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Characterization of Jak, STAT, and Src interactions in Head and Neck Squamous Cell Carcinoma

Reshma Jaseja

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Characterization of Jak, STAT, and Src interactions in Head and Neck Squamous Cell Carcinoma

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Characterization of Jak, STAT, and Src interactions in Head and Neck Squamous Cell Carcinoma

A

THESIS

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

MD Anderson Cancer Center

Graduate School of Biomedical Sciences

In Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Reshma Jaseja, B.S

Houston, Texas

August, 2013
Dedicated to my family
Acknowledgements:

I would like to express my gratitude to my mentor Dr. Faye Johnson for her kindness, patience, and support while she gave me guidance for the past two years. I am also grateful for the help I received from the members of the lab: Dr. Banibrata Sen, Dr. Courtney Nicholas, Dr. Tuhina Mazumdar, Dr. Shaohua Peng, and Dr. Renata Ferrarotto. I would also like to thank my committee members Dr. Gary Gallick, Dr. Pierre McCrea, Dr. Michael Davies, and Dr. Don Gibbons for their advice given during committee meetings.

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Characterization of Jak, STAT and Src Interactions in Head and Neck Squamous Cell Carcinoma
Reshma Jaseja, B.S.
Supervisory Professor: Faye M. Johnson, M.D., Ph.D.

Recurrence of Head and Neck Squamous Cell Carcinoma (HNSCC) is common; thus, it is essential to improve the effectiveness and reduce toxicity of current treatments. Proteins in the Src/Jak/STAT pathway represent potential therapeutic targets, as this pathway is hyperactive in HNSCC and it has roles in cell migration, metastasis, proliferation, survival, and angiogenesis. During short-term Src inhibition, Janus kinase (Jak) 2, and signal transducer and activator of transcription (STAT) 3 and STAT5 are dephosphorylated and inactivated. Following sustained Src inhibition, STAT5 remains inactive, but Jak2 and STAT3 are reactivated following their early inhibition. To further characterize the mechanism of this novel feedback pathway we performed several experiments to look at the interactions between Src, Jak2, STAT5 and STAT3.

We attempted to develop a non-radioactive kinase assay using purified recombinant Jak2 and Src proteins, but found that phospho-tyrosine antibodies were non-specifically binding to purified recombinant proteins. We then performed in vitro kinase assays (IVKAs) using purified recombinant Jak2, Src, STAT3, and STAT5 proteins with and without Src and Jak2 pharmacologic inhibitors. We also examined the interactions of these proteins in intact HNSCC cells. We found that recombinant Jak2, STAT3, and STAT5 are direct substrates of Src and that recombinant Src, STAT3, and STAT5 are direct substrates of Jak2 in the IVKA. To our knowledge, the finding that Src is a Jak substrate is novel and has not been
shown before. In intact HNSCC cells we find that STAT3 can be reactivated despite continuous Src inhibition and that STAT5 continues to be inhibited despite Jak2 reactivation. Also, Jak2 inhibition did not affect Src or STAT5 activity but it did cause STAT3 inhibition. We hypothesized that the differences between the intact cells and the IVKA assays were due to a potential need for binding partners in intact HNSCC cells. One potential binding partner that we examined is the epidermal growth factor receptor (EGFR). We found that EGFR activation caused increased activation of Src and STAT5 but not Jak2.

Our results demonstrate that although STAT3 and STAT5 are capable of being Src and Jak2 substrates, in intact HNSCC cells Src predominantly regulates STAT5 and Jak2 regulates STAT3. Regulation of STAT5 by Src may involve interactions between Src and EGFR. This knowledge along with future studies will better define the mechanisms of STAT regulation in HNSCC cells and ultimately result in an ideal combination of therapeutic agents for HNSCC.
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CHAPTER 1: INTRODUCTION
Head and Neck Squamous Cell Carcinoma Background

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common type of cancer worldwide (1). Approximately 3 to 5% of patients in the United States (US) with cancer suffer from HNSCC. It is estimated that in the US about 53,640 people will acquire head and neck cancer in 2013 and of these, 11,520 people will die. Generally, twice as many men suffer from HNSCC as women and a majority of people are diagnosed after the age of 50. This type of cancer begins in the squamous cells that line the head and neck mucosal surfaces (2). These surfaces include those of the pharynx, larynx, oral cavity, paranasal sinuses, and nasal cavity (Figure 1).

Figure 1. Anatomy of the Head and Neck. Permission to use this image was given by Ms. Terese Winslow LLC.
Many risk factors can contribute to HNSCC. About 85% of head and neck cancers are caused by tobacco use. Human papilloma virus (HPV), particularly subtypes 16 and 18, is a major risk factor for oropharyngeal cancer; the incidence of HPV-related oropharyngeal cancer is increasing in the US (3). Other risk factors include alcohol consumption, exposure to the sun (lip), Epstein-Barr virus (nasopharynx), male gender, advanced age, Asian race, poor oral hygiene, and others (4, 5).

The choice of treatment depends principally on the location of the primary tumor and the stage of the cancer. Treatments include surgery, targeted therapy, radiotherapy, chemotherapy, or a combination of these therapies. All of these therapies can result in uncomfortable and life-altering side effects that may impact speech, eating, hearing, vision, and appearance. For example, surgery may result in the removal of the larynx (voice box); radiation therapy can cause hypothyroidism and severe xerostomia by destruction of the normal thyroid and salivary tissue respectively; chemotherapy can enhance the effects of radiation and can also cause systemic side effects such as infection, hair loss, fatigue, neuropathy, nausea and vomiting (2).

Targeted therapy inhibits specific proteins that allow the cancer cells to grow and survive; there is a continuous need for research to identify specific targets so that inhibitors or other treatments can be used against them. Cetuximab, a monoclonal antibody against epidermal growth factor receptor (EGFR) is the only currently approved targeted therapy against HNSCC (6). Cetuximab is used in
combination with chemotherapy or as monotherapy for metastatic disease or in combination with radiotherapy for locally advanced HNSCC.

**Commonly altered signaling pathways in HNSCC**

Targeted therapy is an important and effective way in which cancer patients are treated. For targeted therapy to be effective, it is essential to know which signaling pathways are activated and drive cancer survival and progression in a specific type of cancer. Previous research has shown that several signaling pathways may contribute to the progression of HNSCC.

EGFR has been targeted for the treatment of HNSCC and other cancer types. In non-small cell lung cancer (NSCLC) EGFR may be activated through mutation. Although activating mutations in EGFR are rare in HNSCC, EGFR is overexpressed, often by gene amplification, in this type of cancer in 80-90% of all cases (7). EGFR is a transmembrane glycoprotein that is part of the ErbB/Her family of tyrosine kinase receptors. When EGFR binds its ligand such as Epidermal Growth Factor (EGF) or transforming growth factor α (TGFα), the receptor experiences a conformational change that exposes two previously occluded sites within EGFR and allows dimerization with another EGFR or ErbB/Her family member. Receptor dimerization allows EGFR autophosphorylation and the activation of its kinase activity. EGFR has several substrates which subsequently cause activation of downstream pathways that are involved in proliferation, survival, and migration of the cancer cell. One of the best-studied pathways downstream of EGFR and other cell surface receptors is the RAS/RAF/MAPK pathway. Previous
studies have shown that EGFR overexpression in HNSCC correlates with poor clinical outcome (8); EGFR tyrosine kinase inhibitors have been approved for clinical use and include gefitinib, erlotinib, and lapatinib (1, 9).

Another important signaling pathway in HNSCC is the PI3K/Akt/mTOR pathway. It has been discovered through whole-exome sequencing that about 20-30% of HNSCC tumors that were analyzed had activating mutations in phosphoinositide 3-kinase (PI3K) (10). Class IA PI3Ks are a family of kinases that phosphorylate phosphatidylinositols such as phosphatidylinositol-4,5,bisphosphate (PI(4,5)P₂) to produce phosphatidylinositol-3,4,5,trisphosphate (PI(3,4,5)P₃), which is a second messenger. This second messenger then binds to 3'-phosphoinositide-dependent kinase 1 (PDK1) at the Plekstrin Homology (PH) domain and Akt. This binding causes their translocation to the cell membrane and allows PDK1 to phosphorylate and activate Akt. Activated Akt has multiple substrates, including mTORC1, that are necessary for many cellular processes such as cell survival (Figure 2). This pathway becomes constitutively active when there is an activating mutation in the PIK3CA gene. Mutations can also occur in other parts of this pathway including Akt, other PI3K family members, and PTEN, a phosphatase which acts to inhibit Akt by dephosphorylating (PI(3,4,5)P₃). Components of this pathway can be altered by mutation, deletion, amplifications, and loss (11).

Other commonly activated pathways in HNSCC are Src and the JAK/STAT pathways described in more detail below. Common somatic mutations include those in FAT1, HRAS, MLL2, CASP8, FBXW7, and CDKN2A (12). Several tumor suppressors are also commonly mutated in HNSCC and these include p53 and NOTCH1(12).
Src Family Kinases in HNSCC

Src belongs to a family of non-receptor tyrosine kinases that includes Yes, Fyn, Lyn, Lck, Blk, Fgr, and Hck in mammals. Src was the first proto-oncogene to be discovered and is an important protein that has roles in cancer cell migration, proliferation, survival, differentiation, invasion and angiogenesis (13).

Figure 3. Src kinase structure showing all domains. Reprinted by permission from Macmillan Publishers Ltd: Oncogene, 2004.

The Src protein has several domains that contribute to and regulate its function as a kinase. Src contains a catalytic domain (SH1 domain), SH2 domain, SH3 domain, SH4 domain, and a unique domain (Figure 3). The catalytic domain or kinase domain (SH1) contains an activation loop where the tyrosine 419 (humans) is located. Src kinases also have a small C-terminal cytoplasmic tail, which contains a phosphorylation site at tyrosine 527. This is an inhibitory phosphorylation site. The SH2 and SH3 domains are non-catalytic domains that
together act as a regulatory unit. These two domains allow Src to be in an inactive confirmation when there is intramolecular binding (13). (Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides). The SH2 and SH3 domains allow for the recognition of other proteins to bind to Src via phosphotyrosine motifs and proline-rich motifs and once this binding occurs, the protein unfolds and becomes activated because the kinase phosphorylation site is accessible (Figure 4) (14).

Figure 4. Active and Inactive conformations of Src. Reprinted from Molecular Cell, Vol 3, Weqing Xu, Amish Doshi, Ming Lei, Michael J Eck, Stephen C Harrison, 629-638., 1999, with permission from Elsevier.
There is increased Src expression in HNSCC compared to the normal mucosal surfaces (15). Previous reports have also shown that Platelet Derived Growth Factor Receptor (PDGFR), EGFR, and Focal Adhesion Kinase (FAK), among many other molecules, can activate Src (16-20). There has also been evidence to suggest that the cytoplasmic domain of beta integrin may have a role in Src activation (21). The integrins are described in more detail below.

Src directly phosphorylates many proteins. Its substrates include STATs, cell adhesion receptors, G-protein coupled receptors, Hepatocyte growth factor receptor (Met), and components of the FAK, PI3K-Akt, MAPK, and Jak/STAT pathways (22-25). It is known that Src activates STATs, particularly STAT3 and STAT5, independently of Jak activation (26). Previous reports from this laboratory also show that with Src inhibition, both STAT5 and STAT3 are initially inhibited (27).

**Integrins**

As mentioned previously, integrins act upstream of Src. Integrins are needed for cell adhesion to the extracellular matrix, which is important for cell survival, differentiation, growth, migration, etc. Integrin signaling occurs through the interaction of protein partners such as FAK, adaptor proteins, and integrin-like kinase (ILK). Integrins are made up of an α chain and a β chain. There are 18 α chains and 8 β chains. One of the most common chains found in integrin heterodimers (in epithelial cells) is the β1 integrin. Cytoplasmic proteins and adaptor proteins bind to the cytoplasmic tails of integrins, which leads to signaling (28).
The Jak-STAT pathway

The main components of the Jak/STAT pathway include a cytokine or growth factor receptor, Janus Kinases (Jaks) and Signal Transducers and Activators of Transcription (STATs). Janus Kinases (Jaks) are a family of non-receptor tyrosine kinases; this family includes Jak1, Jak2, Jak3, and Tyk2. A diagram of the Jak structure is shown below (Figure 5). Jaks contain 7 Jak Homology (JH) domains. The JH1 domain, which is at the C-terminal end, is also the catalytic domain. This domain contains phosphotyrosines that are necessary for the activation of Jaks (tyrosine 1007/1008 for Jak2). The JH2 domain is the “pseudo-kinase” domain. This domain acts to negatively regulate the protein; when tyrosines on this domain are phosphorylated, the activity of the JH1 domain is inhibited. JH3 through JH7 domains are also regulatory domains and are needed for receptor interactions and includes the FERM (F -4.1 protein, E -ezrin, R - radixin and M – moesin) domain (29).
The Jak/STAT pathway is primarily activated by cytokine and growth factors. When the receptor’s ligand binds, receptor dimerization occurs to form homodimers or heterodimers allowing the associated Jaks to trans-phosphorylate each other to become activated, which then allows them to phosphorylate the interacting receptors and STATs. Phosphorylation of STATs causes them to dimerize and translocate to the nucleus, where they can bind to specific DNA sequences and cause transcription of their target genes (Figure 6) (30).
The STAT proteins are transcription factors that regulate cell survival and differentiation. There are 7 STAT family members which are STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Unphosphorylated STATs are located in the cytoplasm. After phosphorylation they dimerize through the interactions of their SH2 domains and move to the nucleus where they bind to specific sequences on the DNA called Gamma-activated sites (GAS) and stimulate
transcription (31) (Figure 7). The two STAT family members that are implicated in HNSCC progression are STAT3 and STAT5 (32, 33). STAT3 is activated principally through phosphorylation of tyrosine 705 and this activation results in its mediation of important cell processes such as cell proliferation and apoptosis. STAT5 has two isoforms, STAT5A and STAT5B and they are activated by phosphorylation of tyrosine 694.

**Figure 7. STAT structure showing different domains and their functions.**

This pathway can be regulated at several levels. For the negative regulation of this pathway, there are three types of negative regulators. These include the Suppressor of Cytokine Signaling (SOCS) proteins, protein inhibitors of activated STATs (PIAs), and Protein Tyrosine Phosphatases (PTPs). SOCS inhibit the kinase activity of Jaks, aid with ubiquitin-mediated degradation, and prevent STATs from
binding to cytokine receptors. The PIAs negatively regulate STAT transcription and PTPs dephosphorylate Jak kinases (27). Although this pathway has been researched extensively, there are still many details of the pathway that are unknown.

**STAT3 Reactivation Following Sustained Src Inhibition in HNSCC**

Previous studies have shown that Src inhibition using an ATP-competitive Src kinase inhibitor such as dasatinib results in rapid (30 min) Jak2, STAT3, and STAT5 dephosphorylation and inactivation (27). During sustained Src inhibition, decreased STAT3 activation is only temporary but STAT5 inhibition was sustained. By 4-7 hours STAT3 becomes reactivated. This is also similar to Jak2 in that sustained Src inhibition results in initial Jak2 inhibition and Jak2 kinase activity recovers by 7 hours. In a previous study, it was shown that STAT3 reactivation occurs via Jak2 kinase activity and Jak-STAT3 binding. The investigators showed that when they depleted Jak2, they also saw an inhibition of STAT3 (Figure 8) (27). Because Jak2 regulates STAT3, when Jak2 is chronically inhibited so is STAT3 (Figure 8) (27).

The reactivation of STAT3 is dependent upon Jak2 and Tyk2. This was shown by depleting both Jak2 or Tyk2 using siRNA. Depletion of Tyk2 or JAK2 caused a partial decrease in STAT3 reactivation after Src inhibition. When both Jak2 and Tyk2 were knockdown at the same time, there was complete inhibition of STAT3 reactivation (27).

The reactivation of STAT3 and Jak2 is biologically important. When STAT3
was completely abrogated along with Src, there was significant cell cycle arrest. There was also an increase in apoptosis when cells were treated with both a Src inhibitor and STAT3 siRNA compared to when cells were just treated with the Src inhibitor. This suggests that STAT3 and Src work together to maintain cell survival and proliferation and that STAT3 reactivation lessens the effects of Src inhibition on cancer cell survival and proliferation.

The reactivation of Jak2, Tyk2, and STAT3 is due to the loss of SOCS2 expression. STAT5 regulates SOCS2 transcription (34). The loss of STAT5 activity following Src inhibition leads to the decrease of SOCS protein levels. This prevents SOCS2 from inhibiting Jak2, interfering with Jak2-STAT3 binding and Jak2 kinase activity.
Figure 8. Schematic of basal, acute, and chronic Src inhibition and its effect on Jak2 and STAT activation. The basal state shows activation of Src, Jak2, STAT3, and STAT5. Acute Src inhibition results in inhibition of Jak2, STAT3, and STAT5 activity. Chronic Src inhibition results in STAT3 and Jak2 reactivation and durable STAT5 inhibition. In a negative feedback pathway, SOCS binds to and inhibits Jak2 from binding to STAT3. Taken from Courtney Nicholas with permission, PhD.
Hypothesis

The model described above provides a framework for a feedback loop in which the inhibition of Src leads to Jak, STAT3, and STAT5 inhibition. The sustained inhibition of STAT5 leads to the loss of SOCS2 transcription. The decrease of SOCS2 protein levels allows for Jak2 and STAT3 reactivation by removing an inhibitory effect on Jak2-STAT3 binding and Jak2 kinase activity. However, this model has several unanswered questions.

Although we know that Src inhibition leads to Jak2, STAT3, and STAT5 inhibition, we do not know if these molecules are direct substrates of Src. We are also uncertain about how STAT5 and STAT3 are regulated within the cells. We hypothesize that they are regulated independently by Src and Jak2 respectively. We also do not know the primary regulator of Src in HNSCC cells. It is possible that EGFR, FAK, integrins, a combination of these, or other upstream pathways may regulate Src activation in HNSCC.

This thesis examines these questions by manipulating the pathway by use of inhibitors and stimulation using recombinant cytokines and growth factors. In vitro kinase assays were also performed to look at the interaction of Jak2, Src, STAT3, and STAT5 in a cell free system to compare these interactions with the interactions that occur in intact HNSCC cells. Better understanding of the Jak/STAT pathway is essential so that ultimately better treatments can be developed in order to improve patient survival and lifestyle.
CHAPTER 2: MATERIALS AND METHODS
Cells and reagents

Human HNSCC cell lines OSC19 and TU167 were obtained from Dr. Jeffrey Myers, MD Anderson (35). Cell lines were authenticated by DNA fingerprinting and tested for mycoplasma and other contaminants by mouse antibody production (MAP) testing. Dasatinib, pyridone 6, saracatinib, PF-00562271, and imatinib were purchased from Selleck Chemicals (Houston, TX) and prepared as stock solutions of 10 or 100 mmol/L in DMSO. Cytokines and Growth factors (PDGF, IGF, EGF, IL-6, and TGF-α) were purchased from Peprotech (Rocky Hill, New Jersey).

Full length human, recombinant His-tagged Src expressed in insect cells (66 kDa) was purchased from Sigma-Aldrich. Human, recombinant Jak2 containing the JH1 and JH2 domains (amino acids 532-1132, 99kDa) expressed in Baculovirus infected Sf9 cells was purchased from Sigma-Aldrich and Invitrogen. Human STAT5A was purchased from Novus Biologicals. This recombinant protein contains full length STAT5A and a GST tag resulting in a protein of about 115 kDa that was expressed in an in vitro wheat germ system. Human STAT3 was purchased from Abcam (Cambridge, MA). This protein is a full-length recombinant protein that was expressed in Sf9 insect cells and contains an N-terminal GST tag resulting in a protein of about 120 kDa.

Antibodies pSrc (Y416) CS2101, total Src CS2110, pJak2(Y1007/1008) CS3771, pSTAT3(Y705) CS9131, Total Stat3 CS9139, pSTAT5(Y694) CS9351, Total STAT5 CS9358, pEGFR (Y1068) CS3777, total EGFR CS4267, pFAK(Y576) CS3281, pFAK(Y925) CS3284, pFAK(Y861) CS3283, total Fak CS3285, total PDGFRβ CS3169, and β1 Integrin CS9699 were all purchased from Cell Signaling.
pPDGFR(Y572/574) #44-1000G and pFAK(Y397) #44624G were purchased from Invitrogen. Total Jak2 (sc-294) was purchased from Santa Cruz. Phospho-Paxillin was purchased from Abcam.

For immunoprecipitations, the Jak2 antibody (AHO1352) was purchased from Invitrogen and the Src antibody (OP07A) was purchased from Calbiochem.

**In Vitro kinase assays**

This assay was done as previously described with the following modifications (27). Recombinant proteins [Src (435 nmol/L), Jak2 (870 nmol/L), STAT5, (870 nmol/L), and/or STAT3 (870 nmol/L)] were incubated with DMSO control, 2 μM saracatinib, 100 nM dasatinib, or 20 mM pyridone 6 for 30 minutes after which HBV buffer [50 mM/L HEPES, 100 μmol/L sodium orthovanadate (pH 7.4), 1 M/L MgCl₂, 1 M/L MnCl₂ and 10 μCi (y-32P)ATP (3000 Ci/mmol; 1 Ci= 37 GBq)] was added and the samples incubated for another 15 min at room temperature. The reaction was stopped with 3X sample buffer (2% SDS, 5% beta-mercaptoethanol, 0.125 mol/L Tris (pH 6.8), 1 mmol/L EDTA, 0.02% bromphenol blue). The samples were then boiled for 5 min and separated on 7.5% SDS-PAGE. The gel was fixed with fixing solution [45% Methanol, 45% distilled water, 10% Acetic Acid] for 20 min two times and boiled with 1 mol/L KOH for 10 minutes. The gel was then placed back in fixing solution for another 20 min and then dried using a gel dryer for 3 hr. The radiolabeled proteins were detected by autoradiography.
**Cell culture**

HNSCC cells were grown in DMEM containing 10% Bovine Serum in a humidified CO₂ incubator at 37 °C. They were grown on 100 mm tissue-culture dishes in a monolayer.

**Western blot analysis:**

Subconfluent HNSCC cells were washed with phosphate-buffered saline without calcium and magnesium. The cells were then incubated for 5 minutes with trypsin (Sigma-Aldrich) and spun down for 5 min at 13000 rpm at 4 °C. The cell pellet was washed with PBS and lysed using lysis buffer containing 50 mmol/L Trizma base (pH8; Sigma), 1 % Triton X-100, 150 mmol/L NaCl, 20 ug/mL leupeptin, 10 ug/mL aprotinin, 1 mmol/L pehnylmethylsulfonyl fluoride, 1 mmol/L sodium vanadate and Protease and phosphatase inhibitors and were incubated for 20 mins at 4 degrees on a rotator. The lysates were centrifuged for 10 minutes at 14,000 rpm and the supernatant was collected. The protein concentration of each sample was determined using BCA reagents as per manufacturer's instructions (Thermo Scientific). Equal amounts of each sample was taken and mixed with the same volume of 2X sample buffer and resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% milk in TBS-T for 1 hr at room temperature and then probed with primary antibody overnight. The primary antibody was incubated overnight at 4°C and then the membrane was washed with TBS-T 3 times for 5 min. The secondary antibody conjugated with horseradish peroxidase in 5% milk in TBS-T was then added for 1-2 hr at room
temperature and the membrane was washed again with TBS-T 3 times for 5 min. Protein bands were detected using Enhanced chemiluminescence reagent (Pierce).

**Immunoprecipitation:**

Tu167 and OSC19 cells were collected and lysed using immunoprecipitation (IP) lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 5% glycerol). Lysates containing 800 μg of protein were pre-cleared using protein A and G sepharose beads for 1 hour. The cleared lysate was incubated with the indicated antibody or IgG control antibody and agarose beads for an hour. The beads were then washed 3 times using IP lysis buffer. Sample buffer was added to each sample and then each sample was boiled for 5 min and loaded onto SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with primary antibodies as indicated. The secondary antibody conjugated with horseradish peroxidase was then added and detected using enhanced chemiluminescence reagent.
CHAPTER 3: RESULTS
Commercially available anti-phosphotyrosine antibodies bind non-specifically to recombinant Jak2 and Src

To facilitate our research examining Src, Jak, and STAT interactions and to enhance lab safety, we attempted to develop an in vitro kinase assay (IVKA) using recombinant purified proteins, Src and Jak2, and non-radiolabeled ATP. Recombinant Jak2 and Src were incubated with the Src inhibitor, dasatinib, the Jak inhibitor, pyridone 6, or vehicle control for 30 min in the presence of 80 mM ATP and the exogenous substrate enolase. Following incubation, the samples were run on SDS-PAGE and the membrane was probed with one of two phospho-tyrosine antibodies (4G10 or pY100). We did not observe a significant band corresponding to enolase. When Src was incubated with dasatinib, there was no reduction in the staining of the 60 kDa band corresponding to Src. Likewise, when Jak2 was incubated with pyridone 6; there was no reduction of the 130 kDa Jak2 band (Figure 9A, B). To investigate the possibility of the recombinant proteins already being phosphorylated prior to performing the assay, a phosphatase, protein-tyrosine phosphatase 1B (PTP1B), was added to the IVKA reaction mixture without the addition of ATP. The results of this assay were similar to the previous one, showing no reduction in the staining of both the Src and Jak2 bands (Figure 9C).

To examine the efficacy of the phosphatase, we immunoprecipitated epidermal growth factor receptor (EGFR) from TU167 cells with and without the addition of exogenous EGF to stimulate phosphorylation of EGFR. After the immunoprecipitation was performed, PTP1B was added to one sample to dephosphorylate the receptor. The lysates were run on SDS-PAGE. The
membrane was then probed with pEGFR (Y1068). As expected, EGF stimulation led to a significant increase in pEGFR. There was no pEGFR band in the sample containing EGF and the phosphatase, indicating that PTP1B dephosphorylated EGFR (Figure 9D). We concluded that the phospho-tyrosine antibodies were non-specifically binding to the recombinant proteins, making this non-radioactive IVKA unusable with currently available reagents.
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![Image of gel electrophoresis](image)
Figure 9. Anti-phosphotyrosine antibodies bind non-specifically to recombinant Jak2 and Src. Potent Src (100 nM dasatinib) and Jak (5µM pyridone 6) inhibitors did not reduce the staining of Src and Jak2 with the anti-phospho tyrosine antibodies 4G10 (A) or pY100 (B). PTP1B phosphatase did not reduce the staining by 4G10 of the bands corresponding to the recombinant proteins (C), but did effectively dephosphorylate EGFR immunoprecipitated from TU167 cells (D).
Src and Jak2 are direct substrates of each other in a cell-free, in vitro kinase assay

The non-radioactive IVKA was unusable, so the established IVKA method was used to explore Src, Jak2, and STAT interactions for the remainder of the study. In this method, recombinant proteins are incubated with the Src inhibitor dasatinib, the Jak inhibitor pyridone 6, or vehicle control for 30 min in the presence of 10 mCi (γ-32P)ATP. As expected, Src acted as its own substrate and Src kinase activity was inhibited by dasatinib (Figure 10A, lanes 2-3). Likewise, Jak2 was auto-phosphorylated and its kinase activity was inhibited with pyridone 6 (Figure 10A, lanes 5 and 7). Surprisingly, dasatinib also partially inhibited Jak2 in this assay (Figure 10A, lane 8) — an effect we had not observed in Jak2 that was immunoprecipitated from intact cells incubated with dasatinib (27). Since dasatinib inhibited recombinant Jak2 we used saracatinib, a more specific Src inhibitor, in subsequent experiments (Figure 10B-D).

When Jak2 was incubated with saracatinib at 200 nM, there was no reduction in Jak2 phosphorylation (Figure 10B, lanes 5 and 8), indicating that Jak2 kinase activity was not being affected by this inhibitor. However, 200 nM saracatinib only partially inhibited Src (Figure 10B, lanes 2-3). Increasing the saracatinib concentration to 2 µM led to full Src inhibition (Figure 10C, lanes 1 and 3) without Jak2 inhibition (Figure 10C, lanes 2 and 4).

To determine if Src could phosphorylate Jak2, we incubated the proteins together in the presence of pyridone 6. When Src and Jak2 were incubated together, we saw that both proteins were phosphorylated (Figure 10A-C, lane 6).
When pyridone 6 was added to the combination, inhibiting Jak2 kinase activity, Jak2 was still phosphorylated (Figure 10A-B, lane 10). This demonstrates that recombinant Jak2 is a direct Src substrate.

When 2 μM saracatinib was added to the combination of Src and Jak2 proteins, Src phosphorylation was observed (Figure 10C, lane 9). The degree of Src phosphorylation was similar to that of the Jak2 and Src combination demonstrating that Jak2 can phosphorylate recombinant Src.
Figure 10. Recombinant Jak2 is a direct Src substrate and Src is a Jak2 substrate. Recombinant Src and Jak2 were incubated with 100 nM dasatinib (A), 200 nM saracatinib (B), 2 µM saracatinib (C), and/or 20 µM pyridone 6 as indicated in an IVKA and phospho proteins detected by autoradiography.
Src regulates STAT5 but not STAT3 activity in intact HNSCC cell lines

Previously published data demonstrated that Src inhibition leads to marked and sustained decreased STAT5 phosphorylation despite recovery of Jak2 activity after 4-7 h (27, 34). This suggests that Src, and not Jak2, regulates STAT5 activity in HNSCC cells. To examine potential activators of STAT5 further, we treated OSC19 and TU167 cells with interleukin-6 (IL-6), epidermal growth factor (EGF), dasatinib, pyridone 6, or saracatinib and examined Src and STAT activation using Western blotting (Figure 11).

As expected, IL-6 had no significant effect upon Src phosphorylation; dasatinib and 1 µM saracatinib inhibited Src completely; and pyridone 6 did not change Src phosphorylation levels. The lower concentration of saracatinib (75 nM) did not completely inhibit Src. STAT5 phosphorylation paralleled that of Src but STAT3 activation was unaffected by Src inhibition demonstrating that Src regulates STAT5 but not STAT3 activity.

Jak regulates STAT3 but not STAT5 in intact HNSCC cell lines

We found a significant increase in the phosphorylation of STAT3 in IL-6 stimulated samples and complete inhibition with pyridone 6. IL6 and pyridone 6 did not affect STAT5 activation (Figure 11). Since pyridone 6 is a pan-Jak inhibitor and IL6 stimulates Jak2, we concluded that Jak was regulating STAT3 but not STAT5 in HNSCC cells.
Figure 11. Src predominately regulates STAT5 and Jak predominately regulates STAT3 in intact HNSCC cells. TU167 and OSC19 cells were treated with 100 ng/ml IL-6 or 10 ng/ml EGF for 5 min; 100 nM dasatinib for 1 h; 5 μM pyridone 6 for 1 h; or 75 nM or 1 μM saracatinib for 1 or 7 h as indicated. Cell lysates were subjected to Western blotting and expression of various proteins was measured using the indicated antibodies.
STAT5 is a direct substrate of both Jak2 and Src in a cell-free, *in vitro* kinase assay

In intact HNSCC cells the inhibition of Src reduced STAT5 activation, but Jak inhibition did not. In contrast, others have demonstrated that STAT5 can be regulated by Jak family members (36, 37). We performed an IVKA to determine if STAT5 is a direct substrate of Src or Jak2. Recombinant Jak2, Src, and STAT5A proteins were incubated with $^{32}$P-labeled ATP both with and without dasatinib or pyridone 6. Upon incubation of Src and STAT5A, STAT5A was phosphorylated indicating that STAT5A can be a direct substrate of Src (Figure 12, lane 2). When Src and STAT5A were incubated with dasatinib, the dasatinib inhibited Src kinase activity and no STAT5A phosphorylation occurred (Figure 12, lane 3). When Jak2 and STAT5A were incubated together, STAT5A was phosphorylated, indicating that STAT5A can also be a direct substrate of Jak2 in a cell free system (Figure 12, lane 4). When Jak2 and STAT5A were incubated with pyridone 6, this inhibitor inhibited Jak2 kinase activity so no STAT5A phosphorylation occurred (Figure 12, lane 5).

STAT3 is a direct substrate of both Jak2 and Src in a cell free, *in vitro* kinase assay

Analogous to the experiments above, we performed an IVKA to determine if STAT3 is a direct substrate of Src or Jak2. Upon incubation of Src and STAT3, STAT3 was phosphorylated indicating that STAT3 can be a direct substrate of Src (Figure 13, lane 4). When saracatinib was added, STAT3 phosphorylation was inhibited (Figure 13, lane 6). When Jak2 and STAT3 were incubated together,
STAT3 was phosphorylated. Likewise, Jak2 phosphorylated STAT3 which was inhibited by pyridone 6 (Figure 13, lanes 5 and 7). Although equimolar protein amounts were used, the degree of STAT5A and STAT3 phosphorylation by Jak2 kinase activity was much lower than that caused by Src.
Figure 12. STAT5A is a direct substrate of both Src and Jak2. Recombinant Jak2, Src, and STAT5A were incubated with dasatinib, pyridone 6, or vehicle control as indicated. The proteins were subjected to an IVKA with $^{32}\text{P}$-labeled ATP and phosphorylated proteins detected by autoradiography.
Figure 13. **STAT3 is a direct substrate of both Jak2 and Src.** Recombinant Jak2, Src, and STAT3 were incubated with dasatinib or pyridone 6 or vehicle control as indicated. The proteins were subjected to an IVKA using with $^{32}$P-labeled ATP and phosphorylated proteins detected by autoradiography.
**EGFR activation increases Src and STAT5 activity in HNSCC cells**

Our data suggest that Src predominantly regulates STAT5 in HNSCC cells but Jak2 can also phosphorylate recombinant STAT5A, suggesting that substrate specificity in intact cells may be regulated by proximity. Furthermore, since STAT5, STAT3, Src and Jak2 are all located primarily in the cytoplasm (C. Nicholas, unpublished data) substrate specificity is likely regulated by differential protein binding partners. To determine the upstream activators of Src and STAT5 in HNSCC we first examined the effect of EGFR stimulation on their activation because it has previously been reported to stimulate Src in HNSCC (38). As expected, we did detect increased EGFR activation in the EGF and TGF-α stimulated cells. Both EGFR ligands led to activation of Src and STAT5 in TU167 and Osc19 cells although the stimulation was more marked in Osc19 cells (Figure 11 and 14). Basal expression of pEGFR (Y1086) was undetectable and not affected by stimulation with IL-6 or incubation with dasatinib, pyridone 6 or saracatinib (Figure 11). Tyrosine 1086 was examined because it is not a Src-dependent phosphorylation site (Jak and Grb2-dependent) (39).

To test for Src and EGFR interactions, we performed a Src immunoprecipitation (IP) from HNSCC cells incubated with EGF, TGF-α, or saracatinib (Figure 14A-B). In all conditions, Src and EGFR co-immunoprecipitated in TU167 cells but this interaction was not detectable in Osc-19 cells. Interestingly, despite robust Src activation detected on the Western blots following EGF and TGF-α stimulation, this activation was not detected on the IP samples suggesting that the
anti-total Src antibody used for the IP was saturated and preferentially bound to phosphorylated Src.

Figure 14. EGFR co-immunoprecipitates with Src in TU167 cells. Src immunoprecipitation of cells treated with EGF, TGF-α, saracatinib for 1 and 7 h in (A) TU167 cells and (B) OSC19 cells.
**Focal Adhesion Kinase may regulate Src Activity in HNSCC**

To further investigate the upstream activators of Src, we examined the effects of FAK activity on Src activation. We treated HNSCC cells with an ATP-competitive FAK inhibitor, PF-00562271, for 4 hours at different concentrations (100 nM, 500 nM, 1 µM, and 3 µM). We saw that with increasing doses, there was a gradual decrease in Src activity, but this decrease was very minimal (Figure 15). Likewise there was also a decrease in pPaxillin, a known substrate of FAK.

![Image of Western Blot](image)

**Figure 8.** FAK may play a role in Src regulation. TU167 cells were treated with increasing concentrations of PF-00562271 and probed with pFAK(Y397), pSrc, and pPaxillin (Y118) (A). (B) shows densitometry.
CHAPTER 4: DISCUSSION
In this study, we examined the interactions of Jak2, STAT3, STAT5 and Src in HNSCC cells. Previous work from our laboratory defined a feedback pathway in which sustained Src inhibition led to the loss of STAT5A-driven SOCS2 expression which allowed Jak2 kinase and STAT3 activities to recover. In this work, we demonstrated that Jak2, STAT3, and STAT5A are direct substrates of Src in an IVKA with purified proteins. Likewise Src, STAT3, and STAT5A are all substrates of Jak2 in an IVKA. In contrast, in intact HNSCC cells, Src inhibition leads primarily to STAT5 inhibition with transient, minimal effects on Jak2 and STAT3. Jak inhibition in intact HNSCC cells does not affect STAT5 or Src activity but does result in STAT3 inhibition. The contrast between the IVKA assays with recombinant proteins suggests that substrate specificity in intact cells may be regulated by differential protein binding partners. One potential protein that may mediate the interactions between these proteins is EGFR and we demonstrated that EGFR activation leads to Src and STAT5 activation.

**Jak and Src Interactions**

Previous studies have shown that in intact breast cancer cells, upon stimulation, Jak2 and Src bind to each other (40). However, it has not yet been shown whether they directly phosphorylate each other. This is the first report in HNSCC that investigates a novel relationship between Jak2 and Src. Our in vitro kinase assays show that Src is a direct substrate of Jak2 and Jak2 is a direct substrate of Src. These results are significant because within intact cells, when Src is inhibited, there is initial inhibition of Jak2 activity as well, but Jak2 is recovered after some time. However when we inhibit Jak2 in intact cells, we do not find Src
inhibition. This leads to the possibility that Jak and Src do not physically interact because they bind to distinct scaffolding proteins. These results demonstrate that Jak2 is a direct Src substrate in intact cells but that Src is not a Jak2 substrate. As discussed below, possible reasons for the discrepancies between the IVKAs and the intact cells are that the Jak2 protein used in the IVKA is a truncated protein or that Src is more easily accessible for phosphorylation by Jak2 in the cell free system.

**Regulation of STAT3 and STAT5 Activation**

The STAT family of transcription factors, especially STAT3 and STAT5, regulates oncogenic signaling in many different tumor types (41). STATs are Src substrates and can mediate Src's biological effects including proliferation, survival, and angiogenesis (42). In HNSCC cells, c-Src interacts with STAT3 and STAT5 (co-immunoprecipitation) and Src inhibition results in reduced STAT3 and STAT5 activation and reduced cell proliferation (43). Inhibition of STAT3 in HNSCC leads to increased apoptosis, decreased proliferation, and decreased tumor size (44, 45). STAT3 activation leads to the increased expression of downstream target genes (e.g., Bcl-XL, cyclin D1, survivin) and increased cell proliferation and tumor growth in vivo (46). STAT3 is essential for Src-mediated angiogenesis; following activation by Src, STAT3 binds to the promoter of vascular endothelial growth factor (VEGF), leading to increased VEGF production (47, 48). Activation of STAT3 is required for v-Src-mediated transformation (49) suggesting that STAT3 is a key mediator of the oncogenic effects of c-Src.
In addition to regulation by Src, STAT3 can be activated by Jaks. Jaks are essential for cytokine signaling. Jaks constitutively bind to cytokine receptors which do not have intrinsic kinase activity. Upon ligand binding, the cytokine receptor undergoes oligomerization resulting in Jak activation by trans-phosphorylation. The activated Jak molecules then phosphorylate the cytokine receptor allowing for the binding of the monomeric, inactive STATs that are present in the cytoplasm. Once bound to the cytokine receptors, STATs become Jak substrates. Phosphorylated STATs undergo dimerization and nuclear translocation (42). The molecular mechanisms leading to Jak activation are not fully understood and this simplified model underestimates the complexity of these pathway interactions. Two key examples are that: activation of Jak2 can occur, albeit at a lower level, in the absence of Y1007/1008 phosphorylation, theoretically obviating the need for activation by an upstream kinase (50); and Jaks can be activated by oncogenic tyrosine kinases, independent of cytokine receptors (51-53). There has been evidence to suggest that Jak does not activate STAT5. After sustained Src inhibition as already mentioned, Jak2 is reactivated. With Jak2 reactivation STAT3 reactivation was also seen, but STAT5 remained inhibited (27).

As part of this study we also looked at the regulation of STAT3 and STAT5 by Src and Jak2. Our results demonstrate that STAT5A can be directly phosphorylated by both Src and Jak2 in an IVKA but that in intact HNSCC cells Src, but not Jak2, regulates STAT5 activity. Similarly, we also found that STAT3 can be phosphorylated by both Jak2 and Src in an IVKA but in intact cells Jak2 regulates STAT3 activity. Another discrepancy between the IVKA and the intact cell data was
that the degree of phosphorylation of the STAT proteins by Jak2 was much lower than that of Src. There are several possible reasons to explain the discrepancies between the two systems. First a receptor and/or adaptor proteins may be needed to complex with Jak2 and STAT3 for STAT3 phosphorylation to occur. This is consistent with what we know about how Jak functions in cells. This complex may be needed to affect conformational changes in the involved proteins or simply to bring them into proximity for enzymatic interactions. Another reason for the lower Jak2 kinase activity toward STAT3 in the IVKA may be due to the recombinant Jak2 being a truncated protein. While the domains that we think are the most important for kinase activity and regulation, the JH1 and JH2 domains, are still present, the recombinant Jak2 lacks the 20 kDa region on the N-terminal end. This loss may affect Jak2 interactions with its substrates. It is also possible that Jak2 may have a slower enzyme kinetics or that the accessibility of both Jak2 and Src to the STAT proteins may be better In Vitro compared to intact cells.

When we investigated the regulation of STAT3 and STAT5 in intact HNSCC cells, we saw that Jak2 was the primary regulator of STAT3. When Src was inhibited with dasatinib and saracatinib, there was no sustained decrease in STAT3 activity, but when Jak2 was inhibited with pyridone 6, we saw complete inhibition of STAT3. When we inhibited Jak2 and looked at STAT5 activation, there was no change; whereas when Src was inhibited, there was complete inhibition of STAT5. These results suggest that Src primarily regulates STAT5.
**Src Activation**

Src participates in several cellular functions in cancer cells including cell cycle progression, immune recognition, adhesion, spreading, migration, apoptosis regulation, and differentiation (54, 55). Although v-Src, which lacks the C-terminal regulatory domain and is constitutively active, transforms fibroblasts (56), c-Src is only weakly transforming. HNSCC cells transfected with a dominant active c-Src showed enhanced tumor growth and invasion (57). Inhibition of c-Src activity using both molecular approaches and pharmacologic inhibitors leads to reduced anchorage-independent growth (58, 59), proliferation (60, 61), tumor growth in vivo (62, 63), invasion, migration (64-71), metastasis (72-76), vascularity (77-79), and survival (59). In HNSCC specifically, inhibition of c-Src leads to decreased invasion, migration and proliferation (38, 43, 57, 64, 80).

Aberrant c-Src expression has been demonstrated in multiple epithelial cancers (81) where its expression and activation correlate with malignant progression (82-88), advanced clinical stage, and poor survival (89). c-Src is expressed in areas of hyperproliferation in HNSCC, dysplastic oral epithelium, and benign oral mucosal lesions (90). Several Src members are expressed and activated in multiple HNSCC cell lines (38, 43, 91). Expression of activated c-Src is higher in human HNSCC tumor tissue than in normal mucosa and correlates with an invasive, poorly differentiated phenotype and advanced nodal stage (15).

Src can be activated by Platelet Derived Growth Factor Receptor (PDGFR), EGFR Focal Adhesion Kinase (FAK), G-coupled protein receptors (GCPRs), and integrins (16-21). We examined the effect of EGFR on Src activation because
previous reports had identified Src and STAT activation downstream of EGFR in HNSCC cells. When we stimulated cells with ligands that are known to activate EGFR (EGF and TGF-α) we observed an increase in Src activation. We also performed an immunoprecipitation and found Src-EGFR interaction in TU167 cells. However the interaction was undetectable in OSC19 cells. We did not detect any association between Src and pEGFR (Y1068). These results (TU167) are similar to previously published data suggesting that EGFR is involved in the direct activation of Src (26). As previously mentioned, there are many possible activators of Src. We also examined FAK in HNSCC and its effect on Src and found that Src activation did not change substantially decrease with the use of a potent FAK inhibitor.

**Future Directions**

We know that STAT3 is an important therapeutic target in HNSCC and that both STAT3 and STAT5 have distinct biological functions and transcriptional profiles. STAT3 activation as mentioned previously, leads to the increased expression of downstream target genes that are involved in cell proliferation, cell survival, and angiogenesis. These transcriptional targets include Bcl-XL, cyclin D1, and survivin (47, 48). STAT5 activation has been mainly examined in hematopoietic malignancies and is also involved in the expression of genes that are needed for hematopoiesis and cell differentiation (33, 36). One of the purposes of this study was to examine the regulation of both STAT3 and STAT5. We hypothesized that the regulation of both proteins was differentially regulated by Src and Jak2 and our results supported this in intact HNSCC cells.
To address the discrepancies between the IVKA results and the signaling results in intact HNSCC cells, future studies should include the purification of a full length Jak2 protein and kinase-dead Jak2 and Src proteins to use in the *in vitro* kinase assays instead of using the Src and Jak inhibitors. These inhibitors may have non-specific effects that could be affecting the interactions and the use of these mutated proteins may eliminate this issue.

Based on the examination of the regulation of STAT3 and STAT5, we concluded and hypothesized that a scaffolding protein may be necessary for the activation of Src and STAT5. In this study we only looked at FAK and EGFR as possible regulators of Src. We could also look at other kinases such as PDGFR, fibroblast growth factor receptor (FGFR), erythropoietin receptor, other ErbB receptors, and IGFR, which are known activators of Src.

We could also use non-biased approaches to identify proteins that bind to Jak2, STAT5A, STAT3 and Src. These could include performing immunoprecipitations to precipitate one of these proteins followed by mass spectrometry to identify proteins that may be co-immunoprecipitating. Another non-biased approach to study proteins that may be involved in STAT3 and STAT5 regulation may include an siRNA screen in which hundreds of genes can be depleted at one time. The cells are then analyzed to examine a specific protein that is affected by the loss of these genes and the siRNA can be identified using different methods including mass spectrometry. This method will not identify a particular scaffold protein, but can narrow down which protein may be necessary for the interactions to occur.
Several biased approaches could also be used to study the interactions between Jak2, STATs, and Src. One example is the proximity ligation assay (PLA). This method is a simpler version of a fluorescence resonance energy transfer assay (FRET) and involves the use of two affinity probes directed against two proteins of interest. If these proteins physically interact with one another, the probes will anneal and the ligated products can then be amplified resulting in their fluorescence (92).

The ultimate goal of cancer research is to prevent cancer or develop treatments for cancer patients that increase survival and improve their quality of life by defining the mechanisms that underlie cancer cell progression and survival. In HNSCC, it is imperative to study the Src/Jak/STAT pathway and the feedback pathways that may be activating STAT3. Because a Src inhibitor has previously failed in a clinical trial, it is important to study the pathway further so that other biomarkers of HNSCC (such as the STATs) can be identified and targeted.
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