increase metastatic properties of PCa as well as Src (107,146). Studies from our laboratory demonstrate that Src regulates IL-8 secretion (138). However, there was no clear understanding of whether Src regulation of IL-8 secretion results in Src activation and whether this contributes to migration and invasion. Studies in Chapter 3 determined that a feed-forward loop in PCa exists whereby IL-8 increased SFK activity increases IL-8 production and expression, which in turn increases IL-8 activity. I also determined that this feed forward loop contributes to migration and invasion of non-metastatic and low metastatic PCa cells.

To determine how IL-8 affected SFK activity, I first determined that the high affinity IL-8 receptor, CXCR1, was present in all cell lines (Figure 6). Expression of CXCR1 was decreased in the highly metastatic PC3-MM2 cells. This result may be explained by studies showing that higher IL-8 levels correlates with rapid internalization of the CXCR1 receptor resulting in decreased CXCR1 expression (221). So, it is likely that the decrease observed in PC3-MM2 is due to the presence of high IL-8 expression and secretory levels in comparison to the other PCa cell lines (Figure 7).

In addition to PC3-MM2 cells having the highest IL-8 levels, they also expressed the highest SFK activity in comparison to the other PCa cell lines (Figure 8). While the levels of IL-8 do not directly correlate with levels of SFK activity, the trend of increased secretion and expression of IL-8 and increased Src activity in PC3-MM2 cells is striking. However, one explanation for there not being a direct correlation may be because IL-8 receptor does not directly associate with SFKs. There is no
information suggesting that IL-8 binding to the CXCR1 receptor elicits direct activation of SFKs. There are numerous reports demonstrating that RTKs activate SFKs, outlined in the introduction. It is likely that there is crosstalk between CXCR1 and RTKs resulting in SFK activation. This is supported by evidence demonstrating that IL-8 binding of CXCR1 promotes the transactivation of RTKs, such as EGFR in ovarian cancer and VEGFR in endothelial cells (175). While EGFR plays less of a role in PCa, VEGFR, PDGFR, MET, IGF-1R and AXL activation, to name a few, have been shown to induce SFK activation in PCa. It would be interesting to investigate whether IL-8/CXCR1 signaling promotes transactivation of these RTKs and whether there are other unknown upstream signaling factors involved in IL-8-mediated SFK activity.

While increased IL-8 expression and Src activity are, individually, known to increase progression, I showed that IL-8 and Src function in the same signaling pathway to increase metastatic properties of PCa. Specifically, I demonstrated that IL-8 stimulation increases SFK activity and migration and proliferation of non-, low and moderately metastatic PCa cells (Figures 9-12), but not that of the highly metastatic PC3-MM2 cells. IL-8 has also been shown to increase PCa cell SFK activity, proliferation and migration in non- to low metastatic PCa cells, while the results from highly metastatic PC3-MM2 cells have never been reported (146,223). One explanation for the unresponsiveness of PC3-MM2 cells to further IL-8 stimulation may be due to their intrinsic high production of IL-8 (Figure 6). In terms of migration, this explanation is supported by Schraufstatter et al. demonstrating that IL-8 stimulation with 30nM or 100nM has the same effect on migration of high IL-8 producing cells suggesting that IL-8 does not have any further effect on migration beyond these
concentrations of IL-8 (227). In addition, Park et al. demonstrated that two SFK members, Src and Lyn, contribute to PCa progression through differential biological roles. Their work shows that Src primarily contributes to migration and invasion, while Lyn contributes to proliferation in PCa cells (107). It is possible that IL-8-mediated changes in Src activity contribute to migration and invasion of PCa, while IL-8 mediated changes in Lyn activity contribute to the proliferation of PCa cells would require further studies. Another explanation for the changes in proliferation stems from studies demonstrating that IL-8-mediated expression of cyclin D1, proliferative marker, is a contributor to the proliferation of PCa cells (146). Further studies will need to be done to determine whether the significant but minor changes in proliferation are due to IL-8-mediated Lyn activation in PCa as well as cyclin D1. While studies demonstrate that IL-8 does not play a role in the proliferation of other cancers, such as melanoma and lung cancer, my studies along with others demonstrate that IL-8 is a contributing factor to the proliferation of low metastatic PCa (147,185,223). However, I note that IL-8’s role in increasing proliferation, while significant are small whereas there were major increases in migration and invasion.

Thus far, I demonstrated that IL-8 production and Src activity are increased in the highly metastatic PC3-MM2 cells and additional IL-8 does not increase Src activity and biological functions in these cells. This work demonstrates that one mechanism contributing to the increases in metastatic properties of PCa is through IL-8 increasing SFK activity in vitro. Likewise, depletion of IL-8 should result in decreased SFK activity, migration and invasion. To test this possibility, I used a lentiviral system to knockdown IL-8 in PC3-MM2 cells (Figure 14). I demonstrate that lentiviral
knockdown of IL-8 decreases Src activity (Figure 15a). In addition, I demonstrate that targets, integral to IL-8 signaling, such as Akt and Erk, are also decreased, while the well-known IL-8 transcription factor, NFk-B, is decreased as well (Figure 15b-c). Many studies provide evidence demonstrating that IL-8 signaling increases Akt and Erk activation in multiple cancer cells supporting my current results (229). While there are other mechanisms of decreasing IL-8 production, such as the use of IL-8 neutralizing antibodies, this study aimed to deplete endogenous levels of IL-8 in a stable context rather than deplete the production of IL-8 (250,251). The use of neutralizing antibodies are beneficial and would be useful in future experiments.

Surprisingly, there was also a 90% decrease in the phosphorylation of NFk-B at p65 after knock down of IL-8 in PC3-MM2 cells. These results are supported by studies from Singh et al. demonstrating that depletion of IL-8 decreases NFk-B activity, and Wilson et al. showing that, upon IL-8 overexpression, IL-8 increases NFk-B activity (174,229). While these studies demonstrate that NFk-B activity is regulated by an IL-8-mediated signaling mechanism, it is possible that IL-8-mediated SFK activation contributes to NFk-B activation. An earlier study demonstrates that SFK-deficient B cells (either Blk -/-, Fyn -/- or Lyn -/-) lack proper IkB phosphorylation and display decreased NF-kB DNA binding activity demonstrating that NFkB activation is, in part, SFK-dependent (230). Future studies would need to be performed to determine if SFK activation mediates NFk-B activity as opposed to expression in PCa cells. It would also be important to determine NFk-B activity in my system by performing an activity assay or assessing whether phosphorylation of proteins involved in NFk-B sequestration, such as IkB members, are associated with NFk-B. The observed reductions in NFk-B may,
partly, explain the feed forward loop because if Src activation is decreased upon IL-8 depletion and contributes to decreased NFk-B function than less IL-8 secretion would be expected.

While NFk-B is an essential transcriptional regulator of IL-8 in PCa, evidence suggests that other transcriptional regulator, STATs, may also regulate IL-8 expression (138,231). This result is supported by a previous study from our laboratory demonstrating that Src activation leads to STAT3 activation, binding to the IL-8 promoter and increased IL-8 secretion (138). More recently, Okutani et al. demonstrates that STAT5 activation induces IL-8 secretion, STAT5 is also directly phosphorylated and activated as a transcription factor by Src (232). Gu et al. shows that STAT5 activity is decreased by Src inhibition and in turn decreases metastatic behaviors of PCa (233). It would be interesting to determine if SFK activity leads to STAT3 or STAT5 transcriptional regulation and whether this promotes IL-8 production and expression demonstrating another mechanism for IL-8-mediated SFK activity in PCa cells.

Overall, this chapter provides, for the first time, a mechanism of IL-8 mediated SFK activation whereby IL-8 stimulates SFK activation that further stimulates IL-8 production increasing migratory and invasive capabilities of metastatic PCa in vitro. The work in this chapter suggests that further investigation into the following areas would be beneficial in understanding PCa progression: 1) investigation of biological functions associated with IL-8 mediated Lyn activity and 2) examining whether STAT3- or STAT5-binding to IL-8 promoter affects transcription dependently or independently of NFk-B transcriptional regulation.
The role of HS5 stromal-produced IL-8 on SFK activity prostate cancer progression in vitro

Studies in chapter 4 demonstrated a feed forward loop whereby production of IL-8 in HS5 stromal cells increases SFK activity which in turn activates IL-8 in these cells. I also demonstrated that, upon silencing IL-8 production in HS5 stromal cells, SFK activation was decreased to baseline levels in PCa as were migration and invasion. This demonstrates a paracrine mechanism whereby IL-8 produced in stromal cells can also affect metastatic properties of PCa.

Prior to work in this dissertation, there has been an increased effort in the investigation of mechanisms that contribute to bidirectional activities in the progression of PCa to metastatic disease (234,235). Studies demonstrate that interactions, such as those between bone marrow stromal cells (BMSCs) and PCa cells, alter gene expression in PCa cells, but there is limited information regarding how stromal cells alter gene and protein expression or whether this affects PCa progression (236,237). In PCa, we know that IL-8 secretory levels and gene expression are elevated as PCa progresses, but previous studies did not address whether paracrine-mediated contributions of stromal produced IL-8 affects PCa progression or whether SFK activation is involved.

To investigate the effects of stromal produced IL-8, I utilized the well-established and commercially available fibroblast HS5 bone marrow-derived cell line as representative of the stromal compartment. Torok and Storob et al. report that the immortalized, non-tumorigenic forming HS5 cells produce high levels of IL-8 and, thus, are an attractive model to explore the effects of stromal production of IL-8 on PCa progression (224).
I first demonstrated that conditioned media from HS5 stromal cells increases migration and invasion of PCa cells (Figure 20) and through successful knockdown of IL-8 in HS5 stromal cells (HS5shIL-8), I then demonstrated that HS5 stromal IL-8 is one factor contributing to increases in migration and invasion of low metastatic PCa cells (Figure 28-29). It is likely that stromal IL-8 production contributes to the production of migratory and invasive factors through other cell types. There is evidence suggesting that IL-8 increases endothelial cell production of MMP-2 and MMP-9, known invasion markers, and this production correlates with tumor invasiveness (173). Endothelial cells are involved in the process of trans-endothelial migration of cancer cells which is correlated with increased metastatic spread of cancer cells (77). Thus, production of stromal IL-8 could stimulate MMP production in endothelial cells which increases the migratory and invasive properties of PCa cells. Further studies addressing whether stromal IL-8-mediated MMP production through SFK activation in PCa cells affects migration and invasion are needed.

There are also other cell types secreting IL-8 that likely contribute to changes in PCa progression because PCa is also known to induce an inflammatory microenvironment whereby IL-8 is also regulated by inflammatory responses (238). While my current study did not address cancer-induced inflammation, I note that increased IL-8 secretion in PCa may be due, in part, to the recruitment of inflammatory cells that also produce IL-8, such as macrophages. Studies show that tumor-infiltrating macrophages were located near non-small cell lung cancer cells expressing high levels of IL-8 (239). An interesting question that may be addressed in future studies is what
role does IL-8 play in prostate cancer-induced inflammation and if it increases progression of PCa.

In the next set of experiments, I demonstrated that HS5 conditioned media increases SFK activity in PCa cells, while SFK activity was decreased in the presence of HS5shIL-8 conditioned media in PCa cells (Figure 25 and 26). I also show that a feed forward loop exists whereby stromal knockdown of IL-8 decreases Src activity while SFK inhibition decreases IL-8 activity (Figure 23 and 24). Prior to studies from this dissertation, investigations studying whether there was an IL-8-mediated SFK signaling mechanism in stromal cells that contributes to SFK activity in PCa cells had not been reported. Work from this dissertation demonstrates that IL-8 production by HS5 stromal cells contributes to increased SFK activity in PCa cells by stromal production of IL-8 which in turn increases IL-8 production.

Although my results provide a mechanism for stromal IL-8-mediation of SFK activation in PCa, I do not propose that all of the changes in SFK activity in PCa cells are mediated by stromal IL-8 because it is likely that other stromal produced factors are involved in SFK activation. In particular, SFK activation is associated with CXCL-12, previously referred to as stromal-derived factor 1α and the production of CXCL-12 in turn affects migration and proliferation (240-242). Further studies are required to determine if CXCL-12 is relevant in paracrine-mediated events in PCa in an IL-8 independent mechanism. In addition, conditioned media from stromal cells also produces cytokine factors, such as IL-2 and 3 also increases migration. It would be interesting to study if these increases were SFK-dependent or independent.
This study provides a better understanding of how IL-8-mediated SFK activation contributes to the crosstalk between stromal and PCa cells in PCa progression. It also suggests that further investigation into the following areas would be beneficial in understanding PCa progression: 1) investigation of other stromal derived factors that contribute to PCa progression and 2) exploring how other IL-8 producing cell models contribute to PCa progression.

**The role of HS5 stromal-produced IL-8 in metastatic potential of prostate cancer**

In Chapter 5 of this dissertation, I determined that increases in tumor growth and metastatic potential are, in part, due to HS5 stromal production of IL-8. There were significant increases in tumor growth, at days 14 and 18, between the PC3:HS5NT cell combination and the PC3 alone group demonstrating that the tumor-stromal interaction contributes to tumor growth (Figure 32). I then demonstrate that mice injected with the combination of PC3 and HS5shIL-8 cells led to tumors that were larger than control but smaller than the combination of PC3 and HS5NT cells, showing that HS5 stromal IL-8 producing cells contributes to tumor growth. This was further confirmed by the significant increases seen in the tumor weights of PC3:HS5NT group when compared to the PC3:HS5shIL-8 group (Table 1). Prior to this investigation, studies investigating the effects of stromal IL-8 on PCa progression in vivo were limited in scope. It is likely that the increases in growth observed with PC3:HS5NT group were due to the secretion and expression of multiple stromal factors, discussed in the previous discussion section.

The next part of this study was to determine if HS5 stromal cells increase the metastatic potential of PCa cells and whether stromal produced IL-8 is a contributing factor. I demonstrated that there was a significant increase in metastatic incidence with
PC3:H5NT stromal cells when compared to PC3 cells (Table 2). There were 4.5 average metastases in the PC3:HS5NT group when compared to 1.5 average metastases in the PC3 group. The results demonstrate increases in metastatic incidence were due, in part, to stromal production of IL-8 whereby the PC3:HS5NT group displayed significantly more metastases than PC3:HS5shIL-8. I demonstrated that stromal contributions favor increased metastatic potential of PCa, but that, specifically, IL-8 production is one contributing factor.

An interesting finding was the presence of both iliac and renal lymph nodes in the PC3:HS5NT and PC3:HS5shIL-8 groups whereas the PC3 group only exhibited iliac lymph node metastases. While it is clear that the tumor-stromal interaction increased metastases, this variability in metastatic site had not been observed prior to this study. While the PC3:HS5NT group demonstrated significantly more metastases relative to PC3:HS5shIL-8 or PC3 groups, there was no significant difference between renal lymph node metastases in PC3:HS5NT and PC3:HS5shIL-8 groups suggesting that IL-8 is not responsible for the variability in lymph node metastases. Other factors that increase metastatic potential need to be investigated. However, this study is supported by Giannoni et al. demonstrating that the co-injection of PCa cells and cancer-associated fibroblasts resulted in ‘spontaneous micrometastases’ in the lungs of mice, not seen in their control groups (235). They suggest that the cancer-associated fibroblasts increase the aggressiveness of PCa cells creating an opportunity for metastasis to other less common sites.

To address metastatic potential, my current study uses an orthotopic in vivo model. While PCa orthotopic mouse models are known to primarily produce lymph
node metastases, but they do not produce bone metastases (107,244). This is discussed because PCa preferentially metastasizes to the bone and it would be interesting to investigate the effects of stromal produced IL-8 in PCa metastasis to the bone. This could be investigated using one model known to produce bone metastases, the intracardiac model, whereby intracardiac injection of stromal cells and PCa cells would be performed. This model could be used to address the specific question of whether stromal production of IL-8 increases PCa metastasis to the bone.

Overall, this study provides novel in vivo evidence whereby HS5 stromal cells producing IL-8 increase tumor growth and metastatic potential. It also suggests that further investigation into the following areas would be beneficial in understanding PCa progression: 1) examining the roles of other IL-8 producing cells in growth, 2) investigating stromal IL-8 mediated markers associated with migration and invasion in metastatic tissue and 3) exploring other mouse models that assess IL-8’s role in metastasis.

Clinical significance of IL-8 and Src in CRPC

Preclinical studies from our laboratory demonstrated that tumor size and lymph node metastases were reduced upon dasatinib treatment (107). It was also demonstrated that after dasatinib treatments in mice with intratibial injections of prostate tumor cells, PSA levels were decreased significantly as well as bone mineral density. This result was attenuated with the addition of docetaxel (226). As a result, a Phase I/II clinical trial with dasatinib+docetaxel trial was completed during my studies (211). The goal of this study was to determine the efficacy of targeting the epithelial and bone compartments of PCa with combination docetaxel + dasatinib therapy.
Response to therapy was determined by PSA levels, but more objectively by the presence of decreased lesions in radiographic bone scans. Numerous cytokines were examined, including IL-8. Figure 33 demonstrates that all patients showed response according to PSA levels, but this response was irrespective of whether there was a decrease in radiographic response. Nearly all patients showed response according to IL-8 levels, but, interestingly, this response was increased in patients who did not respond to treatments. Although this was a very small cohort of samples, studies from chapter 6 suggest that changes in IL-8 from dasatanib + docetaxel treatment may be more predictive of response than changes in PSA.

Several reports demonstrate higher levels of IL-8 in serum and tissue of patients with metastatic disease than in patients without prostate disease or with benign prostatic hyperplasia (165,244). While my current studies demonstrate that IL-8 mediates SFK activity in PCa which contributes to tumor growth and metastatic potential, the Phase II clinical trial results suggest clinical significance for investigating IL-8 and Src activity in PCa. It also suggests that by inhibiting SFK activation IL-8 activity is also targeted. In support of this study, Zabransky et al. provides another study demonstrating that lenalidomide, a thalidomide analog, treatments increased numerous cytokine levels in men with biochemically recurrent PCa, except for one cytokine IL-8. Similar to studies with dasatanib, but through a different therapeutic strategy, this study demonstrates that IL-8 levels were decreased in patients who responded while IL-8 levels were increased in those who had progressive disease (169). These results suggest that there is therapeutic benefit in targeting SFK/IL-8 signaling and that there may be an advantage to using IL-8 as a biomarker of metastatic disease.
Currently, there is a Phase I clinical trial assessing the safety and efficacy of lenalidomide + docetaxel treatments in men with metastatic CRPC. A Phase III study assessing the effects of dasatanib in metastatic CRPC has been completed with results pending. As a result, the assessment of IL-8 levels in responding and non-responding patients may be crucial in understanding how the inhibition of SFK/IL-8 signaling affects metastatic CRPC.
Figure 34 Schematic Representation of IL-8-mediated SFK Activity in Tumor and Stromal Cells
The proposed mechanism shows that IL-8 increases Src activity in low metastatic prostate cancer cells and in turn IL-8 is produced further stimulating Src activation demonstrating a feed forward loop that increases metastatic properties of PCa cells. However, the IL-8/SFK signaling axis in the prostate cancer cells is not completely sufficient in increasing their metastatic potential, but, rather, stromal IL-8 production is contributes to metastatic potential and growth of PCa cells through a paracrine mechanism. Targeting SFK activity by dasatinib will likely decrease SFK activity in the stromal and prostate cancer cells decreasing production of IL-8 and inhibit metastatic spread of PCa.
Summary/Current Perspectives

Collectively, this dissertation demonstrates that: 1) IL-8 mediates SFK activity in the tumor through a feed forward mechanism, 2) a stromal IL-8-mediated paracrine mechanism increases SFK activity and metastatic properties of PCa an 3) IL-8 is one factor in the tumor-stromal interaction contributing to tumor growth and metastatic potential (Figure 34). Thus, I hypothesized that IL-8 is one factor mediating increases in tumor growth and metastatic potential of PCa through SFK activation.

This study demonstrates that IL-8 increases Src activity in PCa cells, but there is increasing evidence suggesting the role of other SFK members in PCa progression. Of the SFK members, there is evidence demonstrating a role for Lyn in PCa progression, but there is increasing evidence suggesting Fyn plays a putative role in PCa progression (245). This is important because as we begin to understand how Fyn contributes to PCa we can explore signaling pathways associated with its expression. Park et al. suggests that SFK members have differential roles in PCa, so it likely that Fyn’s contributions to biological activities of PCa are not exactly the same as other SFK members. As such, the role Fyn plays in the metastatic process should be explored. In addition, it would be interesting to investigate whether Fyn increases the metastatic potential of PCa through an IL-8-mediated mechanism. While studies investigating this would be useful in better understanding how activation of SFKs contribute to the metastatic process, it would also be beneficial in delineating why SFK inhibitors, such as dasatanib, are effective in decreasing tumor growth and metastatic potential.

There are multiple studies that have investigated therapies that target signaling pathways involved with metastatic PCa, outlined in Introduction. While preclinical
models of melanoma and other cancers demonstrated successful inhibition of metastasis by blocking IL-8 using ABX-IL8 the fully human neutralizing antibody against IL-8, this therapy was ineffective in clinical trials as a single therapy. Studies supporting this demonstrate that therapies, such as dacarbazine, elicit cytotoxic effects which attenuated IL-8 and VEGF levels. Further studies suggested that a combination therapy, including dacarbazine and ABX-IL8, may be beneficial by abolishing IL-8 production increasing the effectiveness of dacarbazine (246). This implies that the use of IL-8 neutralizing antibodies would diminish the drug-induced IL-8 response increasing the effectiveness of drug therapies.

As it relates to prostate cancer, Wilson et al. demonstrates that oxaliplatin induces IL-8 production in PCa cells which stabilizes the IL-8-induced changes to PCa progression. In addition, this report demonstrates that the combination of oxaliplatin and the depletion of the secondary IL-8 receptor, CXCR2, increases anti-proliferative signaling mechanisms (247). This suggests that the receptors are also important targets. As a result, the development of small molecule inhibitors targeting CXCR1 and CXCR2 are being investigated, but in vivo studies are also needed to identify their role as a therapeutic strategy (247,248). Future studies would be needed to determine if the combination of IL-8 neutralizing antibody therapies and current drug therapies increases the effectiveness of drug therapies by diminishing IL-8-induced changes to PCa progression.

In addition, these studies also beg the question of the usefulness of IL-8 as a target or as a biomarker for response. Chapter 6 of this thesis demonstrates, in a small cohort of patients, that IL-8 levels are decreased in patients who respond to
docetaxel+dasatanib, while in non-responding patients IL-8 levels are still increased. The IL-8-mediated SFK axis is perturbed through SFK inhibition in responding metastatic CRPC patients. It is likely that by targeting SFK activation IL-8 will be inhibited providing a targetable signaling pathway that exists not only in the tumor but in the microenvironment as well as decreasing metastatic disease. In addition, the sustained high IL-8 levels in patients who did not respond to treatments, such as those in the Phase I/II dasatanib trial of chapter 6, provides reasoning for the use of IL-8 as a marker of response to treatment modalities inhibiting SFKs. While this provides strong implications for IL-8 as a marker of response for bone metastatic disease in the Phase III clinical trials, it is possible that in a larger sample of patients there may not be comparable results. In this context, IL-8 may be useful in assessing disease that has metastasized to a primary metastatic site, such as lymph node. Evidence from my in vivo experiments demonstrates that IL-8 is also involved in PCa metastases to the lymph nodes. The use of therapies inhibiting SFKs can provide benefit for a subset of patients that have lymph node metastases by potentially decreasing spread to the bone.

These studies suggest: 1) that investigating the role of IL-8 and Fyn in PCa progression may be useful in understanding SFK activities in the metastatic process, 2) that therapies targeting PCa through SFK inhibition, such as dasatanib, subsequently decreasing IL-8 levels are needed, 3) studies addressing the significance of the IL-8 receptors in metastatic disease and as a therapeutic strategy will be beneficial and 3) that IL-8 may serve as a predictor of disease progression.
Conclusions

Aberrant activation of SFK activity and overexpression and production of IL-8 have been frequently associated with PCa progression. The regulation of IL-8 by SFK activity has been implicated as a mechanism involved in PCa progression. In addition, the production of IL-8 by tumor-associated cells, such as stromal cells, contributes to tumor progression. This dissertation demonstrates that a feed forward loop exists whereby IL-8 increases SFK activity which in turn increases SFK in PCa cells and this contributes to migration and invasion of PCa. In addition, a paracrine loop exists whereby stromal production of IL-8 increases SFK activity and inhibition of stromal IL-8 decreases SFK activity to baseline levels which was also seen in migration and invasion of PCa cells. The tumor-stromal interaction increased tumor growth and metastases while these increases were decreased upon inhibition of stromal IL-8 demonstrating that stromal IL-8 is one major contributing factor in tumor growth and metastatic potential. My study establishes an IL-8-mediated mechanism in the activation of SFKs in PCa. I also establish that stromal IL-8 contributes to metastatic properties in PCa which are involved in increasing tumor growth and metastatic potential in PCa. This study provides evidence that a feed forward loop whereby IL-8 increases SFK activity exists providing unique evidence that IL-8 mediation of SFK activity involves Src. The paracrine role of stromal IL-8 in vitro and in tumor growth and metastatic potential also provides new insights into how IL-8 production by tumor-associated stromal cells contributes to the metastatic process.
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