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# The Role of ST-18 in Inv16 Leukemogenesis

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# THE ROLE OF ST-18 IN INV16 LEUKEMOGENESIS

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# ABSTRACT

The human gene Suppression of Tumorigenicity-18 (ST-18) encodes a zinc finger protein previously associated with breast carcinoma, however it may have an oncogenic role in the development of Inversion 16 leukemia (inv16). This project assayed the level of ST-18 in inv16 leukemic lines compared to unrelated cancer lines. The data show that ST-18 is more highly expressed in an inv16 acute myeloid leukemic cell line (Me-1) compared to Kasumi-1 and U937 non-inv16 cancer lines. In addition, murine ST-18 was successfully cloned into plasmid vector pMSCV2.2 for use in future over-expression experiments.

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# BACKGROUND

## I. LEUKEMIA

### I.1 DESCRIPTION

Leukemia is a specific type of cancer pertaining to white blood cells. It is the leading cause of death by disease in children and young adults between the ages of 0 and 20 and accounts for approximately 33% of cancer cases in children. Despite its reputation for affecting the young, however, leukemia does not discriminate based on age. The disease can affect any age, any race, any gender, at any time (Leukemia Research, 2011). In the US, more than 30,000 new cases of leukemia are diagnosed every year, and adult onset accounts for 90% of the new cases (Xie Y et al., 2003).

### I.II TYPES

Leukemia can be categorized into four types, according to the type of white blood cell affected and how quickly the disease develops and advances (Vachani, 2007). The four types include Acute Myelogenous Leukemia (AML), Chronic Myelogenous Leukemia (CML), Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL). Types are considered acute when the white blood cells multiply very quickly (AML and ALL). In the chronic types of leukemia (CML and CLL), white blood cells multiply very slowly (Oncolink.org). In the myelogenous types of leukemia (AML and CML), myeloid cells, granulocytes and monocytes are affected. In the lymphocytic types of leukemia (ALL and CLL), lymphocytes are affected (Life Extension, 2011).

## I.III CAUSES

Leukemia is a specific type of cancer pertaining to white blood cells. White blood cells are made in the bone marrow and spend their lives traveling through the blood stream to other parts of the body to defend the body against infections. Normally, all cells in the body regardless of type are created and destroyed at the same rate. This rate can change, depending on what the body requires, but it is always a stable process, keeping the body balanced (Peterson, 2011). Leukemia occurs as a result of DNA damage to white blood cells (Reilly, 2004). One or more DNA mutations can disrupt this balanced process, causing cells to become damaged, mutated and cancerous. The type of cell affected by the mutation dictates the type of cancer. The DNA damage in leukemia is caused by chromosome translocations (shifting and re-arrangement of chromosome segments), mutations, or a combination of both (Irons and Stillman, 1996). The mutation(s) cause cell production in the bone marrow to go awry. The body starts to produce new abnormal white blood cells when they aren't required and when they are not mature. The body arrests blood cell precursors, called "blasts", before they can mature into blood cells capable of fighting infection (National Marrow Donor, 2011). The body also begins inappropriately restricting mature white blood cells from undergoing cell death, or "apoptosis" (**Figure 1**). The abnormal white blood cells gain the unique ability to divide or "proliferate" out of control. They quickly take over the bone marrow and spread to other tissues and organs in the body via the bloodstream, crowding out other healthy cells in their path (MayoClinic, 2011). The body's bone marrow may also begin to make abnormal red blood cells and platelets (National Marrow Donor, 2011).

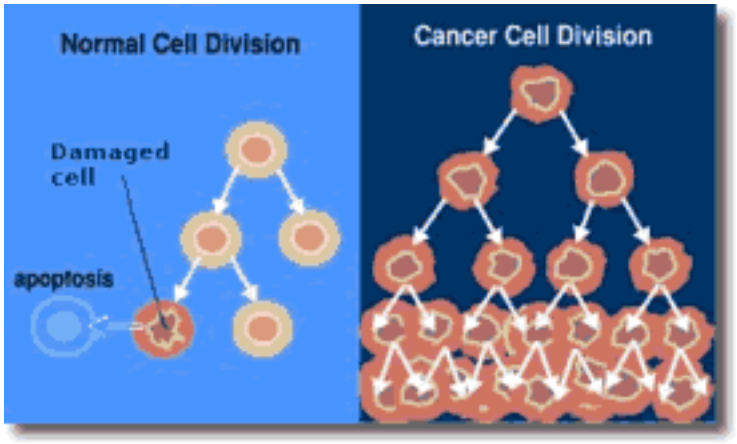


**Figure 1: Comparison of Normal and Cancerous Cell Division.** Normally, mutated cells undergo apoptosis (cell death) to eliminate them from the body (left panel), while cancerous cells (right panel) incorporate the mutation and acquire a cell proliferation advantage (diseaseeducation.com).

# II. AML AND INV(16)

## II.I DESCRIPTION

Acute myeloid leukemia is the most common of the four types of leukemia. AML occurs in both children and adults, but is most common in adults over the age of 65 (Mayo Clinic, 2010). AML affects the myeloid progenitor cells, which are normally capable of renewing themselves and of transforming, or "differentiating," into red blood cells, white blood cells or platelets. DNA mutations arrest myeloid cells in an early stage of development, restricting maturation and causing an overabundance of young mutated cells incapable of fighting infections (National Marrow Donor, 2011). These young abnormal cells acquire a survival advantage and are able to proliferate uncontrollably, allowing them to crowd out the normal healthy cells.

The types of mutations responsible for the abnormal cells are chromosomal

translocations, chromosomal rearrangements and other genetic abnormalities (Mayo Clinic, 2010). Inversion 16 leukemia, or inv(16), is a specific type of leukemia marked by the rearrangement of chromosome 16. Inv(16)(p13;q22), is one of the most frequent recurring chromosomal rearrangements detected in AML, generally marked by abnormal bone marrow eosinophils, deregulated cell proliferation, impaired differentiation, and a survival advantage to leukemic cells (Grardel, 2002).

## II.II SPREAD

After leukemic cells conquer the bone marrow, they begin to spread and proliferate to other parts of the body. Cell signaling is the most important director of leukemia cell spread. The abnormal cells move through the bloodstream and invade other organs, such as the spleen, lymph nodes, liver and central nervous system as a result of three contributing cell-signaling factors: growth factors, cytokines and contact inhibition (Life Extension, 2011).

The body controls cell proliferation with a specific subset of signaling proteins called "growth factors." Cells secrete growth factors, which bind to cell-surface receptors and stimulate cell growth, proliferation and "differentiation", which refers to the capability of less-specialized cell types to become more specialized cell types (Prospec Protein Specialists, 2011). Research suggests that growth factors could have a role in the proliferation-aspect of cancer.

Cytokines are another class of signaling proteins, many members of which also exhibit growth factor activity. They stimulate paracrine, autocrine and endocrine systems when secreted and are produced by hematopoietic and non-hematopoietic cell types.

Many types of leukemia produce specific inflammatory and immunosuppressive cytokines and use cell-signaling pathways early in the disease progression (Life Extension, 2011). The cytokines overexpressed in AML include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) (Life Extension, 2011). VEGF is considered necessary for growth, survival and spread of leukemic cells (Podar and Anderson, 2004). bFGF is usually necessary in the spread of cancer cells (Bieker et al., 2003). Research shows that HGF is overexpressed in myelomonocytic leukemia (Aguayo et al., 2000).

Contact inhibition also contributes to cell spread. In normal cells, contact inhibition is the phenomenon that signals normal cells to stop dividing. Cells discontinue replication when they recognize contact with adjacent cells. Research shows that cancer cells lack contact inhibition, which causes them to divide without regulation (Yongqing et al., 2010).

## II.III RISK FACTORS

One predominant hypothesis is that all cancers begin with just one abnormal cell. This first cell becomes cancerous as a result of abnormal gene function most often caused by DNA mutations. In AML, a small proportion of cases are caused by inherited, abnormal genes (Fong and Brodeur, 1987; Bischof et al., 2001; Alter, 2003). Children with Down's Syndrome, in particular, have a 10 to 20 time higher risk of developing leukemia than normal (Fong and Brodeur, 1987). However, most DNA damage is caused by sporadic mutations. These mutations are not inherited and are caused by lifestyle and environmental factors like weight, exercise, diet, and drug or alcohol use.

Excess weight causes the body to produce and circulate more hormones such as estrogen and insulin, which can stimulate cancer growth. Exercise helps control weight, and regulates hormone levels and immune system function. A compromised immune system cannot detect mutations or adequately direct apoptosis or replication in cells (American Cancer Society, 2011).

A nutritious diet improves health, aids in weight control, and lowers risk of cancers. Insufficient amounts of micronutrients can cause DNA damage associated with leukemia. It can also limit the ability for repair (Ames, 1998; Ames, 1999). A diet high in fruits, vegetables and other antioxidants can help protect against DNA damage (Ames et al., 1993).

Long-term drug use is also a risk-factor in developing AML. Alcohol use raises estrogen levels in the body, slows or hinders DNA repair, causes irritation, and acts as a solvent, helping harmful chemicals enter cells (American Cancer Society, 2011). Cigarettes contain leukemia-causing chemicals such as benzene (Korte, et al., 2000).

Sporadic medical treatments can also cause DNA mutations, which lead to AML. The major cause of AML in children is chemotherapy for the treatment of other cancers (Felix, 1998). Treatment-related AML accounts for up to 20 percent of AML cases (Kaldor, et al., 1990; Smith et al., 1996). High amounts of radiation have been linked to myeloid leukemia by inducing DNA damage through translocations (Kamada et al., 1987; Archer, 1987).

Aside from lifestyle choices, leukemia-causing DNA mutations can occur simply as a result of one's passive environment. Environmental factors include medical treatments, pollution or silent chemicals present in the home or workplace (Irons and

Stillman, 1996; Greaves, 2004). Long-term or occupational exposure to benzene is a cause of AML, as well as agricultural chemicals such as herbicides and pesticides (Austin et al., 1988; Meinert et al., 2000). Hair dyes also contain chemicals that are associated with AML (Sandler, 1995).

Age is considered to be one of the biggest risk factors for developing AML. Seventy-percent of cases occur over the age of 50 (Fenech et al., 1997; Russell, 2000). Older people have more fragile chromosomes in their white blood cells, making them more susceptible to the types of DNA damage known to cause leukemia (Esposito et al., 1989; Mendoza-Nunez et al., 1999). Older people with AML are also more difficult to treat, because older people are more resistant to chemotherapy (Schoch et al., 2001).

## II.IV TREATMENT OPTIONS

Acute myeloid leukemia progresses quickly. Immediate treatment is necessary to provide the best chance of remission. The treatment of AML is complex, and may include chemotherapy, radiation therapy, and bone marrow transplant. Chemotherapy and radiation are generally effective in treating AML, and are often used as concurrent treatments (Life Extension, 2011). Bone marrow transplantation mainly used as a last resort, since the treatment by itself shows 15% mortality, and it otherwise has serious side effects.

Chemotherapy involves administering drugs that attack rapidly-dividing cells, like cancer cells. Chemotherapy agents stop the growth of these cells by either destroying them or keeping them from dividing. But cancer cells are not the only type of rapidly-dividing cell in the body, so chemotherapy also attacks hair follicle cells, platelets, cells

lining the gastrointestinal tract and healthy blood cells (AP John Institute, 2011). This attack on normal, healthy cells contributes to some of the side effects of chemotherapy like hair-loss, fatigue, low blood cell counts, risk of infection, nausea and vomiting, loss of appetite, and diarrhea.

Radiation therapy kills cancer cells by exposing them to ionizing radiation, which damages cell DNA. There are two types of radiation therapy. The most common type of radiation treatment is called "external-beam radiation therapy." This type of therapy involves giving radiation using a machine outside the body (AP John Institute, 2011). External-beam radiation therapy is used for AML that has or may spread to the brain and spinal cord and may also be used to treat bone pain that can occurs from high numbers of leukemia cells in the bone (Naveen et al., 2006; National Marrow, 2011).

For some patients, a bone marrow transplant may offer the best chance for a long-term remission. A bone marrow transplant involves chemotherapy followed by replacing, or "infusing," the abnormal stem cells in the blood with donor stem cells (AP John Institute, 2011). These re-infused stem cells grow into and restore the body's blood cells. A transplant is a strong treatment with risks of serious side effects; it is not used for all patients with AML. A transplant is used when chemotherapy alone is unlikely to provide a long-term remission (National Marrow, 2011). Many patients with AML reach remission, but relapse is common. Most patients need a second phase of treatment to prevent relapse (National Marrow, 2011).

## II.V CAUSE AND MECHANISM

At the genetic level, AML is the best understood of the acute leukemia family.

Research confirms that AML is caused by the collaboration between two types of DNA mutations: proliferative and blocking; either type of mutation alone is usually insufficient to cause AML (Reilly, 2004). Proliferative mutations give a proliferative or survival advantage to cells. Approximately 70% of patients with AML and inv(16) are known to possess proliferative mutations (Reilly, 2004). Research has shown that the proliferative and survival advantage of AML cells is due to a missing signal caused by mutations in either the receptor tyrosine kinase (RTK) class III or the RAS genes (Reilly, 2004). A variety of mutations may occur to cause this signaling disruption (**Figure 2**). Internal tandem duplications, and mutations in the tyrosine kinase domain (TKD) of the FLT3 receptor, are the most common (Cammenga et al., 2005). Mutations may also affect other RTK, for example c-KIT. These mutations contribute to a small percentage of AML cases (Cammenga et al., 2005).

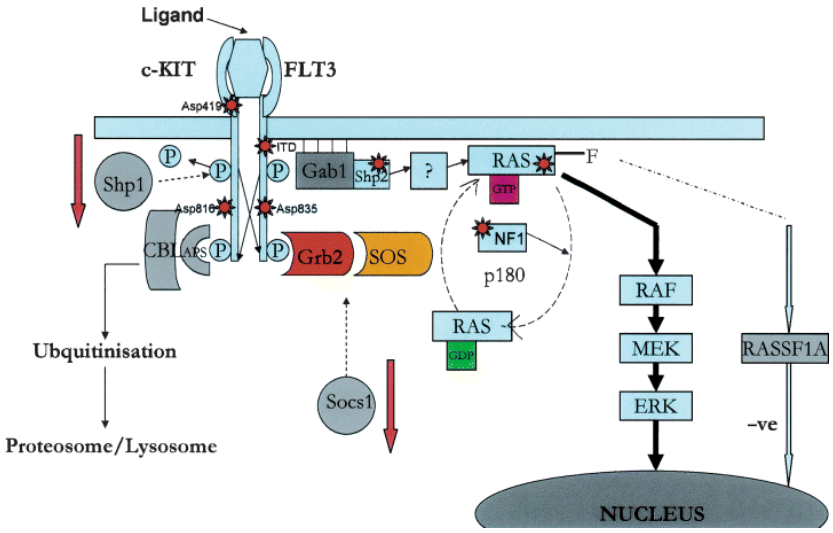


**Figure 2: Diagram of the RTK/RAS Signaling Pathway Showing Mutation Sites in AML.** A red asterisk indicates a known mutation site that causes AML. Note the key role of the FTL3 (c-Kit) receptor to affect the RAS/RAF pathway (Reilly, 2004).

In inv16 leukemia, the inversion of chromosome 16 breaks and joins the CBFB gene with the myosin gene MYH11 to create a CBFB-MYH11 fusion protein. CBFB-MYH11 encodes a fusion oncoprotein CBFB-SMMHC, which is present in 12% of AML cases (Kuo et al., 2006). The expression of CBFB-MYH11 disrupts the normal transcription activity of the core binding factor (CBF) in AML and inv(16) leukemia. CBF is a family of heterodimeric transcription factors containing a common B subunit (CBFB) and a CBFα subunit, which is encoded by one of the three genes in the RUNX family (RUNX1, RUNX2 or RUNX3) (Kuo et al., 2006). CBF is an important transcriptional activator of genes involved in hematopoiesis and bone development (Takahashi et al., 1995; Komori T et al., 1997). Disruption of CBF by the CBFB-MYH11 fusion gene impairs normal CBF activity. The CBFB-MYH11 fusion protein blocks embryonic hematopoiesis at the stem progenitor cell level and impairs differentiation (Kuo et al., 2006).

The DNA binding alpha subunit in CBF is encoded by one of three RUNX members (RUNX1, RUNX2 or RUNX3). All three RUNX proteins are integral in pathways regulating cell growth and differentiation. Studies have shown that functional mutations in RUNX1 cause AML (Kuo et al., 2006) and 5-10% of AML cases involve somatic point mutations in RUNX1 (Blyth et al., 2005).

## III. ST18

Suppression of tumorigenicity-18 (ST-18) is a transcription factor with mostly unknown function (Yang et al., 2008). Previous studies have established that ST-18 represses transcription of a synthetic reporter construct consisting of the consensus

sequence AAAGTTT linked to the thymidine kinase promoter. ST18 is expressed at a low level in a number of normal tissues including mammary epithelial cells (Jandrig et al., 2004). Research has implicated ST-18 in breast cancer, where ST-18 exhibits significantly reduced expression (Yang et al., 2008). ST-18 has been identified as a possible gene involved in t(8;21) leukemia, however minimal research has been done regarding the role of ST18 in leukemia of other types (Steinbach et al., 2006).

# PROJECT PURPOSE

The human gene Suppression of Tumorigenicity-18 (ST-18) is a zinc finger protein previously associated with breast carcinoma, however it has recently been identified as having a possible oncogenic role in the development of Inversion 16 leukemia (inv16). Despite this speculation, the extent of the role of ST18 in inv16 leukemogenesis has yet to be determined. The goal of this project was to study the expression and effect of ST-18 on cell survival, proliferation, and differentiation in inv16 acute myeloid leukemic cells (Me-1) versus non-inv16 cancer cell lines (Kasumi-1 and U937) using gene knock-in and knock-out experiments. This report demonstrates that ST18 is more highly expressed in Me-1 cells compared to Kasumi-1 and U937 cell lines. This report also demonstrates that murine ST-18 was successfully cloned into vector pMSCV2.2 for use in future knock-in experiments.

# METHODS

## CELL CULTURE

Kasumi-1 cells were incubated in RPMI supplemented with 20% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (p/s). U937 cells were incubated in RPMI supplemented with 10% FBS and 1% p/s. Me-1 cells were incubated in RPMI supplemented by 20% FBS with 1% p/s and 2.5% HEPES. All cells were incubated at 37°C.

## QUANTITATIVE REAL TIME -POLYMERASE CHAIN REACTION

Approximately $1x10^6$ cells of each cell type were isolated by centrifugation. RNA isolation was accomplished with use of TRIZOL reagent, chloroform, RNA-free isopropyl alcohol, and RNA-free 75% ethanol. RNA pellets were re-dissolved in 20 uL RNAse-free water. The RNA was synthesized into DNA using a mixture of random primers, dNTP, 5x First Strand buffer, DDT/DTT, RNAse out, SuperScript III RT added to 2 ug of RNA template. The cDNA was used for quantitative real time PCR using SYBR green and forward/reverse primers, to make 3 replicates per gene. The samples were run as a comparative CT ($\Delta\Delta$CT), at a ramp speed of 2 hours standard, using StepOne Software v2.1.

## VECTOR CONSTRUCTION

## RESTRICTION DIGESTS

DNA samples (approximately 1-2 ug) were digested with appropriate restriction

enzyme(s), buffer, 10x BSA and H2O for a total reaction volume of 20 ul. Samples were incubated at 37°C for 60 minutes.

## PCR GEL

Electrophoresis gels were used to confirm that digestions or colony PCR results were complete and correct. The digested samples were mixed with 6x loading buffer and run in comparison to a DNA ladder.

## DNA PURIFICATION

The digested vector DNA was purified using the QIAQuick PCR Purification kit, treated with SAP (to remove 5' phosphate residues to prevent self-ligation) and 10xSAP buffer, and then re-purified.

## BLUNT END LIGATION

The cDNA enzyme digest was mixed with Klenow DNA poly I added to dNTP and appropriate buffer for each enzyme and ddH2O, then incubated for 15 minutes at RT.

## LIGATION

Using the digested cDNA samples, three types of ligation products were produced: one sample containing both vector and insert at a ratio of 1:6, one containing no insert, and one containing no ligase. Approximately 50 ng of vector and 2 ul 10x T4 DNA ligase buffer were added to all three samples. Approximately 150 ng of insert was added to all samples except for the "no insert" sample, and 1 ul ligase was added to all

samples except for the “no ligase” sample. To each sample, water was added to make a reaction volume of 20 ul. Samples were incubated at RT for 60 minutes.

## TRANSFORMATION

Each ligation product was added to a vial of 100 ul competent bacteria, and was incubated on ice for 30 minutes. The mixtures were heat shocked, 1 ml of LB broth was added to each sample, then all samples were incubated at 37°C for 60 minutes in a shaker. The transformed ligation products were transferred to plates with ampicillin or kanamycin-containing media and incubated at 37°C for approximately 16 hours.

## MINIPREP

Bacterial cells were harvested from 5 mL cultures grown in a shaker at 37°C for approximately 16 hours. The bacterial pellet was collected in a microcentrifuge, then buffers P2, P2 and N3 were all added according to the QIAGEN Plasmid DNA Purification kit. The samples were microcentrifuged and the supernatants added to QIAprep spin columns, centrifuged again and resuspended in TE buffer.

## MIDIPREP

A 250 mL bacterial culture containing ampicillin and 100 uL bacteria was incubated in a shaker for approximately 16 hours at 37°C. The DNA from the resulting pellet was re-suspended, lysed and precipitated using a cocktail of kit-included buffers (P1, P2 and P3). The lysate was poured into a QIAfilter Cartridge, incubated at RT for 10 minutes and then filtered through a Buffer QBT-equilibrated QIAGEN-tip 500. The DNA

was eluted in 15 mL of Buffer QF after the QIAGEN-tip was washed twice with 10 mL of Buffer QC. The DNA was precipitated with RT isopropanol and the mixture was centrifuged at 15,000xg. The resulting DNA pellet was washed with 5 mL of RT 70% ethanol, centrifuged at 15,000xg for 10 minutes. This pellet was air-dried for 5 minutes and the DNA was re-dissolved in TE buffer.

## BACTERIAL CELL CULTURE

Using sterile technique, transformed bacterial colonies were individually sampled using a micropipettor tip and grown in 5 mL of LB broth + ampicillin. All samples were incubated at 37°C for approximately 16 hours in glass bacterial culture tubes.

## BACTERIAL COLONY PCR

Specific colonies were sampled using a micropipettor tip, and grown on both kanamycin and ampicillin plates. Each PCR well contained bacteria, forward and reverse primers, dNTP, ThermoPol Buffer, Taq Polymerase and ddH2O for a reaction volume of 20 ul. The plate was run through 30 cycles of temperatures and amounts of time (3 min. at 95°C, 30 sec. at 94°C, 30 sec. at 60°C, 30 sec. at 72°C, 10 min. at 74°C, Forever at 4°C).

## TRANSFECTION

cDNA samples Gag-Pol and VSVG were transformed, added to 250 mL of ampicillin-containing LB broth, and incubated overnight at 37°C. After a midiprep was performed on Gag-Pol and VSVG, 1.67 ug of each ST18 group was added to 1.67 ug

gag-pol, 0.167 ug VSVG and buffer EC. 16 ul enhancer and 60 ul Effectene Transfection Reagent were added to each DNA enhancer mixture. Growth medium was added to each tube, and the transfection complexes were added drop-wise onto 293T cells in 60mm culture dishes. All dishes were incubated at 37°C for approximately 18 hours. The medium was carefully collected at 7-9 hour intervals into 4 bottles for the four viruses. Each virus was vacuum-filtered and centrifuged at 4°C for two hours. Each resulting pellet was re-suspended in new media, separated into 1.5 mL aliquots and stored at -80°C.

## INFECTION

8 ul Polybrene was added to 10 mL 293T cell medium. Three concentrations of virus and Polybrene media were mixed for each virus in 1.5 mL Eppendorf tubes: 1:1, 1:2 and 1:4 (1:1: 0.6 mL virus, 0.6 Polybrene media, 0.3 mL regular media. 1:2: 0.3 mL virus, 0.6 mL Polybrene media, 0.3 mL regular media. 1:4: 0.15 mL virus, 0.6 mL Polybrene media, 0.45 regular media). 1 mL of each mixture was added to a 100 mm plate of 293T cells. After 5 hours, 4 mL regular medium was added to all cells. After 36 hours, each plate was split and transferred to two wells of a 6-well plate. 3 mL of regular medium was added to each well, and then 2 ng Puromycin was added to one of the two wells of each virus at each concentration. Plates were incubated at 37°C for 48 hours, after which the cells in each well were counted.

# RESULTS

The first goal of this project was to assay the expression of human ST-18 in an inv16 leukemia cell line (Me-1) versus other cancerous cell lines Kasumi-1 and U-937. The level of was determined using Quantitative Real Time PCR. Kasumi-1 is a human acute myeloid leukemia cell line of the t(8;21) chromosome translocation, functioning as a comparative cancerous cell line for this assay (Asou et al., 1991). U-937 is a human diffuse histocytic lymphoma cell line, which displays model monocytic characteristics (atcc.org). This cell line serves as the control cell line in this assay. MSLN and CLIPR59 are other genes known to exhibit cancer-related over-expression. CLIPR59 over-expression, in particular, has been linked to inv16 cell lines (Bullinger et al., 2007). MSLN over-expression is already being used as a marker to diagnose mesotheliomas, ovarian cancers and pancreatic cancers (Hucl, 2007). Therefore, the expression of MSLN and CLIPR59 were assayed for comparison.

The data show that ST-18 mRNA expression (red histobars) is notably higher in Me-1 cells than in the control U-937 cell line (**Figure 3**). Additionally, ST-18 expression is slightly higher in Me-1 cells than in the related cell line Kasumi-1. MSLN mRNA expression (green histobars) is down-regulated in Me-1 and Kasumi-1 cells, while CLIPR59 mRNA expression (violet histobars) is slightly up-regulated in Me-1 cells and down-regulated in Kasumi-1 cells (**Figure 3**).

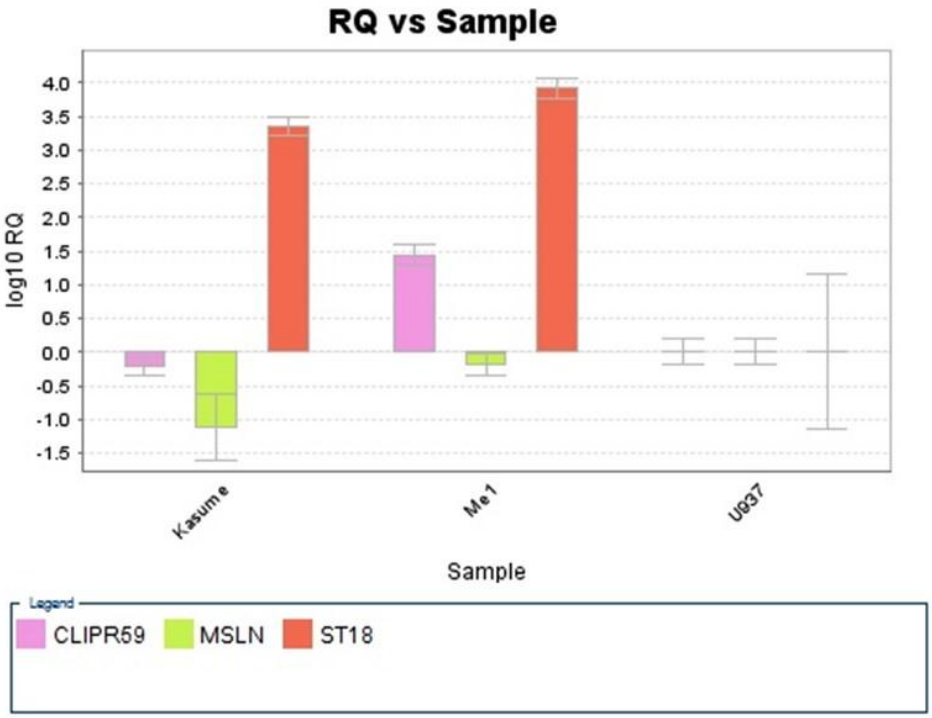


**Figure 3. Assay of Relative Gene Expression in Various Cell Lines by qRT-PCR.** ST-18 (red histobars) is highly upregulated in Me-1 cells compared to control U937 cells. ST-18 is also upregulated in Kasumi-1 cells, but to a lesser extent. CLIPR59 (violet histobars) shows a mix of modest down and up-regulation depending on the cell line. MSLN (green histobars), a marker for some types of cancer, is down-regulated in all cell lines.

Based on the results of the qRT-PCR expression assay, the second goal of this project was to make a viral construct capable of over-expressing ST-18 to explore the effects of ST-18 over-expression in both non-leukemic and inv16 leukemic cells. Although the viral construct could not be finished in the timeframe allowed for this project, the murine ST-18 gene was successfully cloned into plasmid vector pMSCV2.2. Several potential ST-18-containing vectors were digested with SacI (**Figure 4, Lanes 2-5**), identifying one positive clone (lane-4) that released bands of 7.1 kb, 0.35 kb, and 0.30 kb.

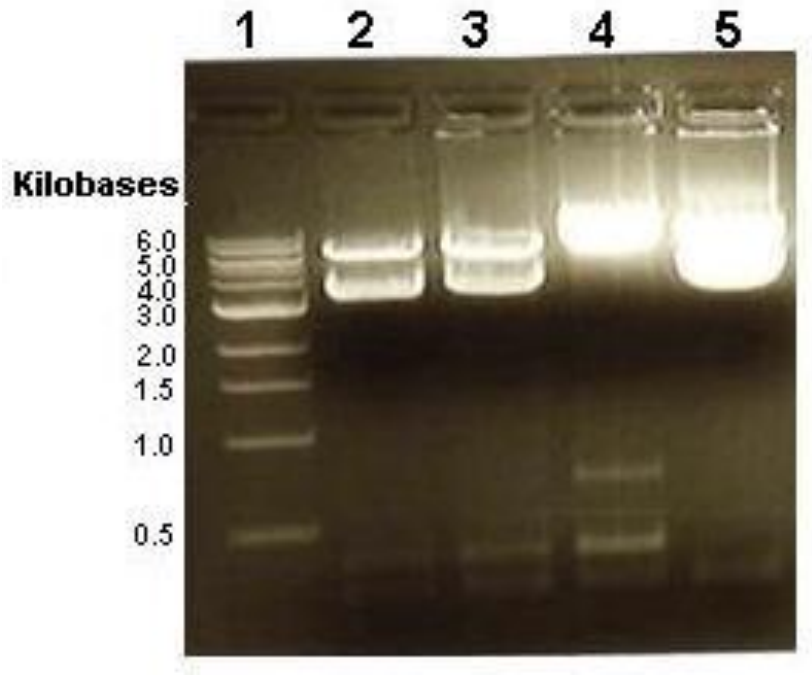


**Figure 4. Restriction Digestion Screening of Plasmid Vector pMSCV2.2+ST-18**. Samples were digested with SacI. One sample was positive for ST-18 insert, yielding bands of 7.1 Kb, 0.35 Kb and 0.30 Kb (Lane 4).

Additional restriction digestions were performed on selected samples (Lanes 3, 4, and 5 from Figure 3) to confirm that the sample in Lane 4 contained the ST-18 insert (**Figure 5**). Samples were digested with Dra1 (red lanes) and also double digested with Xho1/Acc1 (blue lanes). Dra I restriction sites predicted bands of 4.0Kb, two-2.3Kb, 2.3Kb, and 0.04Kb in samples positive for the ST-18 insert, and one positive was observed (red lane-4). The double digestion with XhoI/AccI predicted bands of 7.1Kb and 0.6Kb in samples positive for the ST-18 insert, and one was positive (blue Lane 4). Thus, one successful clone was positively identified using a total of three different restriction digestions schemes.

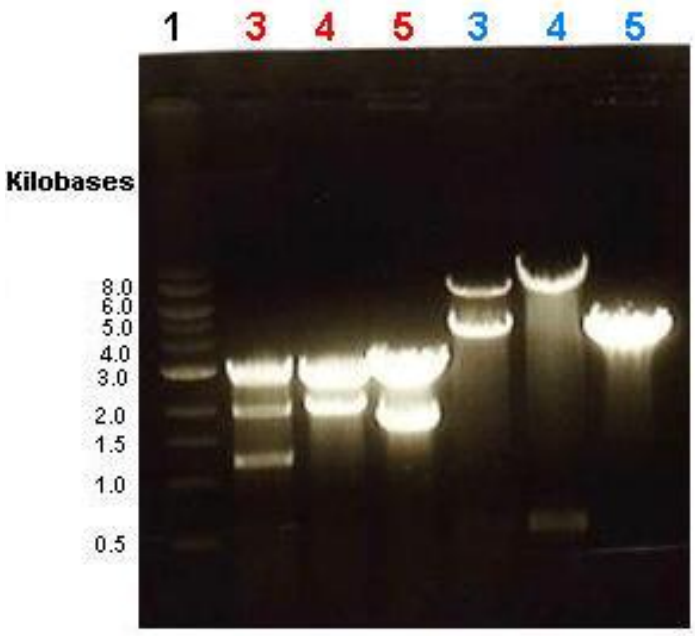


**Figure 5. Continued Restriction Digestion Screenings of Vector pMSCV2.2+ST-18.** Samples were digested with DraI (red lanes) or XhoI/AccI (blue lanes). Samples positive for ST-18 insert yielded bands of 3.0 Kb, 2.3 Kb(2) and 0.04 Kb when cut with DraI (red Lane 4). Samples properly-containing ST-18 insert yielded bands of 7.1 Kb and 0.6 Kb (blue Lane 4).

Several attempts were made to sub-clone the insert from pMSCV2.2 + ST-18 into viral-based vectors several times, but no candidates contained both insert and vector. Ideally, our ST-18-containing vector could be used to sub-clone the murine ST-18 gene into a viral construct for use in future experiments when more time is allowed. In spite of these results, we transfected a lentiviral construct with three ST-18 containing plasmids, and the preliminary screen yielded adequate cell numbers, however no cells survived in the Polybrene wells and the end of the screening process, suggesting that no live virus was produced.

# DISCUSSION

The most reliable results of this paper came from the qRT-PCR expression analysis comparing ST-18 mRNA expression in Me-1 cells (containing the inv16 leukemic mutation) versus Kasumi-1 and U937 cancer cell lines that do not contain the inv16 mutation. Synthesizing cDNA from cells for RT-PCR was the most effective procedure of the methods used in this paper, as the procedure yielded significant results each time it was used. ST-18 expression was markedly higher in inv16 leukemia cells (Me-1 cells) than in Kasumi-1 or U937 cells. This data widens the type of cancers involving ST-18 over-expression, connecting over-expression in breast cancer cell lines to over-expression in inv16 leukemia cells. Other interesting future experiments involving RT-PCR and ST-18 expression should include comparing ST-18 expression in other leukemia cell lines and in other un-related cancer cell types.

The ST-18 DNA cloning procedures were the most challenging procedures in this project. After numerous attempts, I was finally able to yield a positive clone containing the murine ST-18 gene inserted (in the correct orientation and in the correct number of copies) into plasmid pMSCV2.2. The successful cloning of ST-18 into plasmid, and its proper orientation, was determined by three different restriction digestions.

Many additional attempts were made to try to subclone the plasmid ST-18 insert into a viral-based vector to use it in further transfection/infection experiments. However, despite altering restriction enzymes, vector-to-insert ratios, and purification techniques, none of those attempts proved fruitful. I determined that these obstacles are typical of the virus/DNA cloning process. This project revealed that while mastering techniques is

crucial to producing valid results, there are additional elements including constant repetition, procedural patience, and sometimes "luck" that make positive colonies with the correct orientation of genes. More often than not, the ST-18 insert did not insert correctly in my experiments despite best efforts and care. Repeated restriction digestions, ligation procedures and transformation procedures were repeated countless times to finally produce a single colony that correctly expressed ST-18. Part of the project's success involved persevering with experiments despite the fact that the ST-18 insert procedure required perfection to yield perfect results. It was hard to accept that this aspect of the procedure was uncontrollable.

In the transfection/infection experiment, no cells survived in the puromycin wells, suggesting that no intact virus was produced. There are several possible reasons for this. Since this procedure was only completed one time due to time constraints, it is likely that human error occurred. It's possible that too much puromycin was added, killing all cells. Another explanation may be that the 293T cells responsible for producing the virus may have been killed by either not sufficiently warming up the cellular media before adding to the cells or by harvesting the virus after too long of a time period. Additionally, it was necessary to take several viral collections in the middle of the night. Fatigue might have been a negative factor that resulted in error. It was necessary to complete many of the procedures in this project at least once before achieving accurate results. Additional opportunities to complete this procedure may have yielded different, more significant results. My recommendation for future success is that students complete the transfection/infection experiments in groups of two to ensure accuracy and close, round-the-clock monitoring that this procedure requires in order to achieve success.

The unique design of this project would suit two students very well. Other hindrances to this project's success included time required to do routine "housekeeping" work, such as culturing cells or running PCR gels. A team approach might significantly improve the potential for successful results. Therefore, based on my experiences, I would recommend that a project of this type in the future be completed either by two students or at least a two-year time period that would allow a "doubling up" on all fronts-- thereby increasing experimental frequency, decreasing time constraints and ensuring a successful outcome to contribute to the efforts to cure leukemia.

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