Inducing TCF3-PBX1B (t1;13)(q23;p13.3) Fusion Protein Influences Metabolism of Methotrexate in Human Acute Lymphoblastic Leukemia Cell Lines

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Abstract

Acute Lymphoblastic Leukemia (ALL) is the most widespread form of childhood cancer, and methotrexate (MTX) is one of the most commonly used chemotherapeutic agents used to treat pediatric ALL. The antileukemic effects of MTX is partially determined by intracellular concentrations of MTX polyglutamate (MTXPG), the active form of the drug. MTXPG inhibits the folate pathway, leading to cytotoxicity towards leukemic cells. It has been observed that MTXPG concentrations are lower in the Transcription Factor 3 - PBX Homeobox 1B (TCF3-PBX1B) subtype of leukemia patients as compared to other subtypes, but the mechanism through which this occurs is largely unknown. This study aims to establish the REH human ALL cell line as a viable cell line that accurately depicts pediatric ALL patients to allow for future studies to be performed to elucidate the mechanism of action of the TCF3-PBX1B fusion protein.

Preliminary patient data has shown that folate pathway genes, folylpolyglutamate synthetase (*FPGS*) and reduced folate carrier (*SLC19A1* or RFC) are positively correlated with MTXPG concentration. Further analysis has shown that transcription factors Notch Receptor 2 (*NOTCH2*) and SRY-Box Transcription Factor 11 (*SOX11*) have strong negative correlation with *FPGS* and *SLC19A1*. In our experiment, REH human ALL cells were transduced with either doxycycline-inducible LacZ (as control) or TCF3-PBX1, to determine whether the same trends seen in patients hold true in human leukemia cell lines. Data shows similar trends between patients and cell line and that the presence of TCF3-PBX1B fusion protein in REH cell line ultimately decreases intracellular MTXPG concentration. This establishes the REH human ALL cell line as a viable method of studying MTX metabolism in pediatric ALL patients, and future studies performed on the cell line can be incorporated into clinical care to create a personalized MTX treatment.

Key Terms: Acute Lymphoblastic Leukemia, methotrexate, methotrexate polyglutamate, TCF3-PBX1, SOX11, NOTCH2, FPGS, RFC, REH cell line

Introduction

Acute Lymphoblastic Leukemia (ALL) is the most common pediatric cancer, making up almost a quarter of the 20,000 newly diagnosed pediatric cancer patients each year (Board, 2019). The disease originates from the bone marrow where it affects the production of white blood cells, specifically T-lymphocytes, and B-lymphocytes, giving names to the two main classifications as T-ALL and B-ALL (St. Jude Children's Research Hospital, 2020), which refers to the leukemia that affects the T-cell and B-cells in the adaptive immune system. As a part of the adaptive immune system, T-lymphocytes and B-lymphocytes are responsible for identifying and dispatching foreign particles. As a result, this abnormal proliferation of white blood cells leads to a high population of immature white blood cells, which impedes the normal functioning of healthy white blood cells, leading to a compromised immune system (St. Jude Children's Research Hospital, 2020).

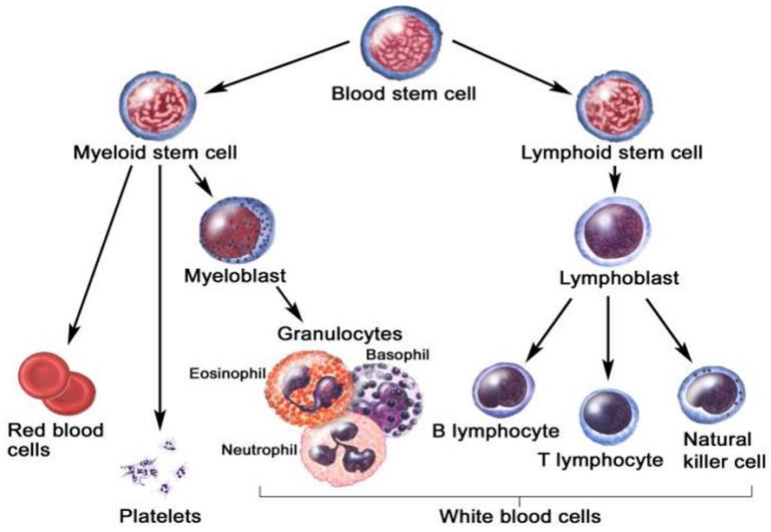

Figure 1. ALL originates in the bone marrow, and it is the result of rapid reproduction of either the B-lymphocyte or T-lymphocyte, which leads to immature white blood cells that can reduce the body's capability of fighting off infection as they compete with the healthy cells (Board, 2019).

As described by Pui and Evans (Pui & Evans, 2013), breakthroughs in cancer treatment over the past half a century have contributed greatly to the improvement in pediatric ALL cure rates. In the 1960s, a diagnosis of ALL was a death sentence as physicians did not have a proper treatment for the disease. The first breakthrough in pediatric ALL treatment came from the discovery of aminopterin in 1948, a folate antagonist, followed by the discovery of methotrexate, which has a similar mechanism to aminopterin (Pui & Evans, 2013). As a folate antagonist, aminopterin and methotrexate have enough structural similarity to folate so that they are able to competitively inhibit the normal function of folate, which is critical in cell growth and nucleic acid synthesis. Without the ability to synthesize new amino acids, the leukemia cells cannot divide further and thus go through apoptosis. Through this, physicians were able to improve two-year survival rates to about 20%. According to Pui and Evans (2013), the next breakthrough came from combination chemotherapy, where multiple drugs were used to improve the antileukemic effects of the drugs. This era of chemotherapy, which took over in the 1960s is still in part used in today's therapies. The 21st century marked the beginning of full-genome profiling of patients. This revolution marked the beginning of individualized treatment through the classification of various crucial genetic markers that affect ALL treatment. Since then, advancements in whole genome sequencing have led to novel treatment protocols that are applied to patients based on known markers in their genes. Using these methods, ALL cure rates improved to over 90% from just 4% half a century ago, but ALL remains as one of the leaders in childhood cancer mortality with over 3,000 patients diagnosed every year (Pui & Evans, 2013).

In order to explain why the remaining 10% of patients are unable to be cured, small, genetic factors seem to be the most likely candidate. These small alterations in the genes of some ALL patients can confer resistance to some of the most popular chemotherapeutic drugs, such as

MTX (Holleman et al., 2004). Holleman and colleagues further elucidate that gene-related drug resistance can be an explanation for the small percentage of patients who do not respond to initial chemotherapeutic treatments.

This drug resistance could possibly be accounted for by few genes that are involved in further subtypes within T-ALL and B-ALL. Many of these subtypes, including *TCF3-PBX1B*, which falls under B-ALL, are chromosomal translocations. A chromosomal translocation is a malfunction in cell division where part of one chromosome is exchanged with part of another chromosome. The TCF3-PBX1B translocation occurs on the 1st and 19th chromosomes in the q23 and p13.3 locations on those chromosomes (t1,19)(q23;p13.3). Of all the subtypes of ALL, the TCF3-PBX1B translocation only occurs in about 4% of patients, as shown in figure 2A (Mullighan, 2012). Although this comprises a small percentage of total ALL patients, the complications that arise due to unique drug resistance and drug interactions in TCF3-PBX1B patients make this subtype an important area of study when trying to improve patient outcomes from the 90% survival rate.

In the case of the TCF3-PBX1B chromosomal translocation, the fusion gene produced leads to the misregulation of downstream genes rather than the complete loss of function of the genes. As shown in figure 2B, pediatric ALL patients with the *TCF3-PBX1B* chromosomal alteration do not respond as well to the common cancer drug, methotrexate (MTX), accumulating a lower concentration of the active form of the drug in the form of methotrexate polyglutamates (MTXPGs) than other B-ALL subtypes (Kager et al., 2005). The researchers were also able to show that lower MTXPG accumulation in patients leads to reduced antileukemic effects of the drug MTX.

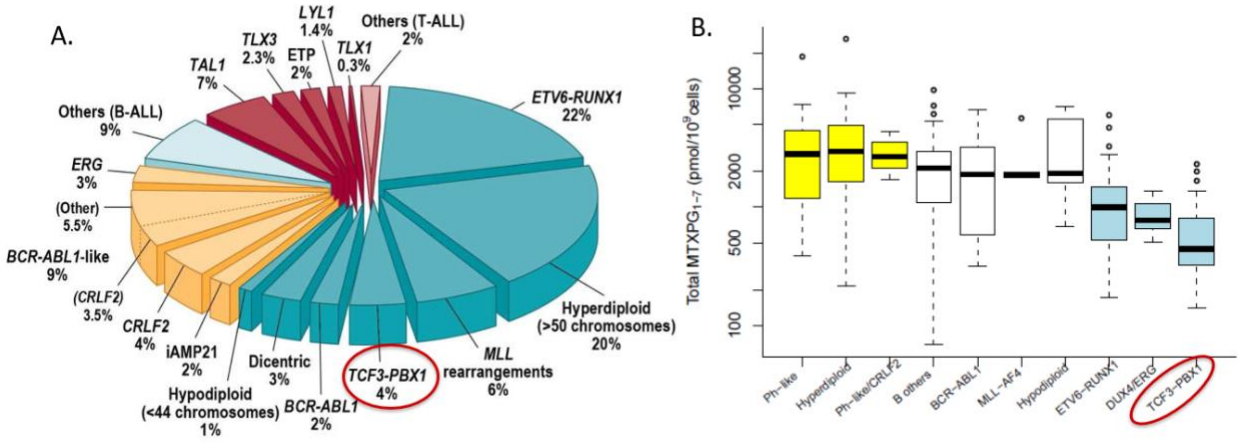

Figure 2A. The blue and yellow regions of the chart represent subtypes of B-ALL, while the red regions of the chart represent subtypes of T-ALL. The TCF3-PBX1 translocation represents 4% of pediatric ALL cases (Board, 2019).

Figure 2B. This graph shows MTXPG concentrations in pediatric ALL patients of various subtypes. The TCF3-PBX1 subtype has the lowest average concentration of MTXPG in patients, which is why this subtype is one of the most resistant to MTX (Board, 2019).

The effect of the TCF3-PBX1B fusion protein on folylpolyglutamate synthetase (FPGS), reduced folate carrier (RFC), and MTXPG concentrations within patients have already been observed, but it is unknown whether these correlations can be seen in human ALL cell lines. In addition, the mechanism of TCF3-PBX1B that leads to the alteration of the expression of FPGS and RFC is unknown. This investigation began as a means to study the effects of the TCF3-PBX1B fusion protein and its downstream effects in human ALL cell lines with intent to improve MTX drug treatment on patients of the TCF3-PBX1B ALL subtype. Through a series of experiments in human ALL cell lines, we hope to elucidate the mechanism behind how TCF3-PBX1B ultimately results in a lower concentration of MTXPGs as well as determine whether the REH human ALL cell line is a viable model to study the TCF3-PBX1B fusion protein. The latter

can be confirmed if there is a match between the preliminary patient data and the experimental data resulting from the cell line.

Key Terms and Abbreviations

Doxycycline. It is derived from tetracycline. In the presence of tetracycline or doxycycline, the TetR repressor, bound to the DNA control sequence upstream of the gene, is released, allowing the gene to be transcribed, or expressed (Ramos et al., 2005). In this project, doxycycline is used to bind to TetR to allow for the expression of LacZ (control) and TCF3-PBX1B fusion protein (experimental).

Folate. A water-soluble B vitamin. It is essential in the formation of nucleic acids and amino acids, making it critical in DNA replication and cell division (National Institutes of Health [NIH], 2020).

Folylpolyglutamate synthase (FPGS). An enzyme responsible for the ATP-dependent addition of glutamate to folate (National Institute for Biotechnology Information, 2020). It is suspected that a decrease in FPGS expression correlates with lower methotrexate polyglutamate concentrations.

Methotrexate (MTX). A common drug used in diseases such as cancer and arthritis. A folate derivative that acts as a folate antagonist (hijacks the folate pathway) to inhibit the natural effects of folate in the body (Malakouti, 2018).

Methotrexate polyglutamates (MTXPGs). The active form of methotrexate containing up to seven glutamate residues added by folylpolyglutamate synthase. Most importantly, the addition of glutamate residues allows the MTXPGs to be retained within the cell longer (U.S. National Library of Medicine, 2010).

Notch receptor 2 (*NOTCH2*). A critical gene in various biological pathways including cellular development, immune system, tissue repair, etc. In this project, it is suspected that Notch2 negatively influences the expression of folylpolyglutamate synthase (U.S. National Library of Medicine, 2020).

Reduced folate carrier (RFC), a.k.a. solute carrier family 19 member 1 (SLC19A1). An intermembrane protein that acts as the main transporter for folate to bring folate into the cells (Matherly & Hou, 2008). In this project, it is suspected the RFC has a direct influence on MTXPG concentrations.

REH human acute lymphoblastic leukemia (ALL) cell line. A cell line derived from the peripheral blood of an ALL patient with specific characteristics such as the absence of the Epstein Barr Nuclear Antigen and presence of Epstein Barr Virus receptors and B-cell leukemia markers (Rosenfeld et al., 1965). This cell line was chosen for this project for its high endogenous *SOX11* expression and low *SOX11* methylation.

SRY-Box transcription factor 11 (*SOX11*). A critical gene in nervous system development and cellular differentiation. Overexpression of *SOX11* can lead to retardation and various cancers (Narurkar et al., 2016). In this project, it is suspected that overexpression of *SOX11* is correlated to lower expression levels of reduced folate carrier.

Transcription factor 3 - PBX homeobox 1B (*TCF3-PBX1B*) chromosomal translocation. A genetic error in which the *TCF3* gene (located on chromosome 1) and *PBX1B* gene (located on chromosome 19) appear next to each other on the same chromosome post cell replication via a translocation (Tirado et al., 2015). The product of this is a TCF3-PBX1B fusion protein where the transcription factor domain (TCF3) and DNA-binding domain (PBX1B) work

synergistically to activate various downstream genes. In this project, this fusion protein is suspected to upregulate the expression of *NOTCH2* and *SOX11*.

Ultra-high performance liquid chromatography-mass spectrometry (LC-MS). A technique in compounds within a mixture separated based on physical characteristics with the ultra-high performance liquid chromatography and further identified using the mass spectrometry Pitt, 2009). Using this technique allows for the separation and identification of MTXPGs (along with the number of glutamate residues attached) from a mixture of other cellular components.

Western blotting. A laboratory technique with the goal of determining the presence and relative abundance of specific proteins (Mahmood & Yang, 2012). Proteins are separated by size and charge, and an antibody with a chemiluminescent tag is used to visualize the relative abundance of the protein when compared to a control (B-actin).

Background

What is Methotrexate?

Methotrexate (MTX), a chemotherapeutic drug that is included in almost every ALL treatment protocol, has been widely successful in treating most patients with ALL. When MTX is taken orally or injected intravenously, it is carried into the cell via active transport by the reduced folate carrier (RFC) and essentially hijacks the folate pathway mechanisms to use to its advantage. Folate acts as an intracellular coenzyme that is responsible for assisting in transferring organic molecules that are critical for the synthesis of genetic material, thus making it essential for cell division, which is rampant in cancerous cells (NIH, 2020). It also plays a role as a cofactor in the process of epigenetics in certain genes. Certain abnormalities, such as neural

tube defects (NTDs) can be developed due to a lack of folate (Ebara, 2017). The RFC is a carrier protein that is specific to folate, however, since MTX is a derivative of folate with similar properties, it can also specifically bind to the RFC carrier protein and enter the cell. Alternatively, MTX is effluxed, or removed, from the cell via the ATP-binding cassette subfamily C1 (ABCC1), which is a protein that is also typically specific to folate (Sorich et al., 2008). Within the cell, MTX is then metabolized by enzyme folylpolyglutamate synthetase (FPGS), which adds up to seven glutamates to MTX, forming methotrexate polyglutamate (MTXPG), the active form of the drug MTX (Mikkelsen et al., 2011). The active MTXPG is able to remain in the cell longer because ABCC1 does not spontaneously efflux MTXPG (Sorich et al., 2008).

The active MTXPG mimics the folate to disrupt the folate pathway. The FPGS protein is specific to folate, but since MTX is structurally similar to folate, it can be modified by FPGS, which adds up to seven glutamate residues to MTX. With the presence of high concentrations of MTX utilizing the machinery of the folate pathway, the cell is not able to uptake and metabolize the necessary amount of folate, which is critical for nucleic acid synthesis, regular cell function, and cell division (Tsukioka et al., 2011). Because MTX is a folate derivative, it can replace the body's endogenous folate, which severely limits the cell to synthesize genetic material. Metabolically active MTXPG is also able to inhibit dihydrofolate reductase (DHFR), which disrupts folate metabolism to hinder *de novo* purine synthesis. This is crucial for the cell's synthesis of new DNA, ultimately leading to cellular apoptosis, or cell death (Masson et al., 1996). This mechanism gives rise to a significant correlation where an increase in MTXPG concentrations in ALL cells leads to a decline in white blood cell counts in these patients due to a higher cytotoxicity (Sorich, 2008), meaning there is a greater antileukemic effect. High levels of

MTXPG accumulation also significantly reduces the likelihood of Leukemia remission (Mikkelsen et al., 2011).

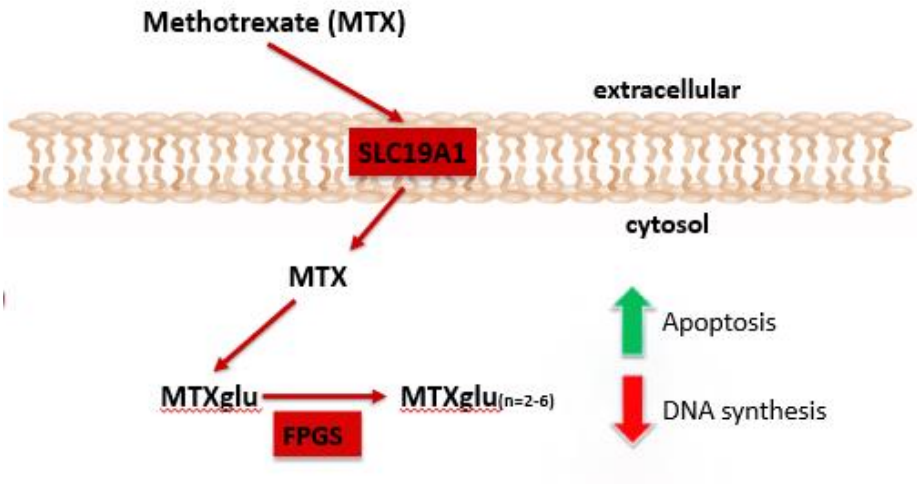

Figure 3. Mechanism of action of MTX once it enters the body. MTX enters the cell through SLC19A1 (RFC) and is metabolized by FPGS. The results of this interaction is DNA synthesis decreases due to the hijacking of the folate pathway by MTX, which ultimately increases cell death, or apoptosis.

Other Genes Involved

Kager and colleagues make the claim that the resistance towards Methotrexate (MTX) in some Acute Lymphoblastic Leukemia (ALL) patients can be attributed to a few genes that are involved in the folate pathway, which is critical for DNA replication (Kager et al., 2005). Prior research shows that MTX has a greater cytotoxic effect on ALL cells when there are more MTXPGs, the active form of MTX, within the cell. In the study by Kager *et al.*, the authors identified critical proteins that correlate with the end production of MTXPGs within the cell. These proteins are the reduced folate carrier (RFC) and folylpolyglutamate synthase (FPGS). Through a correlational study, it was shown that a lower expression of these two genes leads to an overall decrease in MTXPG concentration within the cell (Kager et al., 2005). Researchers in Taiwan echoed this conclusion when they found that patients at a higher risk, such as those with

the TCF3-PBX1 mutation were given higher dosages of the drug to compensate for their drug resistance (Yen et al., 2017). The results showed that there was no significant difference in survival between TCF-PBX1 ALL patients and other ALL patients. Yen and colleagues (2017) showed that patients with the TCF3-PBX1 chromosomal rearrangement need more methotrexate to compensate for their innate resistance to the drug.

Given the importance of RFC and FPGS in the pharmacokinetics of MTX, a strong trend has been shown in patients that expressing higher levels of RFC or FPGS give rise to higher concentrations of MTXPG. Compared to other B-ALL subtypes, the *TCF3-PBX1B* gene fusion subtype in ALL patients translates to significantly lower expression of RFC and marginally lower expression of FPGS (Sorich et al., 2008). Preliminary gene expression profiling in B-ALL patients performed by Evans *et al.* in the Department of Pharmaceutical Sciences at St. Jude Children's Research Hospital searched for genes that are highly correlated to *FPGS* and *SLC19A1*. The researchers found that in TCF3-PBX1B ALL patients, *NOTCH2* and *SOX11* are highly expressed. High expression of *NOTCH2* and *SOX11* have also been found to correlate with lower expression of *FPGS* and *SLC19A1*. In normal day-to-day function, the product of the *NOTCH2* gene is the Notch2 protein, which is a receptor that is critical for development, regulation of the immune system, and various other mechanisms. In the case of the Notch2 protein, when a ligand binds to the receptor, the intracellular domain (ICD) is released to activate the transcription of downstream genes. One of the suspected targets of the ICD Notch2 is the *FPGS* gene, which reduces the production of the FPGS protein (Kraman & McCright, 2005). As for SOX11, once it is activated, it is hypothesized that SOX11 acts as a repressor that deactivates the *SLC19A1* gene, which halts the production of the RFC protein. It is hypothesized that the

TCF3-PBX1B fusion protein acts as an activator for both NOTCH2 and SOX11, which leads to a decrease in FPGS and RFC, which ultimately decreases intracellular MTXPG concentrations.

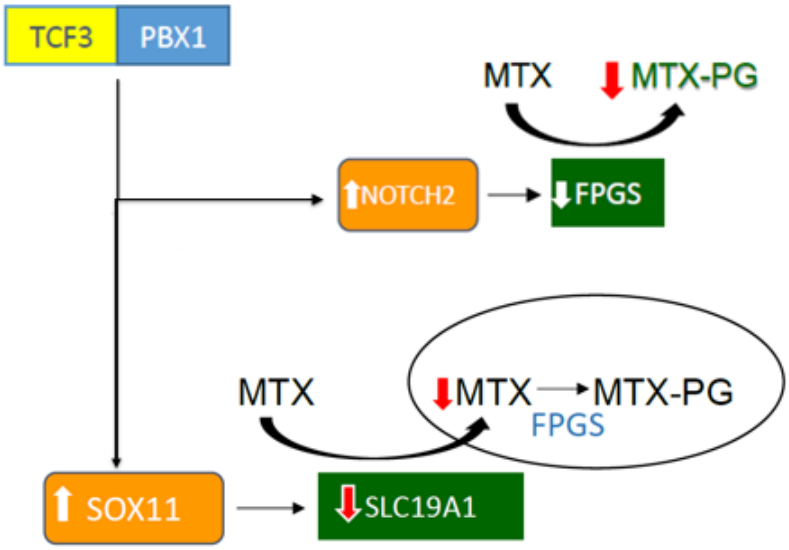

Figure 4. This represents the hypothesis for the mechanism of action of the TCF3-PBX1B fusion protein. In the presence of TCF3-PBX1B, NOTCH2 and SOX11 are expressed at greater levels, which further repress the expression of FPGS and SLC19A1 (RFC) respectively, which contribute to the decline in intracellular MTXPG.

Subtypes of Acute Lymphoblastic Leukemia

A translocation occurring between chromosomes 1 and 19 at q23 and p13.3 leads to the transcription and subsequent translation to form the fusion protein, TCF3-PBX1B. A translocation occurs during chromosomal replication where a section of one chromosome is swapped with a region of another. The exchange of regions of chromosomes creates new connections and gene associations (Griffiths et al., 2000), creating novel protein combinations that have unknown downstream effects. In many cases, an alteration of chromosomes leads to an adverse effect on physiology. In cancer patients, chromosomal translocations are common, as the chromosomal rearrangement can lead to misregulation of downstream genes or loss of function of the gene completely (O'Connor, 2008).

Methods

Cloning of TCF3-PBX1B and LacZ gene into pLenti-Puro.

EcoRV and Pmel restriction enzymes, proteins that make cuts along specific regions of DNA, were used to linearize the pLenti-Puro plasmid DNA. EcoRV and Pmel were also used on TCF3-PBX1B and LacZ, the control vector, to generate 5' and 3' compatible ends to the pLenti-Puro vector fragment. The pLenti-Puro linearized plasmid DNA and TCF3-PBX1B and LacZ fragments, respectively, were ligated with T4 DNA ligase, transformed into chemically competent *E. coli,* and plated on Lanox Broth/agar dishes plus ampicillin. The plates were incubated at 30°C overnight. Several surviving colonies were then transferred to LB broth plus ampicillin to incubate overnight at 30°C in a shaking incubator. Plasmid DNA was then extracted and purified using Qiagen plasmid purification kit and analyzed by restriction enzyme analysis and Sanger sequencing. The final product of this procedure are colonies of lentivirus which contain the TCF3-PBX1B and LacZ genes respectively. In achieving this, the lentivirus can then be used as a vector for the TCF3-PBX1B and LacZ genes to ultimately be inserted into the genome of the REH human ALL cell lines through transduction.

Repressible Gene Engineering and Lentiviral Transduction.

REH human ALL cell lines were transduced with CMV-TetR-blasticidin lentivirus particles. Successfully transduced cells were selected for by culturing cells in complete medium (RPMI-1640 plus 10% Fetal Bovine Serum plus L-Glutamine) plus antibiotic blasticidin. Selected cells were then analyzed for tetracycline repressor (TetR) expression through Western blotting with an anti-TetR antibody. Cells expressing TetR were then transduced with pLenti-Puro-iLacZ and pLenti-Puro-iTCF-PBX1B lentivirus. As a result of transduction, the pLenti-

Puri-iLacZ and pLenti-Puro-iTCF-PBX1B genes are now inserted into the cells that already express TetR. Transduced cells were selected by culturing the cells in complete medium plus antibiotic puromycin, meaning the surviving cells in the antibody medium contain the desired LacZ and TCF3-PBX1B expression was analyzed on Western blots using cells that were cultured overnight in 1 ug/mL Doxycycline to induce expression of target genes. The end goal of a successful lentiviral transduction is to use lentiviruses, a species of retrovirus, to inject and insert specific genetic sequences into the genome of REH human ALL cells.

Human Cells.

Human leukemia cell line REH (containing TetR-iLacZ and TetR-iTCF3-PBX1B and containing Red Fluorescence Protein-Puromycin control, SOX11 overexpressed, PBX1B overexpressed, TetR-iLacZ, and TetR-iTCF3-PBX1B) were cultured in Rosewell Park Memorial Institute-1640 (RPMI-1640) liquid medium containing 10% Fetal Bovine Serum and L-Glutamine, which is a critical amino acid for growth of cells in standard cell culturing. The LacZ and TCF3-PBX1B proteins are inducible through the addition of tetracycline or doxycycline, which are molecules that bind to the repressors on the gene, release the repressors, and allow for the transcription of the target gene. Western blot preliminary analysis was performed on the lentiviral transduced cell lines to ensure expression of the TCF3-PBX1B and LacZ proteins. In confirming the presence of expression of TCF3-PBX1B and LacZ proteins, the cells are prepared for the next step of the experiment in which a variation in expression of the target genes can be induced by doxycycline.

Treating REH Cells with Doxycycline.

REH iLacZ and iTCF3-PBX1B cells were counted using an automated cell counter, centrifuged at 500g for 5 minutes to eliminate the residual cell culture medium, and resuspended in RPMI-1640 +10% Tetracycline-free fetal bovine serum & L Glut at 1 million cells/mL. Tetracycline-free fetal bovine serum was used to ensure that there would be no residual tetracycline to unintentionally activate the expression of the target genes. As tetracycline is analogous in structure to doxycycline, the tetracycline-free fetal bovine serum ensured that there would not be any unintentional activation of the target gene. The cells were then seeded in 12-well plates, 2 mL per well, 6 wells per cell line. Each set of 6 wells were treated without doxycycline, followed by 5 increasing intervals of doxycycline (0, 0.2, 0.4, 0.6 ng/mL doxycycline). The doxycycline binds to the TetR, releasing it from the promoter, and allows for the expression of the downstream gene. The cells were incubated overnight for 22 hours at 37°C. After incubation overnight, the cells were collected, counted, washed in Dulbecco's Phosphate Buffered Saline (D-PBS), aspirated, and the pellets were stored in -80°C for future use.

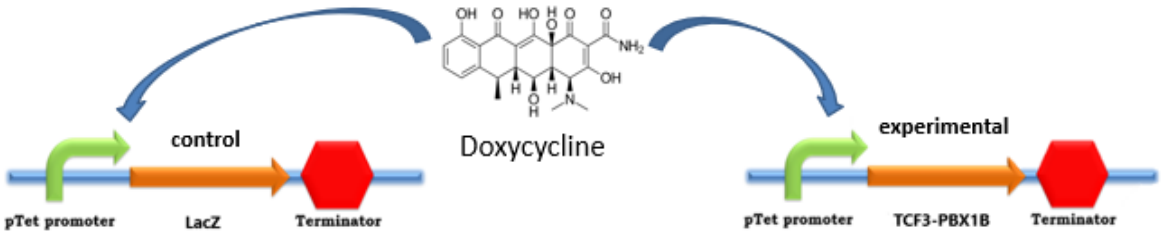

Figure 5. Doxycycline inducible LacZ control and TCF3-PBX1B experimental model. The doxycycline compound binds to the pTet promoter, which allows for the transcription and expression of the downstream gene.

Western Blot Analysis of REH Human ALL Cell Lines.

Western blot followed by analysis for protein quantification was performed. Lysis of cell pellets was performed by using RIPA buffer, and protein concentration of lysates was determined by BCA Assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run using Midi NuPAGE 4-12% acrylamide Bis-Tris 20-Well Gels and 1x 3-(N-morpholino) propane sulfonic acid (MOPS) electrophoresis buffer. The transfer of the proteins to a membrane was performed using 1x NuPAGE Transfer Buffer and polyvinylidene fluoride (PVDF) Blotting Membranes. Duplicate transfers were performed using the iBlot 2 transfer system for a duration of 7 minutes. Tris-Buffered Saline, 0.1% TWEEN (TBS-T) (50 mL 1 M Tris-HCl: pH 8.0, 30 mL 5M NaCl, 920 mL type 1 autoclaved water, and 0.5 mL Tween-20) was used as washing buffer, and blocking buffer was made using TBS-T with 5% (w/v) non-fat dry milk. The following primary antibodies were used for detection: Rabbit anti-NOTCH 2, rabbit anti-TCF3, rabbit anti-SLC19A1, rabbit anti-FPGS, rabbit anti-SOX11, mouse anti-LacZ, and mouse anti-B-actin. Horseradish peroxidase (HRP) conjugated secondary antibodies were used (Donkey-anti-rabbit IgG-HRP and donkey anti-mouse IgG-HRP both reconstituted in 0.5 mL water plus 0.5 mL glycerol). This was followed by Super Signal West Femto Chemiluminescence substrate (Pierce #34096). The primary antibodies attach to the protein on the membrane, and the secondary antibodies attach to the primary antibodies. The Chemiluminescence substrate then activates a luminescence protein attached to the secondary antibody, which can be imaged by Li-cor Odyssey. Mouse anti-B-actin was used as a control in the Western Blot protocol because that gene was not altered and concentrations remain consistent throughout all samples. The strength of each signal was then analyzed by the ImageStudio software, where the strength of the signal was directly proportional to the amount

of protein that existed in the lysed cell. After imaging, antibodies were stripped using NewBlot PVDF 5x Stripping Buffer (Li-Cor #928-40032) and new antibodies were applied.

MTXPG Cell Lysates.

24 hours prior to treatment, REH cells were pelleted and refreshed with complete medium. A cell count was performed, the cells were centrifuged at 500g for 5 minutes, the medium was aspirated, and fresh tetracycline-free medium was added to a concentration of 1 million cells/mL. Glycine, adenosine, thymidine (GAT) amino acid solution (stock: 8.4 mL 100 mM glycine, 4.6 mL 10 mM adenosine, 5.1 mL 10 mM thymidine) was then added at a concentration of 15 mL/million cells. The cells were then plated in a 6-well plate, 6 wells per cell line, 4 mL per well, and 1 million cells/mL. The wells were treated without doxycycline, followed by 5 increasing intervals of doxycycline (0, 0.25, 0.5, 0.75, 1, 5 ng/mL doxycycline). The cells were incubated for 5 hours at 37°C. Following the incubation, MTX was added to each well at a final concentration of 1 uM, and the plates were incubated for 24 hours at 37°C. Cells were then extracted, pelleted, and washed in 1 mL chilled D-PBS. Exactly 1 million cells per sample were extracted to 1.5 mL microcentrifuge tubes, pelleted, and resuspended in 100 uL Type 1 autoclaved water. The tubes were then heated at 100°C for 5 minutes, chilled on wet ice, frozen on dry ice, and stored in -80°C.

MTXPG Analysis using Ultra-high performance liquid chromatography combined with mass spectrometric detection.

Quantification of MTXPG (G1-G7) in cell lysates was performed by using ultra-high performance liquid chromatography (UPLC) with mass spectrometric (MS) detection in a method called liquid chromatography-mass spectrometry (LC-MS). In this method, the liquid chromatography separates molecules in a solution based on their physical features, and the mass

spectrometry analyzes the mass of the substance, ultimately identifying the MTXPG concentrations and the ratios that they exist in a solution. Mobile phase A was 10 mM ammonium bicarbonate adjusted to 10 pH (790mg ammonium bicarbonate, 1 L type 1 water, 30% ammonium hydroxide), and solvent B was MeOH (Fisher Scientific). Six calibration standards were made with 1, 5, 20, 50, 100, and 200 nM G1-G3 and G6-G7 and 6, 10, 20, 50, 100, and 200 nM G4-G5 MTXPG isotopes. Quality Control Samples were made with 5, 30, and 150 nM G1-G3 and G6-G7 and 18, 30, and 150 G4-G5 MTXPG isotopes. Internal standard working solutions were prepared by adding 1100 nM G1-G7 isotope stock solution, prepared in DMSO with a concentration of 1 mg/mL. MTXPG samples were prepared by pipetting 10 uL internal standard solution into each sube. Samples were heated at 95°C for 5 minutes, cooled on ice for 10 minutes, and centrifuged at 15,000 RPM for 10 minutes at 4°C. HPLC parameters included: column (ACQUITY BEH C18 1.7 u 100A), guard column (ACQUITY in-line filter), mobile phase (gradient in Table I), column temperature at 40°C ± 5°C, autosampler temperature at 10°C ± 5°C, flow rate at 0.6 mL/minute, run time for 12 minutes, pre-inject wash at 0 seconds, and post-inject was at 6 seconds. Data collection was performed by MassLynx V1.40 chromatography/mass spectrometric software.

Results

Western Blot Analysis of REH Cells.

Protein detection with anti-TCF3 antibodies showed a dose-dependent effect as a result of increasing the concentration of doxycycline on the inducible TCF3-PBX1B REH human ALL cells. The experimental REH-iTCF3-PBX1B cell line expressed more than two-fold NOTCH2 and SOX11 when contrasted with the REH-iLacZ line, showing that the experimental cell line is

viable, and there is no endogenous TCF3-PBX1B in the control cell line that would have the potential to alter the results.

When controlled for with B-actin, NOTCH2 and SOX11 expression showed a two-fold increase when treated with 0.6 ng mL doxycycline when compared to no treatment. B-actin is used as a control because the protein was not altered in any way, and the protein is necessary for normal cell function, meaning B-actin levels should be similar across all cell samples. This two-fold increase in NOTCH2 and SOX11 expression is consistent with the hypothesis that the TCF3-PBX1B fusion protein induces the expression of these two genes.

The experimental cell line, REH-iTCF3-PBX1B, showed a 46% decline in RFC expression when comparing no doxycycline treatment to treatment with 9.6 ng/mL of doxycycline. FPGS showed decreasing expression with increase in doxycycline concentration, and at 0.6 ng/mL doxycycline concentration, there was no FPGS expression signal (Figure 6). This shows that there is a negative correlation between an increase in TCF3-PBX1B fusion protein and FPGS and RFC expression, which supports the hypothesis of this experiment and the preliminary data that was seen in patients. Although no conclusions of causation can be extrapolated between the effects of NOTCH2 and SOX11 on FPGS and RFC, the hypothesis holds true in that increased levels of TCF3-PBX1B result in lower expression of NOTCH2 and SOX11, decreasing the expression of FPGS and RFC.

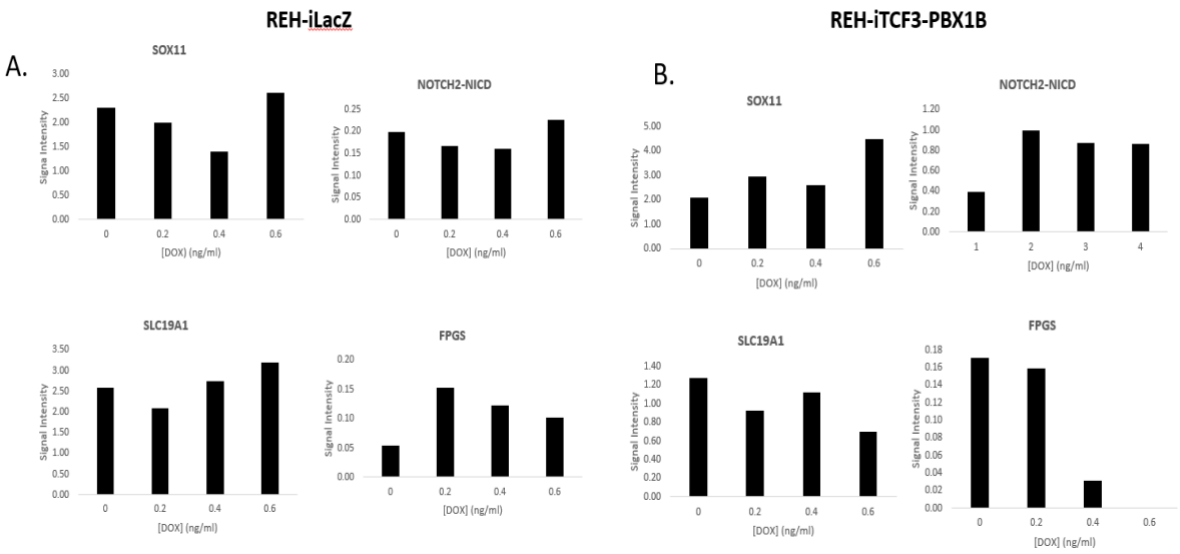

Figure 6. Western Blot results of control REH iLacZ cell line (A) compared to experimental REH iTCF3-PBX1B (B). NOTCH2 intracellular domain (NICD) and SOX11 expression increased as more doxycycline was added while expression of SLC19A1 and FPGS declined as cells were treated with increasing dosage of doxycycline.

MTXPG Analysis Using LC-MS.

MTXPG-4-6 combined are shown in figure 7. After infusion with 1 uM MTX for 24 hours, there was no significant difference in the quantity of MTXPG-4-6 in the REH-iLacZ control cell line with different concentrations of doxycycline treatments. In referring to the Western Blotting results, the induction of the control cell line with different concentrations of doxycycline treatments had no effect on the TCF3-PBX1B expression since the control REH-iLacZ did not contain the gene for the fusion protein.

The iTCF3-PBX1B cell line treated with 5 ng/mL doxycycline showed a 31% decline in MTXPG concentrations when compared to the experimental sample not treated with doxycycline (Figure 7). There was also a 32% decline in MTXPG concentrations from iLacZ to iTCF3-PBX1B cells treated with 5 ng/mL of doxycycline. These results align with the hypothesis, which state in the presence of the TCF3-PBX1B fusion protein, MTXPG formation in REH

human ALL cells will be hindered. These results are comparable to the preliminary data as seen by Evans and his colleagues in the Department of Pharmaceutical Sciences at St. Jude Children's Research Hospital. In combining the results of the western blot data with the LC-MS data, there is a clear trend that increasing the expression of the TCF3-PBX1B fusion protein leads to a decrease in both transcription factors NOTCH2 and SOX11, which decrease the crucial folate pathway proteins FPGS and RFC respectively, which decrease the concentration of active intracellular MTXPGs.

MTX-glu(5) was the most predominant MTXPG in both cell lines and in each treatment of doxycycline, while MTX-glu(6) was the least common. There was a 46.5% decrease in MTX-glu(5) from 0 ng/mL doxycycline treatment to 5 ng/mL doxycycline treatment in the experimental TCF3-PBX1B cell line. There was also a 38.2% decrease in MTX-glu(5) concentration from the control iLacZ cell line to the experimental iTCF3-PBX1B cell line when both were treated with 5 ng/mL of doxycycline (Figure 8).

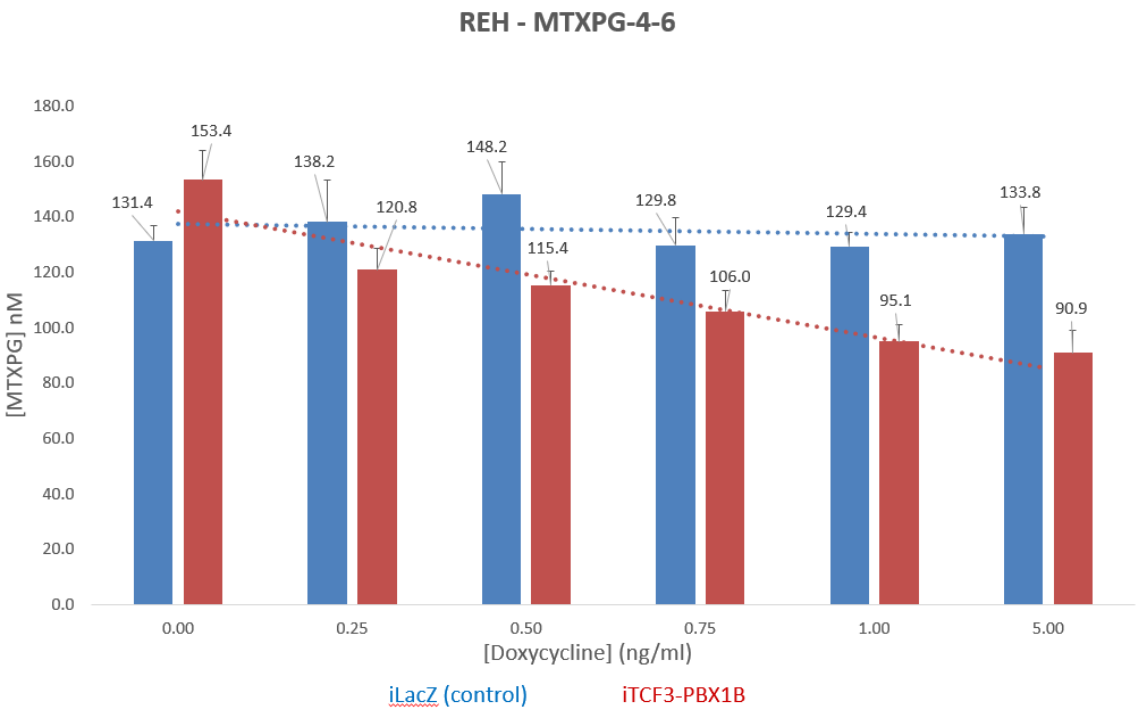

Figure 7. Quantification of long-chain MTXPGs (4-6 glutamate residues) via ultra-high performance liquid chromatography-mass spectrometry (LC-MS) shows a decline of long-chain MTXPGs as higher concentrations of doxycycline is added in REH-iTCF3-PBX1B cell line.

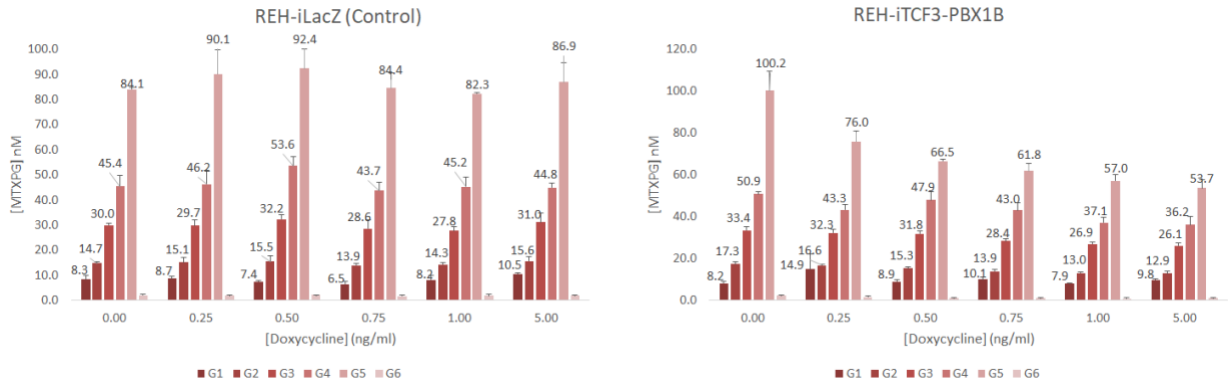

Figure 8. Analysis via LC-MS of MTXPG shows a decline in total MTXPG concentrations. The most common form of MTXPG is that in which there are five glutamates attached, indicating either a mechanistic preference to add 5 glutamates or an increased stability of MTXPG with five glutamates.

Discussion

This study showed that the expression of the TCF3-PBX1B fusion protein through doxycycline induction in the REH human ALL cell line reduces overall MTXPG concentration in cells. Although MTXPGs were detected in both the iTCF3-PBX1B cells treated with doxycycline and untreated with doxycycline, there was a significant difference in MTXPG concentration depending on the presence of doxycycline and the concentration of doxycycline. There was a 31% decline in MTXPG concentration from the iTCF3-PBX1B REH cells unexposed to doxycycline and those treated with 5 ug/mL doxycycline when observing long-chain MTXPGs (4-6 glutamate residues). This difference in MTXPG production is evidence that the TCF3-PBX1B fusion protein has a downstream effect alters MTXPG production in REH human ALL cell lines. This indicates that: 1) REH human ALL cell lines are a viable method of study for the TCF3-PBX1B fusion protein, and 2) the presence of the TCF3-PBX1B fusion protein has a negative effect on MTXPG production.

The genes that have previously been found by Evans and colleagues to have high correlations with MTXPG concentrations in patients (*SLC19A1 and FPGS*) also correlate to MTXPG concentrations in the TCF-PBX1B REH cell line. Lowering expression levels of reduced folate carrier (RFC) corresponded with a decrease in intracellular MTXPG concentrations. This is likely due to the importance of RFC as a carrier protein to allow MTX to enter the cell via active transport. Similarly, lowering expression levels FPGS resulted in a decrease in intracellular MTXPG concentrations as well. FPGS enzyme plays an important role in the ATP-dependent addition of glutamates to MTX. This reduction in MTXPG concentration as a result of decreased expression of *SLC19A1* and *FPGS* is a likely cause of MTX resistance in TCF3-PBX1B ALL subtype patients.

NOTCH2 and *SOX11* also seem to play a critical role in the regulation of *FPGS* and *SLC19A1*. Patient data analyzed by Evans *et al.* showed that FPGS and RFC expression negatively correlated with SOX11 and NOTCH2 expression. In the REH-iTCF3-PBX1B cell line, the two-fold increase in both NOTCH2 and SOX11 expression resulted in a doxycycline-dependent decline in FPGS and RFC expression.

Limitations

Previous studies have shown that the SOX11 transcription factor is highly regulated through DNA methylation (Nordstrom, 2015). Methylation is a process in which a methyl group binds to an area of DNA that contains the gene of interest and prevents the expression of that gene (Phillips, 2008). In our experiment, we only worked with the REH cell line, which is characterized by low DNA methylation and high SOX11 expression (Vegliante et al., 2011). To further elucidate the mechanism of the fusion protein, experiments on various other human ALL cell lines would have to be performed.

By inducing TCF3-PBX1B fusion protein in the REH cell line, the increased expression shown in figure 9 would not likely be caused solely by demethylation of *SOX11*. In other cell lines, such as the Nalm6 human ALL cell line, SOX11 is highly methylated, thus there is low endogenous expression in SOX11. In that case, the results of the experiment could differ from the current results. This is a potential limitation of the study, as the research was only performed on one variant of a cell line, the REH human ALL cell line, and other cell lines may have drastically different endogenous gene expression levels, which may help elucidate the true mechanism of the TCF3_PBX1B fusion protein.

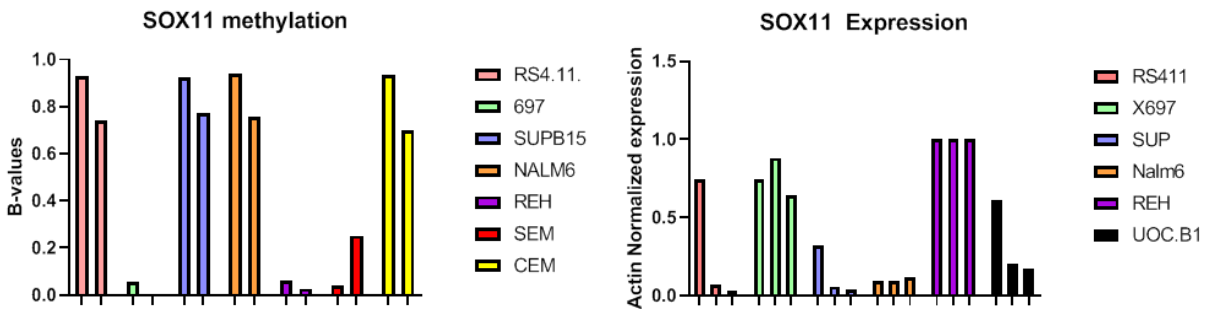

Figure 9. SOX11 methylation (left) and SOX11 expression (right) are negatively correlated with each other. Higher SOX11 methylation is accompanied by lower SOX11 expression levels *in vitro*. In the case of SOX11 in the REH human ALL cell line, there are low methylation levels, which correlate to relatively higher levels of expression when compared to other human ALL cell lines.

In addition, in a study performed by Mangolini and colleagues (2018) identified one of the ways in which NOTCH2 affects patients with leukemia. Through cell sorting, antibody detection, and protein identification techniques, the researchers found that the Notch2 and Wnt pathways are important in combating leukemic cells. An increase in Notch2 production leads to an increase of Wnt signalling, which increases the production of adhesion proteins in leukemic cells, increasing the survivability of the leukemic cells. After inhibiting the Notch2 or Wnt pathways, the opposite effect on the leukemic cells occurs (Mangolini, 2018). This proposes another mechanism in which an increase in the NOTCH2 expression due to the expression of the TCF3-PBX1B fusion protein could lead to drug resistance against MTXPG. Since the presence of NOTCH2 increases the resiliency of leukemia, there may be an inherent need for more drug administration for the patient to get the same results as a patient without the TCF3-PBX1B translocation.

Future Directions

On the contrary, Liu and colleagues examined the role of SOX11 and found that an overexpression of SOX11 leads to the under expression of the Wnt signaling pathway, which is

critical to produce adhesion proteins. This suppresses the tumor cells' ability to function normally. Ultimately, an increase in SOX11 expression could act as a tumor suppressor (Liu 2019). In this case, the effect of SOX11 on the Wnt pathway, which affects cancerous growth, is exactly the opposite of NOTCH2. Therefore, further research needs to be performed on NOTCH2 and SOX11 to determine the combined effects of the two proteins on pediatric ALL patients.

Further experiments on other human ALL cell lines would be necessary to show if the same effects of inducing TCF3-PBX1B fusion protein causes the same effects under different *SOX11* methylation environments. A whole transcriptome analysis through RNA-Seq in multiple human ALL cell lines would also be beneficial in showing whether inducing TCF3-PBX1B fusion protein leads to higher levels of *NOTCH2* and *SOX11* mRNA or lower levels of *SLC19A1* and *FPGS* mRNA and whether mRNA expression directly relates to protein expression in these genes.

Conclusion

It has been demonstrated in the lab that inducing TCF3-PBX1B fusion protein in REH human ALL cell lines would ultimately lead to a decrease in the concentration of MTXPG. This lowering of concentration is achieved through the action of the TCF3-PBX1B fusion protein increasing the expression of NOTCH2 and SOX11 transcription factors, which ultimately lead to a decrease in the expression of FPGS and RFC. This translates to a decline in the active form of MTX, MTXPG. This decline in MTXPG concentration is the driving force behind the MTX drug resistance in TCF3-PBX1B ALL patients.

These results reiterate the preliminary results seen in the pediatric clinic in that there is a negative correlation between SOX11 and RFC, and there is also a negative correlation between NOTCH2 and FPGS. This study serves as a proof of concept for the fact that the preliminary patient data observed can also be replicated in the lab, meaning the REH human ALL cell line is a viable method of study for patients with the TCF3-PBX1B genotype. The ability to test use the REH human ALL cell line as a model to test ALL patients of the TCF3-PBX1B subtype is an important step in understanding how to remedy the MTX drug resistance in TCF3-PBX1B ALL patient subtypes. This study also goes a step further in proving that the TCF3-PBX1B fusion protein plays a role in altering the expression of genes that ultimately affect MTXPG levels.

In performing further experiments, this research can ultimately be translated to clinical practice in that physicians will be able to prescribe a more targeted dosage to patients with the TCF3-PBX1B B-cell ALL genotype. Based on the current preliminary data performed on the REH cell line and based on what has been observed in patients, there is a possibility that patients with the TCF3-PBX1B genotype are more resistant to methotrexate, meaning that clinicians can counteract this by possibly giving patients a longer infusion time or higher dosage of MTX. In understanding the genotypic markers of drug resistance in pediatric ALL patients, there is a chance that each patient can receive individualized treatments specific to their genome, which would yield higher survival rates and an improved post treatment quality of life.

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