INHIBITION OF INFLAMMATORY SIGNALING VIA THE IL-6-STAT3 PATHWAY AND ITS EFFECTS ON GROWTH AND SURVIVAL OF TRIPLE NEGATIVE BREAST CANCER CELLS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelor of Science in Molecular Biology with Distinction

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I would like to thank my mentor, Dr. Sims-Mourtada, and Dr. Arnold for their guidance, as well as my family for their support. I would like to thank God for watching over and guiding me.

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ABSTRACT

Breast cancer is one of the most common types of cancer, and while many types of breast cancer can be treated with anti-estrogen therapy or Trastuzumab, such directed treatment against triple negative breast cancers (TNBC) remains elusive and the prognosis remain poor. Previous evidence would suggest that the inflammatory IL-6-STAT3 pathway may be a viable target for treatment in triple negative breast cancers, as IL-6 is upregulated in TNBC, and STAT3 has been shown to promote dedifferentiation into cancer stem cell and tumor cell proliferation and survival in TNBC. This has led us to test the effects of anti-inflammatory inhibitors on the effects of TNBC cell viability and proliferation. The inhibitors tested were the direct STAT3 inhibitor, Stattic, and the ATP competitive JAK1/2 inhibitor, Baricitinib. Stattic inhibited tumor cell viability and proliferation, whereas Baricitinib did not. These results suggest that STAT3 plays a major role in triple negative breast cancer, but it may not be mediated through the canonical pathway.

Chapter 1

INTRODUCTION

1.1 Breast Cancer

Breast cancer is the most common type of cancer among women, and is the 5th most common cause of death among cancers.[1] Breast cancer is a heterogeneous disease that can be classified into a number of different groups based on the presence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), which can be used as indicators of prognosis.[2, 3] Moreover, these receptors are useful targets for therapeutic breast cancer treatment. Treatments for breast cancers with these markers include tamoxifen and aromatase inhibitors, which interfere with the estrogen pathway, and Trastuzumab, which blocks HER2.[2]

Triple negative breast cancers (TNBC) are characterized by a lack of estrogen receptors, progesterone receptors, and HER2 and make up approximately 15-20% of breast cancers.[4, 5] Due to the absence of these receptors, hormonal therapy is not a treatment option for this type of cancer, and there is no standard treatment aside from chemotherapy.[6] Moreover, TNBC have a high rate of recurrence and death; these attributes make it a prime target for research to identify new treatments that could be tailored specifically to TNBC.[7]

In an effort to identify potential targets for treatment of TNBC, bioinformatics analysis of TNBC gene expression profiles obtained from the Trans Cancer Genome Atlas (TCGA) database was performed by the Sims-Mourtada lab. Analysis revealed overexpression of inflammatory cytokines, which can activate the NF-κB-STAT3

pathways. In particular, inflammatory cytokines IL-1 IL-8, and IL-6 [8-11] were significantly increased in TNBC compared to estrogen receptor-positive breast cancer tumors or normal breast tissues. IL-1 and IL-8 are capable of activating the NF-κB pathway, while IL-6 can activate the IL-6-STAT3 pathway, as well as NF-κB.[8-11] STAT3 expression was also significantly increased. These results were confirmed by immunohistological analysis of normal and malignant breast tissue obtained through surgical resection of TNBC. In particular, overexpression of Il-6 was observed in TNBC compared to normal breast tissues. This is consistent with previous reports sighting overexpression of these cytokines in TNBC.[9, 12-15] Based on this information, these cytokines may play a role in triple negative breast cancer progression and therefore be targets for specific treatment within this tumor subtype.

1.2 IL-6-STAT3 Pathway

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of transcription factors that are commonly activated via tyrosine phosphorylation by Janus kinases, or JAKs, in response to cytokines, including IL-6.[16, 10] The STAT protein family is composed of STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b,and STAT6.[17] STAT3 in particular has been implicated in TNBC and is necessary for the transformation of tumor cells into cancer stem cells, and promotes tumor cell proliferation and survival via the upregulation of such proteins as, survivin, cyclin D1, and the transcription factors Oct-4, Snail, and Twist, which has resulted in it being a target for inhibition in the study of breast cancer.[18-22] The IL-6-STAT3 pathway (Figure 1.1) begins with the release of the cytokine IL-6 from tumor cells or nearby cells, which induces IL-6 production in the tumor cells.[23, 24] IL-6 binds to IL-6 receptor (IL-6R) or soluble IL-6R (IL-6sR), which induces homodimerization

of two membrane bound gp130 proteins, bringing the kinases associated with each IL-6R monomer, JAK1/2 together, and forming a functional receptor.[10] The associated JAK is then activated through autophosphorylation and phosphorylates STAT3 on tyrosine705, which is translocated to the nucleus where it can bind to DNA elements such as interferon-Gamma Activated Sequences(GAS) or Sis-Inducing Elements (SIE) and regulate gene expression.[25, 26] This allows STAT3 to regulate many genes involved in cancer such as, p53, IL-6, vimentin, TGF-β, and numerous other proteins.[25] Activity of STAT3is regulated by nuclear phosphatases by dephosphorylating and deactivating active STAT3 dimers.[27] IL-6 can also act as a part of a positive feedback loop in tumor cells.[11] Ordinarily, a negative feedback loop limits the increase in STAT3 activity to prevent a runaway increase in activity, but there can be mutations that inhibit such regulatory mechanisms in TNBC.[28] For example, suppressor of cytokine signaling 3 (SOCS3) expression is induced by active STAT3 and acts as a part of a negative feedback loop in IL-6-STAT3 pathway by inhibiting the tyrosine kinases, JAK1/2, possibly via induction of a conformation change in the kinase or recruitment of ubiquitin ligases to the receptor to mark it for degradation by the proteasome.[29-31] However lack of p53 and PTEN in triple negative breast cancers appears to result in increased degradation of SOCS3 via the proteasome resulting in inhibition of the negative feedback loop and induction of IL6/Stat3/NF-κB positive feedback loop.[28] This makes the IL6/Stat3/NF-κB positive feedback loop a possible target for treatment, as inhibiting it could potentially reduce STAT3 and NF-κB activity considerably, thereby inhibiting the expression of tumor supporting genes.

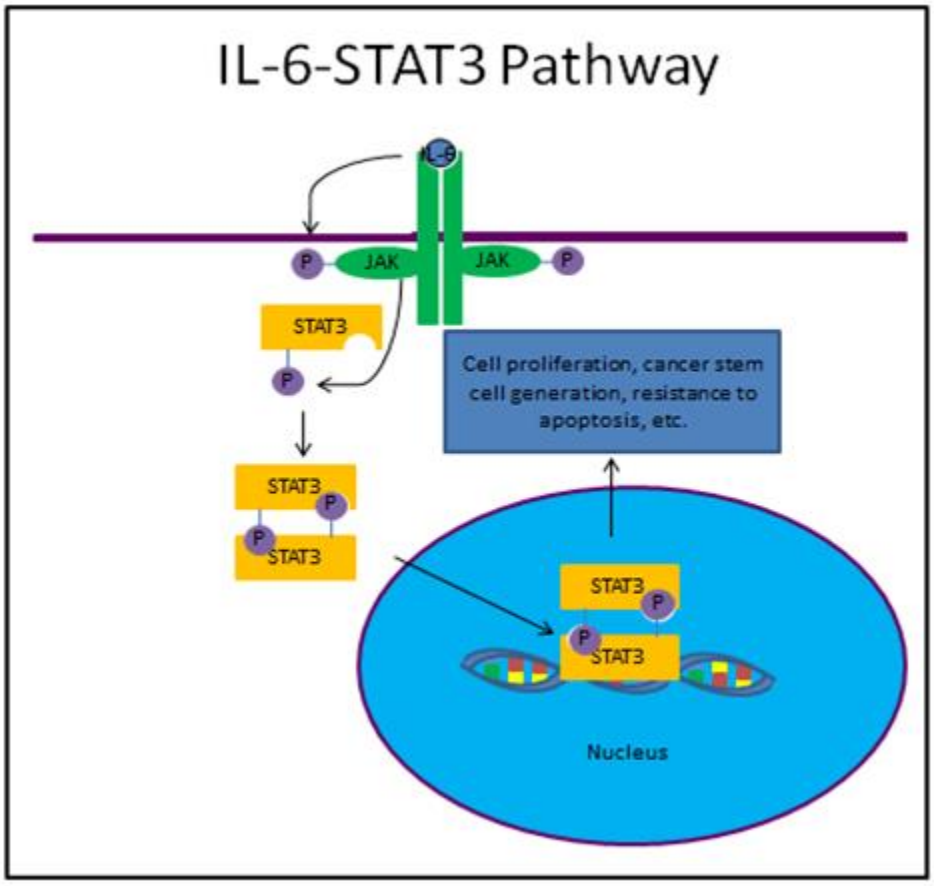

Figure 1.1: **IL-6 STAT3 Pathway**- Il-6 induces IL-6R dimerization, which allows the JAKs to autophosphorylate and activate STAT3, which dimerize and regulate genes in the nucleus.

1.3 STAT3 Effects in Breast Cancer

The IL-6-STAT3 pathway is capable of mediating tumor progression in breast cancer by triggering a variety of mechanisms. For example, the Il-6-STAT3 pathway is a capable of mediating breast cancer cell dedifferentiation into cancer stem cells via the induction of Oct-4 expression, which can induce dedifferentiation, much as inflammatory factors can cause dedifferentiation via epithelial mesenchymal transition (EMT) in normal wound healing.[21, 32-34] EMT is a process of dedifferentiation, which

results in a polarized epithelial cell to transition to a mesenchymal phenotype and is characterized by a decrease in adherens junctions, tight junctions, desmosomes, and E-cadherin, and an increase in matrix metalloproteinases and the mesenchymal cell markers, vimentin, Snail, and N-cadherin, resulting in an increase of motility and invasiveness.[35-38] Ordinarily, EMT occurs in response to epithelial tissue damage and nearby cells undergo EMT to migrate to the wound and replace the lost epithelial cells; however EMT is also linked to cancer, as an upregulation of EMT proteins in tumor cells is associated with dedifferentiation into cancer stem cells.[34] Cancer stem cells are of particular interest as they are more resistant to treatment and could therefore potentially survive chemotherapy treatment and develop into a more malignant tumor by acting as tumor-initiating cells.[39] Breast cancer stem cells can be identified by a high concentration of CD44, the absence or low concentration of CD24, the presence of ESA, or high expression of aldehyde dehydrogenase.[40-42] It is also important to note that cancer stem cells may be able to arise from ordinary tumor cells after they have been exposed to stresses such as wounds from surgery, radiation, or chemotherapy drugs which are often used in cancer treatments.[34, 43,44] Furthermore these cells are also resistant to such therapies due to the expression of ATP-binding cassette transporters, multidrug resistance transporters, and an increased ability to repair DNA.[45-49] Moreover, STAT3 is also capable of inducing chemoresistance in breast cancer cells, via promotion of autophagy, which can also reduce stresses such as starvation, DNA damage, and oxidative stress in tumor cells.[50-52] EMT of cells may also increase the metastatic potential of cells by inhibiting anchorage-dependent apoptosis and increasing tumor cell motility.[53,54] As a result of these properties it is

important that a strategy is developed to combat these cells as a part of cancer treatment.

Another protein promoted by STAT3 in breast cancer is survivin. [55, 19] Survivin is an anti-apoptotic protein that is not normally highly expressed in differentiated tissue, but is frequently expressed in cancer cells.[19, 56] As a result of this these cells have a greater resistance to apoptosis.

STAT3 is also capable of upregulating breast cancer cell motility via upregulation of vimentin and fascin expression.[57, 58] Vimentin belongs to the family intermediate filament proteins and is expressed in embryotic cells but not frequently expressed in differentiated cells.[57] STAT3 has been reported to upregulate vimentin by binding to the gene's antisilencer element and binding to ZBP-89, which inhibits the expression of vimentin. Fascin influences cell adhesion and motility by bundling actin together.[58] STAT3 and NF-κB have been shown to be necessary for IL-6 to induce expression of fascin which is required for breast cancer migration.

1.4 IL-6-STAT3 Pathway Inhibitors

1.4.1 Stattic

Stattic is a small molecule inhibitor that irreversibly inhibits STAT3 phosphorylation and dimerization by binding to the SH2 domain of STAT3 or near it regardless of its phosphorylation state, thus preventing the STAT3 from docking onto gp130 proteins and preventing phosphorylation by JAK1/2 and from binding together via their phosphotyrosines (Figure 1.2).[59] Stattic's inhibition of STAT3 dimerization may also explain its inhibition of STAT3 nuclear translocation, as dimerization appears to promote accessibility to the nuclear localization signal (NLS).[60] Stattic

accomplishes this inhibition via the covalent bonding of up to nine molecules of Stattic to residues in STAT3, 4-5 of which appear to be cysteines.[61] Stattic was shown to inhibit STAT3 by over 80% at 37°C in vitro, as well as STAT1 and STAT5b by approximately 40% at 30°C.[59] Stattic has an IC_{50} value of 5.1 μM as determined in vitro using protein extract with 370 nM of STAT3.[59] Stattic is selectively toxic to tumor cells that have constitutively activated STAT3 including breast tumor cells, and has also been shown to be capable of abolishing the presence of cancer stem cells in $HER2^+$ breast cancer and inhibiting cell viability in breast cancer.[59, 62, 63]

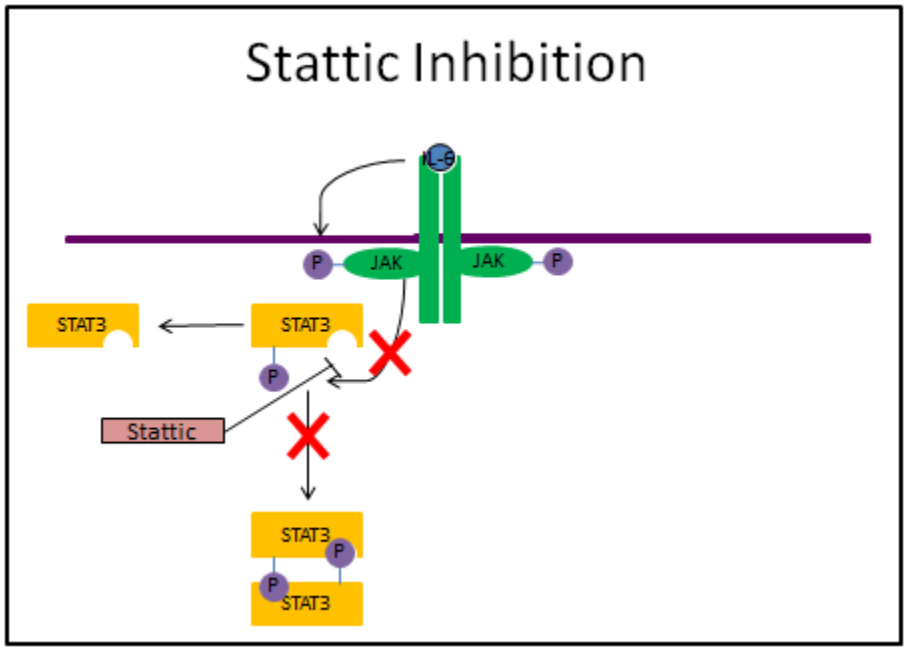

Figure 1.2: **Stattic Inhibition Mechanism**- Stattic irreversibly inhibits STAT3 directly by binding to its SH2 domain, thereby inhibiting its phosphorylation and dimerization.[59]

1.4.2 Baricitinib

Baricitinib is an ATP competitive kinase inhibitor that inhibits STAT3 phosphorylation by inhibiting JAK1 and JAK2 activity (Figure 1.3).[64] As a result of this, it is capable of inhibiting the actions of several cytokines including GM-CSF, IFN-γ, IL-23, IL-12, and IL-6.[65] Baricitinib is currently undergoing phase 3 trials for FDA approval of rheumatoid arthritis treatment. It has IC_{50} values of 5.9 nM and 5.7 nM for JAK1 and JAK2 respectively, as determined by homogenous time-resolved fluorescence assays at ATP concentrations similar to those in cells.[64] Additionally, Baricitinib was also found to have an IC_{50} of 53 nM for Tyk2.

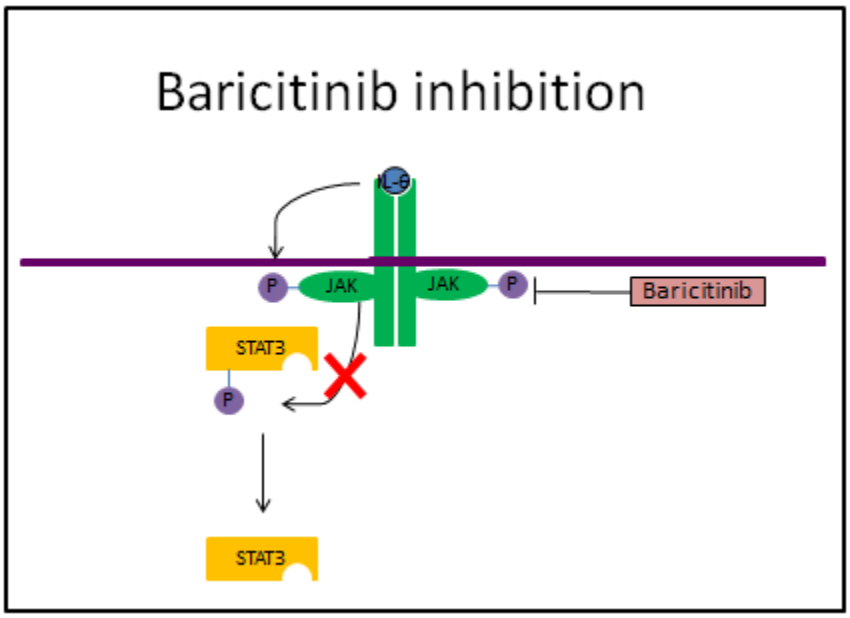

Figure 1.3:**Baricitinib Inhibition Mechanism**- Baricitinib inhibits STAT3 phosphorylation by inhibiting JAK1/2.[66]

1.5 Objective and Hypothesis

The objective of this study was to determine the effects of the inhibitors to the IL-6-STAT3 pathway, on the growth and proliferation of TNBC. Moreover, we also aimed to elucidate the specific mechanism whereby the tumor cells promote their survival. We hypothesized that Baricitinib would inhibit STAT3 activation via JAK1/2 inhibition thereby reducing concentration of phosphorylated STAT3 and reducing tumor cell viability and cell proliferation in TNBC.

The inhibitors chosen for this study were Stattic and Baricitinib. Baricitinib was chosen to study the effects of STAT3 on tumor cell viability and proliferation in TNBC via inhibition of JAK1/2 phosphorylation and activation of STAT3. Stattic directly binds to STAT3's SH2 domain, inhibiting its phosphorylation and activation. Stattic's and Baricitinib's results were compared to determine the differences in effects of inhibiting different points of the IL-6-STAT3 pathway.

To accomplish our goals, we analyzed the inhibitors' effectiveness at inhibiting STAT3 activation by quantifying the relative concentrations of phosphorylated STAT3 and STAT3 via Western blotting, as well as quantifying the inhibitors' effect on triple negative breast cancer cell viability and cell proliferation via MTT assays and clonogenic assays respectively. Should Baricitinib be successful, it may be able to one day help cancer patients stay in remission by inhibiting the growth and survival of the tumor cells.

Chapter 2

METHODS

2.1 Cell Lines and Inhibitors

The TNBC HCC1143, HCC1937, and MDA-MB-231 human breast cancer cells were obtained from ATCC (Manassas, VA) and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg /ml Fungizone) (Life Technologies). The TNBC SUM159 cells were obtained from Asterand (Detroit, MI) and maintained in Hams F12 supplemented with 10% FBS, 1% antibiotic/antimycotic, 0.1 mg/ml hydrocortisone (Life Technologies), and insulin (Life Technologies).The triple negative non-invasive breast epithelial cell MCF12A line was obtained from ATCC and was maintained in DMEM/F12 base with 10 μg epidermal growth factor (EGF) (Thermo Fisher Scientific), cholera toxin (0.1 mg/mL) (Calbiochem), insulin (Thermo Fisher Scientific), human recombinant, zinc (4 mg/mL) (Thermo Fisher Scientific), hydrocortisone (1mg/mL) (Thermo Fisher Scientific), and 25% horse serum (Thermo Fisher Scientific). The triple negative non-invasive epithelial breast cell 184B5 line was obtained from ATCC and maintained in media prepared from the Clonetics™ MEGM™ BulletKit™ (CC-3150) (Lonza) without the GA-1000 and with an addition of cholera toxin (1ng/ml) (Calbiochem). All cells were grown at 37^{o}C in an atmosphere of 5% CO_2. Stattic and Baricitinib were obtained from Tocris Bioscience and Cayman Chemical respectively and reconstituted in DMSO.

2.2 Protein Extraction and Quantification

Cell extracts were harvested using 100 μL of RIPA (Thermo Fisher Scientific) buffer with protease inhibitor (Calbiochem) and phosphatase inhibitor (Thermo Fisher Scientific) after being washed with 1x PBS. The extracts were then scraped and transported into microfuge tubes and chilled on ice for 30 minutes. The extracts were centrifuged for 5 minutes at 5,000 RPM and the DNA was removed and discarded. Protein concentrations were quantified using BCA assays. Cell extracts were diluted in RIPA buffer in a 1:10 dilution, and 10 μL of each sample were placed in two wells in a microplate. A standard curve was generated using a range of bovine serum albumin (BSA) concentrations. 200 μL of working reagent from the BCA protein assay kit (Thermo Fisher Scientific) were added to each well and the plate was covered in tinfoil and briefly mixed. The plate was incubated at 37°C for 30 minutes and the absorbances were read at 550 nm using a microplate reader (Tecan).

2.3 Western Blot

The breast cancer cell lines were screened for phosphorylated STAT3 proteins to ensure STAT3 activation. Western blots were used to confirm that phosphorylated STAT3 proteins were indeed present. The 10% separating gels were created using 5 mL of bis-acrylamide, 5 mL of 1.5 M tris, 200 μL of APS, 200 μL of SDS, 8 μL of temed, and 9.6 mL of deionized water. The 4% stacking gels were created using 1.25 mL of bis-acrylamide, 1.25 mL of 1.0 M tris, 100 μL of APS, 100 μL of SDS, 10 μL of temed, and 7.3 mL of deionized water. The samples were prepared by mixing them with Laemmli buffer in a 1:1 ratio and boiling them at 95°C for 10 minutes. Gels ran at 80 volts for 2 hours in 1x tris-glycine-SDS running buffer. The proteins were then transferred at 100 volts for 1 hour to nitrocellulose membranes. After the transfer the,

nitrocellulose membrane was put in Ponceau S and placed on a shaker for 5 minutes to confirm the protein transfer. Membranes were destained with water. The membrane was then blocked in 5% nonfat dry milk in tribuffered saline and Tween 20 (TBS-T) at 4°C overnight. The blocking solution was removed and the primary antibody solutions were added and incubated at 4°C overnight. The primary antibodies utilized were rabbit anti-STAT3 (ab76315, Cell Signaling Technology, Danvers, Massachusetts), rabbit anti-phosphorylated STAT3 (ab76315, Abcam, Cambridge, Massachusetts), or mouse anti-beta actin (sc-81178, Santa Cruz Biotechnology, Dallas, Texas). The anti-phosphorylated STAT3 and anti-beta actin antibody solutions were made by creating a 1:10,000 dilution using 3% BSA in TBS-T, and the anti-STAT3 antibody solutions were made by creating a 1:2,000 dilution using 3% BSA in TBS-T. Beta actin was used as a loading control. The membrane was then washed with TBS-T and placed on the shaker for 10 minutes 3 times. The secondary antibody was then added and the membrane was placed on the shaker for 1 hour at room temperature. The secondary antibodies utilized were donkey anti-mouse (715-035-150, Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) and donkey anti-rabbit (711-035-152, Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania). The anti-mouse antibody and anti-rabbit antibody solutions were made by creating a 1:10,000 dilution using 3% BSA in TBS-T. The membrane was washed with TBS-T and placed on the shaker for 10 minutes per wash 3 times. The proteins were then detected using an ECL kit (Thermo Fisher Scientific). The relative protein concentrations were quantified using densitometric analysis.

2.4 Cell Viability Assays

Cells were also treated with yellow tetrazolium MTT (Biotum), which was converted by the cells' mitochondria into an ionic purple dye, to quantify cell metabolic activity. Cells were first plated and incubated for 24 hours. 10 μL of MTT were added to the cells, which incubated at 37°C for 3 hours. 100 μL of DMSO was added to the cells to lyse them, and absorbances of the samples were measured using a Tecan microplate reader at 570 nm. The averages of the results for each concentration in a cell line were compared to the average of the untreated samples for that cell line and statistical significance was calculated via one way ANOVA and Dunnett's multiple comparisons test using GraphPad Prism VI (GraphPad Software, Inc.). Statistical significance was considered to be when $p \leq 0.05$.

2.5 Clonogenic Assays

SUM159 cells were seeded in six-well plates with a density of 100 cells/well for the untreated and Baricitinib-treated wells and 400 cells/well for the Stattic-treated wells. Cells were treated with inhibitors and were incubated for seven days. After the incubation period, the cells were fixed with 10% formalin for 15 minutes, washed with 1x PBS, and stained with 0.5% crystal violet for five minutes. The colonies were then counted and the colony forming efficiencies were calculated by dividing the number of colonies by the number of cells plated.

Chapter 3

RESULTS

3.1 Basal STAT3 Activation in Breast Cancer Cell Lines

The basal levels of phosphorylated STAT3 and STAT3 were measured via Western blots relative to β-actin concentrations to determine which cell line had the greatest level of phosphorylated STAT3, which would be used in the experiments testing the effects of the inhibitors on STAT3 phosphorylation (Figure 3.1). The HCC1143, HCC1937, MDA-MB-231 and SUM159 lines are TNBC lines, whereas 184B5 and MCF12A are non-invasive breast epithelial cell lines. All cell lines screened were found to have phosphorylated STAT3. The SUM159 cells showed the greatest levels of phosphorylated STAT3. This is supported by the IL-6 secretion in HCC1143, HCC1937, MDA-MB-231, and SUM159 cells reported by Hartman et al (2013).

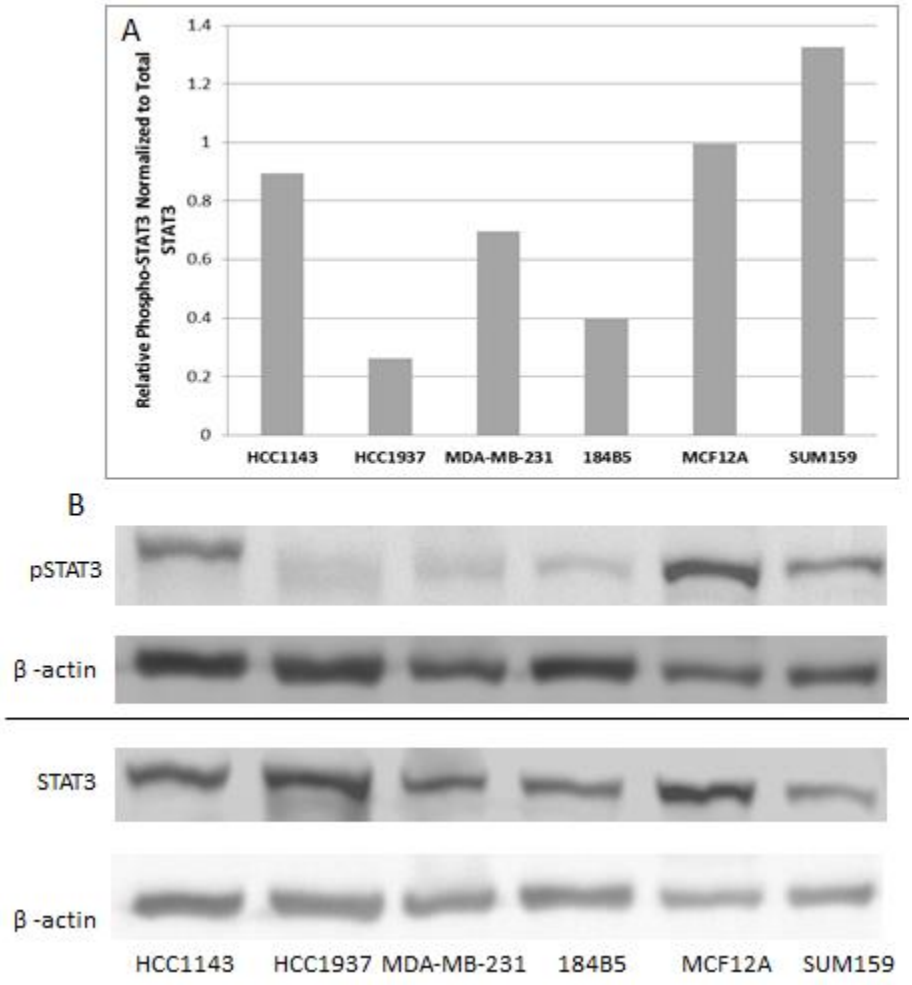

Figure 3.1: **Basal STAT3 Phosphorylation in Breast Cancer Cell Lines**- Shown here are the ratios of phosphorylated STAT3 to STA3 in the cell lines screened (A) the Western blot used for the initial screening of the cell lines (B). The HCC1143, HCC1937, MDA-MB-231, 184B5, MCF12A, and SUM159 lines were used.

3.2 Effects of Stattic and Baricitinib on STAT3 Activation

Western blots were also used to quantify relative STAT3 and phosphorylated STAT3 with varying concentrations for each inhibitor. The Western blots for Stattic show that Stattic inhibits STAT3 phosphorylation in the SUM159 cell line in a dose-dependent manner, as the ratio of phosphorylated STAT3 to STAT3 decreases as the

concentration of Stattic increases (Figure 3.2). The Western blots for Baricitinib appear to show that it reduces STAT3 phosphorylation in the SUM159 cell line in a dose-dependent manner, as the ratio of phosphorylated STAT3 to STAT3 decreases as the concentration of Baricitinib increases (Figure 3.3). Baricitinib also showed a greater degree of STAT3 phosphorylation inhibition than Stattic at lower concentrations, which may be due to its inhibiting STAT3 phosphorylation the more directly than Stattic, as Baricitinib inhibits JAK1/2, which can be directly responsible for STAT3 phosphorylation on tyrosine 705. The Western blots were also used to help characterize the effects of Stattic (Figure 3.4) and Baricitinib (Figure 3.5) on phosphorylation over time. The time courses show that Stattic and Baricitinib have begun to inhibit STAT3 phosphorylation by 4 hours after treatment, appear to reach maximum levels of inhibition at about 24 hours after treatment, and appear to be losing effect by 48 hours after treatment.

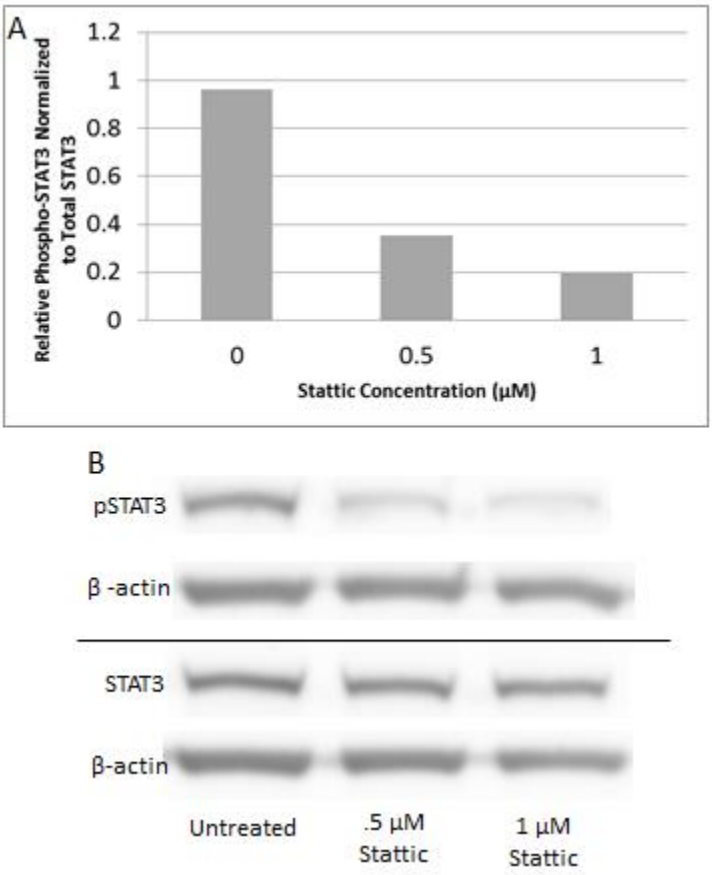

Figure 3.2: **Effects of Stattic on STAT3 Phosphorylation**- Shown here are the ratios of phosphorylated STAT3divided by β-actin to STAT3divided by β-actin in SUM159 cells (A) and the Western blot used to calculate the ratios (B) when treated with Stattic. STAT3 tyrosine 705 phosphorylation was reduced when treated with Stattic.

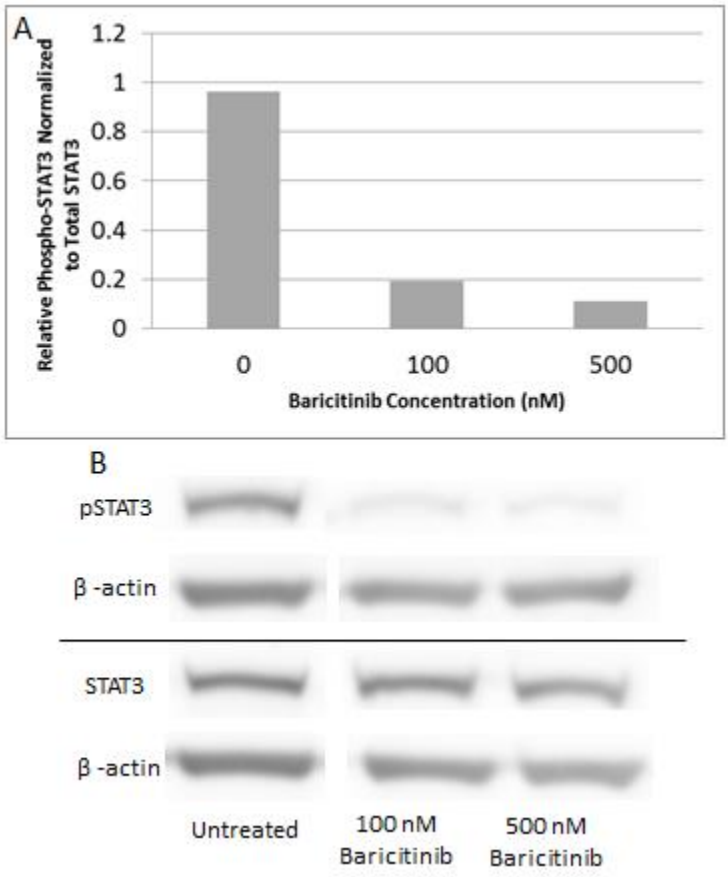

Figure 3.3: **Effects of Baricitinib on STAT3 Phosphorylation**- Shown here are the ratios of phosphorylated STAT3divided by β-actin to STAT3divided by β-actin in SUM159 cells (A) and the Western blot used to calculate the ratios (B) when treated with Baricitinib. STAT3 tyrosine 705 phosphorylation was reduced when treated with Baricitinib.

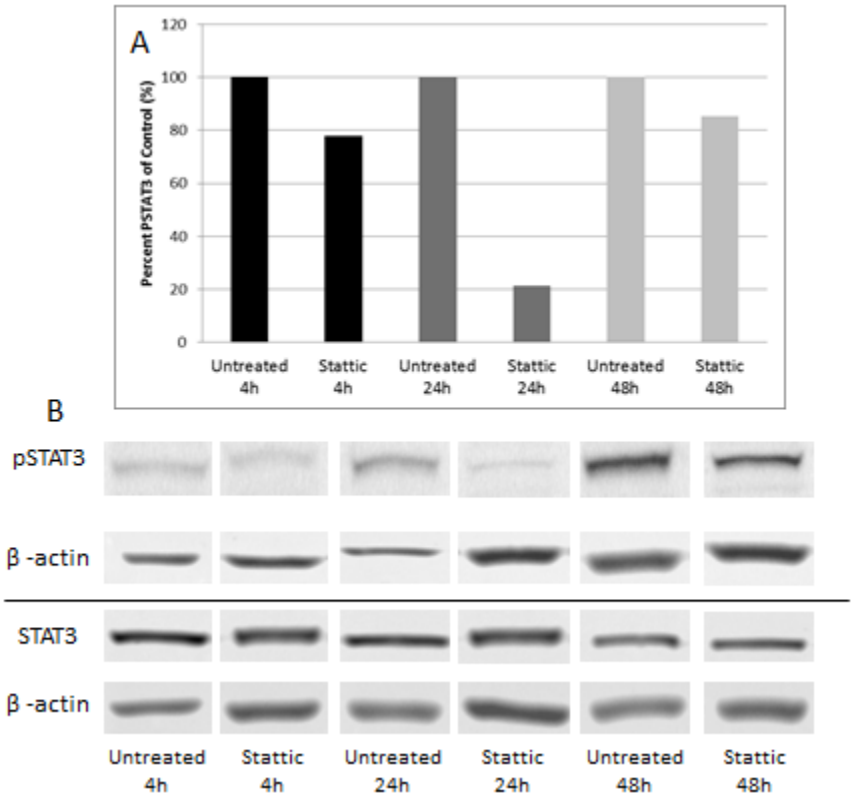

Figure 3.4: **Stattic Time Course**- Stattic (concentration) has begun to take effect on the SUM159 cells by 4 hours, reaches its highest recorded level of effectiveness by 24 hours, and has begun to lose effect by 48 hours.

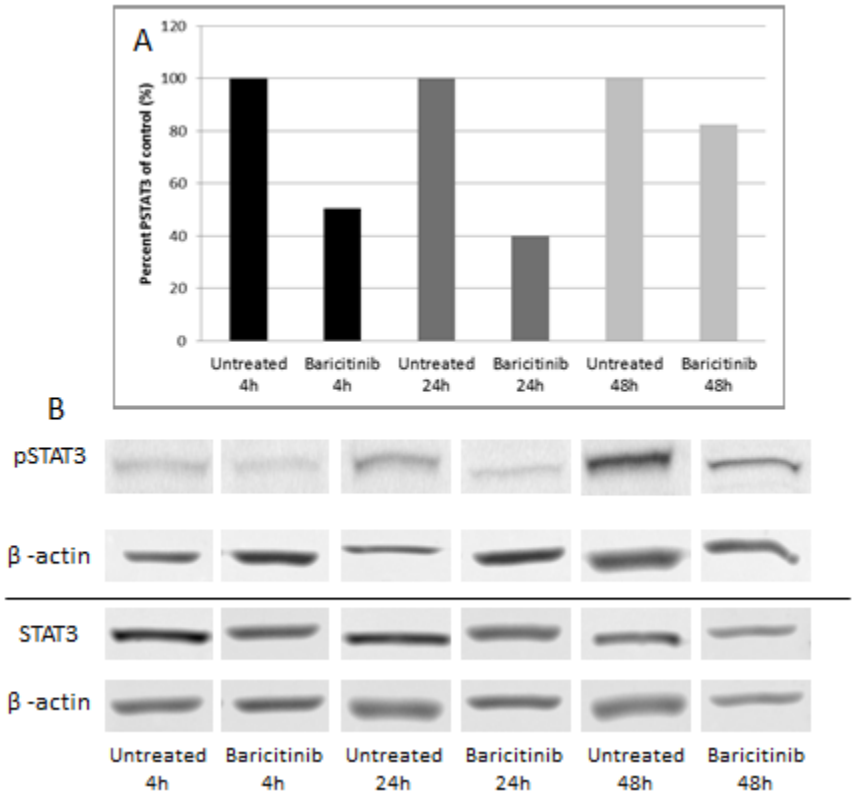

Figure 3.5: **Baricitinib Time Course**- Baricitinib (concentration) has begun to take effect on the SUM159 cells by 4 hours, reaches its highest recorded level of effectiveness by 24 hours, and has begun to lose effect by 48 hours.

3.3 Effect of STAT3 Inhibition on Cell Viability in TNBC Lines

MTT assays were also used to quantify the inhibitors' impact on cell viability in the HCC1143, HCC1937, and SUM159 cell lines, as a decrease in cell viability would indicate a decrease in growth, or possibly cell death. Cells were stained with tetrazolium yellow MTT, which only acts as a dye after it has been modified by the mitochondrion, to quantify the inhibitors' effects on cell viability via inhibition of mitochondrial activity. Cell viability decreased as the Stattic concentration increased starting at a concentration of 1 μM. Stattic significantly ($p<.0001$) reduced cell viability in all cell lines (Figure 3.5). In contrast, Baricitinib did not exhibit a

significant reduction in cell viability when it was used to treat the cell lines (Figure 3.6).

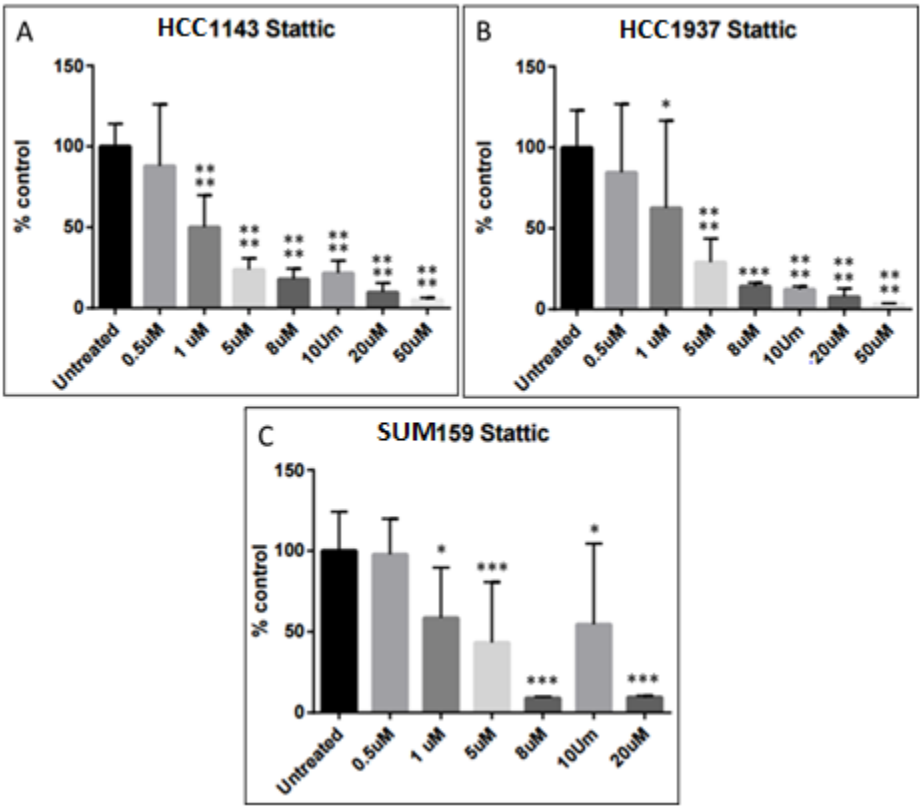

Figure 3.5: **Stattic Effect on Tumor Cell Viability**- Cellular viability of HCC1143 (A), HCC1937 (B), and SUM159 (C) cell lines when treated with increasing concentrations of Stattic as compared to control. Cell viability was reduced when treated with Stattic.

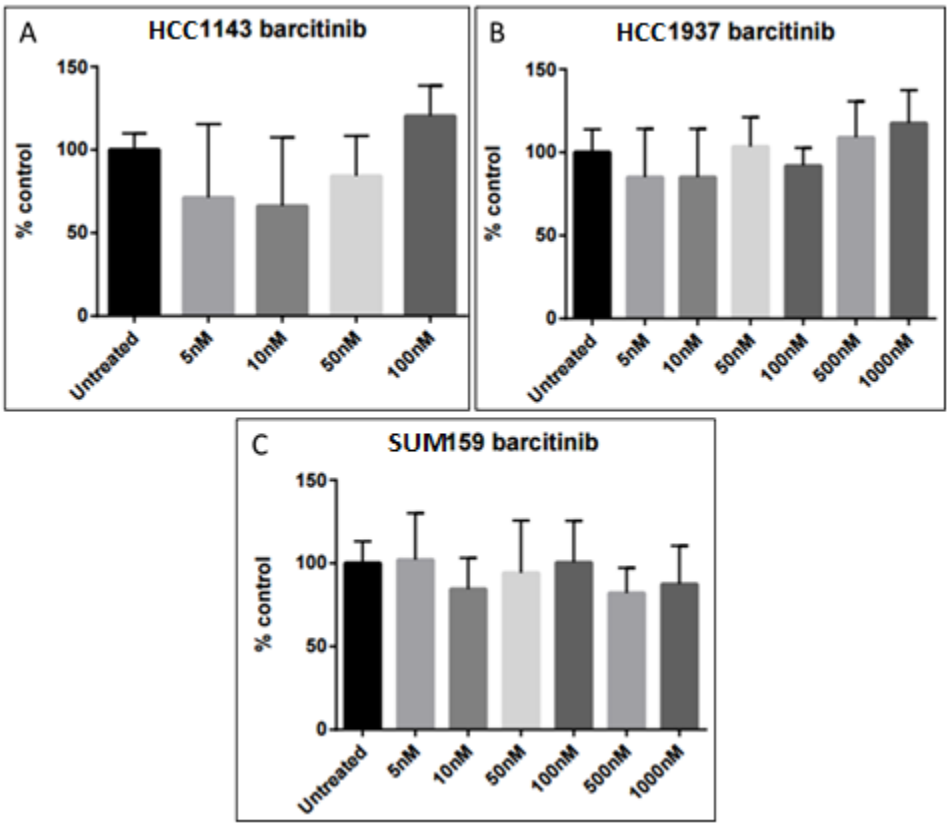

Figure 3.6: **Baricitinib Effect on Tumor Cell Viability**- Cellular viability of HCC1143 (A), HCC1937 (B), and SUM159 (C) cell lines when treated with increasing concentrations of Barcitinib as compared to control. Cell viability was not reduced when treated with Baricitinib.

3.4 Effects of Stattic and Baricitinib on Cell Proliferation

Clonogenic assays were used to quantify changes in cell proliferation when treated with the inhibitors. When treated with Stattic, the average colony forming efficiency of the SUM159 cells, showed a significant decrease to 4% when compared to untreated samples' average of 47%, which indicates a reduction in cell proliferation, possibly due to death (Figure 3.7, Figure 3.8). When treated with Baricitinib, the average colony forming efficiency did not change significantly when compared to

untreated wells, only changing from 57% to 61%, which indicates that Baricitinib did not affect cell proliferation (Figure 3.9, Figure 3.10).

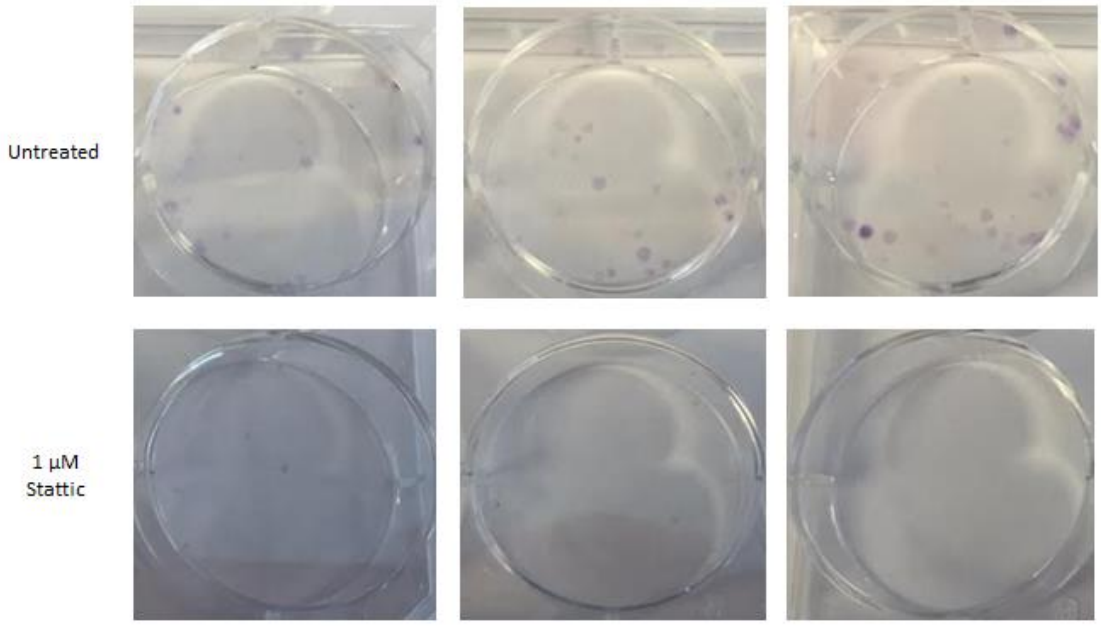

Figure 3.7: **Stattic Clonogenic Images**- The colonies formed when SUM159 cells were left untreated and treated with Stattic are shown here.

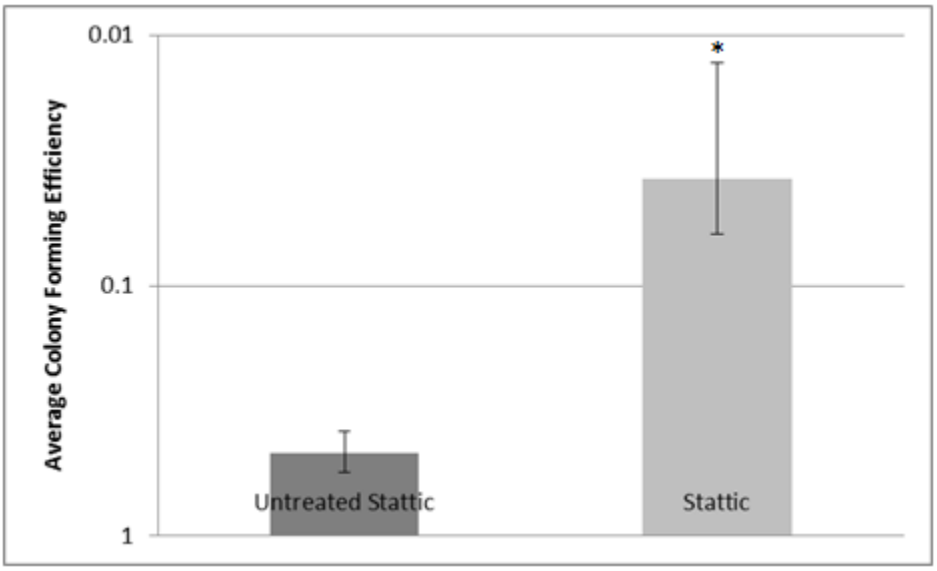

Figure 3.8: **Stattic Effect on Tumor Cell Proliferation**- The average colony forming efficiencies of the Stattic clonogenic experiments are in logarithmic scale. Stattic appears to significantly reduce the average colony forming efficiency of SUM159 cells when compared to untreated wells (P<.001). Error bars represent standard deviations.

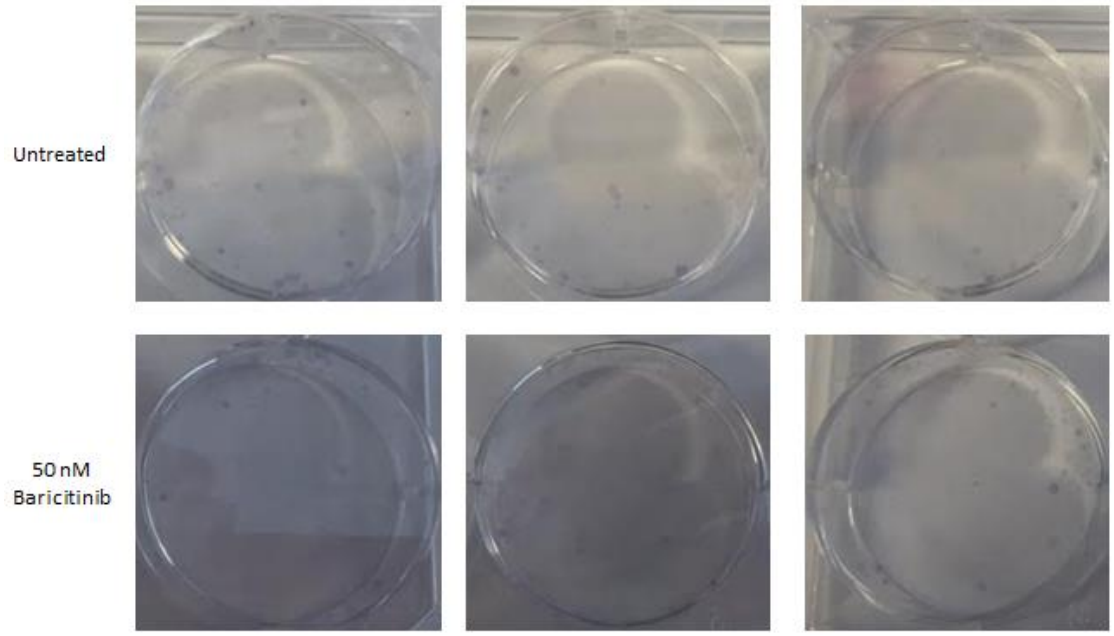

Figure 3.9: **Baricitinib Clonogenic Images**- The colonies formed when SUM159 cells were left untreated and treated with Baricitinib are shown here.

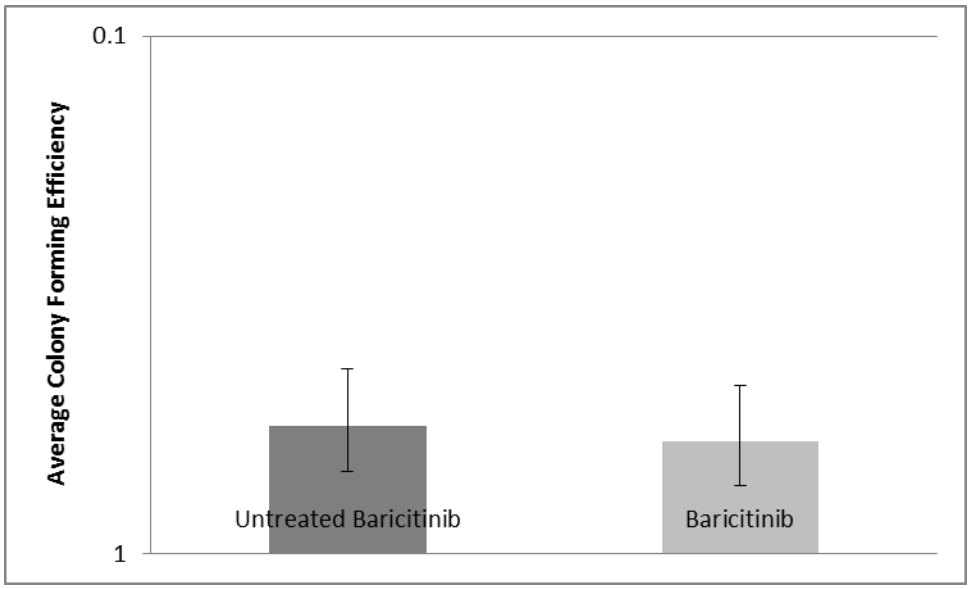

Figure 3.10: **Baricitinib Effect on Tumor Cell Proliferation**- The average colony forming efficiencies of the Baricitinib clonogenic experiments are in logarithmic scale. No significant difference in average colony forming efficiencies of SUM159 cells was observed between wells treated with Baricitinib and those left untreated. Error bars represent standard deviations.

Chapter 4

DISCUSSION

4.1 Interpretation of Results

Stattic and Baricitinib both inhibited STAT3 phosphorylation; however only Stattic appeared to affect tumor cell proliferation or viability. The decrease in colony forming efficiency in response to Stattic seems to indicate that active STAT3 is required for SUM159 proliferation. Baricitinib's lack of an effect on colony forming efficiency would seem to indicate that phosphorylation of STAT3 via JAK1/2 is not necessary for SUM159 proliferation in vitro. Moreover, according to the MTT assays, Baricitinib also did not inhibit tumor cell viability, which seems to indicate that JAK1/2 is also unnecessary for the tumor cells to remain viable in vitro, whereas tumor cells treated with Stattic did show cell viability inhibition. Indeed, these results are supported by Chang et al.'s (2013) in vitro inhibition of JAKs in mouse mammary carcinoma cells, as it did not affect tumor cell growth. This data may indicate that these cells are using a non-canonical STAT3 pathway to survive, as the canonical pathway phosphorylates STAT3 via JAK and inhibition of JAK1/2 via Baricitinib does not reduce the colony forming efficiency as one would expect if the canonical pathway played a role in tumor cell growth or survival, whereas the inhibition of colony formation via Stattic appears to confirm that STAT3 plays a role in tumor cell survival. This may indicate that STAT3 promotes tumor cell survival via a non-canonical pathway.

4.2 Non-Canonical STAT3 Pathways

Multiple non-canonical STAT3 pathways exist that could be involved in tumor progression in breast cancer. For example, STAT3 can be modified in multiple ways including non-canonical phosphorylation, monoubiquitination, and acetylation, which can cause STAT3 to promote the expression of various genes and could potentially influence tumor progression.[67- 69] There are also other kinases that can activate STAT3 in breast cancer which could potentially impact the results of inhibiting JAKs as done in these experiments. EGFR, which can activate STAT3 via tyrosine 705 phosphorylation, has been found to be in approximately 64% of TNBC lines by Siziopikou et al. (2006), which offers another possible alternative method of canonical STAT3 activation.[70] Serine 727 must also be phosphorylated in order to induce maximal gene expression, which may also play a role.[72] Moreover, a decrease in survivability via inhibition of the IL-6-STAT3 pathway may be due to the downregulation of anti-apoptotic proteins and growth factors that STAT3 promotes such as cyclin D1 and survivin.[20, 55] Furthermore, it is possible that Stattic causes a decrease in survivability due to sterically blocking modifications to STAT3, or its binding to other proteins, thereby inhibiting the promotion of anti-apoptotic proteins and growth factors. For example, STAT3 lysine 685 acetylation appears to promote methylation of tumor suppressor genes, as well as therapeutic targets, such as ERα via DNMT1, which may indicate a role in the development of triple negative breast cancers.[73] Importantly, STAT3 activation has also been shown to upregulate the production of STAT3 in cancer, which may help mediate further canonical STAT3 activation, as well as non-canonical STAT3 activity.[74]

4.3 STAT3-NF-κB Crosstalk

One non-canonical STAT3 pathway is in the NF-κB pathway which can result in unphosphorylated STAT3 binding to p65 and upregulating genes with NF-κB activation sites and downregulating genes with overlapping NF-κB and STAT3 activation sites.[8] Furthermore, like the IL-6-STAT3 pathway, the NF-κB pathway may be capable of being activated as part of a wound response to surgery, radiotherapy, or chemotherapy in vivo via the production of IL-1β, thereby allowing the NF-κB pathway to potentially remain as a viable target, even if the IL-1 is not necessarily constitutively secreted in vitro.[34]

IL-1α or IL-1βcan induce the release of NF-κB which allows it to translocate to the nucleus where it can regulate the expression of genes.[75] Alternatively, the p65 subunit of NF-κB may bind to unphosphorylated STAT3 which forms a complex capable of upregulating NF-κB-activated genes and downregulating genes with overlapping STAT3-activated and NF-κB-activated sites by competitively binding to STAT3-binding DNA elements and binding STAT3 that could otherwise be activated (Figure 4.1), as well as regulating genes with novel binding elements, such as serum amyloid A.[8, 76] Activation of NIK in the NF-κB pathway appears to be capable of promoting canonical STAT3 phosphorylation in the basal-like subtype of triple negative breast cancers.[77] Likewise, IKKβ appears to promote STAT3 phosphorylation on tyrosine 705 by recruiting proteins via lysine 63-linked ubiquitination scaffolding.[78] Moreover, cancer mutations in IKKβ's lysine 171 residue can promote constitutive kinase activity, increased polyubiquitination, and increased activation of STAT3.[78, 79]

4.4 Effects of NF-κB Pathway Activation

NF-κB is also capable promoting metastasis in triple negative breast cancer in several ways. NF-κB can induce EMT via the promotion of TrkB synthesis, which transduces survival signals to the cell and initiates EMT by triggering the transcription of factors Twist and Snail, as well as inhibiting anchorage-dependent apoptosis.[53, 80] Moreover, the tumor-suppressing kinase, RKIP which typically inhibits IKK, as a part of a negative feedback loop in the NF-κB pathway appears to be inhibited by NF-κB-induced Snail in SUM159 cells.[81, 82] Upregulation of RKIP in breast cancer tends to result in decreased migration and metastasis, as well as increased sensitivity and apoptosis in response to chemotherapy. NF-κB activation also appears to be able to promote invasive growth in TNBC via the induction of TMOD1, which promotes the secretion of MMP13 via β-catenin activation.[83] Secretion of MMP13 promotes tumor cell migration and proliferation and extracellular matrix degradation. Activation of the NF-κB pathway via IL-1 may also be capable of inducing secretion of both IL-6 and IL-8, thereby contributing to the activation of both the NF-κB pathway and the IL-6-STAT3 pathway and the metastatic processes that result from them.[84]

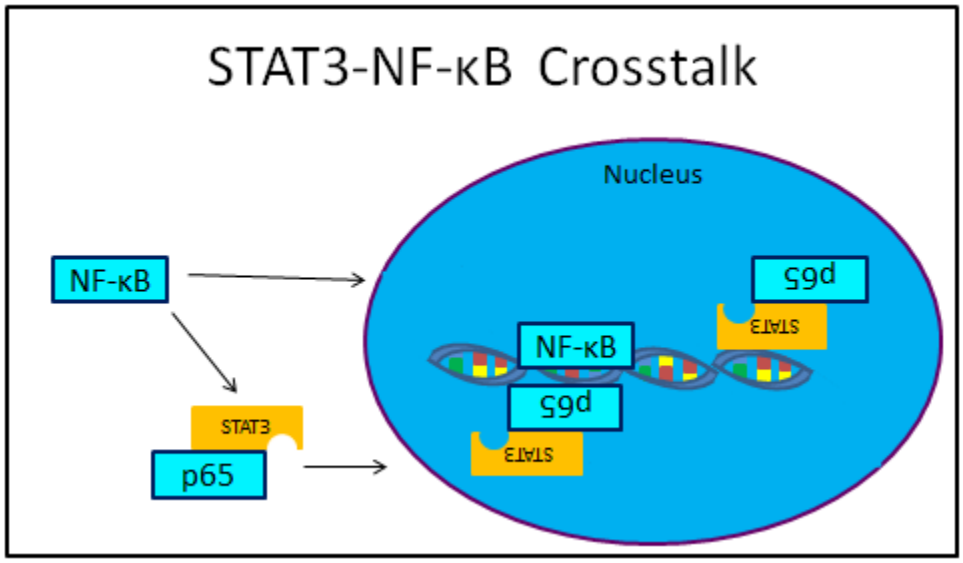

Figure 4.1: **STAT3-NF-κB Crosstalk Mechanism**- After being released from IκB, NF-κB subunit p65 can bind to unphosphorylated STAT3, which allows them to regulate gene expression.[8]

Other research seems to indicate that interactions between the STAT3 and NF-κB pathways can play a role in tumor progression in other cancers.[85] For example, unphosphorylated STAT3 may also be able to induce persistent activation of NF-κB by recruiting the histone acetyltransferase complex, p300/CBP, which can acetylate p65, thereby promoting NF-κB retention in the nucleus and inhibiting IκBα binding to NF-κB.[86, 87] Furthermore, when mice were injected with triple negative breast cancer cells in Hartman et al.'s (2013) study, both Il6 and IL-8 were necessary for the development of tumors. Based on this information and the failure of Baricitinib to decrease cell viability and growth, it is plausible to hypothesize that the crosstalk between the STAT3 and NF-κB pathways play a major role in triple negative breast cancer tumor cell growth and viability.

4.5 Conclusions

According to the results of the experiments, it would appear that STAT3 plays a role in tumor cell viability and growth, but its effects may be mediated by a non-canonical pathway, rather than the IL-6-STAT3 pathway. Since the TCGA reported high levels of IL-1 and IL-8, which can activate the NF-κB pathway, and Baricitinib failed to decrease TNBC cell viability, it is plausible that interactions between the STAT3 and NF-κB pathways play a major role in TNBC tumor cell growth and viability; however more tests are need to determine the role of NF-κB and to confirm the promotion of tumor cell growth and viability via crosstalk between the pathways.[8, 9]

4.6 Future Research

Future research could include further testing to determine if the crosstalk between STAT3 and NF-κB promotes TNBC tumor cell survival, possibly by inducing a STAT3 knockdown in TNBC cells or by treating them with the NF-κB inhibitor, IMD-0354, which has been found to reduce viability and increase apoptosis in non-stem breast cancer cells.[88] Despite the possibility of alternative signaling promoting tumor cell survival, the IL-6-STAT3 pathway may still be a worthy target for inhibition in breast cancer, as it could possibly promote tumor cell dedifferentiation into cancer stem cells, tumor cell invasiveness, and chemoresistance, for example.[21, 58, 50] As a result of this, more tests should be conducted on the effects of Baricitinib on other attributes of TNBC including cell motility assays and flow cytometry to analyze cancer stem cell markers, as inhibiting the IL-6-STAT3 pathway via JAK1/2 inhibition may inhibit tumor progression. Moreover, it is possible for the IL-6-STAT3 pathway to become active as a result of the wound healing and

inflammation after surgery, radiotherapy, or chemotherapy.[34, 89, 43, 44] Therefore, Baricitinib should be tested in vivo to determine if it is capable of inhibiting therapy-induced tumor progression. The effects of Baricitinib should also be studied further in vivo, as studies have shown that JAK inhibition in vivo can impact breast cancer tumor growth.[12]

REFERENCES

1. Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., & Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International Journal of Cancer*, 127(12), 2893-2917.

2. Wu, V. S., Kanaya, N., Lo, C., Mortimer, J., & Chen, S. (2015). From bench to bedside: What do we know about hormone receptor-positive and human epidermal growth factor receptor 2-positive breast cancer? *The Journal of Steroid Biochemistry and Molecular Biology*, 153, 45-53.

3. Karlsson, E., Appelgren, J., Solterbeck, A., Bergenheim, M., Alvariza, V., & Bergh, J. (2014). Breast cancer during follow-up and progression – A population based cohort on new cancers and changed biology. *European Journal of Cancer*, 50(17), 2916-2924.

4. 4. Soady, K. J., Kendrick, H., Gao, Q., Tutt, A., Zvelebil, M., Ordonez, L. D., … Smalley, M. J. (2015). Mouse mammary stem cells express prognostic markers for triple-negative breast cancer. *Breast Cancer Research : BCR*,17(1), 31. http://doi.org/10.1186/s13058-015-0539-6.

5. Barbie, T. U., Alexe, G., Aref, A. R., Li, S., Zhu, Z., Zhang, X., … Gillanders, W. E. (2014). Targeting an IKBKE cytokine network impairs triple-negative breast cancer growth. *The Journal of Clinical Investigation*, 124(12), 5411–5423. http://doi.org/10.1172/JCI75661.

6. Bramati, A., Girelli, S., Torri, V., Farina, G., Galfrascoli, E., Piva, S., … Verde, N. M. (2014). Efficacy of biological agents in metastatic triple-negative breast cancer. *Cancer Treatment Reviews*, 40(5), 605-613.

7. Dent, R., Trudeau, M., Pritchard, K. I., Hanna, W. M., Kahn, H. K., Sawka, C. A., … Narod, S. A. (2007). Triple-Negative Breast Cancer: Clinical Features and Patterns of Recurrence. *Clinical Cancer Research*, 13(15), 4429-4434.

8. Bode, J., Albrecht, U., Häussinger, D., Heinrich, P., & Schaper, F. (2012). Hepatic acute phase proteins – Regulation by IL-6- and IL-1-type cytokines involving STAT3 and its crosstalk with NF-κB-dependent signaling. *European Journal of Cell Biology*, 91(6-7), 496-505. doi:10.1016/j.ejcb.2011.09.008.

9. Hartman, Z. C., Poage, G. M., den Hollander, P., Tsimelzon, A., Hill, J., Panupinthu, N., … Brown, P. H. (2013). Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the pro-inflammatory cytokines IL-6 and IL-8. *Cancer Research*, 73(11), 10.1158/0008–5472.CAN–12–4524–T. http://doi.org/10.1158/0008-5472.CAN-12-4524-T.

10. Kamińska, K., Czarnecka, A., Escudier, B., Lian, F., & Szczylik, C. (2015). Interleukin-6 as an emerging regulator of renal cell cancer. Urologic *Oncology: Seminars and Original Investigations*, 33(11), 476-485. doi:10.1016/j.urolonc.2015.07.010.

11. Iliopoulos, D., Hirsch, H. A., Wang, G., & Struhl, K. (2011). Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. *Proceedings of the National Academy of Sciences of the United States of America*, 108(4), 1397–1402. http://doi.org/10.1073/pnas.1018898108.

12. Chang, Q., Bournazou, E., Sansone, P., Berishaj, M., Gao, S. P., Daly, L., … Bromberg, J. (2013). The IL-6/JAK/Stat3 Feed-Forward Loop Drives Tumorigenesis and Metastasis. *Neoplasia* (New York, N.Y.), 15(7), 848–862.

13. Kim, S., Lee, J., Jeon, M., Lee, J. E., & Nam, S. J. (2015). MEK-dependent IL-8 induction regulates the invasiveness of triple-negative breast cancer cells. *Tumor Biology*, 37(4), 4991-4999. doi:10.1007/s13277-015-4345-7.

14. Maris, P., Blomme, A., Palacios, A. P., Costanza, B., Bellahcène, A., Bianchi, E., … Turtoi, A. (2015). Asporin Is a Fibroblast-Derived TGF-β1 Inhibitor and a Tumor Suppressor Associated with Good Prognosis in Breast Cancer. *PLoS Medicine*, 12(9), e1001871. http://doi.org/10.1371/journal.pmed.1001871.

15. Vyas, D., Deshpande, K., Chaturvedi, L., Gieric, L., & Ching, K. (2016). Rapid Extensive Recurrence of Triple Negative Breast Cancer: Are Both Therapy and Cancer Biology the Culprit? *Journal of Clinical Medicine Research*, 8(2), 162-167. doi:10.14740/jocmr2365w.

16. Darnell, J., Kerr, I., & Stark, G. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science*, 264(5164), 1415-1421. doi:10.1126/science.8197455.

17. Furqan, M., Akinleye, A., Mukhi, N., Mittal, V., Chen, Y., & Liu, D. (2013). STAT inhibitors for cancer therapy. *Journal of Hematology & Oncology*, 6, 90. http://doi.org/10.1186/1756-8722-6-90.

18. Walker, S. R., Xiang, M., & Frank, D. A. (2014). Distinct roles of STAT3 and STAT5 in the pathogenesis and targeted therapy of breast cancer. *Molecular and Cellular Endocrinology*, 382(1), 10.1016/j.mce.2013.03.010. http://doi.org/10.1016/j.mce.2013.03.010.

19. Gritsko, T. (2006). Persistent Activation of Stat3 Signaling Induces Survivin Gene Expression and Confers Resistance to Apoptosis in Human Breast Cancer Cells. *Clinical Cancer Research*, 12(1), 11-19.

20. Ishii, Y., Pirkmaier, A., Alvarez, J. V., Frank, D. A., Keselman, I., Logothetis, D., … Germain, D. (2006). Cyclin D1 Overexpression and Response to Bortezomib Treatment in a Breast Cancer Model. *Journal of the National Cancer Institute*, 98(17), 1238-1247. doi:10.1093/jnci/djj334.

21. Kim, S.-Y., Kang, J. W., Song, X., Kim, B. K., Yoo, Y. D., Kwon, Y. T., & Lee, Y. J. (2013). Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. *Cellular Signalling*, 25(4), 961–969. http://doi.org/10.1016/j.cellsig.2013.01.007.

22. Sullivan, N. J., Sasser, A. K., Axel, A. E., Vesuna, F., Raman, V., Ramirez, N., … Hall, B. M. (2009). Interleukin-6 induces an epithelial–mesenchymal transition phenotype in human breast cancer cells. *Oncogene*, 28(33), 2940-2947. doi:10.1038/onc.2009.180.

23. Berishaj, M., Gao, S. P., Ahmed, S., Leslie, K., Al-Ahmadie, H., Gerald, W. L., … Bromberg, J. F. (2007). Stat3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/Janus kinase pathway in breast cancer. *Breast Cancer Research*, 9(3), R32. http://doi.org/10.1186/bcr1680.

24. Sansone, P., Storci, G., Tavolari, S., Guarnieri, T., Giovannini, C., Taffurelli, M., … Bonafè, M. (2007). IL-6 triggers malignant features in mammospheres from human ductal breast carcinoma and normal mammary gland. *The Journal of Clinical Investigation*, 117(12), 3988–4002. http://doi.org/10.1172/JCI32533.

25. Carpenter, R. L., & Lo, H.-W. (2014). STAT3 Target Genes Relevant to Human Cancers. *Cancers*, 6(2), 897–925. http://doi.org/10.3390/cancers6020897.

26. Smith, P. D., & Crompton, M. R. (1998). Expression of v-src in mammary epithelial cells induces transcription via STAT3. *Biochemical Journal*, 331(Pt 2), 381–385.

27. Herrmann, A., Vogt, M., Monnigmann, M., Clahsen, T., Sommer, U., Haan, S., … Muller-Newen, G. (2007). Nucleocytoplasmic shuttling of persistently activated STAT3. *Journal of Cell Science*, 120(18), 3249-3261.

28. Kim, G., Ouzounova, M., Quraishi, A. A., Davis, A., Tawakkol, N., Clouthier, S. G., … Korkaya, H. (2014). SOCS3-mediated regulation of inflammatory cytokines in PTEN and p53 inactivated triple negative breast cancer model. *Oncogene*, 34(6), 671-680.

29. Naka, T., Narazaki, M., Hirata, M., Masumoto, T., Minamoto, S., Aono, A., … Kishimoto, T. (1997). Structure and function of a new STAT-induced STAT inhibitor. *Nature*, 387(6636), 924-929.

30. Babon, J. J., Kershaw, N. J., Murphy, J. M., Varghese, L. N., Laktyushin, A., Young, S. N., … Nicola, N. A. (2012). Suppression of Cytokine Signalling by SOCS3: Characterisation of the mode of inhibition and the basis of its specificity. *Immunity*, 36(2), 239–250. http://doi.org/10.1016/j.immuni.2011.12.015.

31. Babon, J. J., Sabo, J. K., Zhang, J.-G., Nicola, N. A., & Norton, R. S. (2009). The SOCS box encodes a hierarchy of affinities for Cullin5: implications for ubiquitin ligase formation and cytokine signalling suppression. *Journal of Molecular Biology*, 387(1), 162–174. http://doi.org/10.1016/j.jmb.2009.01.024.

32. Niwa, H., Miyazaki, J., & Smith, A. G. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics*, 24(4), 372-376.

33. 33. Iliopoulos, D., Hirsch, H. A., & Struhl, K. (2009). An epigenetic switch involving NF-κB, Lin28, let-7 microRNA, and IL6 links inflammation to cell transformation. *Cell*, 139(4), 693–706. http://doi.org/10.1016/j.cell.2009.10.014.

34. Arnold, K. M., Opdenaker, L. M., Flynn, D., & Sims-Mourtada, J. (2015). Wound Healing and Cancer Stem Cells: Inflammation as a Driver of Treatment Resistance in Breast Cancer. *Cancer Growth and Metastasis*, 8, 1–13. http://doi.org/10.4137/CGM.S11286.

35. Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of Clinical Investigation*, 119(6), 1420–1428. http://doi.org/10.1172/JCI39104.

36. Kalluri, R., & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *Journal of Clinical Investigation*, 112(12), 1776–1784. http://doi.org/10.1172/JCI200320530.

37. Grant, C. M., & Kyprianou, N. (2013). Epithelial mesenchymal transition (EMT) in prostate growth and tumor progression. *Translational Andrology and Urology*, 2(3), 202–211. http://doi.org/10.3978/j.issn.2223-4683.2013.09.04.

38. Cohen, E. N., Gao, H., Anfossi, S., Mego, M., Reddy, N. G., Debeb, B., … Reuben, J. M. (2015). Inflammation Mediated Metastasis: Immune Induced Epithelial-To-Mesenchymal Transition in Inflammatory Breast Cancer Cells. *PLoS ONE*, 10(7), e0132710. http://doi.org/10.1371/journal.pone.0132710.

39. Bhola, N. E., Balko, J. M., Dugger, T. C., Kuba, M. G., Sánchez, V., Sanders, M., … Arteaga, C. L. (2013). TGF-β inhibition enhances chemotherapy action against triple-negative breast cancer. *The Journal of Clinical Investigation*, 123(3), 1348–1358. http://doi.org/10.1172/JCI65416.

40. Dontu, G., Al-Hajj, M., Abdallah, W.M., Clarke, M.F., & Wicha, M.S. (2003) Stem cells in normal breast development and breast cancer. *Cell Proliferation*, 36(suppl 1), 59–72.

41. Ponti D, Costa A, Zaffaroni N, Pratesi, G., Petrangolini, G., Coradini, D., … Daidone, M.G. (2005). Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Research*, 65(13): 5506–5511.

42. Ginestier, C., Hur, M. H., Charafe-Jauffret, E., Monville, F., Dutcher, J., Brown, M., … Dontu, G. (2007). ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell*, 1(5), 555–567. http://doi.org/10.1016/j.stem.2007.08.014.

43. Ghisolfi, L., Keates, A. C., Hu, X., Lee, D., & Li, C. J. (2012). Ionizing Radiation Induces Stemness in Cancer Cells. *PLoS ONE*, 7(8), e43628. http://doi.org/10.1371/journal.pone.0043628.

44. Sims-Mourtada, J., Opdenaker, L. M., Davis, J., Arnold, K. M., & Flynn, D. (2014). Taxane-induced hedgehog signaling is linked to expansion of breast cancer stem-like populations after chemotherapy. *Molecular Carcinogenesis*, 54(11), 1480-1493.

45. Fillmore, C. M., & Kuperwasser, C. (2008). Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Research : BCR*, 10(2), R25. http://doi.org/10.1186/bcr1982.

46. Phillips, T. M., Mcbride, W. H., & Pajonk, F. (2006). The Response of CD24-/low/CD44 Breast Cancer-Initiating Cells to Radiation. *Journal of the National Cancer Institute*, 98(24), 1777-1785.

47. Gong, Y., Wang, J., Huo, L., Wei, W., Ueno, N. T., & Woodward, W. A. (2014). Aldehyde Dehydrogenase 1 Expression in Inflammatory Breast Cancer as Measured by Immunohistochemical Staining. *Clinical Breast Cancer*, 14(3). doi:10.1016/j.clbc.2013.12.006.

48. Shi, W., & Harris, A. L. (2006). Notch Signaling in Breast Cancer and Tumor Angiogenesis: Cross-Talk and Therapeutic Potentials. *Journal of Mammary Gland Biology and Neoplasia*, 11(1), 41-52. doi:10.1007/s10911-006-9011-7.

49. Al-Hajj, M. (2007). Cancer stem cells and oncology therapeutics. *Current Opinion in Internal Medicine*, 6(2), 178-181. doi:10.1097/cco.0b013e328011a8d6.

50. Tan, Q., Wang, H., Hu, Y., Hu, M., Li, X., , A., . . . Song, L. (2015). Src/STAT3-dependent heme oxygenase-1 induction mediates chemoresistance of breast cancer cells to doxorubicin by promoting autophagy. *Cancer Science*, 106(8), 1023-1032. doi:10.1111/cas.12712.

51. Ryter, S. W., & Choi, A. M. (2013). Regulation of Autophagy in Oxygen-Dependent Cellular Stress. *Current Pharmaceutical Design*, 19(15), 2747-2756. doi:10.2174/1381612811319150010.

52. Pursiheimo, J., Rantanen, K., Heikkinen, P. T., Johansen, T., & Jaakkola, P. M. (2008). Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. *Oncogene*, 28(3), 334-344. doi:10.1038/onc.2008.392.

53. Howe, E. N., Cochrane, D. R., Cittelly, D. M., & Richer, J. K. (2012). miR-200c Targets a NF-κB Up-Regulated TrkB/NTF3 Autocrine Signaling Loop to Enhance Anoikis Sensitivity in Triple Negative Breast Cancer. *PLoS ONE*, 7(11), e49987. http://doi.org/10.1371/journal.pone.0049987.

54. Thompson, E. W. (2005). Carcinoma Invasion and Metastasis: A Role for Epithelial-Mesenchymal Transition? *Cancer Research*, 65(14), 5991-5995.

55. Diaz, N. (2006). Activation of Stat3 in Primary Tumors from High-Risk Breast Cancer Patients Is Associated with Elevated Levels of Activated Src and Survivin Expression. *Clinical Cancer Research*, 12(1), 20-28.

56. Adida, C., Crotty, P. L., McGrath, J., Berrebi, D., Diebold, J., & Altieri, D. C. (1998). Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. *The American Journal of Pathology*, 152(1), 43–49.

57. Wu, Y., Diab, I., Zhang, X., Izmailova, E. S., & Zehner, Z. E. (2004). Stat3 enhances vimentin gene expression by binding to the antisilencer element and interacting with the repressor protein, ZBP-89. *Oncogene*, 23(1), 168-178.

58. Snyder, M., Huang, X.-Y., & Zhang, J. J. (2011). Signal Transducers and Activators of Transcription 3 (STAT3) Directly Regulates Cytokine-induced Fascin Expression and Is Required for Breast Cancer Cell Migration. *The Journal of Biological Chemistry*, 286(45), 38886–38893. http://doi.org/10.1074/jbc.M111.286245.

59. Schust, J., Sperl, B., Hollis, A., Mayer, T., & Berg, T. (2006). Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. *Chemistry & Biology*, 13(11), 1235-1242. doi:10.1016/j.chembiol.2006.09.018.

60. You, L., Wang, Z., Li, H., Shou, J., Jing, Z., Xie, J., … Han, W. (2015). The role of STAT3 in autophagy. *Autophagy*, 11(5), 729–739. http://doi.org/10.1080/15548627.2015.1017192.

61. Heidelberger, S., Zinzalla, G., Antonow, D., Essex, S., Basu, B. P., Palmer, J., … Thurston, D. E. (2013). Investigation of the protein alkylation sites of the STAT3:STAT3 inhibitor Stattic by mass spectrometry. *Bioorganic & Medicinal Chemistry Letters*, 23(16), 4719-4722.

62. Chung, S. S., Giehl, N., Wu, Y., & Vadgama, J. V. (2014). STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. International Journal of *Oncology*, 44(2), 403–411. http://doi.org/10.3892/ijo.2013.2195.

63. Hung, A. C., Lo, S., Hou, M., Lee, Y., Tsai, C., Chen, Y., … Yuan, S. F. (2016). Extracellular Visfatin-Promoted Malignant Behavior in Breast Cancer Is Mediated Through c-Abl and STAT3 Activation. *Clinical Cancer Research*, 2704(2015). doi:10.1158/1078-0432.ccr-15-2704.

64. Fridman, J. S., Scherle, P. A., Collins, R., Burn, T. C., Li, Y., Li, J., … Vaddi, K. (2010). Selective Inhibition of JAK1 and JAK2 Is Efficacious in Rodent Models of Arthritis: Preclinical Characterization of INCB028050. *The Journal of Immunology*, 184(9), 5298-5307. doi:10.4049/jimmunol.0902819.

65. O'shea, J. J., Holland, S. M., & Staudt, L. M. (2013). JAKs and STATs in Immunity, Immunodeficiency, and Cancer. *New England Journal of Medicin*e, 368(2), 161-170.

66. Yamagishi, H., Shirakami, S., Nakajima, Y., Tanaka, A., Takahashi, F., Hamaguchi, H., … Inoue, T. (2015). Discovery of 3,6-dihydroimidazo[4,5-d]pyrrolo[2,3-b]pyridin-2(1H)-one derivatives as novel JAK inhibitors. *Bioorganic & Medicinal Chemistry*, 23(15), 4846-4859.

67. Waitkus, M. S., Chandrasekharan, U. M., Willard, B., Tee, T. L., Hsieh, J. K., Przybycin, C. G., … DiCorleto, P. E. (2014). Signal Integration and Gene Induction by a Functionally Distinct STAT3 Phosphoform. *Molecular and Cellular Biology*, 34(10), 1800–1811. http://doi.org/10.1128/MCB.00034-14.

68. Dasgupta, M., Unal, H., Willard, B., Yang, J., Karnik, S. S., & Stark, G. R. (2014). Critical Role for Lysine 685 in Gene Expression Mediated by Transcription Factor Unphosphorylated STAT3. *The Journal of Biological Chemistry*, 289(44), 30763–30771. http://doi.org/10.1074/jbc.M114.603894.

69. Ray, S., Zhao, Y., Jamaluddin, M., Edeh, C. B., Lee, C., & Brasier, A. R. (2014). Inducible STAT3 NH2 Terminal Mono-ubiquitination Promotes BRD4 Complex Formation to Regulate Apoptosis. *Cellular Signalling*, 26(7), 1445–1455. http://doi.org/10.1016/j.cellsig.2014.03.007.

70. Wang, T., Yuan, J., Zhang, J., Tian, R., Ji, W., Zhou, Y., … Nui, R. (2015). Anxa2 binds to STAT3 and promotes epithelial to mesenchymal transition in breast cancer cells. *Oncotarget*, 6(31), 30975-30992.

71. Siziopikou, K. P., Ariga, R., Proussaloglou, K. E., Gattuso, P., & Cobleigh, M. (2006). The Challenging Estrogen Receptor-Negative/ Progesterone Receptor-Negative/HER-2-Negative Patient: A Promising Candidate for Epidermal Growth Factor Receptor-Targeted Therapy? *The Breast Journal*, 12(4), 360-362. doi:10.1111/j.1075-122x.2006.00276.x.

72. Wen, Z., Zhong, Z., & Darnell, J. E. (1995). Maximal activation of transcription by statl and stat3 requires both tyrosine and serine phosphorylation. *Cell*, 82(2), 241-250.

73. Lee, H., Zhang, P., Herrmann, A., Yang, C., Xin, H., Wang, Z., … Yu, H. (2012). Acetylated STAT3 is crucial for methylation of tumor-suppressor gene promoters and inhibition by resveratrol results in demethylation. *Proceedings of the National Academy of Sciences of the United States of America*, 109(20), 7765–7769. http://doi.org/10.1073/pnas.1205132109.

74. Yang, J., & Stark, G. R. (2008). Roles of unphosphorylated STATs in signaling. *Cell Research*, 18(4), 443-451.

75. Acuner Ozbabacan, S. E., Gursoy, A., Nussinov, R., & Keskin, O. (2014). The Structural Pathway of Interleukin 1 (IL-1) Initiated Signaling Reveals Mechanisms of Oncogenic Mutations and SNPs in Inflammation and Cancer. *PLoS Computational Biology*, *10*(2), e1003470. http://doi.org/10.1371/journal.pcbi.1003470.

76. Hagihara, K., Nishikawa, T., Sugamata, Y., Song, J., Isobe, T., Taga, T., & Yoshizaki, K. (2005). Essential role of STAT3 in cytokine-driven NF-kappaB-mediated serum amyloid A gene expression. *Genes to Cells*, 10(11), 1051-1063.

77. Yamamoto, M., Ito, T., Shimizu, T., Ishida, T., Semba, K., Watanabe, S … Inoue, J. (2010). Epigenetic alteration of the NF-κB-inducing kinase (NIK) gene is involved in enhanced NIK expression in basal-like breast cancer. *Cancer Science*, 101(11), 2391-2397.

78. Gallo, L. H., Meyer, A. N., Motamedchaboki, K., Nelson, K. N., Haas, M., & Donoghue, D. J. (2014). Novel Lys63-linked ubiquitination of IKKβ induces STAT3 signaling. *Cell Cycle*, 13(24), 3964–3976. http://doi.org/10.4161/15384101.2014.988026.

79. Carter, R. S., Pennington, K. N., Arrate, P., Oltz, E. M., & Ballard, D. W. (2005). Site-specific Monoubiquitination of I B Kinase IKKbeta Regulates Its Phosphorylation and Persistent Activation. *Journal of Biological Chemistry*, 280(52), 43272-43279.

80. Smit, M. A., Geiger, T. R., Song, J.-Y., Gitelman, I., & Peeper, D. S. (2009). A Twist-Snail Axis Critical for TrkB-Induced Epithelial-Mesenchymal Transition-Like Transformation, Anoikis Resistance, and Metastasis. *Molecular and Cellular Biology*, 29(13), 3722–3737. http://doi.org/10.1128/MCB.01164-08.

81. Hsu, Y., Chen, C., Lin, I., Tsai, E., Kuo, P., & Hou, M. (2012). 4-Shogaol, an Active Constituent of Dietary Ginger, Inhibits Metastasis of MDA-MB-231 Human Breast Adenocarcinoma Cells by Decreasing the Repression of NF-κB/Snail on RKIP. *Journal of Agricultural and Food Chemistry*, 60(3), 852-861.

82. Labbozzetta, M., Poma, P., Vivona, N., Gulino, A., D'Alessandro, N., & Notarbartolo, M. (2015). Epigenetic changes and nuclear factor-κB activation, but not microRNA-224, downregulate Raf-1 kinase inhibitor protein in triple-negative breast cancer SUM 159 cells. *Oncology Letters*, 10(6), 3807–3815. http://doi.org/10.3892/ol.2015.3787.

83. Ito-Kureha, T., Koshikawa, N., Yamamoto, M., Semba, K., Yamaguchi, N., Yamamoto, T., … Inoue, J. (2014). Tropomodulin 1 Expression Driven by NF- B Enhances Breast Cancer Growth. *Cancer Research*, 75(1), 62-72.

84. Dozin, B., Malpeli, M., Camardella, L., Cancedda, R., & Pietrangelo, A. (2002). Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: Molecular and cellular aspects. *Matrix Biology*, 21(5), 449-459.

85. Di Fusco, D., … Stolfi, C. (2015). Th17-type cytokines, IL-6 and TNF-α synergistically activate STAT3 and NF-kB to promote colorectal cancer cell growth. *Oncogene*, 34(27), 3493–3503. http://doi.org/10.1038/onc.2014.286.

86. Lee, H., Herrmann, A., Deng, J., Kujawski, M., Niu, G., Li, Z., … Yu, H. (2009). Persistently-activated Stat3 maintains constitutive NF-κB activity in tumors. *Cancer Cell*, 15(4), 283–293. http://doi.org/10.1016/j.ccr.2009.02.015.

87. Chen, L., Mu, Y., & Greene, W. C. (2002). Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-κB. *The EMBO Journal*, 21(23), 6539–6548. http://doi.org/10.1093/emboj/cdf660.

88. Gomez-Cabrero, A., Wrasidlo, W., & Reisfeld, R. A. (2013). IMD-0354 Targets Breast Cancer Stem Cells: A Novel Approach for an Adjuvant to Chemotherapy to Prevent Multidrug Resistance in a Murine Model. *PLoS ONE*, 8(8), e73607. http://doi.org/10.1371/journal.pone.0073607

89. Taketo, M. M. (2011). Reflections on the Spread of Metastasis to Cancer Prevention. *Cancer Prevention Research*, 4(3), 324-328.