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Analysis of Post-Translational Modifications and Expression of STAT3 under Desferrioxamine Induced Hypoxia in Human Neuroblastoma Cells

By Veronica Harrison

Submitted in Partial Fulfillment of the Requirement of the
Degree of Master of Science in Biology from the
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ABSTRACT:

Oxygen homeostasis is critical for optimal energy production in eukaryotic organisms. The signal transduction pathways involved with detection and mitigation of hypoxia have been carefully scrutinized in an effort to elucidate how metabolism and survival is maintained. Characterization of these pathways and their involvement in physiological processes such as blood cell production in the bone marrow and microvessel formation in developing embryos provides a model of how these functions are executed. Less well-characterized mechanisms in disease states such as solid tumors still confound researchers whose efforts to find effective treatments are falling short. Cancer cells exploit the activities of fundamental signaling pathways to enhance their survival. Aberrant function of the Janus Activated kinase (JAK) pathway and its protein partner, signal transduction and activator of transcription 3 (STAT3) have been linked to many types of cancer, because they mediate cellular processes such as proliferation, metabolism and survival. Significant contradictions persist regarding how each of these functions are achieved. STAT3 is subject to post-translation modifications on two separate amino acid residues, both of which have been linked to solid tumor proliferation and survival. Using desferrioxamine (DFO), a hypoxic mimetic agent, we examined the reaction of human neuroblastoma cells treated with varying concentrations at different time periods. We found significant differences in the post-translational modifications of STAT3 in the cells that survived treatment using flow cytometry. An inhibitor was also used to more closely identify upstream signaling, and to measure its effect on cell viability. Our preliminary data suggests that alterations in the JAK-STAT3 signaling pathway contributes to cell survival under DFO-induced hypoxic conditions.

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INTRODUCTION

Hypoxia is defined as an insufficient supply of oxygen required to maintain optimum cellular function. In eukaryotic organisms, oxygen homeostasis is so tightly regulated that even the slightest changes result in an immediate cellular response (Pierson, 2000; Michiels, 2004). Neurons within the brain devote the majority of their oxygen supply to maintenance of membrane potential and ATP production, and as such have developed signal transduction pathways strictly devoted to mediation of cell survival until physiologic levels are restored (Michiels, 2004; Zheng, et al, 2016; Belanger, 2011). Hypoxia can develop over time from diseases such as Chronic Obstructive Pulmonary disorder resulting from diminished pulmonary capacity, and if left untreated can result in respiratory failure or cardiovascular death (Kent, 2011). In cancer, solid tumor cells proliferate at such a rapid rate that they exceed their oxygen supply. Vascular tissue formed by unregulated angiogenesis is insufficient to meet increasing metabolic needs, and as such solid tumors have adopted and activated pathways that enhance survival and chemo-resistance, resulting in poor prognosis for patients (Poomthavorn, et al., 2009; Eales, Hollinshead, & Tennant, 2016). Conversely, the prevalence of hypoxia is normal in the bone marrow niche and in the developing embryo, leading scientists to question whether similar mechanisms mediate physiological processes and disease states alike (Ara, et al., 2013; Gao, et al., 2013).

Many conserved pathways and intracellular proteins are devoted to fundamental processes that maintain homeostasis, regulate the supply of oxygen and nutrients, or perform functions unique to cell type. The signal transducers and activators of transcription (STAT) is a family of ubiquitously expressed proteins that participate in homeostatic functions such as metabolism and survival. STAT proteins also mediate immune function, embryological

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development, cell proliferation, differentiation, and angiogenesis (Sherry, 2008; Darnell, 1977; Gorissen, et al, 2011; Liongue and Ward, 2013). This wide range stems from their structural diversity, and the extracellular signaling molecules to which they respond, including cytokines, chemokines, and growth factors (Wierenga, et al., 2001; Gao, 2014). STAT proteins exist in seven different isoforms, and vary in length from 700-850 amino acids, yet they share homology in several functional domains (Becker, et al., 1998). The N-terminal domain contains non-specific DNA binding sites and chaperone protein binding sites. Following is the coiled-coil domain, a linker domain, and the src-homology 2 domain (SH2), required for receptor tyrosine kinase activation and dimerization (Hirai, et al., 2011; Becker, 1998). The trans-activation domain is one in which each STAT isoform shares the least homology. This domain provides for cofactor binding and defines the unique function of each STAT protein (Levy, 2002; Darnell, 1997; Becker, 1998; Rawlings, 2004). Several STAT proteins are expressed in lower vertebrates, and evolutionary evidence exists to suggest that as the complexity of organisms increased, specifically with respect to acquired immunity, each STAT protein developed a unique function (Liongue and Ward, 2013; Gorissen, et al., 2011). Of the seven different isoforms of STAT proteins, STAT3 has received the most scrutiny as it appears to play a central role in innate immunity, embryological development, proliferation, metabolism, inflammation and survival (Gao, 2014; Kamakura, 2004). Most compelling, is the evidence that STAT3 knockouts result in embryonic lethality (Onishi, et al., 2015; Hirai, 2011).

STAT3 is present in the cytoplasm at constant levels; it has the dual role of transducing signals from extra-cellular receptors and transcribing its unique set of target genes (Samavati, 2009; Sriuranpong, 2003). The most well-characterized receptors that activate STAT3 include the Janus Activation Kinase pathway (JAK), the mitogen-activated pathway (MAPK), and the

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non-receptor tyrosine kinases Src and Ras (Wierenga, 2001; Rawlings, 2004). Finally, STAT3 activation can occur via the opioid family of G-protein coupled receptors. Their role is well-established regarding pain modulation and in addiction, however, they are also involved with maintenance of ionic membrane homeostasis, cell proliferation, and immune function (Ram and Iyengar, 2001; Leu, 2003).

Stat3 is activated via phosphorylation, forms homodimers and translocates to the nucleus, where it transcribes genes involved with the cell cycle, embryological development, angiogenesis, metabolism, and survival through prevention of apoptosis. Multiple animal studies showed that STAT3 is the upstream regulator in the pathway that maintains pluripotency (Vinh Do, et al, 2013; Onishi, 2015). Upon receipt of signaling from leukemia inhibitory factor (LIF), a member of the Il-6 family of cytokines, STAT3 promoted the proliferation of mouse ES cells, and mediated appropriate mitochondrial support (Hirai, et al., 2011; Vinh Do, 2013, Huang, et al, 2013). After fertilization the developing embryo relies on glycolysis, however, upon implantation, STAT3 directs the development of microvessels via transcription of vascular endothelial growth factor (VEGF), and induces the mitochondria to begin the switch to oxidative phosphorylation (Carbognin, et al., 2016; Poli and Camporeale, 2015). LIF mediates the reprogramming of endometrial cells to promote acceptance of embryo implantation, and STAT3 was shown to be concentrated in the stromal cells surrounding the implantation site. When either LIF or STAT3 is blocked, implantation fails. Downstream activation of Egr1 and WNT4, whose expression is controlled via LIF-gp130-STAT3 signaling ultimately carries out further development of the embryo by initiating cell differentiation, proliferation and survival (Liang, et al., 2014; Carbognin, et al., 2016).

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The role of STAT3 in survival was first elucidated in its involvement in the acute phase response (Koo, et al., 2011; Levy and Lee, 2014). Cells infected with bacteria release cytokines such as IL-6, IL-1ß, and tumor necrosis factor (TNF-ß), initiating the production of acute phase response proteins (APP) in the liver (Alonzi, et al., 2001). APPs have the dual role of activating the innate immune system while preventing an excessive inflammatory response that could cause tissue and organ damage (Liu, 2010; Alonzi, 2000; Ahyi, 2013). STAT3 is responsible for transcription of APP; it also provides remediation from the stress placed upon organelles responsible for completion of these functions. Specifically, the endoplasmic reticulum (ER) comes under significant stress when processing the extensive amount of proteins required for the acute phase response. Translation of proteins proceeds at such a high rate, that unfolded proteins can accumulate in the ER (Ahyi, et al., 2013). STAT3 phosphorylation is upregulated both before and after the induction of ER stress, and it was found to be responsible for maintaining the integrity of all involved organelles. Most importantly, conditional knockout of STAT3 abrogated the acute phase response in its entirety, providing strong evidence that STAT3 is responsible for all essential survival functions from receipt of initial signal to interpreting the appropriate response, and modulating the cellular environment (Liu, et al., 2010; Alonzi, et al., 2000; Ahyi, et al., 2013).

Aberrant cell proliferation present in transformed cells and in solid tumors have been attributed to constitutive activation of STAT3 (Iwaramaru, et al., 2007). Controversial evidence exists regarding how STAT3 signaling is involved. Many studies have linked tyrosine phosphorylation with its dimerization, nuclear translocation and subsequent transcription of cell cycle genes such as Cyclin D1, and those involved with prevention of apoptosis: Bcl-2, Bcl-xl, and Mcl-1 (Peyser and Grandis, 2013; Lee, et al., 2006). Equally, serine phosphorylation has

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been linked with enhanced proliferation, angiogenesis, and migration and invasion in transformed cells that have acquired a mutation in KRAS or other ERK upstream targets. Regardless, aberrant activation of STAT3 promotes cell transformation and enhances survival and chemoresistance (Chung, 1997; Selvendiran, 2009, Poli, 2015). Induced hypoxia in cultured cells provides an ideal setting to isolate and identify the exact mechanisms by which STAT3 carries out its role in these cellular processes. The iron chelator, desferrioxamine (DFO), is a well-studied chemical inducer of hypoxia. It does so by interfering with prolyl hydroxylase proteins that are required to inactivate HIF-1α via Von Hippel Lindau ubiquitination, and contributes to a pro-apoptotic cellular environment by upregulating p21 and p27 (Siriwardana and Seligman, 2013; Fu, et al., 2007; Bedessem, et al., 2015).

In this study, we set out to more clearly define the role of STAT3 in mediation of cell survival. Characterization of this pathway could lead to targeted therapies in the treatment of immune disorders, cancer, and to more effective intervention in ischemic injury. We used DFO to create a hypoxic environment in cultured NMB cells. DFO has demonstrated a significant reduction in cell viability in previous studies, however, there are cells that evade its effects (Cook, et al., 2010). It was our goal to more clearly define how these cells are able to survive, and to determine exactly what role STAT3 plays in their survival. We used Flow cytometry to measure the activation levels of STAT3 at both the tyrosine and serine phosphorylation sites to determine whether there is a correlation between either of them and survival. Flow Cytometry was also used to quantify any change in the overall expression of STAT3. An inhibitor was used to more closely pinpoint the origin of the survival signal. Stat proteins receive intracellular signals that originate from a variety of extracellular receptors, however, the use of an inhibitor that targets a JAK receptor could narrow the scope and could provide more direct evidence in

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how cell survival is achieved. Finally, cell viability testing was used to evaluate the combined effects of DFO and the inhibitor on cell survival rate.

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METHODS AND MATERIALS

Cell Culture:

Human neuroblastoma (NMB) cells were maintained in Roswell Park Memorial Institute Media (RPMI) supplemented with 10% FBS, 1% Pen-Strep, and 0.01% gentamycin in 75ml flasks, and incubated at 37˚C/ 5% CO_2. When cultures reached 100% confluency, cells were passaged for maintenance or they were seeded into 10cm culture plates for treatment. Flasks used for seeding were washed with 5ml of RPMI and cells were removed using 5ml of PBS/EDTA/1% trypsin. The suspension was placed in 50 ml conical tubes and centrifuged at 1000RPM at 4˚ C for 5 minutes. The supernatant was aspirated, and cells were resuspended in RPMI/10% FBS.

Cell Treatment:

For treatment with hypoxic mimetic DFO, 10 ml of RPMI/10% FBS was added to each culture plate, 2 plates each were used for control, and for DFO. Media suspension with 1 x 10^6 cells were added to each plate which were then incubated for 24 or 48 hours. Cells were then harvested and used in Flow cytometry or Cell Viability protocol. Analysis that included the inhibitor required an additional 2 plates each with 4µM of WP1066 and for combined treatment of 300µM of DFO and 4 µM of WP1066.

Cell Viability:

NMB cells were maintained as described above. One ml of RPMI/10%FBS was added to 12-well plates. Approximately 100,000 cells were added to each treatment in triplicate: 100 µM, 200µM, an 300µM of DFO with and without 4µM of WP1066, and control and incubated for 24

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hours. Cells were washed with RPMI and harvested using PBS/EDTA on ice. A 15 µl sample of each well was stained with Trypan blue and cells were counted using a hemacytometer. Counting was repeated four times for each well.

Flow Cytometry:

NMB cells were maintained as described above. 10 ml of RPMI/10% FBS was added to 10cm plates to which 1 x 10^6 cells were added in duplicate for the following conditions: control, 300µM of DFO with or without 4µM of WP1066. Plates were incubated at 37˚ C/5% CO_2 for 24 and 48 hours. Cells were then washed with 5 ml of RPMI and harvested using 1 ml 0.25% trypsin and 10 ml RPMI/10% FBS. The cells were placed in 50 ml conical tubes and centrifuged at 1200rpm for 5 minutes at 4˚C. The supernatant was aspirated and resuspended in 4 ml of DPBS for the control and 3ml of DPBS for each conical tube containing the treated cells. 1 ml of suspension was added to 3 Eppendorf tubes, and centrifuged as described. The supernatant was aspirated, 250 µl of Cytofix buffer (BD Biosciences) was added and incubated at RT for 25 minutes. The suspension was then centrifuged as described, supernatant aspirated and washed with 500 mL of FACS buffer (in house), followed by centrifuge and treatment with Perm buffer (BD Biosciences) for thirty minutes. After incubation, cells were washed and centrifuged as described, and treated with 20µl of either pY705-anti-STAT3, pS727-anti-STAT3, or p-STAT3, (BD Biosciences) and incubated for 30 minutes. Cells were the washed and resuspended in 500µl of FACS buffer and analyzed using a MACSQUANT flow cytometer.

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RESULTS

DFO-induced Hypoxia Resulted in Significant Changes in the Phosphorylation State of STAT3.

STAT3 can be activated via phosphorylation on two amino acid residues: tyrosine 705 and serine 727. A study conducted by Tierney, et al, found constitutively activated pSTAT-727 localized in the cytoplasm of an endometrial cancer cell line, while expression of total STAT3 remained unchanged. Conversely, a study conducted by Selvendiran, et al., determined that survival and chemo-resistance in an ovarian cancer cell line was attributed to elevated levels of pSTAT3-705 while total STAT3 expression also remained unchanged. To determine the effect of chemically-induced hypoxia on the activation of STAT3, human neuronal NMB cells were treated with 300μM of DFO for 24 and 48 hours. After treatment, the dead cells were removed, and those that survived treatment were stained with anti-pSTAT3-705 and anti-p-STAT3-727 fluorescent antibodies to measure changes in phosphorylation levels. Surviving cells were also treated with anti-STAT3 fluorescent antibodies to detect any changes in overall expression, and all were analyzed using Flow Cytometry. These levels were measured against activation levels of cells not treated with DFO. As shown in Figure 1, tyrosine 705 phosphorylation levels increased by 44% when treated with DFO for 24 hours versus control, and serine 727 phosphorylation levels decreased by 23%. This indicates that Stat3 is constitutively activated at its serine residue, and when exposed to hypoxic stress, the activation levels shift. Total Stat3 expression levels increased by 24% in treated versus control cells.

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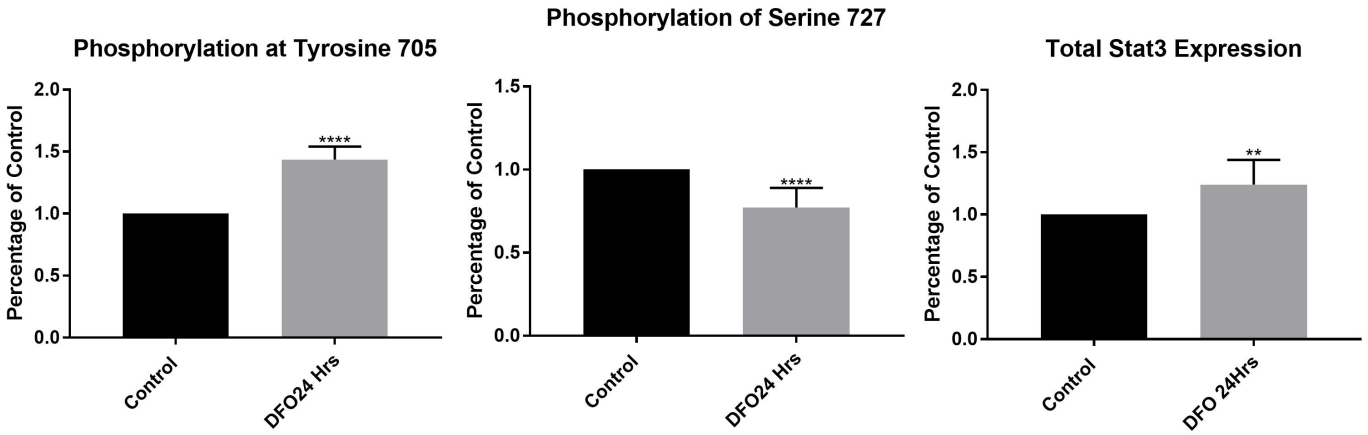

Figure 1: Quantification of the changes in phosphorylation of STAT3 using Flow Cytometry. NMB cells were treated with DFO for 24 hours and surviving cells were isolated and stained with anti-pSTAT3-705, anti-pSTAT3-727, and total STAT3 antibodies. Significant increases in pSTAT3-705 and subsequent decreases in pSTAT3-727 were revealed. DFO-induced hypoxia also resulted in upregulation of STAT3 expression. Data analyzed using un-paired test: p-STAT-705 significance measured $p < 0.0001$; p-STAT-727 significance measured $p < 0.0001$; Total STAT3 measured $p < 0.042$.

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Treatment of human neuronal NMB cells with 300μM of DFO for 48 hours (Figure 2) produced a more profound difference in serine phosphorylation levels, decreasing by 45% in treated versus control cells. Tyrosine phosphorylation increased by 45%, and total STAT3 expression increased by 34%. This indicates that phosphorylation and expression events occur in a time-dependent manner.

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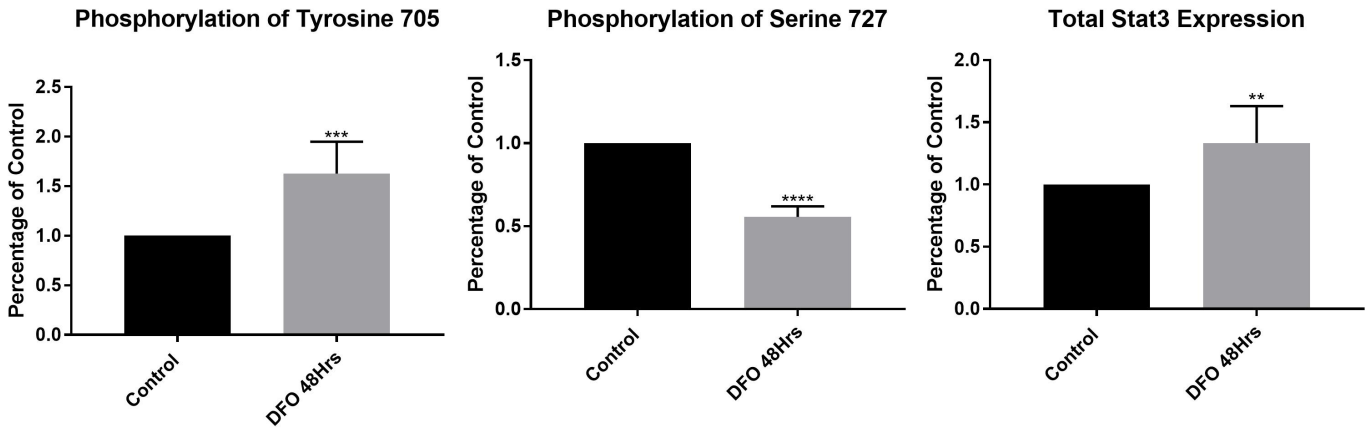

Figure 2: Quantification of the changes in phosphorylation of STAT3 using Flow Cytometry. NMB cells were treated with DFO for 48 hours and surviving cells were isolated and stained with anti-pSTAT3-705, anti-pSTAT3-727, and total STAT3 antibodies. Treatment for 48 hours shows that changes in post-translation modifications and in expression of total STAT3 occurred in a time-dependent manner. Significant increases in pSTAT3-705 and subsequent decreases in pSTAT3-727 were revealed. DFO-induced hypoxia also resulted in upregulation of STAT3 expression. Data analyzed using un-paired test: p-STAT-705 significance measured $p < 0.0002$; p-STAT-727 significance measured $p < 0.0001$; Total STAT3 measured $p < 0.001$.

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JAK/STAT3 Pathway Initiated and Transduced the Survival Signal in DFO-induced Hypoxia

Aberrant STAT3 activation leads to cell transformation and the development of tumors of the kidney, breast, and colon, among others (Iwaramura, et al., 2007). Solid tumors are comprised of cells residing in varying degrees of hypoxia; those close to a constant blood supply exist in a relatively normoxic environment, while those in the deepest portion of the tumor exist in severe hypoxia (Eales, 2016; Peyser 2013; Lee, 2006). In order to more closely pinpoint where the upstream survival signal for STAT3 activation originated, we used a known JAK2 inhibitor. Human neuronal NMB cells were incubated with 300μM of DFO and 4μM of WP1066 for 24 and 48 hours. Anti-pSTAT3-705 and anti-pSTAT3-727 fluorescent antibodies were used to quantify the activation of each site, and anti-STAT3 antibodies were used to measure total Stat3 expression via Flow Cytometry. As shown in Figure 3, combined DFO/WP treatment for 24 and 48 hours resulted in a 45% decrease in tyrosine 705 phosphorylation when compared with control.

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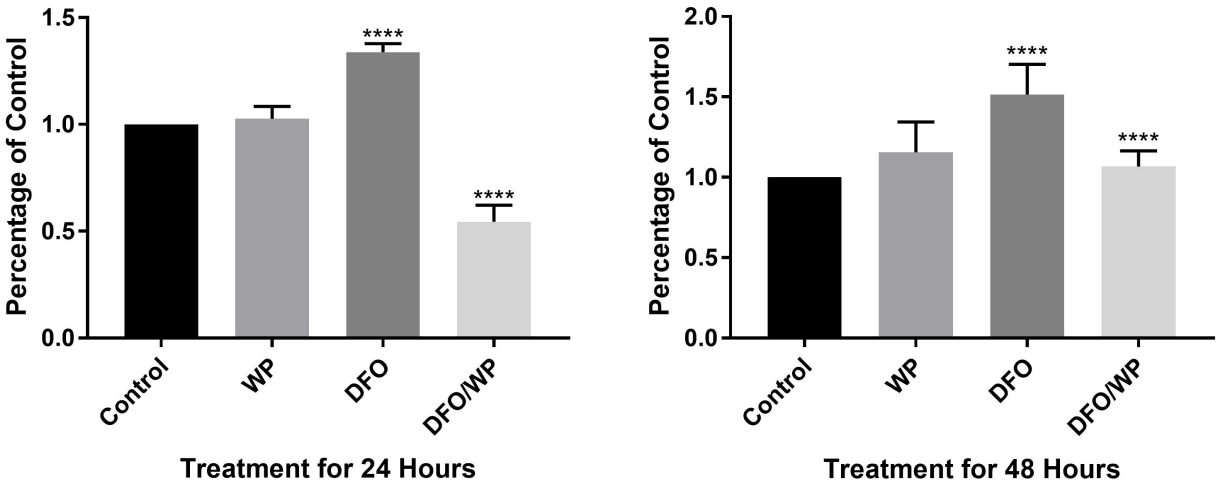

Figure 3: Analysis of the effect of phosphorylation of Tyrosine 705 on STAT3 after treatment of NMB cells with DFO and WP1066 for 24 hours and 48 hours. After treatment, surviving cells were isolated and treated with anti-pSTAT-705 antibodies. Phosphorylation levels were analyzed using Flow Cytometry. Quantification of significance using ONE-WAY ANOVA: p-STAT-705: $p < 0.0001$ for both 24 and 48 hours.

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Figure 4 depicts the quantification of changes in phosphorylation at serine 727, and Figure 5 depicts the quantification of total STAT3 expression. Analysis of the activation of serine resulted in a decrease of phosphorylation of 36% for both 24 and 48 hour treatments. Total STAT3 expression resulted in a 36% increase for both 24 and 48 hour treatments as well, indicating that NMB cells reacted to hypoxic injury by increased expression of STAT3.

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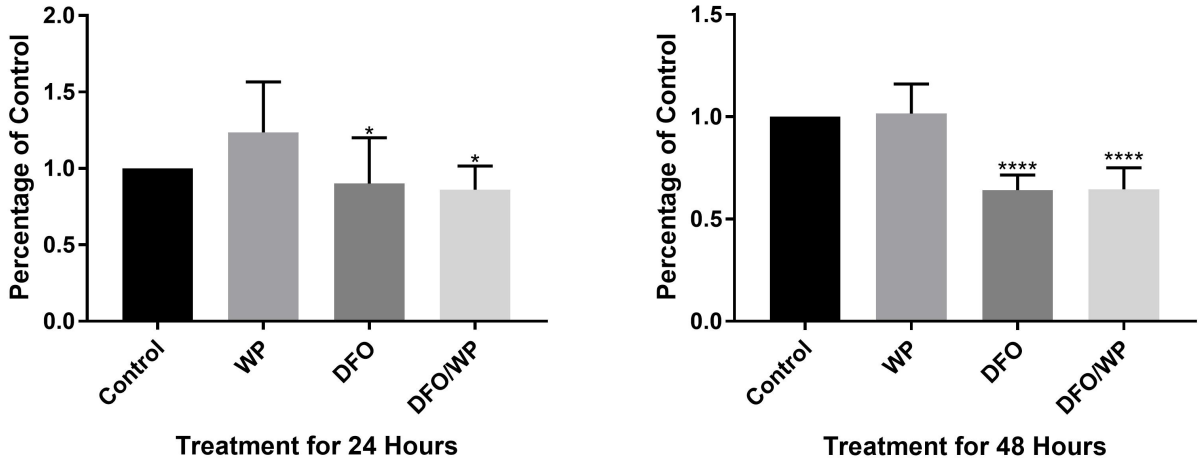

Figure 4: Analysis of the effect of phosphorylation of Serine 727 on STAT3 after treatment of NMB cells with DFO and WP1066 for 24 hours and 48 hours. After treatment, surviving cells were isolated and treated with anti-pSTAT-727 antibodies. Phosphorylation levels were analyzed using Flow Cytometry. Quantification of significance using ONE-WAY ANOVA: p-STAT-727: $p=0.02$ for 24 hours and $p<0.0001$ for 48 hours.

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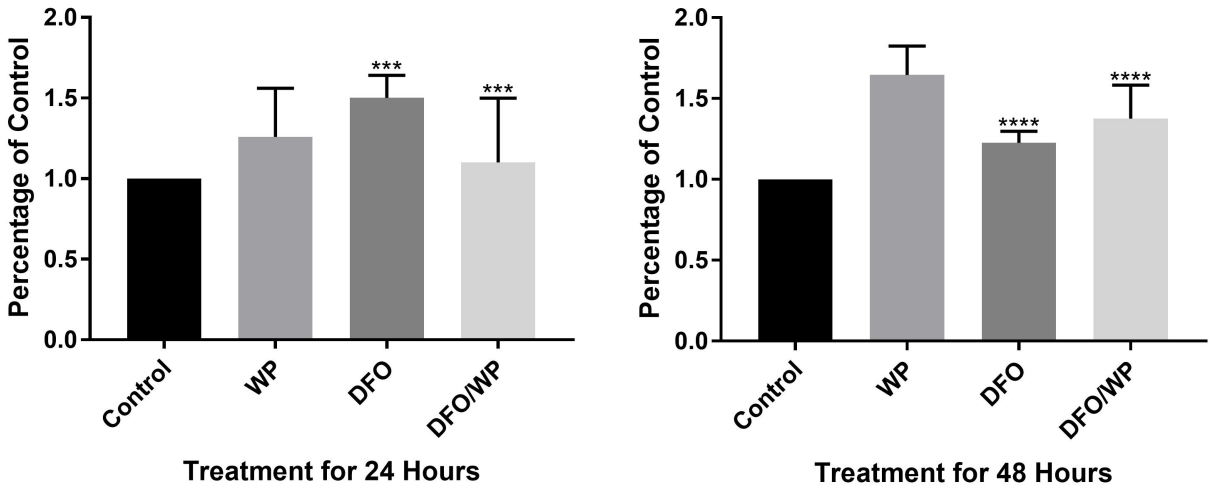

Figure 5: Analysis of the expression of STAT3 after treatment of NMB cells with DFO and WP1066 for 24 and 48 hours. After treatment, surviving cells were isolated and stained with anti-STAT3 antibodies. Expression levels were analyzed using Flow Cytometry. Quantification of significance using ONE-WAY ANOVA: STAT3 expression $p=0.001$ for 24 hours and $p<0.0001$ for 48 hours.

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DFO-induced Hypoxia and Treatment with Inhibitor Resulted in Significant Decrease in Cell Viability.

Cultured cells exposed to low oxygen or chemically-induced hypoxia are subject to decreased cell viability via apoptosis (Zeng, et al., 2011; Bedessem, 2015). To determine the extent to which cell viability is compromised in NMB cells, we used increasing concentrations of DFO (100µM-300µM) and incubated them at 37˚ C for 24 hours. Following incubation cells were washed with PBS/RPMI, and were harvested using 0.01% Trypsin. Cells were resuspended in RPMI/10%FBS solution, stained with Trypan Blue and counted on a Hemacytometer. As shown in Figures 6-8, DFO-induced hypoxia resulted in a 35-64% reduction in cell viability when compared with control. Combined treatment with varying concentrations of DFO and the JAK inhibitor, WP1066 resulted in 60-80% decrease in cell viability.

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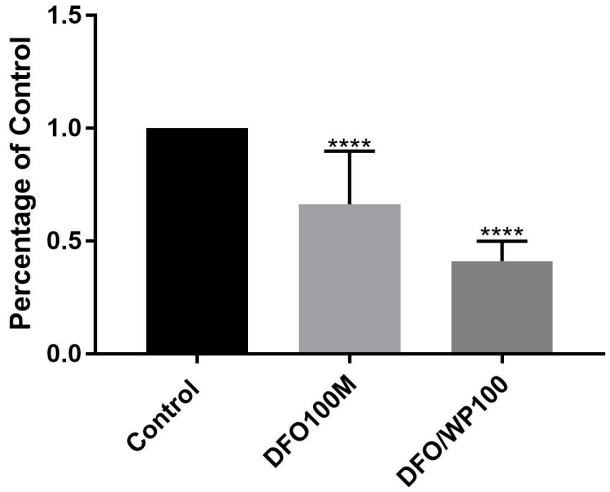

Figure 6: Analysis of Cell Viability after combined treatment with hypoxic mimetic and JAK inhibitor WP1066. NMB cells were cultured with a concentration of 100µM DFO and 4µM of WP1066 for 24 hours. Treatment resulted in a significant decrease in cell viability. One-Way Anova resulted in p value <0.0001.

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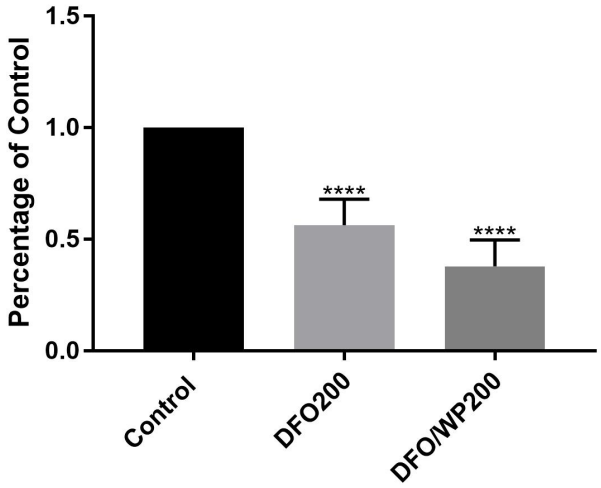

Figure 7: Analysis of Cell Viability after combined treatment with hypoxic mimetic and JAK inhibitor WP1066. NMB cells were cultured with a concentration of 200µM DFO and 4µM of WP1066 for 24 hours. Treatment resulted in a significant decrease in cell viability. One-Way ANOVA: p value <0.0001.

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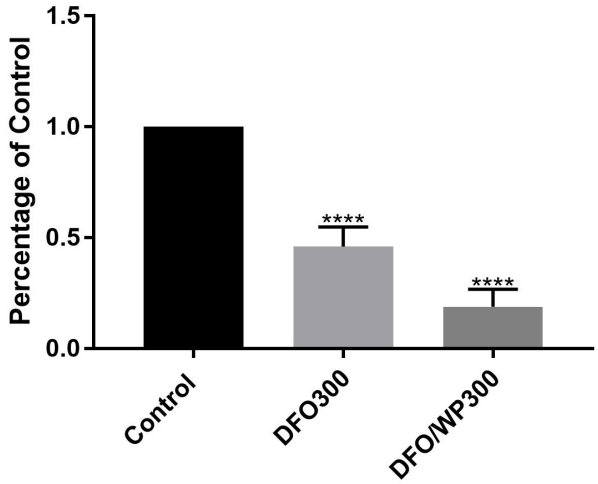

Figure 8: Analysis of Cell Viability after combined treatment with hypoxic mimetic and JAK inhibitor WP1066. NMB cells were cultured with a concentration of 300µM DFO and 4µM of WP1066 for 24 hours. Treatment resulted in a significant decrease in cell viability. One-Way ANOVA: p value <0.0001.

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DISCUSSION

The STAT family of proteins has demonstrated its evolutionary importance in its involvement with both maintenance of homeostasis and in cellular functions unique to cell type (Gorissen, et al., 2011). STAT3 in particular mediates fundamental cellular processes such as embryological development, proliferation, angiogenesis, metabolism and cell survival (Liongue and Ward, 2013; Rawlings, et al., 2004). The functions of this protein are exploited in cells that have accumulated mutations rendering its upstream partners constitutively active, resulting in STAT3 itself to become constitutively active (Verstovsek, et al., 2008; Liu, et al., 2010). Such aberrant signaling is responsible for the survival and proliferation of cells in many types of solid tumors and in several types of leukemias and lymphomas (Hazan-Halevy, et al., 2009; Ferrajoli, et al., 2007). STAT3 has also been linked to unregulated vascular formation and chemotherapy resistance (Ara, 2013; Eales, 2016). Many studies have postulated that tyrosine phosphorylation is necessary for STAT3 function, while others have claimed a direct relationship between serine phosphorylation and the progression of certain cancers (Sruiranpong, et al., 2003; Hazan-Halevy, et al., 2009). These contradicting views shaped this study to determine whether a specific phosphorylation event that occurs in STAT3 has a direct link to survival in our hypoxic cell model system. We then set out to determine the origin of the upstream survival signal, as this has implications for treatment of many tumors that evade current therapies.

In this study, STAT3 was phosphorylated at its serine residue in NMB control cells. Analysis of the cells that survived treatment with DFO revealed a decrease in serine signaling in a time-dependent manner. More importantly, the signal of tyrosine phosphorylation increased within that same time frame. STAT proteins are known to be activated via the JAK pathway, so WP1066, a well-documented JAK2 inhibitor, was used to determine the source of the upstream

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signal for tyrosine phosphorylation (Verstovsek, 2008; Horiguchi, et al., 2010). Using the combined treatment of DFO and WP1066 for 24 to 48 hours, a significant decrease in pSTAT-705 was observed, suggesting that tyrosine phosphorylation results from JAK2 activity in the surviving cells. Additional support for the origin of the survival signal was provided by the cell viability study conducted in this lab, which demonstrated a decrease in cell viability when treated with DFO in a dose dependent manner, further decreasing when WP1066 was added to the treatment. These results are supported by a study conducted by Sriuranpong, et al found that STAT3 is constitutively active in Head and Neck Squamous cell Carcinoma (HNSCC). They used multiple HNSCC cell lines to determine the origin of the signal. Several were found to have active STAT3 independent of the EGF receptor. The addition of a JAK inhibitor led to a decrease in STAT3 activation via tyrosine phosphorylation accompanied by a concurrent decrease in cell survival and proliferation (Sriuranpong, 2003). It was concluded that pSTAT3-705 activation was the result of an autocrine/paracrine loop through the production of IL-6 within several of the HNSCC cell lines, which led to subsequent activation of the gp130/JAK receptor.

This study also demonstrated that STAT3 was phosphorylated at its serine residue in non-treated NMB control cells, while cells surviving DFO treatment resulted in a significant decrease at pSTAT-727 in a time-dependent manner. A study conducted with Chronic Lymphocytic Leukemia cells found STAT3 to be constitutively active at its serine phosphorylation site, and that this activation leads to DNA binding and transcription of Bcl-2, Pim1, Bcl-X_L, Cyclin D_1, p21, and c-Myc, genes involved with the completion of cell cycle and prevention of apoptosis. Furthermore, a study conducted with human endometrial cancer cells documented constitutive activation of STAT3 at its serine residue. The use of the STAT3 inhibitor, HO-3867, resulted in

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a decrease in pSTAT3-727, and a subsequent 50% decrease in cell viability, all while expression levels of total STAT3 remained unchanged, and no changes in the level of pSTAT3-705 were recorded (Tierney, et al., 2014).

Interestingly, in this study, the changes of pSTAT-727 in NMB cells with DFO treatment alone was the same as that measured with combined treatment of DFO and WP1066. The evidence presented in our study demonstrates that tyrosine phosphorylation is related to cell viability and survival. However, it is not clear what function relates to the change in serine phosphorylation. Further study will be required to pinpoint this more accurately. DFO mimics hypoxia by preventing the degradation of the HIF1- α protein, and it negatively impacts cell viability by upregulation of the p21 and p27 proteins in breast cancer cells (Fu, 2007; Siriwardana, 2013). Its interference in the cell cycle is induced by direct inhibition of DNA synthesis via inactivation of the enzyme ribonucleotide reductase, and down regulation of Cyclin D1. Both of these events occurred at the intracellular level, as DFO can penetrate the cell membrane as demonstrated in Hela-Fucci cells (Bedessem, 2015; Guo, 2006).

This study demonstrated a definitive shift between pSTAT3-727 and pSTAT3-705 in human neuronal NMB cells when they encounter a stressor in the form of hypoxia, suggesting our cell model is shifting from proliferation to survival. We also documented a concurrent increase in total STAT3 expression in the cells that survived treatment. Future studies quantifying the expression panel of STAT3 target genes both before and after treatment of DFO and DFO/WP1066 could provide additional evidence that each phosphorylation event carries out a specific cellular function. Since only one cell type was used, measuring the secretion of cytokines such as IL-6 and TNF-α in the media before and after treatment could determine whether an autocrine/paracrine signaling loop is active in our system, and if this type of signaling

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influences the levels of pSTAT3-705 and pSTAT3-727 as with those studies previously mentioned.

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