CHARACTERIZATION OF A GP130 SIGNALING RECEPTOR POLYMORPHISM

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ABSTRACT

We previously reported an association between human herpesvirus 8 (HHV-8) seroprevalence and increased prostate cancer risk among men on the Caribbean island of Tobago. More recently, we have found a single nucleotide polymorphism (SNP) in the IL-6 gp130 signaling receptor at position 148 associated with increased prostate cancer risk among HHV-8 seropositive men. The high risk genotype (Gly) was associated with increased prostate cancer risk among HHV-8 seropositive men (OR= 3.1) compared to the low risk genotype (Arg). This research aims to further explore the effect of this SNP on gp130 function.

The gp130 genotype at position 148 was determined in lymphoblastoid B cell lines (LCLs) derived from Tobago men (representing high and low risk genotypes) and two prostate cancer cell lines (PC3 and DU 145; high risk and low risk respectively). Growth curves were performed by Dr. Jill Henning for LCLs by using 25ng/mL of Interleukin-6 (IL-6), Interleukin-11 (IL-11), or Oncostatin M (OSM). It was discovered that IL-6 had an effect on the growth of LCLs, but IL-11 and OSM did not. I repeated growth curves on LCLs using a concentration of 10ng/mL, and found that there was still a difference in growth at this lower concentration. Levels of phosphorylated STAT3 were measured on cells treated with 10 or 100ng/mL IL-6, IL-11, or OSM for various times (2-60 minutes). Comparative IL-6-mediated downstream signaling between the two genotypes was analyzed in LCLs at 10 minutes post-treatment using the

JAK/STAT Pathway PCR Array Plates (SABiosciences, Valencia, CA), and the Human IL-6 Pathway PCR Array Plates (Life Technologies, Grand Island, NY).

LCLs homozygous for the high-risk gp130 genotype grew significantly faster compared to LCLs homozygous for the low-risk genotype in response to IL-6 but not IL-11 or OSM. LCLs homozygous for both high-risk gp130 genotype as well as LCLs homozygous for the low-risk genotype both showed activation of STAT3 in response to IL-6 by 10 minutes post-treatment. DU 145 (low-risk genotype) cells showed STAT3 activation following IL-6 treatment while PC3 (high-risk genotype) failed to show any STAT3 activation even after 1hr of IL-6 treatment. Both cell lines showed STAT3 activation after OSM treatment while neither line showed activation following IL-11 treatment. RT-PCR analyses of JAK/STAT pathway genes and IL-6 pathway genes in LCLs following IL-6 treatment showed differential gene regulation between genotypes. For example, using the IL-6 pathway plates, the high-risk genotype showed a down-regulation of TP53BP2 (apoptosis stimulating protein of p53-2), while the low-risk genotype showed an up-regulation of this gene. This protein is known to inhibit cell growth and stimulate apoptosis, and is frequently suppressed in human cancers. This differential gene regulation of TP53BP2 is one example of a gene that is differentially regulated between the high- and low-risk genotypes. These data suggest that the G148R SNP of gp130 is involved in cell proliferation mediated by IL-6 downstream signaling.

The public health relevance observed by these results suggests that the resulting genotypes in the G148R polymorphism exhibit different biological affects upon treatment with cytokines that utilize the gp130 signaling receptor. The high-risk genotype could result in an increase in inflammation, which could ultimately contribute to the development or advancement of prostate cancer.

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1.0 INTRODUCTION

The idea that inflammation and cancer are associated is more than a century old. In 1863, Virchow hypothesized that the origin of cancer occurred at sites of inflammation[1]. Today, although the relationship between inflammation and cancer is more widely accepted and understood, the molecular and cellular mechanisms that mediate this relationship are still unclear. What we do know is that excessive, uncontrolled inflammation (chronic inflammation), increases your risk for developing cancer[2]. In response to infection or injury, the body's immune cells release cytokines and chemokines that attract more immune cells in an attempt to repair damage or eliminate pathogens. However, a failure to strictly control this immune response could result in a chronic inflammatory environment that damages DNA, and ultimately might favor the initiation of cancer[3].

Since chronic inflammation is associated with increased risk for cancer, and cytokines help to regulate the response to a stressor, several studies have looked for potential polymorphisms in cytokine genes or their cellular receptors for associations with cancer risk. Hussain et al. reported that polymorphisms in some cytokine genes are associated with an increased risk for prostate cancer[3]. For example, polymorphisms in interleukin-1 are associated with an increased risk for gastric cancer[4], while polymorphisms in IL-6 have been associated with colon and rectal cancers[5]. In addition, persistent or chronic viral infections can often set up a chronic inflammatory environment, and many viruses have been associated with an increased

risk for cancer. For example, hepatitis B virus and hepatitis C virus are both associated with an increased risk for liver cancer[1]. In addition, human papillomavirus has been shown to be the causative agent of cervical cancer[6], and is also associated with head and neck cancers[7]. The following research focuses on a specific polymorphism in the IL-6 cytokine signaling receptor, gp130 and how genotypes of this receptor, in conjunction with a herpes viral infection, could lead to an increased risk of prostate cancer.

1.1 CYTOKINES AND SIGNALING

1.1.1 TYPE I CYTOKINES

Cytokines are proteins that are secreted by cells and exert autocrine or paracrine functions by binding to receptors on cell surfaces and as a result transduce signals[8]. Cytokines have a broad range of actions on the target cell, including effects on cellular growth, differentiation, cell cycle control, apoptosis, cytolytic activity, and chemotaxis[8]. Although there is a diverse repertoire of cytokines, they can be divided into type I and type II cytokines. Type I cytokines (such as IL-6, TGF-β, and IFN-γ) have four α helices, denoted A, B, C, and D, and aid in enhancing cellular immune responses[8]. Type II cytokines (such as IL-4, IL-10, and IL-13) favor antibody responses[8]. Type I cytokines are further categorized by their cellular receptor[9]. Of particular interest to our research are the type I cytokines that share the glycoprotein 130 (gp130) signaling receptor.

1.1.2 IL-6 FAMILY OF CYTOKINES

Cytokines that share the gp130 signaling receptor are often referred to as being in the IL-6 family of cytokines. The interleukin-6 (IL-6) family of cytokines includes 8 cytokines: IL-6, IL-11, OSM (oncostatin M), LIF (leukemia inhibitory factor), CNTF (ciliary neurotrophic factor), CT-1 (cardiotrophin-1), IL-27 and CLC (cardiotrophin-like cytokine)[9,10]. These cytokines are pleiotropic and exhibit overlapping biological functions[11]. The cytokine receptors involved in binding and signaling can be divided into the non-signaling primary receptors (such as IL-6RAα, IL-11RA, and OSMR) and the signal transducing receptor gp130. The gp130 receptor is expressed by most, if not all, cells in the body[12] whereas the binding receptor expression is limited to cells that are known to respond to the respective cytokine. The IL-6 family of cytokines needs both their respective primary receptor and gp130 to induce intracellular signaling[10]. Binding of the cytokines to their primary receptors induces binding to and dimerization of gp130, and begins a cascade of events that lead to the induction of cytokine-responsive target genes[11]. One of the initial events following the association of cytokine, primary receptor, and gp130 is the activation of the JAK/STAT pathway.

1.1.3 JAK/STAT PATHWAY

The first step in the activation of the JAK/STAT pathway involves the activation of Janus tyrosine kinases (JAKs) in the cytoplasm, which occurs upon homodimerization of gp130 or herterodimerization of gp130 with LIFR or OSMR. The JAK family includes JAK1, JAK2, and TYK2[11], with JAK1 being essential for IL-6 signal transduction[10]. The activated JAKs phosphorylate tyrosine residues on the cytoplasmic end of gp130. These phosphorylated tyrosine

residues serve as docking sites for the Src homology 2 (SH2) domains of STAT proteins[13]. STAT denotes "signal transducer and activator of transcription"[9], and these proteins serve as substrates for tyrosine-phosphorylation by JAKs. Once phosphorylated, they are considered "activated", dissociate from the receptor, homo-or heterodimerize, and translocate to the nucleus to induce expression of target genes[14]. After termination of the signal, STATs translocate back to the cytoplasm[15]. There are seven major STATs that have been identified: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. This family of proteins is named for two reasons: they transduce signals through the cytoplasm, and also function as transcription factors once in the nucleus[16]. Of these, STAT3 is the most strongly activated by cytokines that utilize gp130 as their signal transducer. STAT1 is also activated, but to a much lesser extent[11].

STAT3 is structurally similar to the other STAT proteins, yet is the only member of the STAT family that leads to embryonic lethality when ablated, which suggests that it plays a crucial role in development[17]. STAT3 has a conserved amino-terminus, a DNA-binding domain, a SH2 domain that binds to tyrosine-phosphorylated gp130, and a carboxy-terminal transcriptional activation domain[18]. STAT3 becomes activated by tyrosine phosphorylation, like the other STAT proteins, at a single site by the carboxy-terminus (Y705)[10]. STAT3 can also become activated by serine phosphorylation at site S727, which is located within the transactivation domain[10]. Phosphorylation at either site triggers dimerization of STAT proteins and allows translocation to the nucleus[10].

1.1.4 GENES ACTIVATED BY THE JAK/STAT PATHWAY

Once the STAT proteins enter the nucleus they bind to target gene sequences through the transcriptional activation domain, which is poorly conserved among STAT proteins and allows

for unique transcriptional responses dependent upon which STAT protein is activated[15]. STAT3 has been implicated in a variety of cell systems and processes, and seems to have different functions in different cell types[19]. Traditionally, STAT3 was thought to induce a limited set of target genes in response to IL-6 during inflammation. The list of genes has grown considerably since its discovery and STAT3 since has been shown to regulate a baffling complexity of responses, and depending upon the target tissue is capable of inducing proliferation, survival, or apoptosis[19]. For instance, STAT3 stimulates B cell proliferation and inhibits apoptosis in B cells by induction of the antiapoptotic gene Bcl-2. However, in monocytes, STAT3 is known to down-regulate c-Myc and c-Myb and induce jun-B and IRF-1, which triggers differentiation and growth arrest in these cells. STAT3 also induces a number of genes that produce proteins necessary for the acute-phase response of the immune system including C-reactive protein, mannose-binding protein, serum amyloid A, and others[20,19].

Constitutive STAT3 activation by phosphorylation is present in a large majority of human neoplasms[21]. Most of the target genes of STAT3 are involved in tumorigenesis and metastasis[21]. It has been discovered that STAT3 is constitutively active in a number of tumor-derived cell lines, and this activation is necessary in order to maintain the transformed phenotype[22]. STAT3 has also shown to be involved in cell motility and migration under both normal and abnormal biological situations[21]. Overall, STAT3 seems to play a crucial role in both inducing and maintaining and environment that favors malignant transformation. The IL-6 family of cytokines are potent inducers of STAT3 and as a result, have been implicated in numerous human cancers, such as breast and prostate cancer, as well as many leukemias and lymphomas[23,16].

1.1.5 INTERLEUKIN-6

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by both lymphoid cells and non-lymphoid cells[24]. It is a 212 amino acid glycoprotein with a molecular mass ranging from 21 kDa to 28kDa, dependent upon the level of glycosylation and phosphorylation[24]. Traditionally, the cytokine binds to the interleukin-6 receptor (IL-6RA/gp80), and then this complex associates with gp130, allowing it to homodimerize with another molecule of gp130. Together, this complex initiates intracellular signal transduction, something that the IL-6/IL-6RA complex cannot do without gp130. The IL-6RA is mainly expressed on hepatocytes, neutrophils, monocytes, macrophages, some leukocytes, and a number of epithelial cells (such as prostatic epithelial cells)[12]. IL-6 acts as a B cell differentiation factor, and plays an essential role in immunoglobulin induction in these cells[24]. It has also been shown to act as a paracrine growth factor for EBV-immortalized B cells (lymphoblastoid cells)[25]. In hepatocytes, IL-6 acts as a hepatocyte-stimulating factor, inducing the production of proteins such as C-reactive protein, β_2-fibrinogen, and other acute-phase proteins produced in the liver[24]. IL-6 also promotes proliferation and differentiation of cytotoxic T cells[26]. IL-6 can also signal by utilizing a naturally occurring, soluble form of IL-6RA (sIL-6RA), which is found in various body fluids, such as urine and blood[12,27]. This complex of IL-6 and sIL-6RA binds and signals through gp130, a process termed trans-signaling[12]. Trans-signaling allows IL-6 to act on cells that would normally be unresponsive to the cytokine due to the lack of expression of the IL-6RA binding receptor. It has been shown that upon IL-6 binding to the IL-6RA, both IL-6 and the IL-6RA are internalized and degraded[28]. The internalization of IL-6/IL-6RA complex is largely dependent upon the cytoplasmic domain of gp130. Internalization of this complex, however, is not necessary for signal attenuation, and it is currently believed that *de novo* synthesized proteins are

responsible for the down-modulation of the STAT activity. The SOCS (suppressor of cytokine signaling) family of proteins has been implicated in this down-modulation[29].

IL-6 is considered both an inflammatory and anti-inflammatory cytokine and plays a central role in host defense mechanisms[26]. The activities of IL-6 are critical for balancing the interplay between acute and chronic immunologic responses, and recently it has proven to be a key player in the switch between innate and adaptive immune responses[24]. Unfortunately, like many other cytokines, an uncontrolled production of IL-6 can lead to altered cell growth and differentiation. Polymorphisms in cytokine genes and cytokine receptors are often linked to inflammation and cancer[3]. A SNP in a cytokine gene could potentially result in the overproduction of that protein, while a SNP in a cytokine receptor could lead to a heightened response by the cell or extended signaling. In both these scenarios, either overproduction of a cytokine or over sensitization by the receptor, chronic inflammation is likely to occur.

1.1.6 INTERLEUKIN-11

Interleukin-11 (IL-11) is a highly conserved, 178 amino acid protein that is also a member of the IL-6 family of cytokines[30]. IL-11, after binding to the primary receptor (IL-11RAα), also utilizes gp130 for signal transduction. It is a multifunctional cytokine that is produced throughout the body, although the in vivo source of IL-11 remains unclear[31,30]. Some functions of IL-11 include stimulation of megakaryocyte maturation and platelet production, growth of $CD34^+$ hematopoietic progenitor cells, inhibition of adipogenesis, stimulation of osteoclasts, and protection of cells from apoptosis and cellular necrosis in the gut mucosa[32,33]. The protein is rarely detected in serum of healthy subjects, but is readily detected in response to virus during viral-induced inflammation and in many cancers, such as breast and ovarian

cancers[31]. There are two isoforms of IL-11RA; the first is expressed in low levels in the brain, lung, spleen, heart, bladder, kidney, muscle, intestines, salivary glands, bone marrow, testis, ovary, and uterus, the second is expressed in the thymus, lymph nodes, and testis[34]. There is no evidence of a soluble form of the IL-11RA[34].

1.1.7 ONCOSTATIN M

Oncostatin M (OSM) is a secreted 28 kDa glycoprotein[35]. It is a multifunctional cytokine produced by activated T lymphocytes and monocytes[35]. Structurally and functionally, it is related to the IL-6 family of cytokines. OSM acts on a wide variety of cell types including endothelial cells, lung cells, hepatic cells, and many tumor cell lines, and elicits different responses depending upon cell type[35]. It has been implicated in cellular processes such as gene activation, cell survival, differentiation, and proliferation[36]. The OSM signaling receptor complex differs slightly from that of IL-6 and IL-11. OSM can utilize both the OSM receptor subunit and the LIF receptor subunit in combination with gp130. For example, OSM only needs one alpha receptor (either LIFR or OSMR) and one secondary signaling receptor (gp130) to form a heterodimer-signaling complex, whereas both IL-6 and IL-11 require two of their respective alpha receptors (IL-6RA or IL-11RA) and two secondary signaling receptors (gp130) to perpetuate signaling. The individual cytokine structures may have an effect on the difference in composition of signaling complexes. As stated previously, all IL-6 type cytokines are made up of four α-helices: A, B, C, and D. IL-6 and IL-11 have α-helices that are all arranged in the same direction, whereas for OSM the A α-helix is kinked[10]. This structural difference may explain the difference in the composition of the signaling complexes[10].

1.1.8 GLYCOPROTEIN 130

Glycoprotein 130 (gp130) is also known as the interleukin-6 signal transducer (IL6ST), and is known to be a key mediator in regulating inflammation and immune responses[3]. It is a 130 kDa glycoprotein, and a shared cytokine signaling receptor. Gp130 has six domains: an Ig-like domain (D1), cytokine binding domains (D2-D3), and three membrane-proximal fibronectin type III (FNIII) domains (D4-D6)[21]. The gp130 protein forms oligomeric signaling complexes with cytokine receptors and their respective cytokines, such as IL-6/IL-6RA, IL-11/IL-11RA, OSM/OSMR, etc[37]. The complexes of cytokine, the respective alpha-receptor, and gp130 must form in a 2:2:2 ratio in order to be competent for signaling for IL-6 and IL-11[38]. As stated previously, this is not the case for OSM, which is capable of signaling upon association of OSM, OSMR or LIFR, and gp130. In certain cell types, the localization of gp130 is concentrated in microdomains, such as lipid rafts and caveolae[10]. Some cells can produce a soluble form of gp130, (sgp130) using differential splicing. The sgp130 protein has been detected in human serum and shown to be a natural inhibitor of IL-6 trans-signaling by binding to IL-6/sIL-6RA complexes and preventing these complexes from binding to membrane-bound gp130[12]. The gp130 receptor is necessary for IL-6/IL-6RA internalization, and it is strictly dependent upon a di-leucine motif in the cytoplasmic domain of gp130[29]. However, internalization of gp130 is independent of signal transduction (IL-6/IL-6RA binding) and is most likely constitutively internalized via clathrin coated pits[39]. Since internalization of gp130 is not ligation-dependent, it is present on the cell surface at all times[29].

1.2 PROSTATE CANCER

1.2.1 EPIDEMIOGY OF PROSTATE CANCER

Prostate cancer is the most frequently diagnosed cancer in men in the United States[40]. In the United States in 2013, it is estimated that there will be 238,590 new cases of prostate cancer and 29,720 deaths[41]. Among the men alive today, it is estimated that 1 in 6 will be diagnosed with prostate cancer, and 1 in 33 will die from the disease[42]. Risk factors for prostate cancer include age, family history, and race[43]. The incidence and mortality rate of prostate cancer within the United States varies across ethnic groups. African American men and Jamaican men of African descent have the highest incidence and mortality rate of men in the U.S., compared to Caucasian men and men of Asian descent[42]. This trend is not limited to the United States, as a study completed in Trinidad and Tobago also shows that the prevalence of screening-detected prostate cancer was 3-fold higher in the men that were of African descent compared to the men who were of Asian Indian descent[44].

With prostate cancer being the second leading cause of cancer deaths in the United States, there is a necessity to better understand the risk factors for prostate cancer through both epidemiological and molecular research[45]. Although the cause of the difference in prostate cancer incidence and mortality between populations is not clear, there are several investigators who have suggested a genetic or hereditary component for prostate cancer susceptibility[40].

1.2.2 CYTOKINES AND PROSTATE CANCER

Human prostate cancer is a neoplasm, and its growth is initially dependent upon the androgen hormones, 5α-dihydrotestosterone and testosterone[13]. Hormones are chemical messengers released by a gland in one part of the body and typically carried to another organ or part, via blood, to act upon that part of the body[46]. Androgens are typically associated with the development of male sexual behavior, as well as maintenance of the male sex organs and the development of muscle mass[47]. Hormone therapy (androgen ablation) works to reduce the overall circulating levels of androgen in the body, and as a result alleviates the symptoms of prostate cancer by inhibiting prostate cancer growth and proliferation. Hormone therapy has been the standard treatment for prostate cancer for many years[14], but has far from cured the disease. Many prostate cancers return after hormone therapy, as hormone-insensitive metastases (meaning that they do not need androgen to continue to grow and metastasize), and the prognosis after relapse is very poor[14]. The mechanisms through which androgen-independence develops is unknown, however, interleukin-6 (IL-6) seems to play a role[43]. Up-regulation of IL-6 and the IL-6RA has been detected in malignant cancers[48]. Circulating levels of this cytokine are also increased in patients with hormone-insensitive cancers, suggesting that IL-6 may be an alternate marker of the androgen independent, metastatic phenotype[43,48].

There is currently no effective therapy or treatment for metastatic prostate cancer, and a large part of that is due to the fact that the underlying mechanisms that lead to the development and spread of metastatic prostate cancer are unclear. With that being said, a number of *in vitro* studies have been completed in an attempt to understand better the mechanism through which IL-6 may promote metastasis[49,50]. While multiple prostate cancer cell lines have been created for *in vitro* study, there are three that are most commonly used: LNCaP, PC3, and DU 145. LNCaP is

a hormone-sensitive cell line that does not produce IL-6 but responds to it in a paracrine manner, while PC3 and DU 145 are both cell lines from hormone-insensitive cancers that produce IL-6, and respond to IL-6 in both a paracrine and autocrine manner[48]. PC3 and DU 145 cell lines were used in this research. Both cell lines are epithelial cell lines developed from human prostatic adenocarcinomas that metastasized to other parts of the body, have a doubling time of about 34 hours[51,52], and do not express androgen receptors, PSA (prostate-sepcific antigen) or 5α-reductase (the enzyme that converts testosterone to its more active form, 5α-dihydrotestosterone, or DHT)[53]. All three of the prostate cancer cell lines, LNCaP, PC3, and DU 145, express the IL-6RA, IL-11Rα, OSMR, and gp130 receptors[54].

Because IL-6 has drawn considerable attention in the link between inflammation and cancer, the other members of the IL-6 family of cytokines are often overlooked. Recently, however, IL-11 is gaining attention as a potential player in the development of cancer as well. IL-11 has been implicated as a crucial cytokine in the promotion of gastric inflammation and associated tumorigenesis[55], as well as a serum marker of bladder cancer[56]. Interleukin-11 serum levels have also been associated with hormone-resistant prostate cancer, and has the potential to be an indicator for prostate cancer progression[57]. The IL-11Rα has also been involved in cancer development, and several studies have shown that there is increased expression of IL-11Rα in prostate cancers[58, 59].

Oncostatin M is also gaining considerable attention in the link between inflammation and cancer. OSM has been implicated in mammary tumor metastasis[60], as well as promoting proliferation in ovarian cancer cells[61]. It has been shown *in vitro* that OSM is capable of acting as a growth factor for prostate cancer cells[62]. *In vivo*, patients with metastasizing prostate cancer were found to have significantly higher levels of serum OSM when compared to benign

hyperplasia and non-metastasizing prostate cancer[63,64]. OSM has been suggested to be a better target than IL-6 for prostate cancer therapy in late stages of the disease due to lack of side effects associated with OSM depletion compared to IL-6 depletion[64].

Since all three of the cytokines described above seem to play a role in prostate cancer progression, it seems likely that polymorphisms in any of the cytokine genes, their primary receptors, or gp130 could affect the risk for developing advanced metastatic prostate cancer by altering the body's normal responses to inflammatory stimuli. Altering the normal responses could result in chronic inflammation, DNA damage, and ultimately an environment that favors the initiation of cancer.

1.2.3 INFLAMMATION AND PROSTATE CANCER

When effective, an inflammatory response can properly alert the body to an infection or injury and aids in the healing process. However, this natural defense mechanism, when not controlled properly, can lead to chronic inflammation and possible progression to chronic diseases[36]. As stated previously, a sustained inflammatory microenvironment favors the initiation of cancer. This is done through a number of mechanisms. Inflammation provides a constant supply of reactive oxygen and nitrogen species, cytokines, chemokines, and growth factors[3]. These elements are capable of altering crucial biological processes, such as cell proliferation, apoptosis, and cell cycle control, which are responsible for maintaining a homeostatic cellular environment. Continuous alterations in these processes can lead to genomic instability and mutations within the genome, which increases the risk of cancer development[3].

In addition to genetic factors playing a role in the etiology of prostate cancer, there have also been some reports of an infectious agent cofactor. Infectious agents have been more

commonly reported to be causative agents of cancer, and in 2008 it was estimated that worldwide, about 16.1% of cancers were attributable to infections[65]. Hayes et al. reported an elevated risk for prostate cancer among men that are sexually active, or have sexually transmitted infections[34]. The mechanism through which sexual activity and sexually transmitted infections is related to prostate cancer remains unclear[66], but one hypothesis would be that sexually transmitted infections could contribute to prostatic inflammation, and eventually lead to prostate cancer. Chronic inflammation in the prostate due to infections, hormonal changes, dietary factors, and other unknown environmental exposures could all contribute to prostatic inflammation, and in turn prostate cancer[43]. With this knowledge, there were a series of investigations completed to examine possible associations between sexually transmitted infections and sexual behavior in relation to the development of prostate cancer. There have been a number of human viruses investigated as causative agents or cofactors, such as herpes simplex virus (HSV), cytomegalovirus (CMV), papillomaviruses, and Epstein Barr virus (EBV), none of which showed a significant increase in risk for the development of prostate cancer[67].

1.3 HUMAN HERPESVIRUS 8

Human herpesvirus 8 (HHV-8/KSHV) is the causative agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD), which are all different types of cancers. It is a double-stranded DNA virus in the subfamily *gammaherpesvirus*. The HHV-8 genome encodes over 80 genes, and like all herpeviruses, is capable of establishing latency in the host, where it is incapable of being recognized by the host's immune system. The virus does not usually cause clinical symptoms in most healthy

humans, and in fact most primary infections in healthy individuals only result in mild symptoms such as diarrhea, fatigue, and localized rash[68]. Typically, it is when the immune system is compromised that this herpesvirus causes severe disease, however the virus is capable of reactivating in immunocompetent individuals as well[69]. When HHV-8 reactivates, it switches from latent to lytic infection in the host, and begins producing infectious virions[69]. Production of infectious virus particles can kill the host cell. Both the lytic and latent phases of HHV-8 replication are thought to be important in the development of HHV-8-associated cancers[69]. The mode of transmission for HHV-8 is still being defined[68]. HHV-8 DNA can be detected in semen and in the female genital tract, as well as saliva and blood[70].

The HHV-8 genome is approximately 165 kb in length with over 85 open reading frames. There are currently 20 known genes that are specific to HHV-8, many of which are homologs to cellular genes, and a number of these are related to known oncogenes[69]. Viral Bcl-2, cyclin D, interleukin-6, G-protein-coupled receptor, and ribonucleotide reductase are a few of the cellular homologs encoded by the HHV-8 genome[68].

1.3.1 VIRAL IL-6

Viral IL-6 (vIL-6) is a protein encoded by ORF K2 of HHV-8. It is 204 AA in length and is predicted to be 24.3 kDa[71]. The vIL-6 shares about 25% identity with human IL-6, but it has been reported that it can stimulate all of the known human IL-6 signaling pathways[72]. Viral IL-6 is of particular interest because it activates IL-6 signaling pathways and continues transmitting growth stimulatory signals. Normally, once the IL-6 signaling pathway is activated, IL-6RA is down-regulated. However, vIL-6 is capable of bypassing the need for IL-6RA and binding directly to gp130, keeping the growth stimulatory signal operating by activating STAT1, 3, and 5

as well as the MAP kinase-signaling cascade[69]. Constitutive activation of STAT3 has been shown to have oncogenic properties (cell transformation and tumorigenesis)[69], and has been implicated with promoting metastatic progression of prostate cancer[73].

Due to the fact that HHV-8 has oncogenic potential, is sexually transmitted, is found in some prostate tissues, and has an interleukin-6 homolog, it is a practical candidate for an infectious agent cofactor for the development of prostate cancer.

1.3.2 HHV-8 SEROPREVALENCE AMONG MEN FROM TOBAGO WITH PROSTATE CANCER

Our laboratory completed a case-control study on participants from the Caribbean nation of Trinidad and Tobago, and also from the United States, to test whether HHV-8 seropositivity was associated with an increased risk for prostate cancer. Our findings showed a significant increase in risk among HHV-8 seropositive men when compared to corresponding control groups. In Tobago, men with prostate cancer were more likely to be HHV-8 seropositive when compared to the control subjects without cancer (OR 2.24; 95% CI 1.29-3.90), or to healthy men from Trinidad (OR 2.63; 95% CI 1.54-4.50). Among United States men, there was also a difference reported: Pittsburgh men with advanced prostate cancer had a higher seroprevelance rate when compared to blood donors from across the U.S. (OR 4.67; 95% CI 1.9-11.65)[45]. However, additional studies from our laboratory comparing African American men from the Washington D.C. area and Caucasians from Italy reported that there was not a significant difference in HHV-8 seroprevalence between patients with prostate cancer and controls[74]. It should be noted that there was a difference in the sampling of groups between these different studies. In the Tobago cohort, the controls consisted of men with normal DRE and PSA values

(clean controls)[45] while in the Washington D.C. / Italy study, the controls consisted of men with benign prostatic hyperplasia (BPH)[74]. Thus the controls were not the same. What is particularly troubling is that the control group in the Washington D.C. / Italy study consisted of men with BPH, since HHV-8 could be setting up a chronic inflammatory environment in the prostate, resulting in BPH. Our laboratory collaborated in an additional study, which similarly did not rule out men who had BPH in the control group[75]. As a result, the major differences between these studies were what the researchers were using as controls, which can drastically affect how the results are interpreted.

In addition to being suggestive of an androgen-independent phenotype, elevated IL-6 levels have also been associated with increased prostate cancer morbidity, as well as a number of other chronic diseases[48]. As a result, specific polymorphisms in cytokine genes and their receptors have been associated with an increased risk of cancer[3]. This information, together with the findings that men of African descent are at an increased risk for prostate cancer compared to Caucasian men or men of Asian descent led our laboratory and collaborators to search for a polymorphism in some aspect of the IL-6 signaling pathway that could lead to an increased risk for prostate cancer development.

1.4 R148G POLYMORPHISM IN GP130

The laboratory of Dr. Robert Ferrell at the University of Pittsburgh, Graduate School of Public Health sequenced nine reputed single nucleotide polymorphisms (SNPs) in gp130. Of the nine, two were informative: rs3730293 and rs3729960. Rs3729960 is a glycine (GGT) to arginine (CGT) transition at position 148 in the extracellular ligand-binding domain of gp130.

This SNP was genotyped in 221 cases of prostate cancer and 260 controls. The initial findings from Dr. Ferrell's laboratory showed that there was not an increased risk for prostate cancer among any of the genotyped SNPs. However, further analysis that included both the HHV-8 seropositivity along with genotype combinations (GG, CG, CC) showed an increased OR of 3.41 (95% CI 1.36-8.55, p=0.009) for prostate cancer in men who are HHV-8 seropositive and have the GG genotype compared to men who are HHV-8 seronegative with the GG genotype (Table 1).

Table 1: HHV-8 gp130 Association with Prostate Cancer Risk

Table 1: HHV-8 gp130 Association (Logistic Regression Analysis) with Prostate Cancer Risk								
		Case		Non-Case				
HHV-8	gp130	N	%	N	%	OR	95% CI	p-value
-	GG	37	16.7	48	18.5	1.0		
-	CG	67	30.3	95	36.5	0.92	0.54-1.56	0.74
-	CC	41	18.6	57	21.9	0.93	0.52-1.68	0.82
+	GG	21	9.5	8	3.1	3.41	1.36-8.55	0.009
+	CG	38	17.2	38	14.6	1.3	0.70-2.42	0.41
+	CC	17	7.7	14	5.4	1.58	0.69-3.60	0.28
Total		221		260				

Structural modeling of this polymorphism shows that exchanging a glycine for an arginine does not disrupt the overall integrity of the molecule, but may affect the stability and function (Figure 1). It is not apparent whether or not the polymorphism affects the binding of gp130 to its respective primary cytokine receptors (IL-6RA, IL-11RA, or OSMR), but the

location of the polymorphism (in the D2 domain) is located within the cytokine-binding region of gp130. *Benrick et al.* suggest a few mechanisms for how this polymorphism might affect the stability and function of gp130: Arg148 hydrogen bonding may stabilize the surface loop in domain 2 of the receptor. The more stabilized loop could influence the orientations of the cytokine binding domains, and affect the formation of a hexameric complex between the cytokine, the alpha receptor, and gp130[37].

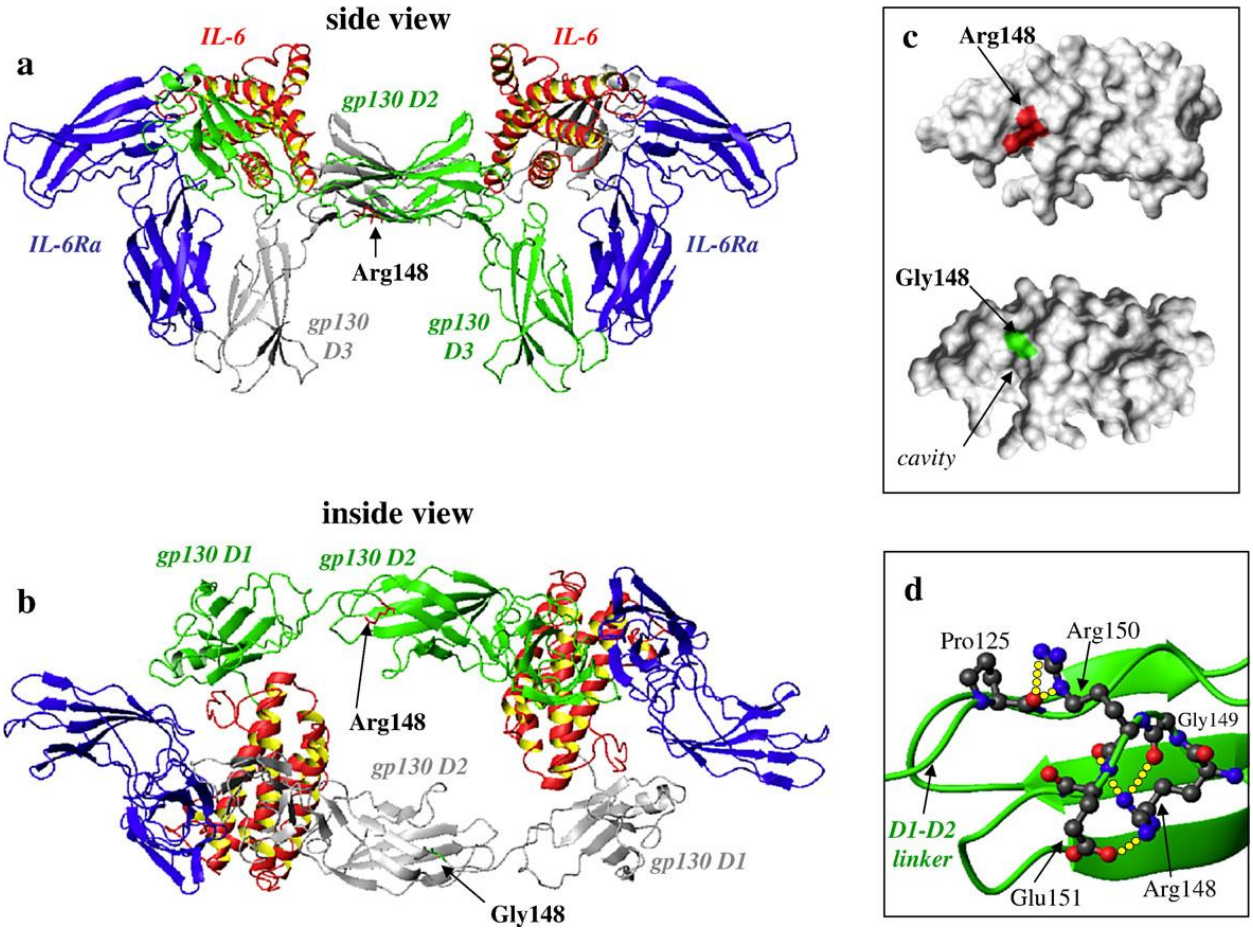


Figure 1: Location of gp130 148Gly/Arg. Figure and legend reprinted with permission from *Regulatory Peptides*, Vol 146, *Benrick et al*., A non-conconservative polymorphism in the IL-6 signal transducer (IL6ST)/gp130 is associated with myocardial infarction in a hypertensive population, 189-196, 2008, with permission from Elsevier: Location of gp130 148Gly/Arg in the structure of a hexameric complex between human IL-6, the cytokine homology region of IL-

6RAα, and the D1, D2 and D3 domains of gp130. (a) Backbone ribbon representation of the complex (side view) with the two IL-6 molecules coloured in red/yellow, the IL-6RAα fragments colored in blue, an the two gp130 fragments colored in yellow and grey, respectively. (b) Inside view of the complex following a 90° rotation about a horizontal asix; the complex is observed from 'underneath' as compared to the view in (a). The position of Gly148/Arg148 in the D2 domain of gp130 is indicated by showing an arginine substitution modeled into position 148 (in red) in one of the gp130 fragments and the original glycine (in green) in the second fragment. (c) Surface representation of the two symmetry-related D2 domains of gp130. The upper molecule shows a model of the D2 surface with substituted Arg148 (in red) and how the substitution might fill out a cavity next to Gly148 (in green) in the lower molecule. (d) Detailed view of how the Arg148 substitution can be modeled into the gp130 D2 domain so that three hydrogen bonds (yellow dotted lines) are formed to the backbone residues 149 and 150 and so that a hydrogen-bond/salt bridge is formed between Arg158 and the side chain of Glu151. The side chain of Arg150 supports the backbone of the D1-D2 linker peptide at the position of Pro125, as indicated. Molecular graphics illustrations were created using MolMol 2K.2"[37].

1.4.1 R148G AND INCREASED RISK FOR MYOCARDIAL INFARCTION

This particular SNP, a glycine to arginine transition at position 148 in the extracellular ligand-binding domain of gp130 (Gly148Arg) has been implicated in other diseases and conditions as well. A prospective randomized clinical trial completed by *Benrick et al.* demonstrated that there was a significantly decreased odds ratio for getting a myocardial infarction (MI) in individuals with the arginine variant (OR=0.56, p=0.02). They suggest that the arginine variant of gp130 is not completely void of activity, but rather that the substitution for arginine in place of the glycine could influence the cytokine binding domains of gp130, and perhaps result in a decreased responsiveness to cytokines utilizing gp130, and as a result a decrease in inflammation[37]. They followed up their statistical analysis with *in vitro* work. After transfecting BAF/3 cells with either the gp130 148Gly variant or the gp130 148Arg variant, *Benrick et al.* measured cellular proliferation and activation of STAT3 in response to Hyper-IL-6

(HIL-6), which can stimulate gp130 even in the absence of membrane-bound IL-6RA. Although there was no difference in gp130 surface expression between the two cell lines, there was a significant difference in their responsiveness to HIL-6, as shown by a difference in cellular proliferation and activation of STAT3, suggesting that the gp130 148Arg variant is associated with decreased biological activity[37].

1.4.2 R148G AND INCREASED RISK FOR HYPERANDROGENISM

A study completed by *Escobar-Morreale et al.* found a connection between the Gly148Arg polymorphism and hyperandrogenism (an endocrine disorder characterized by excessive production and/or secretion of androgens). In addition to a decreased risk of myocardial infarction, it was also found that the 148Arg allele was more frequent in controls than in hyperandrogenic patients (0.17 vs. 0.08, p=0.026). *Escobar-Morreale et al.* found that the basal levels of 11-deoxycortisol and 17-hydroxyprogesterone were lower in controls with the 148Arg allele. These subjects also had a decreased response to adrenocorticotropin and a decrease in free testosterone levels. These results support the notion that proinflammatory genotypes influence the pathogenesis of hyperandrogenism[76]. In this study, the 148Arg allele was again associated with a decrease in biological activity.

In the studies completed by *Benrick et al.* and *Escobar-Morreale et al.*, the 148Arg allele was associated with decreased biological activity. Both studies show an association to a physiological outcome, mycocardial infarction and hyperandrogenism, respectively[37,76]. However, neither study examined the mechanism of action of this polymorphism. The decreased biological activity could be due to a number of factors, and this project sought to further characterize this polymorphism in various cell types.

1.5 HYPOTHESIS

We previously showed an increased OR for developing prostate cancer is 2.24 among men who are HHV-8 seropositive. In addition, the OR for developing prostate cancer increases to 3.41 among men who are HHV-8 positive and have the GG allele of the G148R SNP. We hypothesize that the GG allele (high-risk) of this SNP has an increased biological activity compared to the CC allele (low-risk) in response to cytokines that utilize this receptor. This increased biological activity results in an amplified inflammatory response, which ultimately favors the initiation of cancer. We believe that the G148R SNP in gp130 could also interact with the viral IL-6 produced by HHV-8, which also utilizes the gp130 receptor.

2.0 MATERIALS AND METHODS

2.1 CELL CULTURE

Lymphoblastoid cell lines (LCLs) (see below) were maintained in RPMI 1640 (Cellgro® Cat # 10-040) with 50µg/mL gentamycin, 10% fetal bovine serum (FBS), and supplemented with 10ng/mL of rhIL-6 (Gibco Cat. # PHC004).

Prostate cancer cell lines (PC3 and DU 145) were maintained in DMEM (Cellgro® Cat # 10-013) with 50µg/mL gentamycin and 10% FBS.

2.2 GENERATION OF LCLS

LCLs were generated by *in vitro* transformation of peripheral B cells with Epstein Barr Virus (EBV). Briefly, $5x10^5$ peripheral blood mononuclear cells (PBMCs) in 1 mL of B-cell media [BCM; 500mL of RPMI 1640, 10% FBS, 5.5mL of 100x L-glutamine (Gibco Cat. # 10-040-CV), 10,000 U/mL Pen/10,000µg/mL Strep (Gibco), 100mM Sodium Pyruvate (Gibco), 100x MEM Non-essential amino acids (Gibco), and 1M Hepes (Gibco)] were mixed with 1mL of EBV virus supernatant and incubated at 37°C for 20 minutes in a 15mL conical tube. An additional 8mLs of BCM was added along with cyclosporine (final concentration 1µg/mL). The cells were plated at 100µL per well in a 96 well plate (final concentration 5,000 PBMCs per well

of a 96 well plate, approximately 250 B cells). On day 13, 75µL of BCM was added. At 7 weeks, the cells from each well were transferred to a well in a 24 well plate. At 10 weeks the cells from each well in the 24 well plates were transferred to a T-25 flask. The LCLs were made in the laboratory of Dr. Giovanna Rappocciolo at the University of Pittsburgh Graduate School of Public Health.

2.3 GP130 GENOTYPING

The gp130 genotype of each cell line was determined using a TaqMan® Sample-to-SNP™ kit specifically designed to analyze the G148R SNP according to the manufacturer's instructions (Applied Biosystems Cat. # 4403313). Cells (either LCLs or prostate cancer cell lines) were spun down and resuspended in fresh media. 20µL of lysis solution (supplied in the kit) was added to 2µL of cells, vortexed, pipetted up and down, and incubated at room temperature for three minutes. Next, 20µL of stabilizing solution (supplied in the kit) was added to this mixture and mixed well. Master mix solution was made by adding 5µL of Taqman GTXpress master mix, 0.5µL of the gp130 SNP assay primers, and 2.5µL of nuclease-free water. 2µL of the DNA lysate was added to 8µL of the master mix solution. Each 10µL sample was added to appropriate wells of a 96-well plate, sealed, centrifuged and subject to PCR amplification under the following cycling conditions:

Table 2: PCR Cycling Conditions for gp130 Genotyping

Step	Temperature	Duration	Cycles
Pre-PCR Read	25°C	30 seconds	Hold
Enzyme Activation	95°C	20 seconds	Hold
Denature	95°C	3 seconds	40 cycles
Anneal/Extend	60°C	20 seconds	
Post-PCR Holding Stage	25°C	30 seconds	Hold

Two dyes were used to indicate each allele: VIC indicates a C (cytosine) allele, while FAM indicates a G (guanine) allele. Based off of the allele frequency, the StepOnePlus™ software plots the graph as an Allele 1 versus Allele 2, with each well of the 96-well plate treated as an individual point on the plot. Where each sample clusters on the allelic discrimination plot determines the genotype. There are three possible genotypes (CC, GC, and GG). Nuclease-free water was used as a negative control.

2.4 GROWTH CURVES

2.4.1 GROWTH CURVES BY TRYPAN BLUE EXCLUSION

Growth curves of the LCLs were performed by trypan blue exclusion. Cells were diluted 1:10 in a 0.2% trypan blue solution (Roche Cat. # 05650640001) and cell counts determined using a hemocytometer. Trypan blue stains dead cells blue, while living cells remain unstained. Thus, a trypan blue exclusion assay permits determination of the number of live and dead cells in

each sample. LCLs were IL-6 starved overnight and seeded the following morning at $2.5x10^5$cells/mL. Cells were either left untreated or given 10ng/mL of rhIL-6 (Gibco Cat. # PHC004). This serves as time zero, and an aliquot of cells from each flask (untreated or rhIL-6-treated) were collected at 24, 48, 72, 96, and 120 hours post treatment and counted. Graphs were plotted using GraphPad Prism software.

Growth curves of the prostate cancer cell lines were performed using the method described for the LCLs. Cells were seeded at $7.5x10^4$ cells/well in 2 mLs in a 6 well plate and allowed to adhere to the wells for 8 hours. Regular media with serum was aspirated off, and serum-free DMEM was added to the cells for overnight serum-starvation. The next morning, cells were either treated with DMEM with serum but without cytokines or with 10ng/mL of rhIL-6, rhIL-11, or hOSM (Gibco Cat. # PHC004, R&D systems Cat. # 218-IL, and Gibco Cat. # PHC5015 respectively) diluted in DMEM with serum. This served as time zero, and cells from 2 wells were harvested by trypsin detachment and counted at 24, 48, 72, and 96 hours post treatment. Experiments were completed in duplicate, and graphs were plotted on GraphPad Prism software.

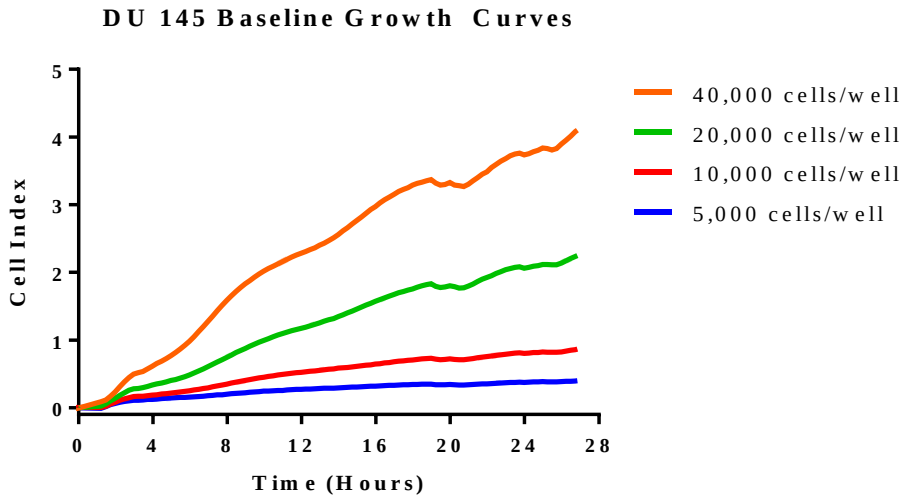
2.4.2 GROWTH CURVES USING XCELLIGENCE

The Xcelligence machine (Roche, Inc) is a real-time cell analyzer, which measures the electrical impedance of cells in real time by electrodes on specialized *E-plates*. As cells grow and spread over the electrodes, the impedance of each well increases. The Xcelligence computer program can be designed to take impedance measurements at regular intervals providing real-time measurements of cell growth. The measurement is termed “cell index”, and an increase in

cell index correlates directly with electrical impedance, which is indicative of the biological activity of cells, and changes based on cellular growth, morphology, and viability.

Prior to treatment with cytokines, we needed to determine the appropriate number of cells to add per well in the *E-plates*, which can vary based on cell size and growth rate. The Xcelligence machine is only capable of being used with adherent cells and as a result only PC3 and DU 145 were used in these experiments (LCLs are non-adherent). Cells were seeded at densities of 10, 20, 30, or 40,000 cells/well in quadruplicates in *E-plates*. The attachment and proliferation was monitored every 30 minutes for 72 hours. Cell-sensor impedance was expressed as an arbitrary unit called the cell index. Each cell type has its own unique size, morphology, and growth rate, so this step is necessary to define a reasonable number of cells to use in the growth assay in response to cytokines. For DU 145 cells, 40,000 cells/well was chosen, and for PC3 cells, 10,000 cells/well was chosen based on the initial cell titration growth curves (Figure 2). It was recommended that we use a concentration of cells that allowed us to see changes in growth over a specified period of time, without plateauing too quickly. We chose these concentrations (40,000 and 10,000 cells/well, respectively) because this concentration allowed for linear growth in terms of cell index. Since different starting concentrations are used for each cell line, raw data comparisons should not be made between cell lines, but rather between treatments for each cell line.

A.



B.

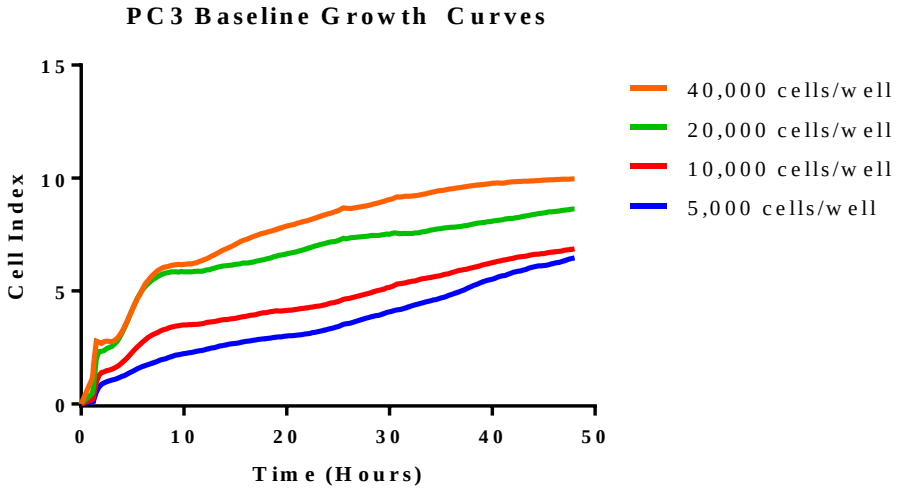


Figure 2: Baseline Growth Curves of DU 145 and PC3. Cells were plotted at 40, 20, 10, or 5,000 cells/well and allowed to settle for 30 minutes before placing them in the Xcelligence machine. Wells were run in triplicate, and cell index values were plotted and graphed using GraphPad Prism.

The growth curves in response to 10, 25, or 100ng/mL of IL-6, IL-11, or OSM were performed on both PC3 and DU 145 cells. Briefly, cells were seeded at the appropriate densities,

and allowed to adhere for approximately 4 hours before increasing amounts of cytokines were added. Impedance measurements were taken from each well every 30 minutes for the next 48 hours.

2.5 WESTERN BLOTTING

Phosphorylation of STAT3 (STAT3 activation) was measured by western blot using an antibody specific for the phosphorylated form of STAT-3. LCLs were IL-6 starved overnight and seeded into T-25 flasks so that the final concentration of cells was 10,000 cells per µL, and increasing concentrations (10 or 100 ng/mL) of cytokines (rhIL-6, rhIL-11, or hOSM) were added to the cells, or they were left untreated. At timepoints, aliquots of cells were removed and lysed in 4X lysis buffer (4X SDS Page Lysis Buffer: 6.5% SDS, 166mM Tris-HCl pH 6.8, 16% β-mercaptoethanol, 13% glycerol, 3% Bromophenol blue), to give a final concentration of 1X lysis buffer. For example, if we removed 300µL of cells, we would add it to 100µL of the lysis buffer. The final concentrations of all cell lysates for experiments were 10,000 cells/µL. This solution was boiled for 10 minutes, sonicated, and loaded into NuPAGE® Novex® 3-8% Tris-Acetate Gels (Life Technologies Cat. # EA03785BOX). Gels were run for approximately 2 hours at constant voltage of 120V and electrotransferred onto a nitrocellulose membrane (Amersham Cat. # 45-000-930) overnight at 14V. The membranes were analyzed for the presence of Tyr705-phosphorylated STAT3 using an anti-pSTAT3 antibody (Santa Cruz Biotechnology Cat. # sc-8059) and for actin levels using an anti-actin antibody (Santa Cruz Biotechnology Cat. # sc-1616). After incubation with the appropriate antibody, membranes were washed and exposed to SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific Cat. # 34080) for 10

minutes at room temperature in the dark. After exposure, membranes were washed and X-ray film (GeneMate, Blue Lite Autorad Film, Cat. # F-9023-8X10) was exposed to the membranes for desired time (differed depending on antibody concentration), and then developed with an imaging device.

2.6 IMAGEJ SOFTWARE ANALYSIS

ImageJ software (National Institute of Health) was used to analyze and quantify the results obtained from the western blots. Exposed X-ray film produced in the western blot procedures was scanned and images uploaded to ImageJ software. Bands were analyzed and quantitated using the software. Each pSTAT3 band was normalized first to its respective actin band, and then this ratio was normalized to the untreated control for each time point. For example, each sample or lane has an actin value and a pSTAT3 value. If actin was 10,000 and pSTAT3 was 250 for the untreated control, the ratio was 250/10,000, or 0.025. If the treated sample values were 20,000 for actin, and 1,500 for pSTAT3, the treated ratio was 1,500/20,000, or 0.075. Now we can normalize to the untreated control by dividing the ratio for the treated by the untreated (0.075/0.025) and we get a value of 3. This indicates that the treated sample had 3 times the amount of pSTAT3 compared to the untreated. Graphs were plotted using GraphPad Prism software.

2.7 GENE EXPRESSION ANALYSIS

2.7.1 RNA ISOLATION AND CDNA SYNTHESIS

$3x10^6$ LCLs were used per timepoint and treatment. For example, we used 10 minutes as a treatment time, so we needed $3x10^6$ cells for treatment with IL-6, and we needed $3x10^6$ cells that were left untreated. $6x10^6$ cells were IL-6-starved overnight in RPMI with 50µg/mL gentamycin and 10% FBS. The next day, cells were collected, centrifuged and resuspended in fresh RPMI at a concentration of $3x10^6$ cells/mL in 1 mL for each sample (treated and untreated). At this time, cells were either left untreated or treated with 10ng/mL of rhIL-6. At 10 minutes post treatment, cells were spun down, supernatants removed, and cells were frozen at -80°C for later RNA isolation. RNA isolation was performed using RNeasy Mini Kit (Qiagen Cat. # 74104) according to the manufacturer's instructions and the amount of RNA present was quantitated using a nanodrop machine (Thermo Scientific). 2µg of total RNA was used for each sample for cDNA synthesis. The cDNA synthesis was completed using the RT^2 First Strand Kit (Qiagen Cat. # 330421) according to the manufacturer's instructions. The cDNA was stored at -20°C until use. The cDNA made from 2µg of total RNA was divided equally into each well of a 96 well plate.

2.7.2 JAK/STAT PATHWAY PCR ARRAY

The JAK/STAT Pathway PCR Array was performed using plates from SABiosciences (Cat. # PAHS-039Z), which contains 84 primers for genes related to JAK/STAT mediated signaling, five housekeeping genes, and seven quality control wells. The isolated cDNA, made

from 2μg total RNA, was added to RT^2 SYBR Green/ROX qPCR Mastermix (SABiosciences Cat. # 330522), and then plated into each of the 96 wells. The following table shows the volumes used per reaction:

Table 3: PCR Reaction Volumes for Gene Expression Analysis using JAK/STAT Plates

Component	Volume (μL) per 1 reaction
Master Mix (2X RT^2 SYBR Green Master Mix)	12.5
cDNA+ Nuclease-free water	12.5
Total volume per reaction (well)	25.0

Plates were sealed with optical adhesive film and centrifuged to remove any air bubbles. The following thermal-cycling profile was used:

Table 4: PCR Cycling Conditions for Gene Expression Analysis using JAK/STAT Plates

Thermal-cycling profile			
Parameter	Polymerase activation	PCR (40 cycles)	
	Hold	Denature	Anneal/Extend
Temperature (°C)	95	95	60
Time (mm:ss)	10:00	00:15	01:00

2.7.3 HUMAN IL-6 PATHWAY PCR ARRAY

The IL-6 Pathway PCR Array was performed using plates from Applied Biosystems (Cat. # 4418794), which contains 92 primers for genes associated with IL-6 pathways and four primers of genes for candidate endogenous controls. The isolated cDNA, made from 2µg of total RNA, was added to Taqman® Fast Advanced Mastermix (Cat. # 4444556), and then plated into each of the 96 wells. The following table shows the volumes used per reaction:

Table 5: PCR Reaction Volumes for Gene Expression Analysis using IL-6 Plates

Component	Volume (µL) for 1 reaction
cDNA template + Nuclease-free water	10.0
TaqMan® Fast Advanced Master Mix (2X)	10.0
Total volume per reaction (well)	20.0

Plates were sealed with optical adhesive film and centrifuged to remove any air bubbles. The following thermal-cycling profile was used:

Table 6: PCR Cycling Conditions for Gene Expression Analysis using IL-6 Plates

Thermal-cycling profile				
Parameter	UNG incubation	Polymerase activation	PCR (40 cycles)	
	Hold	Hold	Denature	Anneal/Extend
Temperature (°C)	50	95	95	60
Time (mm:ss)	02:00	00:20	00:01	00:20

2.7.4 EXPRESSION SUITE ANALYSIS

Gene Expression Analysis was completed using ExpressionSuite Software from Life Technologies™. This software uses the $2^{(-\Delta\Delta Ct)}$ method in order to quantify relative gene expression across a large number of genes. There are two different ways to analyze data from real-time PCR: absolute and relative. Absolute quantification determines the input copy number of the transcript of interest by using a standard curve. We used relative quantification, which describes the change in expression of a target gene in a treated sample relative to a reference group (untreated). For our purposes, using relative quantification is sufficient. For example, from these data we are able to determine the fold induction or reduction of gene expression following 10ng/mL of IL-6 treatment. In order to perform relative quantification, the cycle threshold (Ct) values for each target gene were calculated by the RT-PCR machine (StepOnePlus™) based on a threshold value deemed appropriate for each plate. When each Ct is calculated, the $2^{(-\Delta\Delta Ct)}$ method can be used, as described below[77].

For these experiments, both internal and external controls are necessary. The internal controls are the housekeeping genes, in order to normalize for the amount of RNA added to each well. The external controls are the untreated plates, so that the normalized gene expression can be compared between treated and untreated samples. The first step is to calculate the ΔCt (change in Ct) for each gene of interest (GOI). This is achieved by taking the GOI Ct and subtracting from it the housekeeping gene (HKG) Ct. In some scenarios, more than one HKG is used, and in these cases, an average of the HKG Cts are used. The ΔCt is determined for each gene to normalize the amount of RNA added to each well. Once the ΔCt is calculated for each GOI on each plate, the ΔΔCt can be calculated by taking the ΔCt for each GOI of the treated plates and subtracting from it the ΔCt for each GOI of the untreated plates. This represents the

ΔΔCt value. In order to calculate the fold change for each GOI, the formula $2^{(-\Delta\Delta Ct)}$ was used. This value represents the relative quantity of the GOI in the treated samples compared to the untreated samples. For our experiments, our treatment was 10ng/mL of rhIL-6, and we measured relative fold changes compared to untreated samples at 10 minutes post-treatment. The formulas used and an example of these calculations are shown below:

$\Delta Ct_{treated} = \Delta Ct\ (GOI)_{treated} - \Delta Ct\ (HKG)_{treated}$

$\Delta Ct_{untreated} = \Delta Ct\ (GOI)_{untreated} - \Delta Ct\ (HKG)_{untreated}$

$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{untreated}$

Fold change = $2^{(-\Delta\Delta Ct)}$

Example:

Table 7: Sample ΔΔCt to Fold Change Calculation

Sample	GOI Ct	HKG Ct	ΔCt [ΔCt (GOI)– ΔCt (HKG)]
Treated	25.2	18.5	6.7
Untreated	27.4	19.2	8.2
$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{untreated} = 6.7 - 8.2 = -1.5$			
Fold Change = $2^{(-\Delta\Delta Ct)} = 2^{-(-1.5)} = 2^{(1.5)} = 2.8$			
Conclusion: The treated GOI was up-regulated by 2.8 fold when compared to untreated GOI			

2.8 FLOW CYTOMETRY

Flow cytometry was performed on both the LCLs and prostate cell lines to determine the expression of the IL-6 receptors gp80 and gp130. LCLs were collected by centrifugation and resuspended at a concentration of $1x10^6$ cells/mL in 4% formaldehyde (1 mL 37% formaldehyde,

1 mL 9X PBS, 7 mL ddH_2O) for 15 minutes at room temperature. After fixation, cells were washed twice and resuspended in 1X PBS at $1x10^6$ cells/mL. 100µL of the cell mixture was added to the desired number of wells of a 96 well plate, based on how many staining variables were needed. Cells were either left unstained, stained with isotype control antibodies for gp130 and gp80, or stained with antibodies for gp130 and gp80. For the isotype controls, 10µL of antibodies were used as isotype controls for gp130 or gp80 (IL-6Rα) (isotype control, mouse IgG1, PE conjugated BD Pharmingen™ Cat. # 555749 or isotype control, mouse IgG1, APC conjugated BD Pharmingen™ Cat. # 555751, respectively). In order to stain for IL-6Rα, 5 µL of APC mouse anti-human gp80 (BD Pharmingen™ Cat. # 562090) was used on each desired sample. To stain for gp130, 20 µL of PE mouse anti-human gp130 (BD Pharmingen™ Cat. # 555757) was used. Cells with antibodies were incubated for 30 minutes at room temperature. After incubation, cells were spun down, supernatant was aspirated off, cells were washed twice in 1XPBS and resuspended at a final volume of 1mL 1XPBS in flow cytometry tubes (BD Falcon™ 5mL polystyrene round-bottom tube Cat. #352058). Samples for flow cytometry were read on the BD Accuri™ C6. Samples were first gated on a live population of cells based on the forward and side scatter properties, and then a histogram was made using the correct optical filter (FL4 for gp80 and FL2 for gp130). Histograms for unstained, isotype controls, and antibody of interest were overlayed to determine whether or not the receptor was present.

The table below (Table 2) was made as a reference to the reader so that it is less confusing to remember the cell lines we used and their respective gp130 G148R alleles.

Table 8: Cell Lines used in this Research & their gp130 Status

Cell Line	Cell Type	gp130 Status
Low-Risk LCL	Lymphoblastoid Cell Line	CC
High-Risk LCL	Lymphoblastoid Cell Line	GG
DU 145	Prostate Cancer Cell Line	CC
PC3	Prostate Cancer Cell Line	GG

3.0 RESULTS

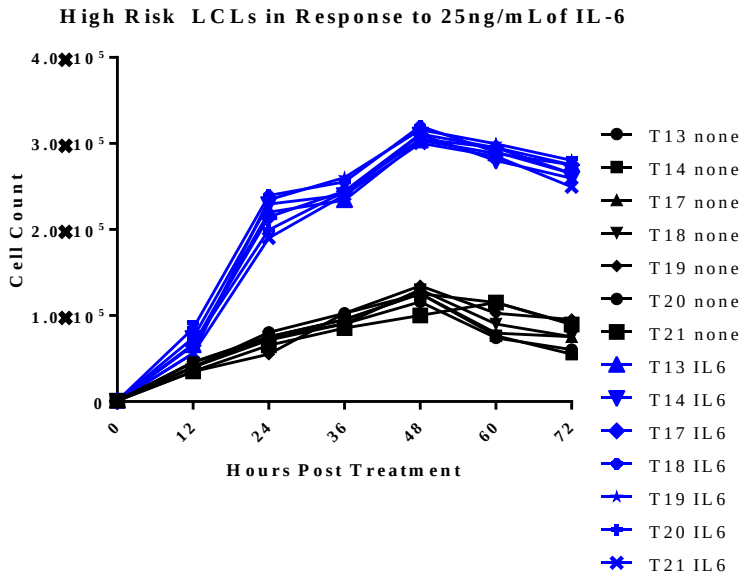
3.1 SPECIFIC AIM I

Characterize the high-risk and low-risk genotypes in LCLs by examining growth curves, downstream signaling events, and gene expression in response to various concentrations of IL-6, IL-11, and OSM. This was completed through growth curves, Western blot analysis, gene expression analysis, and flow cytometry.

3.1.1 LCLS GROWTH CURVES

Dr. Jill Henning had previously showed that high- and low-risk LCLs responded differently to IL-6. In her experiment, she treated the cells with 25ng/mL of rhIL-6 and measured their growth over 72 hours (Figure 3). The high-risk (GG) LCLs responded much earlier and to a much greater extent than the low-risk LCLs when treated with IL-6. Since the LCLs were maintained in 10ng/mL of IL-6, we wanted to see how the cells responded to this dose of IL-6 rather than the 25ng/mL. Also, other studies completed have shown responsiveness to 10ng/mL of IL-6[78,79]. I repeated the growth curve experiments with 10ng/mL of IL-6 instead of 25ng/mL and measured cellular proliferation out to 120 hours (5 days) post treatment. We saw a similar response to 10ng/mL of IL-6; the high-risk cell line responded faster to the cytokine than the low-risk cell line compared to untreated controls (Figure 4).

A.



B.

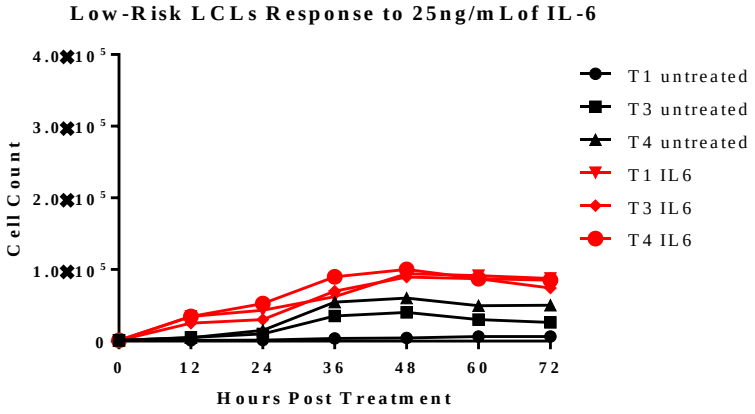
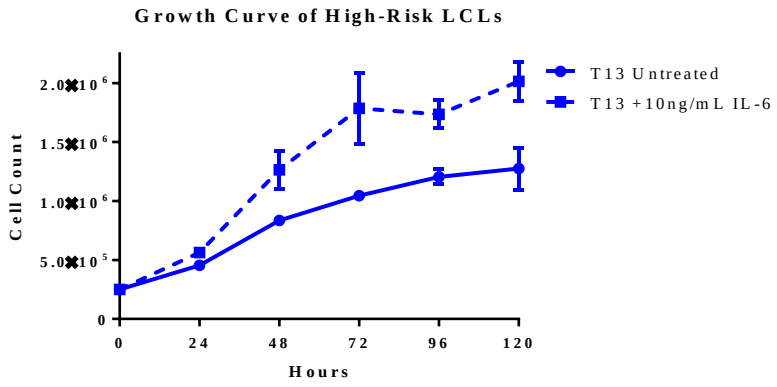


Figure 3: LCLs Growth Curves in Response to 25ng/mL of IL-6. Used with permission from Dr. Jill Henning: High-risk LCLs (A) respond faster and to a greater extent in response to 25ng/mL of IL-6 than low-risk LCLs (B) do, as measured by cell proliferation. Cells were plated at 1x104/well in a 96 well plate in AIM V media and incubated at 37°C overnight. The next day each well was either left untreated or given 25ng of IL-6, IL-11 or OSM (R&D systems Cat. # 206-IL, 218-IL, and 295-OM respectively). Cells from three wells for each treatment were collected and counted by trypan blue exclusion cell count on a hemocytometer at 0, 12, 24, 36, 48, 60, and 72 hours post-treatment. Each line represents an LCL from a different individual[80].

As you can see in figure 3 there are three different low-risk LCLs used and seven different high-risk LCLs used. Unfortunately, after being cultured without IL-6, a majority of these lines no longer responded to IL-6 because by not culturing them in IL-6 we essentially were selecting for the LCLs which do not need IL-6 to grow, and as a result no longer responded to IL-6. We managed to find one high-risk and one low-risk cell line from our original stock. We first needed to test the responsiveness of each cell line to IL-6. Since we were maintaining the lines in 10ng/mL of IL-6, we decided to perform growth curves using this concentration (Figure 4).

A.



B.

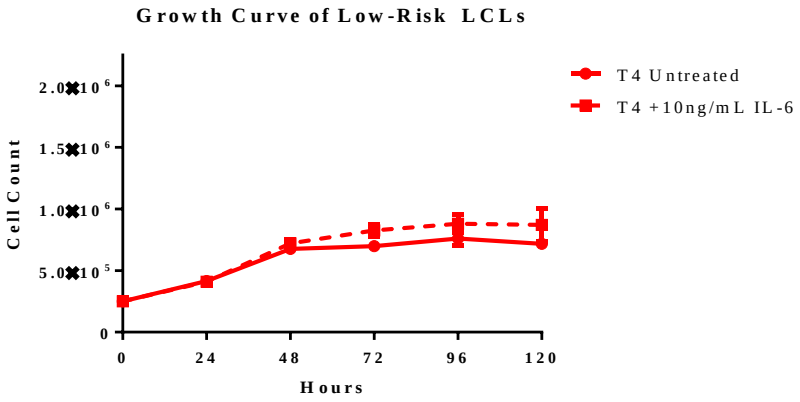
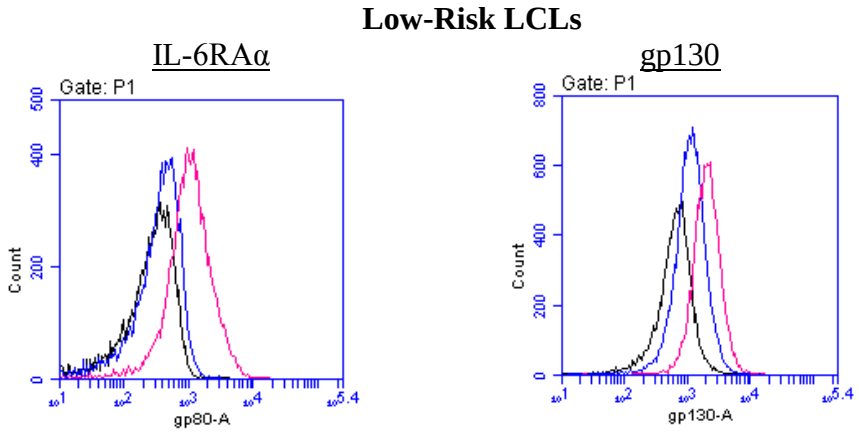


Figure 4: LCLs Growth Curves in Response to 10ng/mL of IL-6. High-risk LCLs (A) respond faster and to a greater extent in response to 10ng/mL of IL-6 than low-risk LCLs (B) do, as measured by cell proliferation. Cells were seeded at 2.5 x 10^5 cells/flask and either treated with 10ng/mL of IL-6 or left untreated. Cell proliferation was measured by trypan blue exclusion every 24 hours. Experiments were completed in duplicate over the course of 5 days. Solid line indicates untreated while dotted line indicates treated with 10ng/mL of IL-6. Graphs were made using GraphPad Prism software.

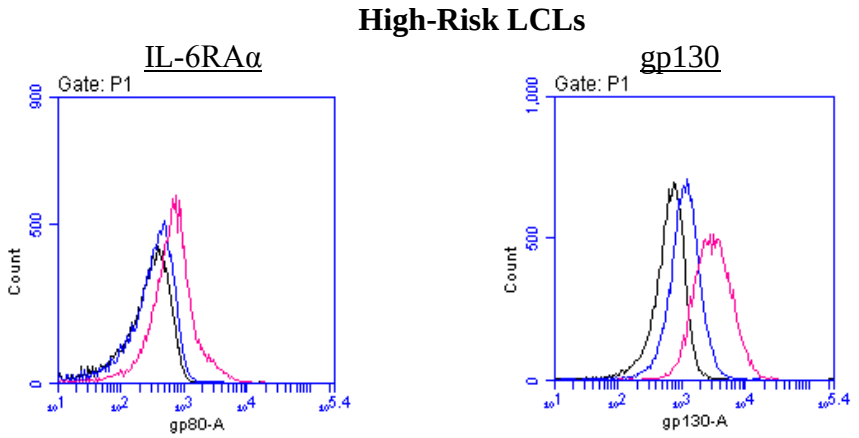
3.1.2 IL-6RAα AND GP130 DISTRIBUTION ON LCLS

Since only the high-risk LCLs showed a major responsiveness to IL-6 as shown by growth curves, we wanted to ensure that both cell lines still had the primary and secondary receptors on the cell surface. To test for this, flow cytometry for the receptors was completed. Both the high- and low-risk cell lines had both receptors on the cell surface (Figure 5), as you can see by the slight shift to the right compared to both the unstained and the isotype control. Since we only have one low-risk and one high-risk LCL line, we do not know if all of the LCLs that Dr. Henning had used have these receptors as well. However, Dr. Henning's growth curves suggest that all of the LCLs of the same genotype act similarly, and led us to believe that these cell lines are representative of all of the high- and low-risk genotypes.

A.



B.



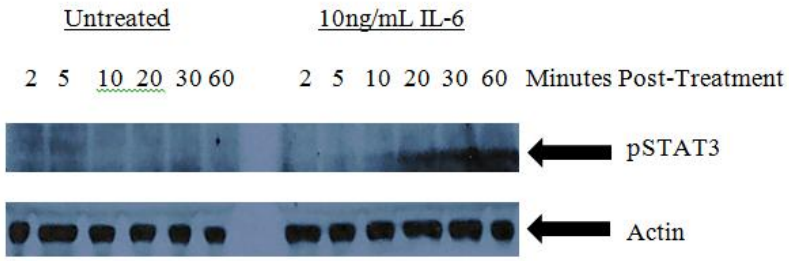
Key:
Black= Unstained
Blue = Isotype Control
Pink = Antibodv

Figure 5: LCLs Receptor Distribution on the Membrane. High- and low-risk LCLs both express the IL-6RAα and gp130 receptors. Low-risk LCLs (A) and high-risk LCLs (B) were seeded at $1x10^6$ cell/mL. 100µL aliquots were seeded and either left unstained (black), stained with the appropriate isotype control antibody (blue), or stained with anti-IL-6RAα or anti-gp130 antibody (pink). Graphs were made using AccuriC6 software.

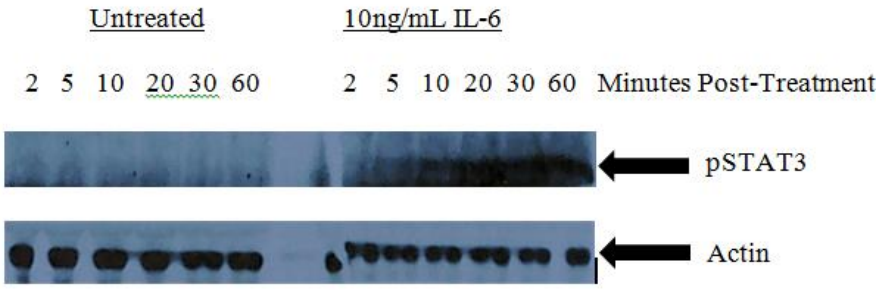
3.1.3 STAT3 ACTIVATION

Since both the primary and secondary receptors were present on the cell surfaces of the LCLs, we decided to look at the proximal signaling events that occur after IL-6 binds. Once IL-6 binds, the JAK/STAT pathway is initiated. To see whether the difference in growth is a result of a defect in this pathway, we next measured the accumulation of phosphorylated STAT3 (pSTAT3) in the cells after treatment with IL-6. In untreated cells, there is little to no pSTAT3 accumulated in the cells. After addition of 10ng/mL of IL-6, pSTAT3 accumulated within the cells within 10 minutes in each cell line (Figure 6). The top band shows phosphorylated STAT3 while the bottom band shows actin, which is used as a loading control. Phosphorylated STAT3 is an intracellular signaling event that occurs once the JAK/STAT pathway is initiated.

A. Low-Risk LCLs



B. High-Risk LCLs



C.

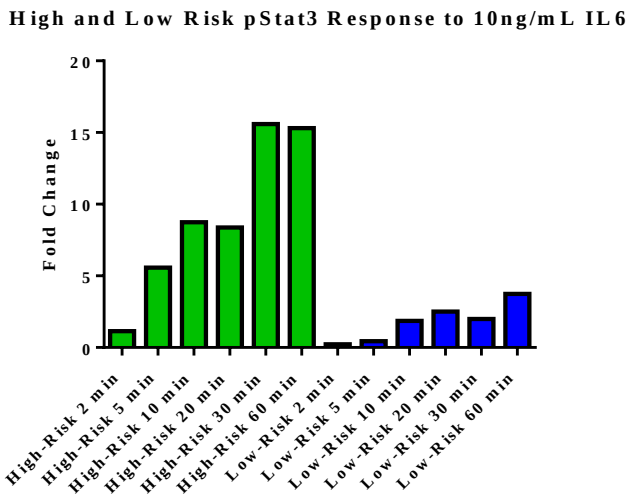
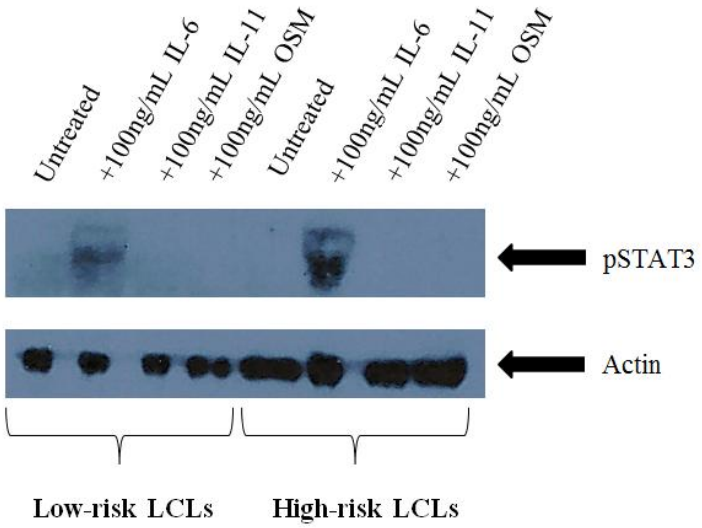


Figure 6: LCLs STAT3 Activation upon Treatment with 10ng/mL of IL-6. High- and low-risk LCLs both show STAT3 activation upon treatment with 10ng/mL of IL-6. Cells were treated for times indicated with 10ng/mL of IL-6 and then a Western blots were performed for pSTAT3, and actin was used as a loading control. Image J software and GraphPad Prism was used to quantify and graph data (C). This experiment was repeated twice using 10ng/mL of IL-6.

Although Dr. Jill Henning previously showed that there was no difference in growth in either the high- or low-risk LCLs when treated with IL-11 or OSM[80], we decided to confirm this by looking at STAT3 activation in response to these cytokines. Cells were seeded and treated with a higher concentration (100ng/mL) of IL-6, IL-11, OSM, or left untreated. After treatment with 100ng/mL of IL-6 for 30 minutes, cells were harvested and cell lysates were analyzed by Western blot for phosphorylation of STAT3 (Figure 7). There was no STAT3 activation in response to IL-11 or OSM in either cell line, but both showed activation of STAT3 in response to IL-6, which is consistent with the results shown in Figure 6.

A.



B.

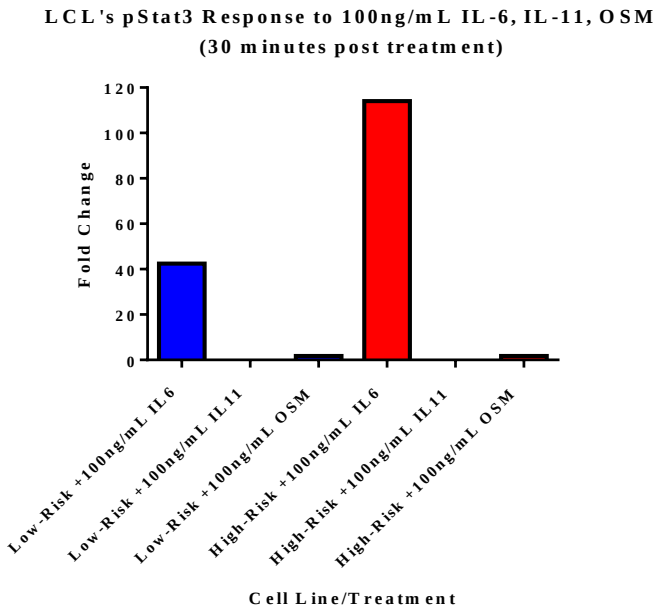
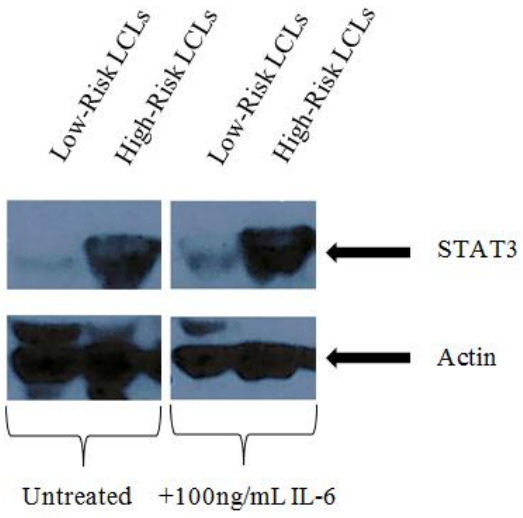


Figure 7: LCLs STAT3 Activation upon Treatment with 100ng/mL of Cytokines. High- and low-risk LCLs show STAT3 activation in response to 100ng/mL of IL-6, but not IL-11 or OSM. Cells were either left untreated, or treated for 30 minutes with 100ng/mL of IL-6, IL-11, or OSM. Western blots were completed for pSTAT3, and actin was used as a loading control. Image J software and GraphPad Prism was used to quantify and graph data.

The same cell lysates that were used in Figure 7 were also used in Figure 8, where we analyzed the amount of STAT3 constitutively present in the cells. The STAT3 in the top blot of figure 8 represents the total STAT3 present, which includes both phosphorylated and unphosphorylated STAT3. This represents the pool of STAT3 proteins in the cell that act as substrates for phosphorylation. There seem to be dramatic differences in the amount of total STAT3 protein present in the cells between the high- and low-risk LCLs. However, the differences between treated and untreated STAT3 levels do not differ very much in the respective cell lines. For example, between untreated high-risk LCLs and treated high-risk LCLs, there are not major differences in the amount of total STAT3 protein present (Figure 8).

A.



B.

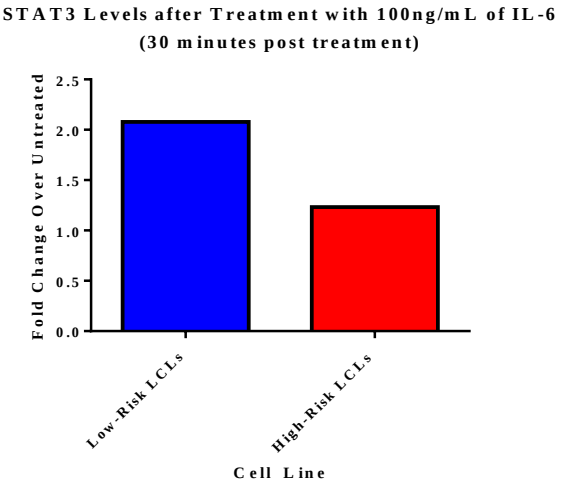


Figure 8: Basal STAT3 Levels in LCLs. High- and low-risk LCLs show minimal changes in STAT3 expression upon treatment with 100ng/mL of IL-6. Using the same cell lysates shown in Figure 7, Western blots were completed for total STAT3 levels and actin was used as a loading control. Image J software and GraphPad Prism was used to quantify and graph data.

3.1.4 GENE EXPRESSION ANALYSIS

The small differences in timing in the activation of STAT3 would not result in the drastic differences seen between the growth curves of the high- and low-risk cell lines in response to IL-6. We feel like a difference of 5 or 10 minutes between the activation of STAT3 would not result in the growth differences we see at 4-5 days post-treatment. Therefore, we hypothesize that as a result of IL-6 signaling and STAT3 activation, there are differences in the genes that are activated downstream. Our hypothesis was that the genes that are activated in the high-risk LCLs are genes involved in inflammation, proliferation, or differentiation, and the genes that are activated in the low-risk LCLs are genes that could perhaps induce apoptosis or genes involved in maintaining the current cellular state. The findings by using the gene expression analysis might help explain the differences seen between the growth curves of the two cell lines.

There are a large number of genes that are regulated by the JAK/STAT pathway. In order to examine a large number of genes that are turned on or off through this pathway, we decided to use pathway-focused, RT-PCR array plates. The first array plate used was the JAK/STAT pathway array from SABiosciences, which uses SYBR Green-based, real-time, RT-PCR technology to amplify cDNA using primers specific for genes regulated by this pathway. SYBR green technology works via SYBR green dye binding to amplified double-stranded DNA sequences. The binding, however, is nonspecific. This means that if there is nonspecific amplification, SYBR green still binds, and the readout is the same whether it bound to the target gene or to non-specific amplification.

To analyze this phenomenon and ensure that there is specific amplification, melt curve analysis was performed on the samples. Because an exponential number of the same fragment of DNA was produced through PCR reactions, you can slowly increase the temperature to see at what point the DNA dissociates, or "melts". As temperature increases, double-stranded DNA separates during denaturation by breaking the hydrogen bonds between base pairs. The complementary bases adenine and thymine form two hydrogen bonds, while guanine and cytosine form three hydrogen bonds. Consequently, the melting point of complementary fragments of DNA is dependent upon the content of DNA in that fragment. For example, an amplified fragment of DNA with a higher guanine and cytosine content will have a higher melting point than an amplified fragment of DNA with a lower guanine and cytosine content. As the DNA is denatured, the SYBR Green fluorescence is also decreased. The melting temperature is then calculated at the point which maximum fluorescence is lost. If there was any nonspecific amplification or primer dimers (amplification of the two primers hybridized to each other), the melt curves would show multiple peaks, or a very low melting temperature. This indicates that

the amplification you see may be nonspecific, and should not be trusted. Of the JAK/STAT pathway array, we could only use a small number of genes due to the unreliable results of the SYBR Green array plates (Figure 9 A and B, full gene listing in Appendix A). High- and low-risk LCLs were treated for 10 minutes with 10ng/mL of IL-6, and then the cDNA made from these cells were used in the JAK/STAT pathway array plates.

A.

JAK/STAT Gene Table

A2M	SH2B2	BCL2L1	CCND1	CDKN1A	CEBPB	CRK	CRP	CSF1R	CSF2RB	CXCL9	EGFR
EPOR	F2	F2R	FAS	FCER1A	FCGR1A	ISG15	GATA3	GBP1	GHR	HMGA1	IFNAR1
IFNG	IFNGR1	IL10RA	IL20	IL2RA	IL2RG	IL4	IL4R	IL6ST	INSR	IRF1	IRF9
JAK1	JAK2	JAK3	JUN	JUNB	MMP3	MPL	MYC	NFKB1	NOS2	NR3C1	OAS1
OSM	PDGFRA	PIAS1	PIAS2	PPP2R1A	PRLR	PTPN1	PTPRC	SH2B1	SIT1	SLA2	SMAD1
SMAD2	SMAD3	SMAD4	SMAD5	SOCS1	SOCS2	SOCS3	SOCS4	SOCS5	SP1	SPI1	SRC
STAM	STAT1	STAT2	STAT3	STAT4	STAT5A	STAT5B	STAT6	STUB1	TYK2	USF1	YY1
B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

B.

JAK/STAT Gene Table

A2M	SH2B2	BCL2L1	CCND1	CDKN1A	CEBPB	CRK	CRP	CSF1R	CSF2RB	CXCL9	EGFR
EPOR	F2	F2R	FAS	FCER1A	FCGR1A	ISG15	GATA3	GBP1	GHR	HMGA1	IFNAR1
IFNG	IFNGR1	IL10RA	IL20	IL2RA	IL2RG	IL4	IL4R	IL6ST	INSR	IRF1	IRF9
JAK1	JAK2	JAK3	JUN	JUNB	MMP3	MPL	MYC	NFKB1	NOS2	NR3C1	OAS1
OSM	PDGFRA	PIAS1	PIAS2	PPP2R1A	PRLR	PTPN1	PTPRC	SH2B1	SIT1	SLA2	SMAD1
SMAD2	SMAD3	SMAD4	SMAD5	SOCS1	SOCS2	SOCS3	SOCS4	SOCS5	SP1	SPI1	SRC
STAM	STAT1	STAT2	STAT3	STAT4	STAT5A	STAT5B	STAT6	STUB1	TYK2	USF1	YY1
B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Key:

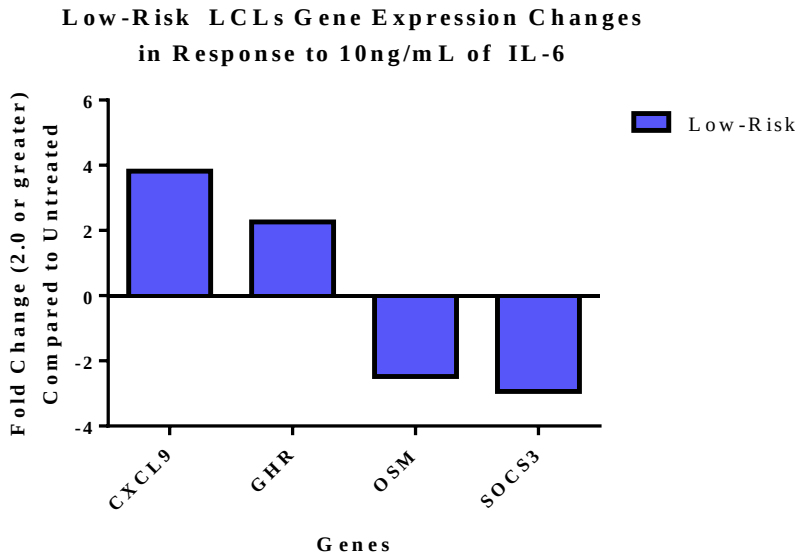
Green = Housekeeping Genes

Purple = Genes with bad melt curves

Grey = Genes with less than 2-fold changes in expression

Red = Genes with greater than 2-fold changes in expression

C.



D.

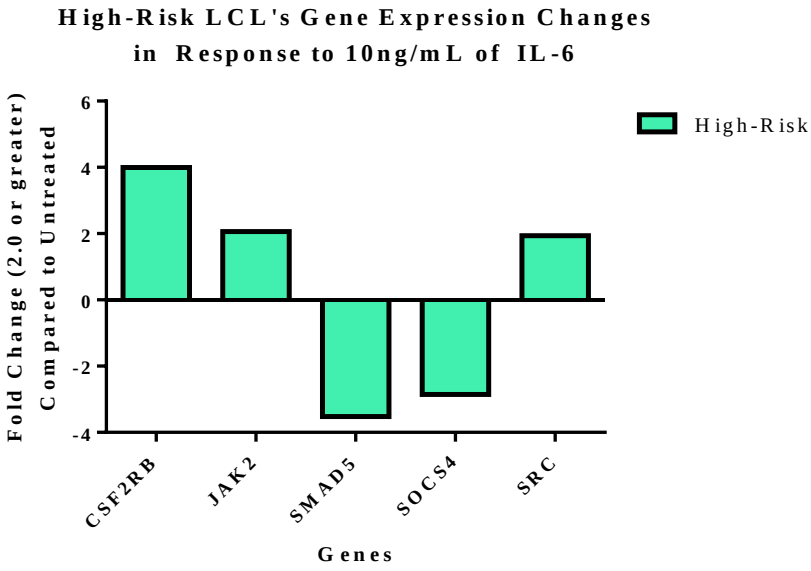


Figure 9: LCLs Gene Expression Analysis using JAK/STAT Plates. Gene expression of high- and low-risk LCLs differs in response to 10ng/mL of IL-6 at 10 minutes post treatment. Gene expression was analyzed on isolated RNAs from low-risk and high-risk LCLs either left untreated or treated with 10ng/mL of IL-6 for 10 minutes using the JAK/STAT Pathway Plate Array from SAbiosciences™. (A) Gene table for the JAK/STAT Pathway array plates. (B) Gene table showing the housekeeping genes (green), the genes that were excluded due to bad melt

curves (purple), the genes with less than a 2-fold change in regulation (grey), and the genes that show a 2-fold change or greater (red). (C and D) Graphical representation of the high-risk and low-risk up- and down-regulated genes, graphed using GraphPad Prism. This figure shows the results from one experiment, so significant differences cannot be calculated.

The genes that are up- or down-regulated in response to 10ng/mL of IL-6 based off of the JAK/STAT pathway PCR array differ between the high- and low-risk LCLs (Figure 9 C and D), where we originally thought that there would be somewhat of an overlap. If we analyze the specific genes, there are a number that are involved in growth and proliferation, and could explain the differences seen in the growth curves. In the low-risk LCLs, we see an up-regulation of CXCL9 (chemokine (C-X-C motif) ligand 9) and GHR (growth hormone receptor), while OSM (oncostatin M) and SOCS3 (suppressor of cytokine signaling 3) are down-regulated. In the high-risk LCLs, CSF2RB (colony stimulating factor 2, receptor beta), JAK2 (janus kinase 2), and SRC (v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog) are up-regulated, while SMAD5 (SMAD family member 5) and SOCS4 (suppressor of cytokine signaling 4) are both down-regulated.

After obtaining these data, we wanted to repeat the experiment. This time, however, we wanted to use a technology that was more reliable than SYBR Green. This way, we would not have to ignore over half of the data due to poor melt curve analysis. Taqman probes are used to increase specificity in real time PCR arrays. Taqman probes work by relying on the Taq polymerase exonuclease activity to cleave a sequence specific oligonucleotide probe from the target DNA sequences. As long as the reporter fluorescent molecule and the quencher are within close distance to one another, the quencher prevents the reporter from fluorescing. Once the probe is cleaved, the fluorescent marker is able to produce fluorescence without it being quenched by the quencher molecule attached to the other end of the probe. The fluorescence

intensity is again indicative of the amount of template cDNA present in the sample. Taqman technology is more specific because the fluorescence is only generated when the probe gets separated from the quencher on the target cDNA sequence, and thus nonspecific amplification is not an issue, and melt curves are not necessary. There are, however, a few genes or wells in which there was no amplification or it was undetectable. Figure 10 below shows the gene table for the Human IL-6 Pathway PCR Array plate, followed by a gene table showing the wells that were excluded due to undetectable amplification, and thus results cannot be compared.

We used Taqman plates from Applied Biosystems. However, they did not have a plate specific to the JAK/STAT pathway. We chose to use an IL-6 pathway-specific plate. Again, cells were treated with 10ng/mL of IL-6 for 10 minutes and cDNA was isolated to be used on the plates. Fold change was analyzed in the treated samples compared to the untreated control samples. The entire experiment (i.e. from treatment to cDNA synthesis) was repeated twice, so experiments were analyzed in duplicate.

A.

IL-6 Gene Table

18S	GAPDH	HPRT1	GUSB	AKT1	ARAF	BAX	BCL2	BRAF	CCL2	CD4	CHUK
CXCL12	FRAP1	GRB2	HRAS	IKBKB	IKBKE	IKBKG	IL10	IL18	IL1A	IL1B	IL2
IL4	IL6	IL6R	IL6ST	IL8	JAK3	KRAS	MAP2K1	MAP2K2	MAP2K3	MAP2K4	MAP2K5
MAP2K6	MAPK1	MAPK12	MAPK3	MAPK6	MAPK7	MRAS	MYC	NFKB1	NFKB2	NFKBIA	NFKBIB
NFKBIE	NRAS	PIAS3	PIK3C2A	PIK3C2B	PIK3C2G	PIK3C3	PIK3CA	PIK3CB	PIK3CD	PIK3R1	PIK3R2
PIK3R3	PIK3R4	PIK3R5	PIM1	PPP1R12A	PPP1R15A	PPP1R1B	PPP2CA	PPP2CB	PPP2R1A	PPP2R1B	PPP2R2A
PPP2R2B	PPP2R4	PPP2R5A	PPP2R5C	PTPN11	PTPN6	RAF1	REL	RELA	RELB	RRAS	RRAS2
SHC1	SHC2	SHC3	SOCS1	SOCS2	SOCS3	SOCS5	SOCS6	STAT3	TIMP1	TNF	TP53BP2

B.

IL-6 Gene Table

18S	GAPDH	HPRT1	GUSB	AKT1	ARAF	BAX	BCL2	BRAF	CCL2	CD4	CHUK
CXCL12	FRAP1	GRB2	HRAS	IKBKB	IKBKE	IKBKG	IL10	IL18	IL1A	IL1B	IL2
IL4	IL6	IL6R	IL6ST	IL8	JAK3	KRAS	MAP2K1	MAP2K2	MAP2K3	MAP2K4	MAP2K5
MAP2K6	MAPK1	MAPK12	MAPK3	MAPK6	MAPK7	MRAS	MYC	NFKB1	NFKB2	NFKBIA	NFKBIB
NFKBIE	NRAS	PIAS3	PIK3C2A	PIK3C2B	PIK3C2G	PIK3C3	PIK3CA	PIK3CB	PIK3CD	PIK3R1	PIK3R2
PIK3R3	PIK3R4	PIK3R5	PIM1	PPP1R12A	PPP1R15A	PPP1R1B	PPP2CA	PPP2CB	PPP2R1A	PPP2R1B	PPP2R2A
PPP2R2B	PPP2R4	PPP2R5A	PPP2R5C	PTPN11	PTPN6	RAF1	REL	RELA	RELB	RRAS	RRAS2
SHC1	SHC2	SHC3	SOCS1	SOCS2	SOCS3	SOCS5	SOCS6	STAT3	TIMP1	TNF	TP53BP2

Key:

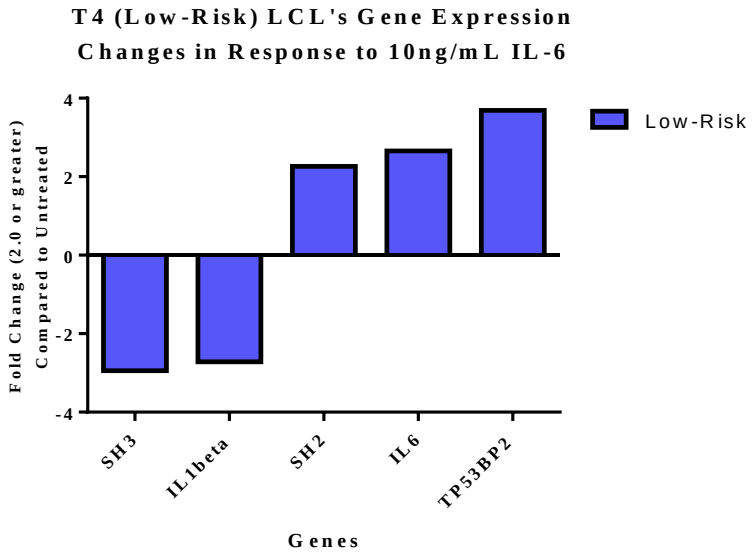
Green = Housekeeping Genes

Purple = No amplification

Grey = Genes with less than 2-fold changes in expression

Red = Genes with greater than 2-fold changes in expression

C.



D.

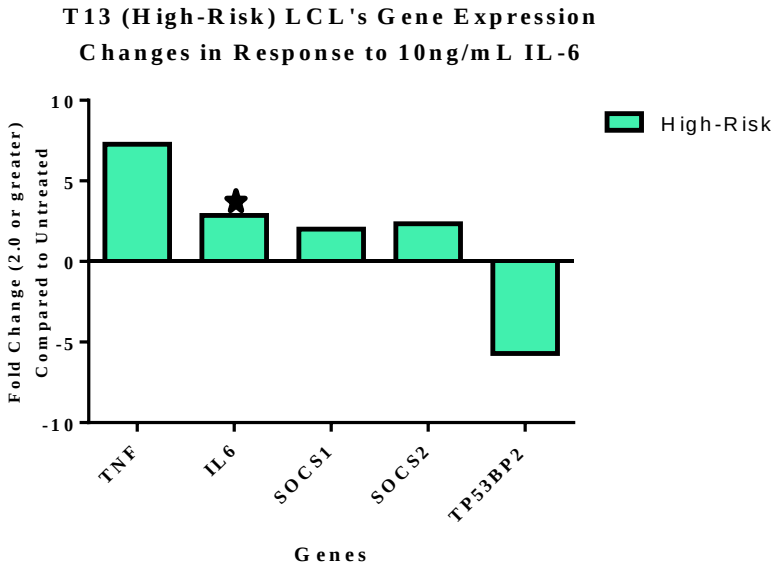


Figure 10: LCLs Gene Expression Analysis using Human IL-6 Plates. Gene expression of high- and low-risk LCLs differs in response to 10ng/mL of IL-6 at 10 minutes post treatment. Gene expression was analyzed on isolated RNA from low-risk and high-risk LCLs either left untreated or treated with 10ng/mL of IL-6 for 10 minutes using the Human IL-6 Pathway PCR Array from Applied Biosystems®. (A) Gene table for the IL-6 Pathway array plates. (B) Gene table showing the housekeeping genes (green), the genes that were excluded due to insufficient amplification (purple), the genes with less than a 2-fold change in regulation (grey), and the genes that show a 2-fold change or greater (red). (C and D) Graphical representation of genes that were up- or down-regulated by 2-fold or greater, graphed using GraphPad Prism. The star denotes only one experiment was compared, so results could not be statistically compared.

Results showed that there were significant changes in a few genes from both high- and low-risk cell lines (Figure 10 C and D). In the high-risk LCLs, TNF, IL-6, SOCS1 and SOCS2 were all up-regulated by a fold change of two or more, while TP53BP2 was down-regulated by a fold change of two or more. In the low-risk LCLs, SH2, IL-6, TP53BP2 were up-regulated by a fold change of two or more, while SH3 and IL1β were down-regulated by a fold change of two or more.

3.2 SPECIFIC AIM II

Although we see differences between the high- and low-risk LCLs, we wanted to see if this would hold true for different kinds of cells. We switched our interest to prostate cancer cells, and had two well-characterized cell lines, PC3 and DU 145, readily available. The gp130 status was determined and it was found that PC3 expressed the high-risk gp130 allele (GG), while DU 145 expressed the low-risk gp130 allele (CC). A benefit of using prostate cancer cell lines was that these cell lines were known to express primary receptors for other cytokines that also utilize the gp130 signaling receptor, namely IL-11 and OSM. Seeing how the high- and low-risk prostate cancer cell lines respond to IL-6, IL-11, and OSM would help us to better characterize the nature of the gp130 G148R polymorphism.

Aim II was to translate experiments to prostate cancer cell lines, and develop and characterize the GG and CC genotypes in response to various concentrations of IL-6, IL-11, and OSM. This will be completed by growth curves, Western blot analysis, and flow cytometry.

3.2.1 IL-6 PRODUCTION BY PC3 AND DU 145

Clearly, there is a large difference between B lymphocytes and prostate cancer epithelial cells. One of the main differences is that while B lymphocytes respond to IL-6, they do not produce IL-6. However, both PC3 and DU 145 produce low levels of IL-6. We wanted to ensure that the amount of IL-6 we were adding (10ng/mL-100ng/mL) was significantly greater than the amount of IL-6 that they produce. To do this, we performed an ELISA for IL-6, which showed that although PC3 and DU 145 produce IL-6, it is in the 10-20pg/mL range, about 1,000-fold less than the amount of IL-6 that we were adding to the cells exogenously (Figure 11). If IL-6 had an

effect on growth, we should still be able to see it by using the concentrations that we used with the LCLs.

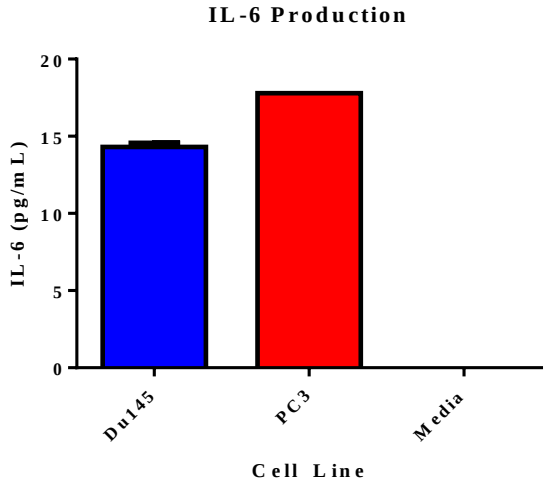


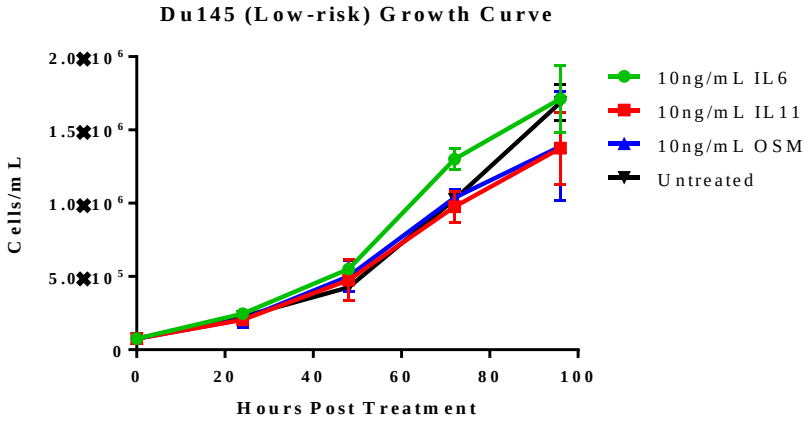
Figure 11: PC3 and DU 145 ELISA for IL-6 Production. PC3 (high-risk) and DU 145 (low-risk) both produce low levels of IL-6. Fresh media or spent media removed from cells that were cultured to 80-90% confluency was analyzed for IL-6 production by ELISA. Both PC3 and DU 145 prostate cancer cells produced low levels (between 10-20 pg/mL of IL-6). Experiments were run in duplicate, and graphed using GraphPad Prism.

3.2.2 GROWTH CURVES

We performed growth curves on PC3 and DU 145 using 10ng/mL of IL-6, IL-11, and OSM using trypan blue exclusion over the course of four days. We saw no significant differences in growth from the untreated cells (Figure 12). However, we hypothesized that although 10ng/mL was a high enough concentration for the LCLs, it was not high enough to see significant differences for the prostate cancer lines. Also, it might be hard to see small differences in growth by using trypan blue exclusion. Also, by only taking timepoints every 24 hours it is possible to miss important changes in growth that may have occurred between timepoints. To remedy this,

we decided to repeat the growth curves using various increasing concentrations of cytokines using an Xcelligence machine.

A.



B.

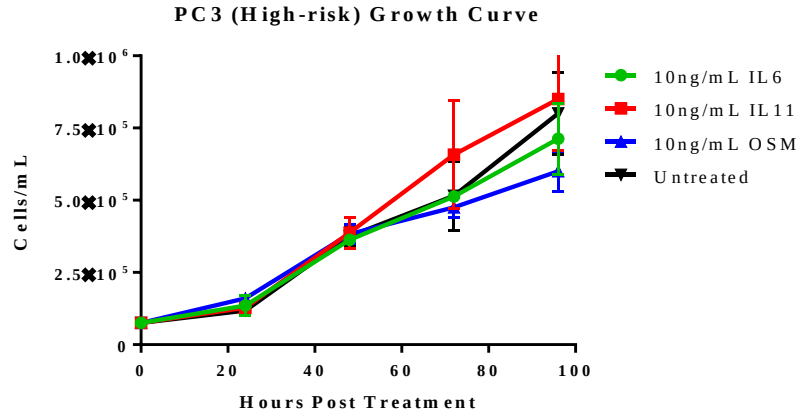
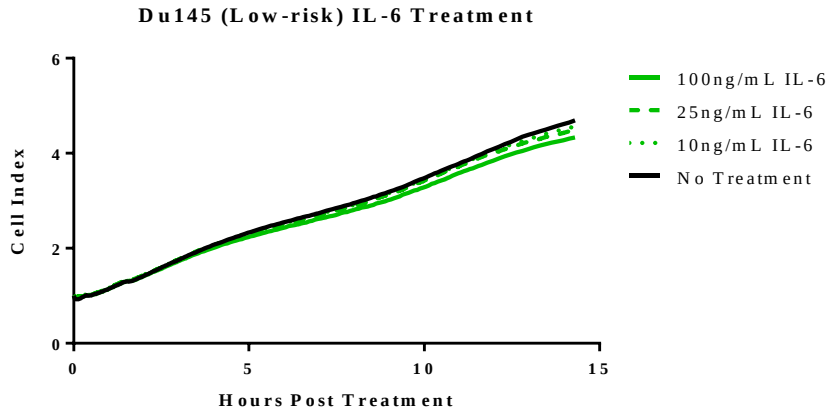
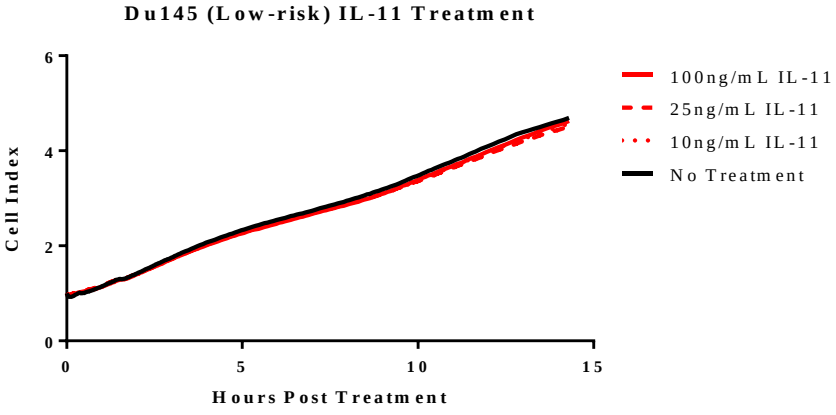


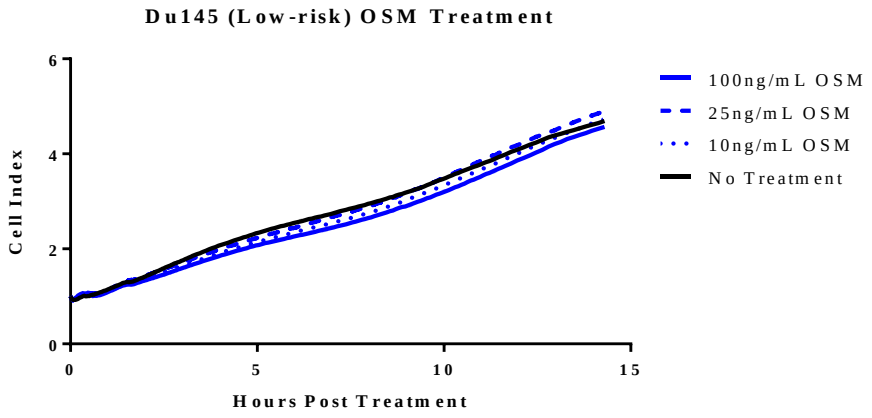
Figure 12: PC3 and DU 145 Growth Curves using Trypan Blue Exclusion Count. DU 145 (A) and PC3 (B) did not show any significant changes in growth when treated with 10ng/mL of indicated cytokine compared to untreated cells. Cells were seeded into 6 well plates and treated with 10ng/mL of indicated cytokine, harvested every 24 hours for 5 days, and counted using trypan blue exclusion. Experiments were completed in duplicate, and graphed using GraphPad Prism.

An Xcelligence machine measures real-time changes in morphology, proliferation and attachment. It allows us to see small changes at more frequent timepoints without disturbing the cells. After deciding the optimal concentration of cells to use, PC3 and DU 145 cells were treated with 10, 25, and 100ng/mL of IL-6, IL-11, or OSM. Untreated and vehicle controls were used, and experiments were completed in quadruplicate. No significant differences were seen between untreated and cells treated with varying concentrations (10, 25, or 100ng/mL) of cytokines in either of the cell lines (Figure 13).

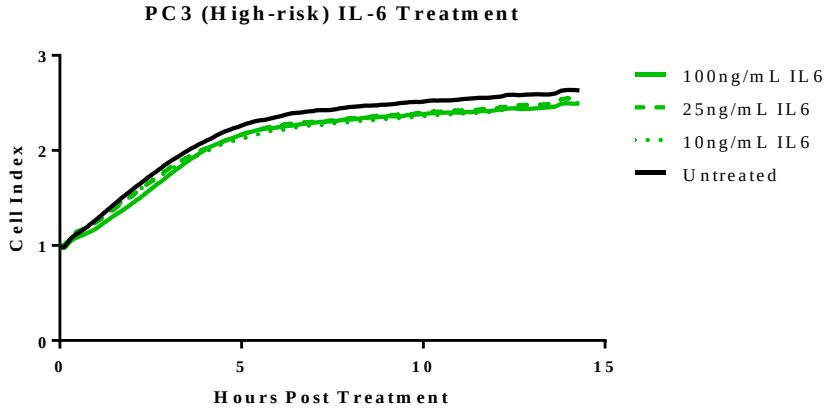
A.

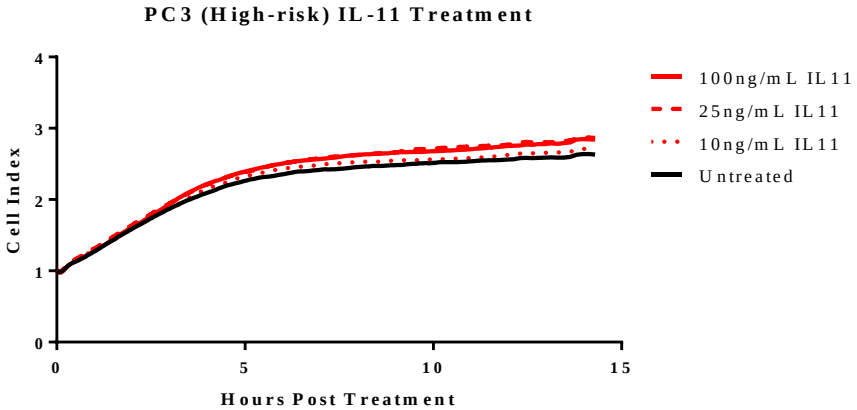





Du145 (Low-risk) OSM Treatment
Cell Index
Hours Post Treatment
100ng/mL OSM
25ng/mL OSM
10ng/mL OSM
No Treatment

B.


PC3 (High-risk) IL-6 Treatment
Cell Index
Hours Post Treatment
100ng/mL IL6
25ng/mL IL6
10ng/mL IL6
Untreated


PC3 (High-risk) IL-11 Treatment
Cell Index
Hours Post Treatment
100ng/mL IL11
25ng/mL IL11
10ng/mL IL11
Untreated

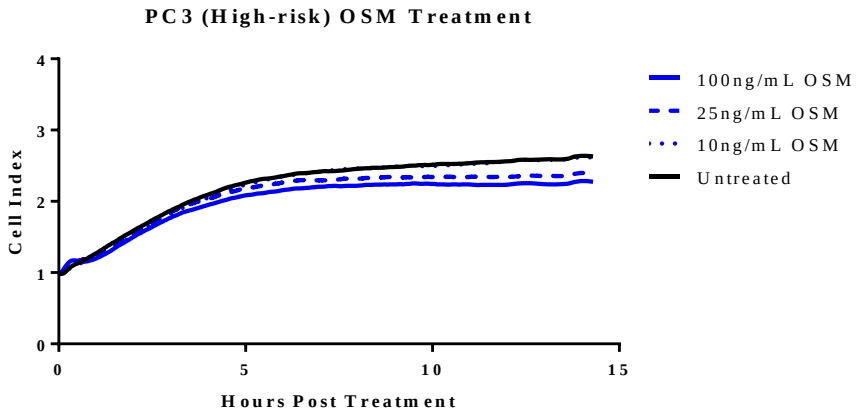
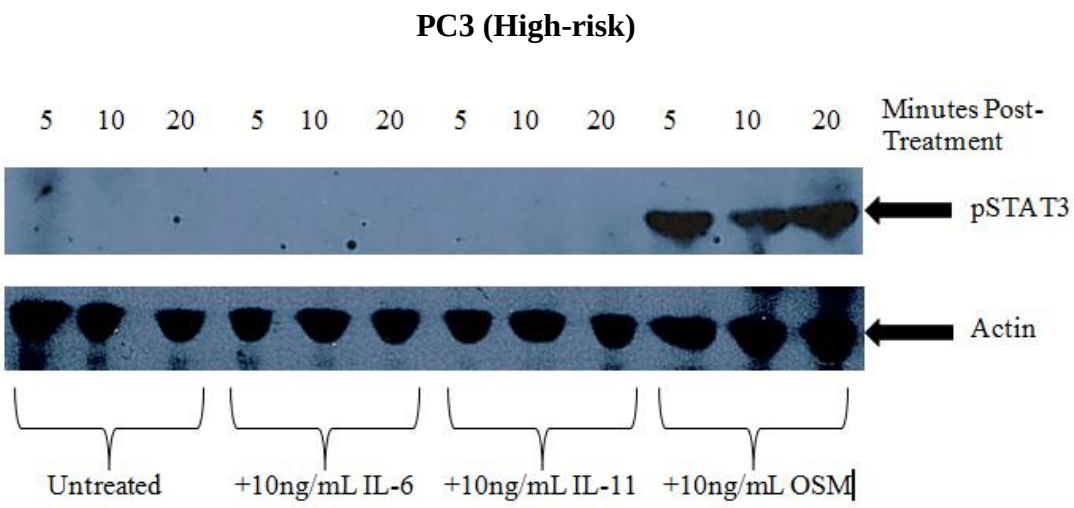


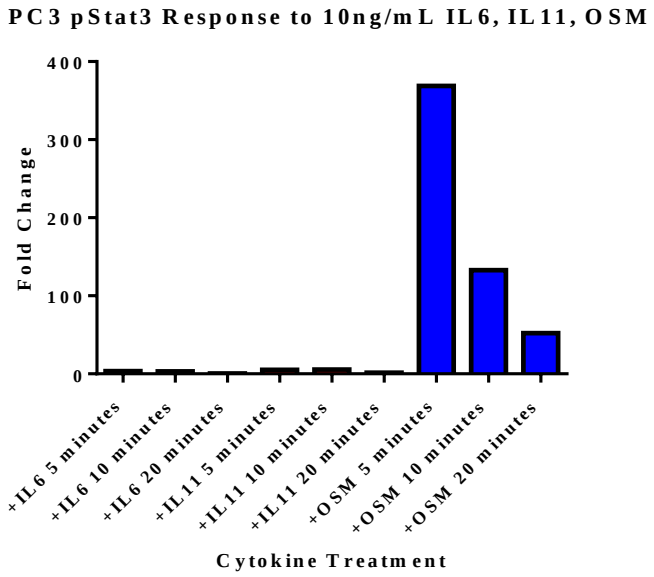
Figure 13: PC3 and DU 145 Growth Curves Using Xcelligence Machine. DU 145 (A) and PC3 (B) did not show any significant changes in growth when treated with various concentrations of indicated cytokine when compared to untreated cells. Cells were seeded at 40,000 cells/well (DU 145) or 10,000 cells/well (PC3) based off of initial growth curves (data not shown). Four hours after cells were seeded, various concentrations of cytokines (IL-6, IL-11, or OSM) were added. Real-time growth was measured for 14 hours post treatment with cytokines. Experiments were completed in triplicate and graphed using GraphPad Prism.

3.2.3 STAT3 ACTIVATION

Western blot analysis of STAT3 activation (pSTAT3) was completed in a similar way with PC3 and DU 145 as with the LCLs. In response to 10ng/mL of IL-11 there was no activation of STAT3 in either of the cell lines, while both cell lines showed a strong response to 10ng/mL of OSM. There was a dramatic difference in how PC3 (high-risk) and DU 145 (low-risk) responded to 10ng/mL of IL-6. The low-risk DU 145s responded to IL-6 (as measured by STAT3 activation) by 10 minutes post treatment. The high-risk PC3s, however, did not show any STAT3 activation in response to IL-6 even by 20 minutes post treatment (Figure 14).

A.


PC3 (High-risk)
5 10 20 5 10 20 5 10 20 5 10 20
Minutes Post-Treatment
pSTAT3
Actin
Untreated
+10ng/mL IL-6
+10ng/mL IL-11
+10ng/mL OSM


PC3 pStat3 Response to 10ng/mL IL6, IL11, OSM
Fold Change
400
300
200
100
0
+IL6 5 minutes
+IL6 10 minutes
+IL6 20 minutes
+IL11 5 minutes
+IL11 10 minutes
+IL11 20 minutes
+OSM 5 minutes
+OSM 10 minutes
+OSM 20 minutes
Cytokine Treatment

B.

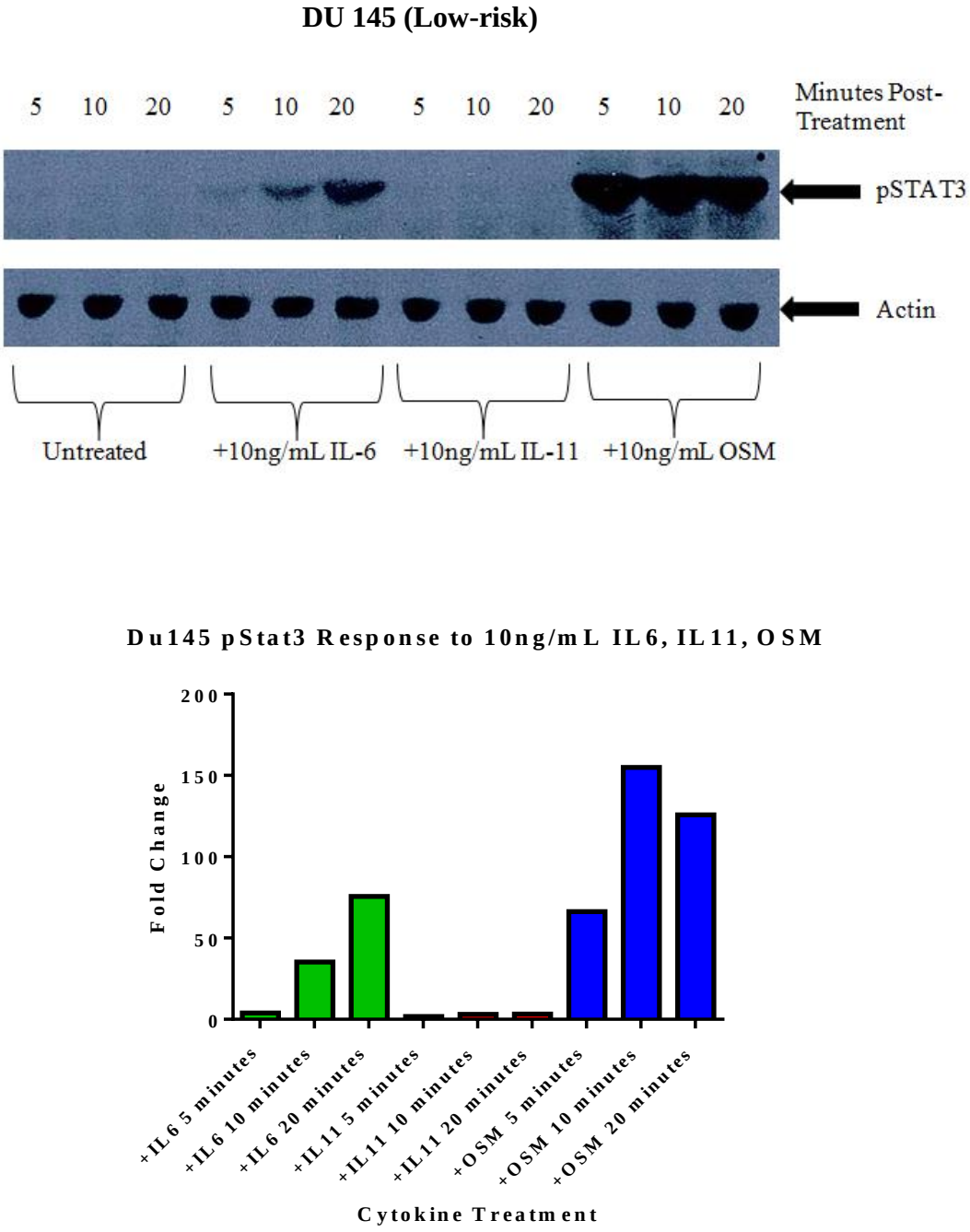


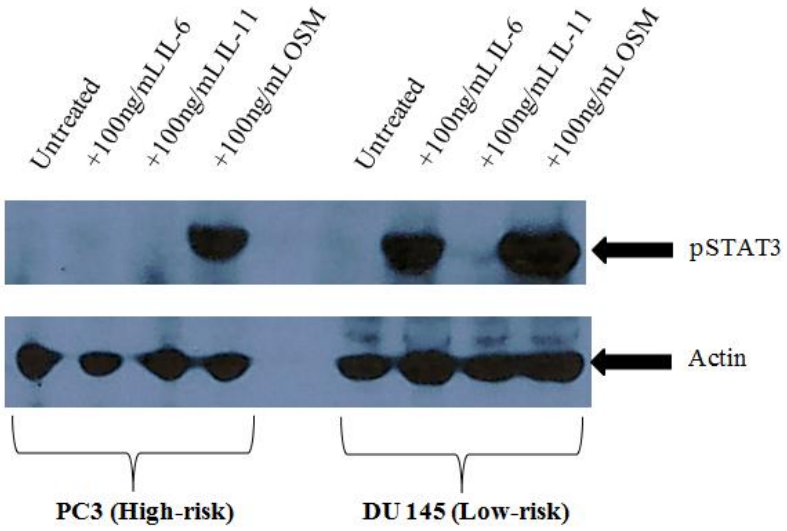
Figure 14: PC3 and DU 145 STAT3 Activation upon Treatment with 10ng/mL Cytokines. The PC3 cell line (high-risk) responded to OSM, but not IL-6 or IL-11, while the DU 145 cell line (low-risk) responded to IL-6 and OSM, but not IL-11 as measured by activation of STAT3. High-risk PC3s (A) and low-risk DU 145s (B) were seeded in 6 well plates and serum-starved

overnight. Cells were treated with regular media, or media with 10ng/mL of indicated cytokine and harvested 5, 10, or 20 minutes post treatment and cell lysates were analyzed for pSTAT3. Actin was used as a loading control. Image J software was used to quantify bands and GraphPad Prism was used to graph the data.

We hypothesized that perhaps the 10ng/mL that was sufficient to activate STAT3 in the LCLs might not be a high enough concentration to see a response from the prostate cancer cell lines in the cases of IL-6 for PC3s and IL-11 for both the PC3s and DU 145s. Cytokines have different optimal concentrations, and thus perhaps 10ng/mL was not the optimal concentration for IL-11 and for IL-6. We increased the concentration of all three cytokines used to 100ng/mL.

In response to 100ng/mL of IL-6, IL-11, and OSM, we still saw a strong STAT3 activation in both high- and low-risk cell lines to OSM by 30 minutes post treatment. When 100ng/mL of IL-11 was used, we still did not see any STAT3 activation in either the high- or low-risk cell lines by 30 minutes post treatment. Interestingly, in response to 100ng/mL of IL-6, there was no STAT3 activation in the PC3 (high-risk) cell line, while DU 145 saw a strong activation of STAT3 by 30 minutes post treatment (Figure 15). This experiment was repeated three times, and each time we still saw the same response. This is particularly puzzling, since there have been a number of studies that have shown that STAT3 is activated in response to IL-6 *in vitro* in both PC3 and DU 145 cells[81].

A.



B.

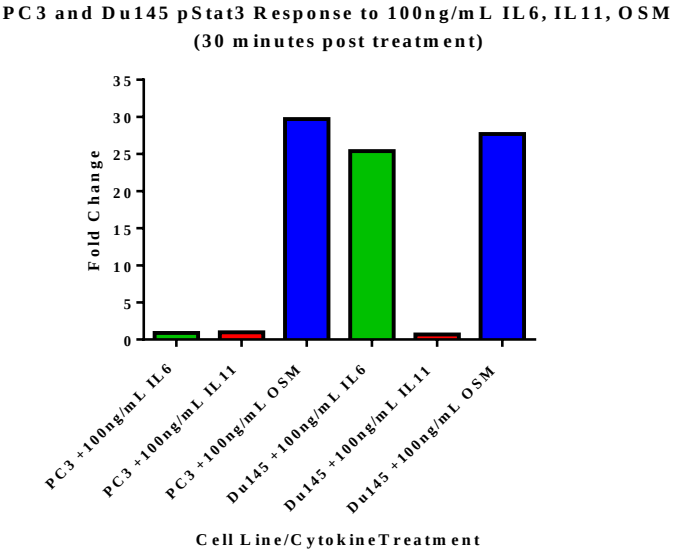
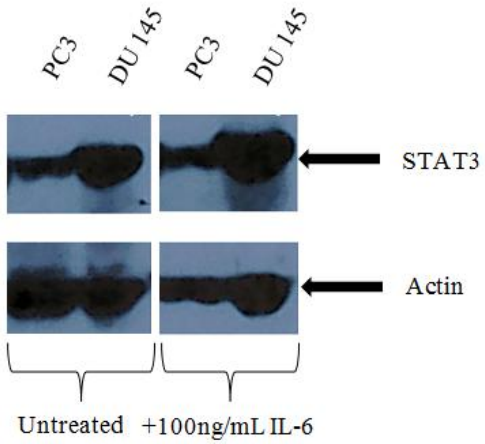


Figure 15: PC3 and DU 145 STAT3 Activation upon Treatment with 100ng/mL Cytokines. The PC3 cell line (high-risk) responded to 100ng/mL of OSM, but not IL-6 or IL-11, while the DU 145 cell line (low-risk) responded to 100ng/mL of OSM and IL-6, but not IL-11 as evidenced by activation of STAT3. Prostate cancer cell lines were seeded into 6 well plates and treated with 100ng/mL of indicated cytokine or left untreated, and harvested at 30 minutes post treatment. Cell lysates were analyzed for pSTAT3 by Western blot (A), and actin was examined as a loading control. ImageJ software was used to quantify bands and GraphPad Prism was used to graph the data (B).

We also decided to look at levels of total STAT3 before and after 30 minutes of treatment with 100ng/mL of IL-6 (Figure 16). We see that in both PC3 and DU 145 cells, STAT3 is constitutively active in untreated cells. In addition, levels of STAT3 do not change very much (less than 2-fold) when treated with 100ng/mL of IL-6 for 30 minutes. This experiment was not repeated.

A.



B.

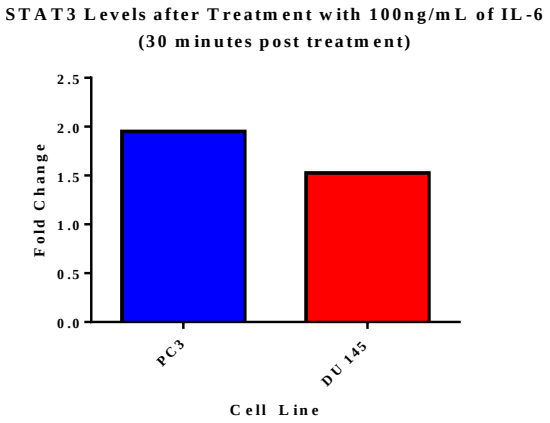
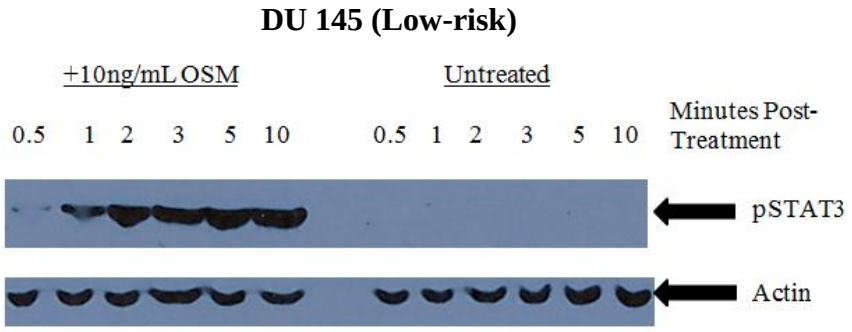
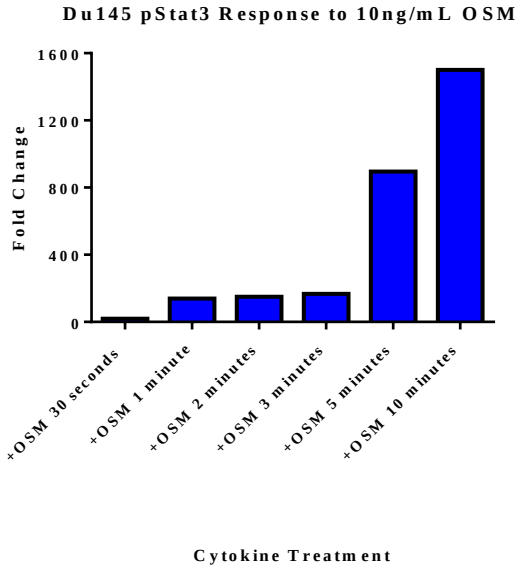


Figure 16: Basal STAT3 Levels in PC3 and DU 145. The PC3 cell line and the DU 145 cell line show minimal changes in total STAT3 expression upon treatment with 100ng/mL of IL-6. Using the same cell lysates shown in Figure 15, Western blots were completed for total STAT3 levels and actin was used as a loading control. Image J software and GraphPad Prism was used to quantify and graph data.

Since both PC3 and DU 145 responded to 10ng/mL of OSM as early as 5 minutes post treatment, we wanted to see if there was a difference in signaling at earlier timepoints in response to OSM. In response to 10ng/mL of OSM, we took timepoints as early as 30 seconds and went out to 10 minutes. Both cell lines showed STAT3 activation by 2 minutes post treatment, but there was not a substantial difference between the two (Figure 17).

A.





B.

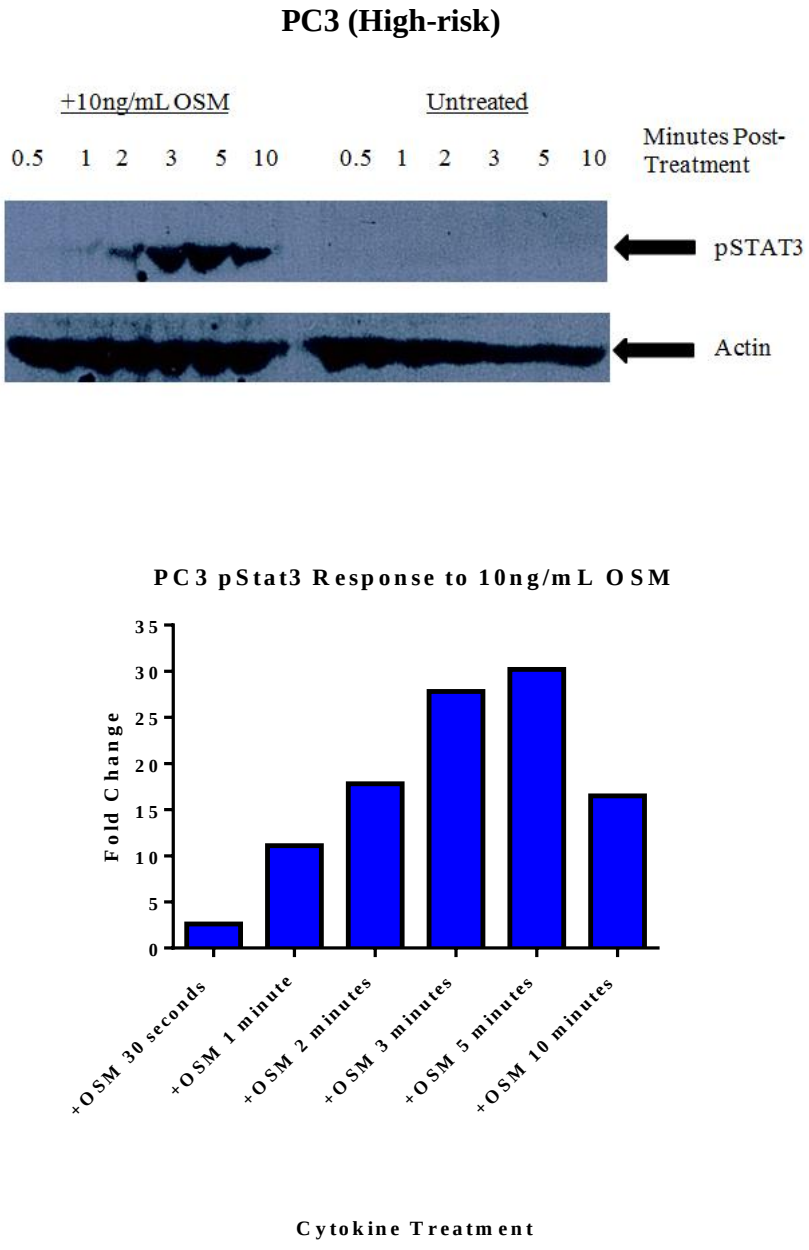


Figure 17: PC3 and DU 145 STAT3 Activation upon Treatment with 10ng/mL OSM. The PC3 cell line (high-risk) and the DU 145 cell line (low-risk) both show particularly early activation of STAT3 in response to 10ng/mL of OSM. Cells were seeded in 6 well plates and treated with 10ng/mL of OSM for indicated times, harvested, and cell lysates were analyzed for pSTAT3 by Western blotting. Actin was used as a loading control. ImageJ software was used to quantify bands and GraphPad Prism was used to graph the data.

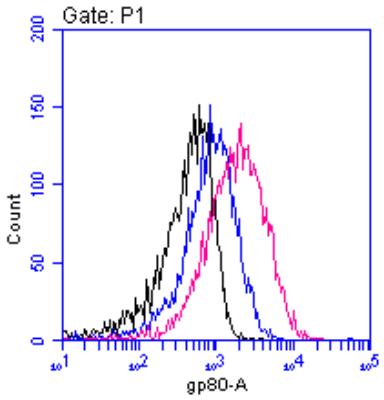
3.2.4 IL-6Rα AND GP130 DISTRIBUTION ON PC3 AND DU 145

Although there is literature that states that both PC3s and DU 145s express the primary and secondary receptors for IL-6[82], we wanted to clarify that the receptors were still present on both of the cells lines, and the lack of signaling in PC3s was not due to lack of receptor presence on the cell surface. Flow cytometry analysis for the receptors showed that both PC3s and DU 145s expressed the primary (IL-6RAα) and secondary receptors (gp130) for IL-6, and the lack of STAT3 activation in PC3s was not due to a lack of receptor presence (Figure 18).

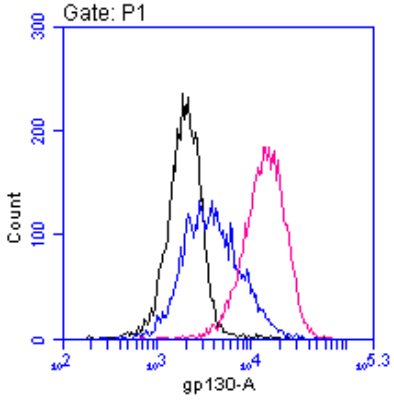
A.

DU 145 (Low-risk)

IL-6RAα



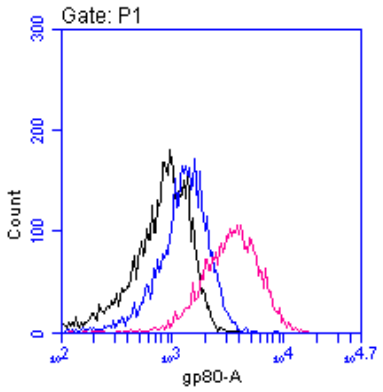
gp130



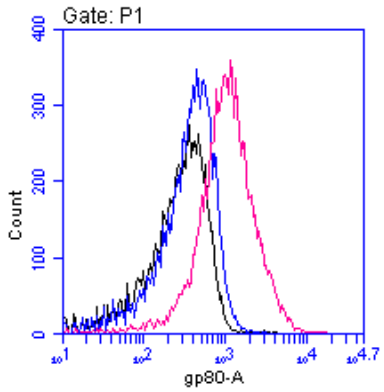
B.

PC3 (High-risk)

IL-6RAα



gp130



Key:
Black= Unstained
Blue = Isotype Control
Pink = Antibody

Figure 18: PC3 and DU 145 Receptor Distribution on the Membrane. The PC3 cell line and the DU 145 cell line both express the IL-6RAα and gp130 receptors. DU 145 (low-risk) (A) and PC3 (high-risk) (B) were set up at $1x10^6$ cell/mL. 100 µL aliquots were removed from this and placed into 96 well plates and either left unstained (black), stained with the desired isotype control antibody (blue), or stained with anti-IL-6RAα or anti-gp130 (pink). Graphs were made using AccuriC6 software.

4.0 DISCUSSION

4.1 AIM I

4.1.1 LCL GROWTH CURVE ANALYSIS

As seen in Figures 3 and 4, low- and high-risk LCLs show a marked difference in their growth responsiveness to IL-6. We hypothesized that this difference in response could be due to a defect in the signaling receptor or in the downstream signaling pathway. Figure 5 shows that both the high- and low-risk LCLs express the primary (IL-6Rα) and the secondary receptors (gp130) on their cell surfaces. This indicates that although they showed a difference in cellular proliferation in response to IL-6, this is not due to a lack of receptor presence on the membrane. However, we do not know whether or not the location of the receptors on the membrane is affecting how the receptors signal. As stated previously, gp130 is commonly concentrated in lipid rafts and caveolae[10], and although LCLs are expressing the primary receptor for IL-6 (Figure 5), we do not know where this binding receptor is localizing on the membrane in relation to its signaling receptor gp130. As seen in Figure 3, however, we can see that the growth curves of LCLs in response to IL-6 remain consistent across all LCLs developed from Tobago men. We hypothesize that this indicates that there is something about the receptor polymorphism G148R that specifically affects how these cells respond to IL-6.

4.1.2 LCL STAT3 ANALYSIS

STAT3 activation analysis, as measured by phosphorylation of STAT3 at tyrosine residue 705, shows that although the low-risk LCLs do not proliferate as well in response to IL-6 as the high-risk LCLs, there is still STAT3 activation occurring (Figures 6 and 7). Together, Figures 6 and 7 indicate that for both the high- and low-risk LCLs, there is no defect in the JAK/STAT pathway, and both activate this pathway in response to IL-6. Although there was a slight timing difference between the two cell lines, this difference would not account for the different responses to IL-6 seen in the growth curves. If the differences in growth were simply due to a 10-minute slower signaling in the low-risk cell line, a natural assumption would be that the low-risk cell line would eventually catch up and proliferate in the same manner as the high-risk LCLs. These data led us to believe that the differences in growth may be due to differential gene activation as a result of JAK/STAT signaling. For example, perhaps in the high-risk cell line, genes that induce proliferation and survival are being turned on, while in the low-risk cell line, genes that induce differentiation (which slows cellular growth and proliferation), or genes that induce apoptosis may be turned on.

The lack of response to IL-11 and OSM in both cell lines (Figure 7) could be due to lack of primary receptor on the cell lines. We have not found any literature to suggest that lymphoblastoid cell lines possess the primary receptors for either IL-11 or OSM. Since Dr. Jill Henning previously showed that IL-11 and OSM do not have any effect on growth in response to either of these cytokines[80], and we saw no STAT3 activation, we did not investigate these cytokines any further in relation to LCLs.

Figure 8 shows that high- and low-risk LCLs have differing levels of constitutive total STAT3 in the cells, but neither cell line showed a dramatic increase in total STAT3 upon

treatment with 100ng/mL of IL-6. Levels of total STAT3 exhibited around a 2-fold change or less, while upon 100ng/mL of IL-6 treatment, levels of pSTAT3 in the same cellular lysate exhibited a 40 to over 100-fold increase. To summarize, upon IL-6 treatment, we see dramatic increases in pSTAT3 levels, but only very small increases in overall STAT3 levels. This indicates that upon treatment, STAT3 that is already present in the cytoplasm is being converted to pSTAT3, and there is very little *de novo* protein synthesis. In addition, cells were IL-6 starved overnight, and yet we are still seeing constitutive STAT3 present in the cells, most notably in the high-risk LCLs. Although studies have shown that EBV-transformed B cells produce IL-6 as an autocrine growth factor[83], our particular LCLs were tested and found that they did not produce IL-6 as measured by ELISA (Jenkins, unpublished data). As a consequence, we do not fully understand why the high-risk LCLs have a higher constitutive amount of STAT3 than the low-risk LCLs do, and it is possible that the difference in STAT3 levels could be contributing to the differences in growth. However, if the differences in the levels of STAT3 in the LCLs is resulting in the differences in their growth phenotypes, we expect that we would not see differences in gene expression.

4.1.3 LCL GENE EXPRESSION ANALYSIS

When we looked at the JAK/STAT Pathway PCR Array plates, we saw that there were up and down-regulation of a number of genes, and they were differentially regulated between the high- and low-risk LCLs. It is difficult to paint a complete picture with just the few genes that were up- or down-regulated. However, it did give us a few ideas for genes that might be responsible, at least in part, for the differences in growth that we see from the LCLs in response to IL-6.

CXCL9 (also known as Mig, which denotes 'monokine induced by gamma interferon') is up-regulated in the low-risk LCLs (Figure 9). CXCL9 has been known to be a T cell chemoattractant. Studies have shown that CXCL9 specifically attracts anti-tumoral T lymphocytes, and could therefore lead to a reduced tumor growth *in vivo*[84]. GHR (growth hormone receptor) gene encodes for the transmembrane receptor for growth hormone. Binding of the growth hormone to the growth hormone receptor leads to intracellular signaling through the JAK/STAT pathway leading to growth[85]. Although it may seem counter intuitive for the low-risk cell line to up-regulate this gene, it may be somehow trying to compensate for the lack or defect in signaling through the IL-6 pathway. OSM is down-regulated in the low-risk LCLs. OSM has been described previously, but notably it has been shown to act as a growth factor for an HHV-8 related cancer called Kaposi sarcoma. OSM has also been shown to enhance the development of certain cells such as endothelial cells and hematopoietic cells[8]. A down-regulation of OSM would result in less secreted OSM by the low-risk LCLs, and as a result would not have an enhanced effect on the surrounding target cells or tissues. SOCS3 is a suppressor of cytokine signaling, as the name suggests. It works to negatively regulate cytokine signaling within the cell. It is down-regulated in the low-risk LCLs, which does not match the lack of growth seen in response to IL-6.

CSF2RB, JAK2, and SRC are all up-regulated in the high-risk LCLs. CSF2RB is a subunit of receptors to the following cytokines: IL-3, IL-5, and GM-CSF. Deregulated signaling in these cytokine pathways is often associated with chronic inflammatory diseases and hematological malignancies. This receptor system seems to be primarily involved in emergency situations, where a large amount of hemopoietic cells are required to elevate the immune response[86]. An up-regulation of this gene could lead to inflammation in the high-risk LCLs.

JAK2 is widely expressed in most cells and is involved in signaling of IL-3, GM-CSF, IL-5, and IFY-γ, but not IL-6, IFN-α or IFN-β[87]. It is interesting that both JAK2 and CSF2RB are up-regulated, considering that both of these genes are involved in GM-CSF/IL-3/IL-5 signaling. SRC is also up-regulated in the high-risk LCLs, and is part of the SRC family kinases (SFK). When not controlled properly, these SFKs show oncogenic activity and have been known to induce malignant transformation of cells. Additionally, SFK deregulation has been found in about 80% of colorectal cancers[88]. The down-regulated genes, SMAD5 and SOCS4 are both negative regulators of signaling. SMAD5 works with other SMAD proteins to transduce signals from TGF-β that regulate cell proliferation, differentiation and death. Ultimately, SMAD5 works to inhibit proliferation of hematopoietic progenitor cells[89]. SOCS4 works very similarly to SOCS3 (explained previously), by inhibiting cytokine signaling. By down-regulating inhibitory signals, you allow the cell to respond to growth stimulatory signals, which is seen in the high-risk LCLs.

These data encouraged us to repeat the experiments. The JAK/STAT plates were only completed once, and since we used SYBR green technology, we had to delete or exclude a majority of genes due to melt curves that caused us to mistrust the data. By using Taqman plates, we could ensure that a majority of the data is usable. The Human IL-6 Pathway PCR Array plates were performed in duplicates, and also showed a number of genes that were significantly up- or down-regulated in response to IL-6 10 minutes post-treatment.

In the high-risk LCLs, TNF, IL-6, SOCS1, and SOCS2 are all up-regulated, while TP53BP2 is down-regulated (Figure 10). TNF is a pro-inflammatory cytokine that exerts multiple biological effects. Locally, low-levels of TNF expression is helpful to the host defense in response to injury or infection. However, if TNF expression is not tightly controlled,

overproduction of this cytokine results in systemic inflammation and tissue damage[90]. An increased gene expression of 7.1-fold over untreated controls in the high-risk LCLs could be leading to the increased growth we saw in the growth curves. TNF is not significantly increased or decreased in the low-risk LCLs. TNF has also been shown to enhance IL-6 production[91]. IL-6 was up-regulated in both the high- and low-risk LCLs. IL-6, as mentioned previously, has a wide variety of biological activities: IL-6 both promotes and inhibits growth, induces differentiation and regulates gene expression. IL-6 has been known to enhance proliferation of B-cells in vitro, and also induce antibody production of B-cells. Overall, IL-6 has been shown to have a central role in host defense mechanisms by regulating the immune response[91]. An increased gene expression of two-fold or greater in both the high- and low-risk cell lines could induce proliferation of LCLs in vivo. SOCS1 and SOCS2 (Suppressor of Cytokine Signaling 1 and 2) are both up-regulated in the high-risk LCLs. SOCS proteins are negative regulators of cytokine signaling, and SOCS 1 and SOCS2 have been shown to specifically inhibit IL-6 cytokine signaling[92]. As a result, it The SOCS proteins were not significantly up- or down-regulated in the low-risk LCLs. TP53BP2 encodes a protein known as apoptosis stimulating protein of p53-2, which is also known as ASPP2 and 53BP2L. This protein typically inhibits cell growth and stimulates apoptosis through a p53-mediated pathway. The expression of this protein is frequently suppressed in human cancers; without the protein expression, cells are less likely to undergo apoptosis and more likely to grow uncontrollably and favor the initiation of cancer [93]. This gene is up-regulated in the low-risk LCLs and down-regulated in the high-risk LCLs. This is an interesting find because this would favor the initiation of cancer in the high-risk LCLs and not in the low-risk LCLs. The TP53BP2 gene is up-regulated by almost 4-fold compared to the

untreated in the low-risk LCLs, while it is down-regulated by greater than 4-fold in the high-risk LCLs.

In the low-risk LCLs, SH2, IL-6, and TP53BP2 are up-regulated, while both SH3 and IL-1β are down-regulated (Figure 10). SHC3 is also known as the Src homology 2 domain containing transforming proteins 3. SHC proteins are adaptor proteins that serve as substrates for tyrosine kinases. Once phosphorylated, these proteins associate with receptor tyrosine kinases and Grb2. The association with Grb2 leads to the activation of the Ras pathway[94] and the MAP kinase cascade[95]. The activation of these pathways results in cell proliferation, differentiation, migration and survival[95]. SHC3 is down-regulated in the low-risk cell line, and there is no significant up or down-regulation of any of the SHC family member proteins in the high-risk cell line. Since SHC3 is down-regulated in the low-risk cell line, this would result in less Ras and MAP kinase cascade activation, and as a result less proliferation and differentiation. A down-regulation of SHC3 in the low-risk cell line also supports the idea that the genes regulated by IL-6 signaling in the low-risk cell line do not favor the initiation or progression of cancer. IL-1β is a member of the interleukin-1 family of cytokines, which is comprised of 11 total members. IL-1β is a proinflammatory cytokine, and has been implicated in a number of autoimmune diseases. When IL-1β is blocked, there is a dramatic decrease in inflammation[96]. IL-1β is down regulated in the low-risk LCLs, which would induce less inflammation in the low-risk genotype than in the high-risk genotype. These data fit with our growth curves, which show that the low-risk LCLs grow much slower and do not respond as well to IL-6 as the high-risk cell line. IL-1β was not significantly up or down regulated in the high-risk LCLs.

Using the array plates is a good way to survey a large amount of genes involved in this pathway in a short amount of time. However, once certain genes are designated as important,

further work could be done to look deeper into the differences between genes deemed important in this pathway. Future work could look into the differences found using the IL-6 pathway array plate. Specifically, looking into the differences seen in the regulation of the TP53BP2 gene would be particularly interesting. This gene could be inducing apoptosis in the low-risk cell line and preventing such in the high-risk cell line. This would result in an increased survival and proliferation in the high-risk cell LCLs.

There are a number of reasons why we could be seeing differential activation of genes between the high- and low-risk LCLs upon IL-6 stimulation. IL-6 has been shown to activate both the JAK/STAT and MAPK pathways in B cells[97,98]. It is possible that the high- or low-risk cell line is also activating alternate pathways upon IL-6 stimulation. STAT3 has also been known to be modified after activation by other proteins in the cell, which could alter its' binding activity, or cause it to preferentially bind to certain DNA sequences rather than others[99].

4.1.4 AIM I CONCLUDING REMARKS

We saw that there was a difference in growth and downstream gene expression as a result of IL-6 stimulation in the high- and low-risk lymphoblastoid cell lines. Based off of our data, we were able to conclude that these differences may be the result of the G148R SNP in the gp130 extracellular ligand binding domain. Although gp130 is expressed on most, if not all, cells of the body, we reasoned the effect that the G148R SNP has on IL-6 signaling seen in the LCLs might not be identical on all cells of the body. In another kind of cell, it is likely that this SNP could have different effects, particularly a SNP involved in cytokine signaling, since we do know that the same cytokine can activate different components in different cell types[100]. In addition to wanting to look at other cells, we also wanted cells that expressed the primary receptors to IL-11

and OSM, to investigate whether the G148R SNP has an effect on these cytokine signaling pathways as well.

4.2 AIM II

4.2.1 PROSTATE CANCER LINES GROWTH CURVE ANALYSIS

Prostate cancer cells PC3 and DU 145 are adherent cell lines that have been used extensively in prostate cancer research, and are well characterized[53]. As stated previously, they are both androgen insensitive, and lack the androgen receptor, 5α-reductase, and PSA[53]. We had both of these cell lines in our lab, and when genotyped for the gp130 G148R SNP, we found that PC3s expressed the high-risk genotype, and DU 145s expressed the low-risk genotype. In addition, literature has shown that both of these cell lines express the primary receptors for IL-6, IL-11, OSM[59,62,101]. To add to this even further, IL-6 has been shown to be in neoplastic prostate tissues at concentrations 18-fold higher when compared to benign or normal prostate tissue[102].

Previous studies have shown that exogenous IL-6 has a significant effect on growth of PC3 and DU 145 cells, at concentrations ranging from 10-100ng/mL[50], while other studies showed that androgen insensitive cell lines, PC3 and DU 145, did not exhibit any growth increase when treated with exogenous IL-6[49]. Another study has shown that OSM is capable of enhancing the growth of DU 145 cells, but not PC3s[62]. In our research, we have shown that 10, 25, or 100ng/mL of cytokines IL-6, IL-11, or OSM do not have a significant effect on the growth of DU 145s or PC3s (Figures 12 and 13).

Although this indicates that IL-6, IL-11, and OSM do not have any effect on growth in either PC3 or DU 145 cell lines, it does not necessarily mean that there is not a difference in downstream signaling due to the R148G polymorphism. IL-6 is a growth and differentiation factor for B lymphocytes[103], so we would expect that when exogenous IL-6 is added, that there is an effect on growth. Even though none of the cytokines had a differential effect on growth, we decided to move forward and see if the polymorphism resulted in a difference in downstream signaling.

4.2.2 PROSTATE CANCER LINES STAT3 ANALYSIS

By looking at STAT3 activation (phosphorylation of STAT3), we are able to see the effects of cytokines on proximal signaling events. Figures 13-15 show that both PC3 and DU 145 respond as early as 2 minutes post treatment to 10ng/mL of OSM, that neither cell line responds to 10-100ng/mL of IL-11, and only DU 145 shows STAT3 activation to 10-100ng/mL of IL-6. These data contrast data in the literature, which have shown a number of different things involving these cell lines. One study completed by *Mori et al.* stated that in PC3s, STAT3 failed to become tyrosine phosphorylated after treatment with OSM[62]. Other research has shown that in DU 145 cells, STAT3 is activated by 25-200ng/mL of IL-11 as early as 10 minutes post treatment[59]. *Pitulis et al.* showed that PC3 cells phosphorylated STAT3 upon treatment with 25ng/mL IL-6[81], however this is not what we saw in our results when treated with 10 or 100ng/mL.

There are a number of reasons that PC3s may not be responding to IL-6. First, the receptor may be deficient in signaling. Although IL-6RAα and gp130 are both present on the cell surface (Figure 16), it may be incapable of inducing signaling for some reason. To test this

hypothesis, you could transfect the cells with a plasmid expressing the high-risk receptor, and then repeat the experiments. If signaling is restored, it would mean that there is something wrong with the receptor that is inhibiting signaling through the JAK/STAT pathway. Second, it is possible that PC3 (high-risk) are signaling through STAT1 rather than the more common STAT3. This hypothesis can also be easily tested by looking at activation of STAT1 (phosphorylation of STAT1) rather than STAT3. It is also possible that after being cultured for a lengthy period of time, any cells are capable of mutating and changing over a period of time. In fact, our PC3 or DU 145 cells might behave differently than cells in other laboratories, and this seems to be evident by the discrepancies surrounding this research that can be found in the literature.

4.2.3 AIM II CONCLUDING REMARKS

The reasoning for the signaling deficiency in the high-risk prostate cancer cell line PC3 is unclear, however, it is obvious that researchers need to look into this further. PC3 and DU 145 are often used to study hormone insensitive prostate cancer, and IL-6 is often implicated in helping the hormone insensitive phenotype develop after androgen ablation by interacting with the androgen-signaling pathway[13,104,105]. Analyzing this pathway in cells that are unresponsive to IL-6 is problematic.

Researchers have also shown that STAT3 is constitutively active in PC3 and DU 145 cells[78]. Our research shown in Figure 16 supports these findings. Both DU 145 and PC3 have constitutive levels of STAT3 in the cells, and upon 100ng/mL of IL-6 stimulation, levels of STAT3 showed less than a two-fold change. This can be compared to Figure 15 which shows a 25-30 fold change in pSTAT3 levels upon IL-6 stimulation by 30 minutes post treatment.

Similarly to the LCLs, this indicates that a majority of the phosphorylated STAT3 is simply being converted from STAT3 that is already present, and there is not a lot of *de novo* STAT3 protein synthesis. It is also a possibility that STAT3 may be constitutively present because of the autocrine feedback loop present in these prostate cancer cells, since they produce low levels of IL-6 (Figure 11)[106].

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

The R148G polymorphism in the gp130 signaling receptor has various effects on different cell types, as evidenced by our research using lymphoblastoid cell lines and prostate cancer cell lines. In LCLs, IL-6 plays a role in growth and proliferation, and the R148G SNP may alter the way the LCLs respond to IL-6. High- and low-risk LCLs show differences in growth in response to IL-6, as evidenced by the growth curves. Upon IL-6 stimulation, both the high- and low-risk LCLs phosphorylate STAT3 by 10 minutes post-treatment, but activate a different set of genes upon STAT3 activation. The genes that are up- or down-regulated may explain the various responses we see in growth upon IL-6 treatment. We cannot make any conclusions on how this SNP affects the response to IL-11 or OSM in the LCLs, since we do not believe that they express the primary receptors for these cytokines. Another interpretation of the results could be that the constitutive STAT3 levels differ between the high- and low-risk genotype, and this is resulting in the differences in growth that are seen. However, we still see a significant amount of phosphorylation in the low-risk LCLs, which would indicate that despite the differences in STAT3 levels, it is still being phosphorylated, and should result in cell proliferation which is slower, but eventually catches up to the high-risk LCLs. This most likely indicates that there is a more downstream mechanism that is being differentially regulated to result in growth differences between the high- and low-risk LCLs.

Additional studies need to be completed to further elucidate which genes are activated in each gp130 genotype, why they are differentially regulated in the same type of cells, and how these genes work to regulate growth responsiveness to IL-6 in LCLs.

When we switched to prostate cancer cells, we did not see any effect on growth in either the high- or low-risk genotype (PC3 and DU 145, respectively) in response to IL-6, IL-11, or OSM. We have discovered that PC3s do not activate STAT3 in response to IL-6 treatment, despite the fact that they have both the primary and secondary receptors for IL-6. It is possible that they are activating STAT1 or STAT3 at another less common residue. Additionally, it is accurate to say that research using PC3 and DU 145 cell lines often show discrepancies between studies on how these cell lines respond to IL-6, IL-11, or OSM. Consequently, investigators should be more cautious when trying to draw conclusions using these cell lines in relation to the role of these cytokines in development of prostate cancer. It would be ideal in future studies to use primary prostate or primary prostate cancer cells with a high- and low-risk genotype when analyzing how the G148R SNP affects the responsiveness to IL-6, IL-11, or OSM.

6.0 STRENGTHS AND LIMITATIONS

As with many research projects, we would like to recognize that there were some limitations to this research. We would like to recognize that a few of the Western blots were only performed once, and not repeated. In particular, the Western blots on total STAT3 (Figures 8 and 16) were only performed once. In addition, although we had replicates for the ELISA and growth curve experiments, they were not repeated. The strength of Dr. Jill Henning's research was that she was able to use three low-risk cell lines and seven high-risk cell lines, while we were limited to one of each genotype. This research would be strengthened if we used multiple cell lines of each genotype.

We do believe that by utilizing multiple array plates each which utilize different technologies, we were able to verify with certainty that there are different genes that were up- or down-regulated by 10 minutes post treatment with IL-6. Another very strong asset to our project was the ability to use B cells that were transformed from the Tobago men that the prostate cancer studies were performed on. The Xcelligence machine was also a very big advantage, as we were able to confirm the growth curve analysis using a much more specific and sensitive method.

7.0 PUBLIC HEALTH SIGNIFICANCE

The cause of prostate cancer still remains largely unknown. What is known is that age, family history, and race are all risk factors for prostate cancer[43]. We have previously demonstrated that there was a positive association between prostate cancer and human herpesvirus 8 (HHV-8) (OR=2.24; 95% CI 1.29-3.90), and further showed a positive association between HHV-8, GG allele of gp130, and prostate cancer (OR=3.41, 95% CI 1.36-8.55, p=0.009). We further elucidated the role that the G148R SNP has in inflammation in response to various cytokines. By further understanding the role that this SNP has in the development of an inflammatory state and possibly establishing an environment for the initiation of cancer, we will also be able to understand how to lower the risk for prostate cancer. In addition, by genotyping men with beginning stages of prostate cancer, we would be able to inform them that they may be at a higher risk for prostate cancer, and they can be more carefully followed over the course of their lives. By no means is this the only way that prostate cancer develops. Rather, there are many different situations, pathways, risk factors, environmental factors, and cellular factors that may all contribute to the development of prostate cancer. By better understanding each one, hopefully the scientific community can work together to ease the burden prostate cancer has on men in the United States as well as other nations.

APPENDIX A: LIST OF GENES IN JAK/STAT PLATE ARRAY

JAK/STAT Plate Array from SABiosciences (Cat. # PAHS-039Z)

Symbol	Description
A2M	Alpha-2-macroglobulin
SH2B2	SH2B adaptor protein 2
BCL2L1	BCL2-like 1
CCND1	Cyclin D1
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
CRK	V-crk sarcoma virus CT10 oncogene homolog (avian)
CRP	C-reactive protein, pentraxin-related
CSF1R	Colony stimulating factor 1 receptor
CSF2RB	Colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
CXCL9	Chemokine (C-X-C motif) ligand 9
EGFR	Epidermal growth factor receptor
EPOR	Erythropoietin receptor
F2	Coagulation factor II (thrombin)
F2R	Coagulation factor II (thrombin) receptor
FAS	Fas (TNF receptor superfamily, member 6)
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide
FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)
ISG15	ISG15 ubiquitin-like modifier
GATA3	GATA binding protein 3
GBP1	Guanylate binding protein 1, interferon-inducible
GHR	Growth hormone receptor
HMGA1	High mobility group AT-hook 1
IFNAR1	Interferon (alpha, beta and omega) receptor 1
IFNG	Interferon, gamma
IFNGR1	Interferon gamma receptor 1
IL10RA	Interleukin 10 receptor, alpha
IL20	Interleukin 20
IL2RA	Interleukin 2 receptor, alpha
IL2RG	Interleukin 2 receptor, gamma
IL4	Interleukin 4
IL4R	Interleukin 4 receptor
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
INSR	Insulin receptor
IRF1	Interferon regulatory factor 1
IRF9	Interferon regulatory factor 9
JAK1	Janus kinase 1

JAK2	Janus kinase 2
JAK3	Janus kinase 3
JUN	Jun proto-oncogene
JUNB	Jun B proto-oncogene
MMP3	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)
MPL	Myeloproliferative leukemia virus oncogene
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NOS2	Nitric oxide synthase 2, inducible
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)
OAS1	2'-5'-oligoadenylate synthetase 1, 40/46kDa
OSM	Oncostatin M
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide
PIAS1	Protein inhibitor of activated STAT, 1
PIAS2	Protein inhibitor of activated STAT, 2
PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha
PRLR	Prolactin receptor
PTPN1	Protein tyrosine phosphatase, non-receptor type 1
PTPRC	Protein tyrosine phosphatase, receptor type, C
SH2B1	SH2B adaptor protein 1
SIT1	Signaling threshold regulating transmembrane adaptor 1
SLA2	Src-like-adaptor 2
SMAD1	SMAD family member 1
SMAD2	SMAD family member 2
SMAD3	SMAD family member 3
SMAD4	SMAD family member 4
SMAD5	SMAD family member 5
SOCS1	Suppressor of cytokine signaling 1
SOCS2	Suppressor of cytokine signaling 2
SOCS3	Suppressor of cytokine signaling 3
SOCS4	Suppressor of cytokine signaling 4
SOCS5	Suppressor of cytokine signaling 5
SP1	Sp1 transcription factor
SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1
SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
STAM	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 1
STAT1	Signal transducer and activator of transcription 1, 91kDa
STAT2	Signal transducer and activator of transcription 2, 113kDa
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
STAT4	Signal transducer and activator of transcription 4
STAT5A	Signal transducer and activator of transcription 5A
STAT5B	Signal transducer and activator of transcription 5B
STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced

STUB1	STIP1 homology and U-box containing protein 1, E3 ubiquitin protein ligase
TYK2	Tyrosine kinase 2
USF1	Upstream transcription factor 1
YY1	YY1 transcription factor
B2M	Beta-2-microglobulin
HPRT1	Hypoxanthine phosphoribosyltransferase 1
RPL13A	Ribosomal protein L13a
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACTB	Actin, beta
HGDC	Human Genomic DNA Contamination
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
RTC	Reverse Transcription Control
PPC	Positive PCR Control
PPC	Positive PCR Control
PPC	Positive PCR Control

APPENDIX B: LIST OF GENES IN IL-6 PLATE ARRAY

Human IL-6 Pathway Plate Array from Life Technologies (Cat. # 4418794)

Symbol	Description
18S	DIM1 dimethyladenosine transferase 1 homolog
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
GUSB	Glucuronidase, beta
AKT1	V-akt murine thymoma viral oncogene homolog 1
ARAF	V-raf murine sarcoma 3611 viral ongoene homolog
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CCL2	Chemokine (C-C motif) ligand 2
CD4	CD4 molecule
CHUK	Conserved helix-loop-helix ubiquitious kinase
CXCL12	Chemokine (C-X-C motif) ligand 12
FRAP1	Mechanistic target of rapamycin
GRB2	Growth factor receptor-bound protein 2
HRAS	Harvey rat sarcoma viral oncogene homolog
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
IKBKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
IL10	Interleukin-10
IL18	Interleukin-18
IL1A	Interleukin-1 alpha
IL1B	Interleukin-1 beta
IL2	Interleukin-2
IL4	Interleukin-4
IL6	Interleukin-6
IL6R	Interleukin-6
IL6ST	Interleukin-6 Signal Transducer
IL8	Interleukin-8
JAK3	Janus kinase 3
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAP2K1	Dual-specificity mitogen-activated protein kinase 1

MAP2K2	Dual-specificity mitogen-activated protein kinase 2
MAP2K3	Dual-specificity mitogen-activated protein kinase 3
MAP2K4	Dual-specificity mitogen-activated protein kinase 4
MAP2K5	Dual-specificity mitogen-activated protein kinase 5
MAP2K6	Dual-specificity mitogen-activated protein kinase 6
MAPK1	Mitogen-activated protein kinase 1
MAPK12	Mitogen-activated protein kinase 12
MAPK3	Mitogen-activated protein kinase 3
MAPK6	Mitogen-activated protein kinase 6
MAPK7	Mitogen-activated protein kinase 7
MRAS	Muscle RAS oncogene homolog
MYC	V-myc myelocytomatosis viral oncogene homolog
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-Cells 1
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-Cells 2
NFKBIA	Nucelar factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
NFKBIB	Nucelar factor of kappa light polypeptide gene enhancer in B-cells inhibitor beta
NFKBIE	Nucelar factor of kappa light polypeptide gene enhancer in B-cells inhibitor epsilon
NRAS	Neuroblastoma RAS viral oncogene homolog
PIAS3	Protein inhibitor of activated STAT3
PIK3C2A	Phosphadtidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 alpha
PIK3C2B	Phosphadtidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 beta
PIK3C2G	Phosphadtidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma
PIK3C3	Phosphadtidylinositol 3-kinase, catalytic subunit type 3
PIK3CA	Phosphadtidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIK3CB	Phosphadtidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta
PIK3CD	Phosphatidylinositol 3-kinase catalytic delta polypeptide
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2
PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3
PIK3R4	Phosphoinositide-3-kinase, regulatory subunit 4
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5
PIM1	Pim-1 oncogene
PPP1R12A	Protein phosphatase 1, regulatory subunit 12A
PPP1R15A	Protein phosphatase 1, regulatory subunit 15A
PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme
PPP2CB	Protein phosphatase 2, catalytic subunit, beta isozyme

PPP2R1A	Protein phosphatase 2, regulatory subunit A, alpha
PPP2R1B	Protein phosphatase 2, regulatory subunit A, beta
PPP2R2A	Protein phosphatase 2, regulatory subunit B, alpha
PPP2R2B	Protein phosphatase 2, regulatory subunit B, beta
PPP2R4	Protein phosphatase 2A activator, regulatory subunit 4
PPP2R5A	Protein phosphatase 2, regulatory subunit B', alpha
PPP2R5C	Protein phosphatase 2, regulatory subunit B', gamma
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
PTPN6	Protein tyrosine phosphatase, non-receptor type 6
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1
REL	V-rel reticuloendotheliosis viral oncogene homolog
RELA	V-rel reticuloendotheliosis viral oncogene homolog A
RELB	Avian reticuloendotheliosis viral (v-rel) oncogene related B
RRAS	Related RAS viral (r-ras) ongoene homolog
RRAS2	Related RAS viral (r-ras) ongoene homolog 2
SHC1	SHC (Src homology 2 domain containing) transforming protein 1
SHC2	SHC (Src homology 2 domain containing) transforming protein 2
SHC3	SHC (Src homology 2 domain containing) transforming protein 3
SOCS1	Suppressor of cytokine signaling 1
SOCS2	Suppressor of cytokine signaling 2
SOCS3	Suppressor of cytokine signaling 3
SOCS5	Suppressor of cytokine signaling 5
SOCS6	Suppressor of cytokine signaling 6
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)
TIMP1	TIMP metallopeptidase inhibitor 1
TNF	Tumor necrosis factor
TP53BP2	Tumor protein p53 binding protein, 2

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