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CBF REGULATION ON RAG GENES VIA ERAG ENHANCER

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ABSTRACT

This project explores the function of Core Binding Factor (CBF) in B-cell development, and its regulation on the recombination activating genes (RAG) that encode the B-cell antibody gene recombinase via the *Erag* enhancer. A GFP reporter construct containing *Erag* and the RAG2 promoter was transfected into pro-B cells, and was mutated to block CBF binding. Leukemia cells and transfected pro-B cells whose growth depends on CBF were treated with CBF inhibitor to measure the effects of disrupted CBF function. The transcript levels of B-cell specific proteins of inhibitor-treated cells were measured by qPCR. The data indicate that cell viability and key B cell protein expressions were decreased, but reporter activity was not decreased after inhibitor treatment. The mutant was obtained but was not yet transfected.

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BACKGROUND

B cells are one of the two major types of lymphocytes that play an essential role in mammal adaptive immune responses (Janeway et al., 2004, 6). B cells differentiate from common lymphoid progenitor (CLP) which originates from hematopoietic stem cells (Janeway et al., 2004, 6). When activated by foreign antigens, B cells differentiate into plasma cells that secrete antibodies that bind pathogens or their toxic products in extracellular spaces of the body (Janeway et al., 2004, 6 and 103). B cells develop in the bone marrow as a result of hematopoiesis: hematopoietic stem cells (HSC) give rise to CLPs which then differentiate into pro-B cells with help of stromal cells (Janeway et al., 2004, 245). Pro-B cells then develop into pre-B cells which continue to differentiate to become immature B cells by generating immunoglobulin M (IgM) (Janeway et al., 2004, 248).

Developing B-cell progenitors undergo a sequence of molecular events to create a variety of mature B cells that have diverse immunoglobulins (Igs) (Janeway et al., 2004, 248). The diversity of Ig is a result of V(D)J gene rearrangements that produce many millions of distinct V (various) regions of the Ig protein (Janeway et al., 2004, 137). The Ig protein is composed of a heavy chain and a light chain. The heavy chain V region is encoded by a (V), joining (J) and diversity (D) sub-exon, whereas the light chain V region is encoded by a V and a J. Each of these types of sub-exons has multiple different segments in the germ-line genome prior to rearrangements, but only one segment of each gene is jointed together via V(D)J recombination to form the variable region expm (Janeway et al., 2004, 139). V(D)J recombination is carried out by a complex of enzymes termed the V(D)J recombinase, of which the lymphoid-specific components are encoded by genes RAG1 and RAG2 (**r**ecombination-**a**ctivating **g**enes) (Janeway

et al., 2004, 142). In developing B cells, RAG genes are expressed in pro-B, pre-B and immature B cells in which the recombination of Ig heavy and light chains takes place (Li et al., 1993; Graunder et al., 1995; Pelanda et al., 1997; Radic & Zouali, 1996). The RAG expression is essential for B-cell development and maturation (Janeway et al., 2004, 143). The specific mechanisms responsible for regulating RAG gene expression during B cell development are only partially understood.

The differentiation from HSC to pro-B cells and the eventually mature B cells are known to be regulated by a variety of proteins, some of which are transcription factors that regulate expression of other genes that are essential for B cell development (Janeway et al., 2004, 265). A transcription factor is a sequence-specific DNA-binding protein that controls the transcription of some genes to mRNA by binding to certain regions of DNA (Latchman, 1997). Several transcription factors are essential for B-cell development, including the products of the *E2A* gene as well as the early B-cell factor (EBF). EBF regulates the transcription of *mb-1* which encodes CD79α (Igα), a protein constantly expressed from the pro-B cell stage and throughout the cell life (Janeway et al., 2004, 265). E2A regulates RAG1/2 expression as well as EBF expression (Janeway et al., 2004, 265). EBF also activates the expression of the transcription factor *pax-5*, which is expressed in pro-B cells and enable proper functioning of antibody heavy chain enhancer (Janeway et al., 2004, 266). Together, E2A, EBF and *pax-5* regulate most of the B cell specific genes required for B cell development and production of Ig. There regulators also bind to the regulatory sites in the genes for λ5, VpreB and other B-cell-specific proteins which can be measured as indicators for B-cell development and differentiation (Janeway et al., 2004, 266). These transcription factors are believed to regulate the order of events in gene rearrangement

during B-cell development and direct the developmental program of B-lineage cells (Janeway et al., 2004, 266). This is an intricate process of which all the details are not known well.

Core binding factors (CBFs) are a class of transcription factors that regulate a variety of genes associated with hematopoiesis and B cell development (Kuo et al., 2008; Speck and Gilliland, 2002). The CBF has a DNA binding subunit, which is encoded by one of 3 genes: *RUNX1*, *RUNX2* and *RUNX3,* and a non-DNA binding CBFβ subunit which is encoded by gene *CBFB* (Kuo et al., 2008). Runx1, the transcription factor encoded by gene RUNX1, marks all functional hematopoietic stem cells in the embryo and is required for their generation (North et al., 2004). Runx1 binds to DNA through a conserved sequence, TGT/cGGT, and forms a heterodimeric transcription factor with CBFβ (North et al., 2004; Meyers et al., 1993). Mutating the consensus site to TGTTAG efficiently blocks the binding of Runx1 (Meyers et al., 1993). The non-DNA binding CBFβ associates with Runx1 and significantly increase its affinity of binding to DNA, and is also the catalytic subunit that protects the heterodimer from proteolysis. It also recruits other proteins to promoters is essential for Runx1 function *in vivo* (Speck & Gilliland, 2002). Runx1 and CBFβ play essential role in hematopoiesis, especially during embryogenesis, and lymphocyte development (Brujin et al., 2004). Both *CBFB* and *RUNX1* genes are targets of chromosomal translocations and inversions that cause the mutations in human acute myeloid leukemia (AML) (Kuo et al., 2008; Hsu et al., 2003).

A number of studies have demonstrated the crucial roles of Runx1 and CBFβ in the generation of hematopoietic stem cells during embryogenesis and lymphocyte development (Bruijn & Speck, 2004). Correct CBF gene dosage was found essential for hematopoiesis (Speck & Gilliland, 2002). The homozygous disruption of Runx1 or Cbfβ in mice results in identical developmental defects, including mid-gestation embryonic lethality during embryogenesis and a

profound block in hematopoiesis and B lymphocyte development (Okuda et al., 1998; Wang et al., 1996; Sasaki et al., 1996). *RUNX1*- or *CBFB*- deficient mouse embryos were found to have no (*RUNX1*$^{-/-}$) or very few (*CBFB*$^{-/-}$) definitive progenitors in their fetal livers or yolk sacs, and they died between embryonic days 12.5 and 13.5 (Okuda et al., 1998; Wang et al., 1996; Sasaki et al., 1996; Cai et al., 2000). This defect was found as result of significantly compromised emergence of definitive hematopoietic progenitors and stem cells in the RUNX1$^{-/-}$ and CBFB$^{-/-}$ mice (North et al., 2004; Mukouyama et al., 2000; Yokomizo et al., 2001). Deletion of RUNX1 in adult mice with *Mx1-Cre* didn't abolish HSC function and the bone marrow (BM) of *RUNX1*$^{-/-}$ mice was able to reconstitute hematopoiesis in irradiated recipient mice (Ichikawa et al., 2004). The deletion of *RUNX1* in BM also didn't seem to affect the presence of the CLP population (Ichikawa et al., 2004). However, it was shown that Runx1 is required for the formation of CD45^{+} cells; and the CD45R (B220), an isoform of CD45, is specifically expressed by B lymphocytes from the stages of pro-B cells after CLP to mature B cells (North et al., 2004; Janeway et al., 2004, 250). The number of B220^{+} cells was under-represented in the spleen of *RUNX1*$^{-/-}$ mice and the BM cells did not give rise to B220^{+} cells upon transplantation to irradiated recipient mice (Ichikawa et al., 2004). It suggested a block that resulted from a RUNX1 deletion occurred in B lymphocyte development later than CLP (Ichikawa et al., 2004). CBFβ was also found to play essential role in hematopoiesis and B cell development. Restoration of CBFβ function in endothelium and endothelia progenitors in CBFβ-deficient mice rescued the appearance of definitive hematopoietic progenitors (Miller et al., 2002). Other studies have shown that the CBF function is essential for hematopoiesis and B lymphocyte development by using a conditional *Cbfb-MYH11* knock-in allele. *Cbfb-MYH11* is a fusion gene created by the chromosome 16 inversion which disrupts the *CBFB* and smooth muscle myosin heavy chain

MYH11 genes, and it is found in approximately 10% of patients with AML (Look 1997; Liu et al., 1993). The encoded Cbfβ-SMMHC protein is a dominant repressor of CBF function, and it was found to impair myeloid differentiation in adult hematopoiesis, creating an abnormal myeloid progenitor population that can be the origin of leukemia stem cells (Kuo et al., 2006; Kuo et al., 2008). Furthermore, the study has shown that Cbfβ-SMMHC expression reduces the expression of Ebf1 and E2A, early B-linage specifying transcription factors, in the CLPs, and also reduces *Rag1/2* expression and V(D)J recombinase activity, and impairs the differentiation and survival of pre-pro-B cells and pro-B cells in adult mice (Kuo et al., 2008). Kuo et al. found Cbfb-SMMHC deregulates the B lineage specifying transcription program and impairs early B lineage differentiation as early as the commitment step from CLPs to pre-pro B cells (2008). Furthermore, *Rag1* expression was 75% reduced and *Rag2* expression was 50% reduced in CLPs of the *Cbfb-MYH11* knock-in mice; and by using VEX as a reporter for recombinase activity, the percentage of VEX-positive cells was reduced 70% in both the CLPs and pre-pro B cells (Kuo et al., 2008). These data provided functional evidence that CFB function is required for normal levels of *Rag1/2* expression during B-cell development (Kuo et al., 2008).

An allosteric inhibitor of the protein-protein interaction between RUNX1 and Cbfb-SMMHC fusion protein was developed by Gorczynski et al (2007). The treatment of the inhibitor on ME-1 cells, a leukemia cell line expressing Cbfb-SMMHC which was crucial to ME-1 growth, showed a significant decrease of the proliferation of ME-1 cells (Gorczynski et al, 2007). These results confirmed the disruptive effect of the inhibitor on Cbfb-SMMHC and RUNX1 interaction, suggesting the inhibitor may also disrupt the interaction between RUNX1 and normal Cbfb, and therefore hindering hematopoiesis and B cell development. The inhibitor

has been tested to increase its specificity against Cbfb-SMMHC while decreasing the toxicity on normal CBFβ.

The expression of RAG1 and RAG2 genes is essential for both T and B lymphocyte development, but the mechanisms of the regulation of the RAG genes are poorly understood. RAG1 and RAG2 are closely linked genes and reside in a locus that is coordinately regulated. A novel conserved transcriptional enhancer in the RAG locus, named *Erag*, was identified by Hsu et al. (2003). Hsu et al. tested restriction fragments generated from clone of the murine RAG locus for the ability to enhance RAG2 promoter activity in a transient luciferase assay and identified a 2.3 kb Xbal fragment located about 22 kb 5' of the RAG2 first exon that gave about 2-fold stimulation of promoter activity in a transfected murine pro-B cell line, 220-8 (2003).

This enhancer was found essential for the expression of a chromosomal reporter gene driven by either RAG promoter (Hsu et al., 2003). The reporter construct was primarily composed of a neomycin resistance gene for selection, insulator genes to prevent the neomycin resistance gene from influencing reporter construct activity, the *Erag* enhancer, a RAG promoter and a GFP gene for analysis (Hsu et al., 2003). 30% to 40% of 220-8 mouse pro-B cells stably transfected with the construct that contained RAG2 promoter were GFP positive (Hsu et al., 2003). A construct composed of human RAG promoter had similar results after being transfected into 220-8 cells, suggesting a conservation of *Erag* in different species; later analysis showed the sequence was highly homologous (~80%) among human, mouse, cow, sheep and pig (Hsu et al., 2003). In order to determine the role of *Erag* in the developmental regulation of endogenous RAG locus expression, *Erag*-deficient mice were generated. As a result, *Erag* knock-out mice were found to have 5- to 15-fold decrease in RAG1 transcript level and 2- to 3-fold decrease in RAG2 transcript level in their pro- and pre-B cells (Hsu et al., 2003). The bone marrows of

homozygous Erag$^{-/-}$ mice were found to have equivalent number of pro-B cells, but about 2.5-fold fewer B220^{+} cells, 3-fold fewer pre-B cells, and 3-fold fewer IgM^{+} B cells than those of wild types (Hsu et al., 2003). Insterstingly, *Erag* deletion had no effect on RAG expression during T cell development (Hsu et al., 2003).

Double-stranded DNA breaks at V(D)J recombination signal sequences (located next to each V, D and J segment) are termed signal broken signal ends or SBE, and the V(D)J recombinase activity within a population of cells can be determined by measuring the level of SBEs (Hsu et al., 2003). By this method, the *Erag*$^{-/-}$ mice were found to have 5- to 10-fold decrease in V(D)J recombinase activity (Hsu et al., 2003). Another experiment measuring the level of J_H1 (joining segment 1 of heavy chain) which is invariably lost upon D_H-J_H rearrangements found that *Erag*$^{-/-}$ pro-B cells had about 9-fold increase in retention of J_H1, suggesting a significant loss of D_H-J_H recombination (Hsu et al., 2003). Also, using the H2SVEX transgenic reporter of V(D)J recombinase activity, the Gerstein lab showed that *Erag*-deficient CLPs have a 4-fold reduction in recombination. As shown by all the results above, the *Erag* enhancer is critical for B lymphocyte development by regulating the transcript level of RAG1/2 which determines the recombinase activity and V(D)J rearrangements of Ig genes.

In the *Erag* sequence obtained by Hsu et al., the enhancer was found to contain two consensus sequences that match the RUNX1 binding sequence, suggesting a possible regulatory role of CBF in *Erag* regulation of RAG expression (Hsu et al., 2003).

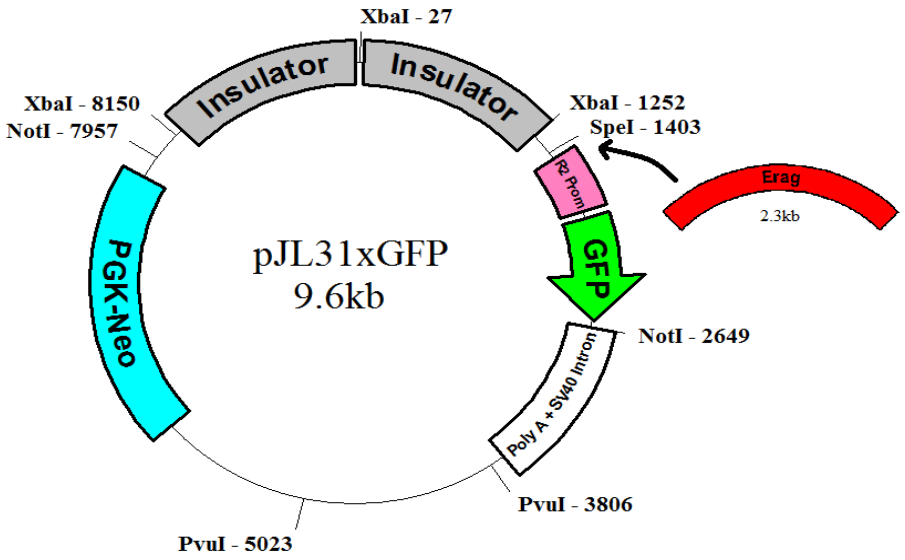
PROJECT PURPOSE

This project explores the regulation of CBF on *Erag* as well as contribution of CBF to expression of B-cell specific genes. The construct plasmids (*Erag* inserted at either orientation) used in the study of Hsu et al. were first used to transfect a pro-B cell line to measure the expression of GFP as an indicator of *Erag*-dependent RAG2 promoter function. Then the transfected cells were challenged by a CBF inhibitor that interrupts CBF function, and were analyzed for cell viability and GFP expression by flow cytometry at different times points after treatment. The mRNA of CBF-inhibitor challenged cells was obtained to measure the level of transcripts of B-cell specific genes regulated CBF by qPCR. The inserted *Erag* sequence was then cloned and mutated to disrupt RUNX1 binding – before inserting to the plasmids again. Pro-B cells were then transfected with mutated constructs and analyzed for their cell viability and GFP expression.

MATERIALS AND METHODOLOGY

Erag Constructs

From the Schlissel lab, the ERAG enhancer was inserted in either orientation at a SpeI site of a plasmid composed of Geneticin Resistance gene (PGK-NEO), two insulator sequences to separate *Erag* from PGK-Neo, RAG2 promoter and GFP reporter. The plasmid with ERAG inserted in the genomic orientation was called JL13×GFP #24, and the opposite orientation was called JL13×GFP #12.



Pro-B Cell Culture and Stable Transfection Assay

Mouse pro-B cell line 1-8 cells were cultured in media made up of 10.0% sterilized fetal bovine serum (FBS), 0.2% 2-mercaptoethanol, and 89.8% Invitrogen media RPMI 1640 enriched with 1.0% glutamine (Q), 1.0% penicillin/streptomycin (P/S) and 1.0% fungizone. Cells were maintained at a concentration between 5.0×10^5 and 35.0×10^5 cells/ml.

The construct used in the study of Hsu et al. was obtained from Professor Mark Schlissel. The plasmids are named JL13×GFP#12 (*Erag* in the reverse direction of the GFP gene) or #24 (*Erag* in the same direction of the GFP gene). Transfection of the construct into 1-8 cells was done by electroporation. 1-8 cells were first counted for their viability and spun down at 1,100 G for 10 min at room temperature. 1.0×10^7 cells were collected and resuspended in 300 µl of complete 1-8 cell media. 12 µl of Hepes and 5-10 µg (≤20 µl) of DNA were added to the cells which were then transferred to a 0.4 cm cuvette. The cells were then electroporated with 270 V and 975 C before putting the cells on ice for 10 min. Cells were then transferred to growth condition and were returned to the incubator. GFP analysis was done by Flow Cytometry 48 hours later.

Leukemia Cell Line Culture

ME-1 cells, an AML leukemia cell line, were cultured in 25mM Hepes 20% FBS and otherwise the same media as the 1-8 cells. Cells were centrifuged and fed every three or four days and the cell concentration was kept between 5.0×10^5/ml to 1.0×10^6/ml.

CBF Inhibitor Treatment Assay

A CBFβ inhibitor that disrupts the protein-protein interaction between Runx1 and CBFβ was developed by Gorczynski et al (EMD product number 219505-10MG, production information can be found at the manufacturer's website (EMD, 2011). The inhibitor was dissolved in DMSO. 1-8 cells and ME-1 cells were challenged with inhibitor. ME-1 cells were treated with 25µM, 50µM and 100µM CBFβ inhibitor or equivalent concentration of DMSO for 72 hours before flow cytometry analysis. 1-8 transfected cells were treated with 200µM, 250µM,

275μM and 300μM CBFβ inhibitor or equivalent concentration of DMSO for 48 hours and with 200μM CBF inhibitor or equivalent concentration of DMSO for 24 hours.

Restriction Digestion of DNA

New England Biolab restriction endonucleases were used to digest plasmids for isolation and identification. Reactions were performed according to New England Biolab protocols with the manufacturer's recommended constituents of NEB buffer and BSA buffer.

Phosphatase Digested Plasmids

Digested vectors, pUC19 and JL13xGFP were phosphatased using NEB Antarctic Phosphatase and its protocol. Phosphatased plasmids were precipitated by adding 3X 100% ethanol, 10 µl sodium acetate and 1 µg glycogen followed by storage at -20°C overnight.

DNA Phenol/Chloroform Purification

DNA solutions were put into 0.5 ml eppendorf tubes, and the volumes were adjusted to 100 µl with TE. Equal volumes of 1:1 phenol:chloroform were added to the solutions and mixed for 2 min before being centrifuged at 13,000xg at 4°C for 5 min. Upon centrifugation, the aqueous phases of the solutions were transferred to new tubes. Equal volumes of chloroform were added to the samples and mixed for 2 minutes before being centrifuged at 13,000 G at 4°C for 2 minutes. The upper layers of the solutions were transferred to new tubes. Then 1 µg of glycogen, one tenth volume of 3M NaOAC pH 6.4, and 2.5 times volumes of 100% ethanol were added to the samples which were put at -20°C overnight or on dry ice for at least 30 minutes to precipitate the DNA. The solutions were then centrifuged at 14,000 G at 4°C for 30 minutes. The

supernatants were discarded and 200 µl of 70% ethanol was added to each tube. Solutions were then centrifuged at 13,000 G at 4°C for 15 minutes before removing the supernatants and being dried. The DNA pellets were resuspended in TE after drying.

Transformation and Minipreps

The competent cells used were Stratagene XL10-Gold Untracompetent Cells, and the transformation was done by the protocol which can be found at http://www.genomics.agilent.com/files/Manual/200314.pdf. The NZY^+ media referred in the protocol was replaced with SOD media. Bacteria cultures were then plated on Amp^+ plates and incubated at 37°C overnight.

Single-Colony Bacteria Culture and Minipreps

Plates incubated at 37°C overnight were taken out and single colonies were selected and put in 2 ml LB broth with ampicillin or the analog carbanicillin. The cultures were then incubated at 37°C overnight. Cultures were then taken out and used for Qiagen minipreps which were done by the protocol of Qiagene miniprep Kit (found at http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAprepMiniprepKit_EN.pdf).

Mutagenesis

The Erag sequence was first isolated from the plasmids obtained from Hse et al. The Erag sequence was amplified though PCR with primers including XbaI recognition site. Then the PCR products were digested with XbaI and cloned into pUC19 which had been digested with XbaI and phosphatased. Mutagenesis of Runx1 consensus sites on Erag sequence were then

mutated using FINNZYNES' Phusion™ Site-Directed Mutagenesis Kit (found at http://www.finnzymes.fi/sequencing_mutagenesis/phusion_site-directed_mutagenesis_kit.html) and pre-designed primers.

The primers for the sites are:

-GGAGGGAATTAAATGACTACAACAACAGCACAGAGCTGTGGTACCACAGCCACCT-
Primer 1 (P)CAGCACAGAGCTGTTAGACCACAGCCACCT
CCTCCCTTAATTTACTGACTGTTGTT(P) Primer 2
-GTAAGAGGGCAGCTGCTTGCTATATTTTTCTCTGTGGTATTTTTCTACAGCA-
Primer 1 (P)ATATTTTTCTCTGTTAGATTTTTCTACAGCA
CATTCTCCCGTCGACGAACGA(P)

The primers contain phosphate group at 5'- or 3'- ends to be ligated back after mutagenesis reaction. After mutagenesis PCR, the plasmids were isolated by using low melt getl. The isolated *Erag* fragment was then ligated to partially SpeI digested JL13xGFP vector.

Low Melt Gel and Recovery of DNA

1% low melt gel in 1X TAE was made for isolation of plasmids. Plasmids were loaded in the gel which was run at 70V 4°C. The gel was then inspected by irradiating the gel with long-wave ultraviolet (wave length: 355-360 nm). The corresponding region of the gel that contained the wanted DNA was cut out and put in a 1.5 clear eppendorf tube. One to three times volumes of TE and 0.3 (total) volumes of 3M sodium acetate pH7 were added to the tube before heating it at 65°C. After the gel dissolved into the solution, an equal volume of TE-saturated phenol was added to the solution which was then mixed for 30 seconds and centrifuged at 13,000G for 7 minutes at room temperature. The aqueous phase was then transferred to a new tube with equal volume of TE-saturated phenol, and the solution was mixed and centrifuged for another 5 min at room temperature. After transferring the aqueous phase into a new tube, an equal volume of chloroform was added to remove the trace of phenol before being centrifuged at 13,000G for 2

minutes at room temperature. Then the aqueous phase was removed and mixed with 2.5 volumes of 100% ethanol. Solution was then put at -20°C to precipitate the DNA. After precipitation, the solution was centrifuged at 14,000G for 30 minutes, and the aqueous phase was then removed. 200µl of 70% ethanol was added to the tube which was centrifuged at 13,000G for 15 minutes. The supernatant was then removed and the DNA dried. TE was added to resolve the DNA after drying.

Ligation of Plasmids

Plasmids were ligated using NEB T4 DNA Ligase, using the manufacturer's buffer and protocol. Vectors were phosphatased before ligation.

mRNA and cDNA Making, qPCR

2×10^6 live cells were collected to prepare one tube of mRNA. The cells were first put in a 1.5 ml eppendorf tube with 1 ml media. The samples were then centrifuged at 1500 for 5 min at 4°C. The supernatant was removed and the cells were resuspended in 1ml Trizol and mixed. The samples were then incubated at room temperature for 5 minutes. 200 µl of chloroform was then added to each sample which was then incubated at room temperature for 2 minutes. The samples were then centrifuged at 12,000G for 10 minutes at 4°C. Upon centrifugation, the upper aqueous phase was transferred to new tube. 500 µl of isopropyl alcohol was then added to each tube and the samples were then incubated at room temperature for 10 minutes before being centrifuged at 12,000G for 10 minutes at 4°C. The RNA pellet should be visible and was washed with 1 ml 75% ethanol. The samples were then centrifuged again at 12,000G for 5 minutes at 4°C. The

supernatant was then removed and the pellet dried in desiccators. The pellet was resuspended in 20 µl RNase-free water and was incubated at 55°C for 10 minutes before being stored in -80°C.

cDNA was made from the mRNA prepared before by using Invitrogen® SuperScript™ III Reverse Transcriptase Kit whose protocol can be found at http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Nucleic-Acid-Amplification-and-Expression-Profiling/Reverse-Transcription-and-cDNA-Synthesis/RT___cDNA_Synthesis-Misc/SuperScript.html.

qPCR was done by using the prepared cDNA, pre-designed primers and Promega GoTaq® qPCR Master Mix (http://www.promega.com/products/pm/genomics/gotaq-qpcr)

RESULTS

Erag constructs, JL13xGFP #12 and #24 were first transfected into the murine pro-B cell line, 1-8 cells, to establish a system with which to measure *Erag*-dependent gene expression. Later experiments were planned, in which mutations would be made in *Erag*, and then the mutant versions would be compared to intact *Erag*, for thei ability to drive *Erag*-dependent GFP expression in murine pro-B cells. Transfected 1-8 cells were selected by 400 µM Geneticin for one week. After selection, the cells were collected and analyzed by flow cytometry. The analysis showed that about 48% of 1-8 cells transfected with JL13xGFP #12 showed elevated GFP expression and about 41% of 1-8 cells transfected with JL13xGFP #24 showed elevated GFP expression (**Fig. 1**). The results were consistent with the data shown by Hsu et al. in which they claimed transfected pro-B cells had more than 30%-40% reporter construct activity than pro-B cells transfected with plain vector (2003).

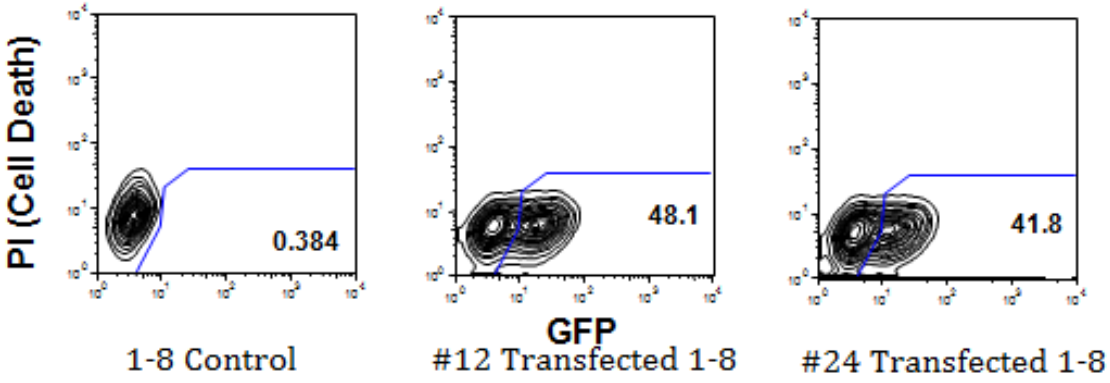


Figure 1. FACS Analysis of JL13xGFP #12 or #24 Transfected 1-8 Cells. Y axis, PI signal for cell death; X axis, GFP signal. Transfected 1-8 cells with Erag construct showed elevated GFP expression.

ME-1 cells, an AML leukemia B cell line whose growth is CBF-dependent, and transfected 1-8 cells were treated with CBFβ inhibitor to measure its effects on the cells and its impact on GFP expression of transfected 1-8 cells. In these experiments, the goal was to determine whether *Erag*-dependent GFP is increased, decreased, or not affected when CBF is inhibited. Because RAG expression was decreased in a mouse genetic model where CBF function is disrupted, it is predicated that CBF inhibitor might generate a similar effect.

ME-1 cells were treated with 25 μM, 50 μM and 100 μM CBFβ inhibitor or equivalent concentration of DMSO for 72 hours before flow cytometry analysis. ME-1 cells showed increased cell death in an inhibitor-concentration-dependent manner (**Fig. 2**). After treatment ME-1 cells were stained with PI for cell death signal – dead and dying cells cannot exclude PI whereas live cells are PI-negative. Control ME-1 cells treated with DMSO showed about 75% live cells and DMSO concentration showed no detectable effect on ME-1 cells viability (**Fig. 2** lane A). ME-1 cells treated with inhibitor showed significantly less live cells, in an inhibitor-concentration-dependent manner (**Fig. 2** lane B and C; $P<0.5$). On average, ME-1 cells treated with 25 μM inhibitor for 72 hours showed 65.9% live cells, ME-1 cells treated with 50 μM inhibitor showed 41.8% live cells, and ME-1 cells treated with 75 μM inhibitor showed 6.125% live cells. The differences between live cell percentages are significant ($P<0.005$). The results confirmed that CBFβ inhibitor disturbed leukemia cell growth which was very likely to result from disrupted CBF function.

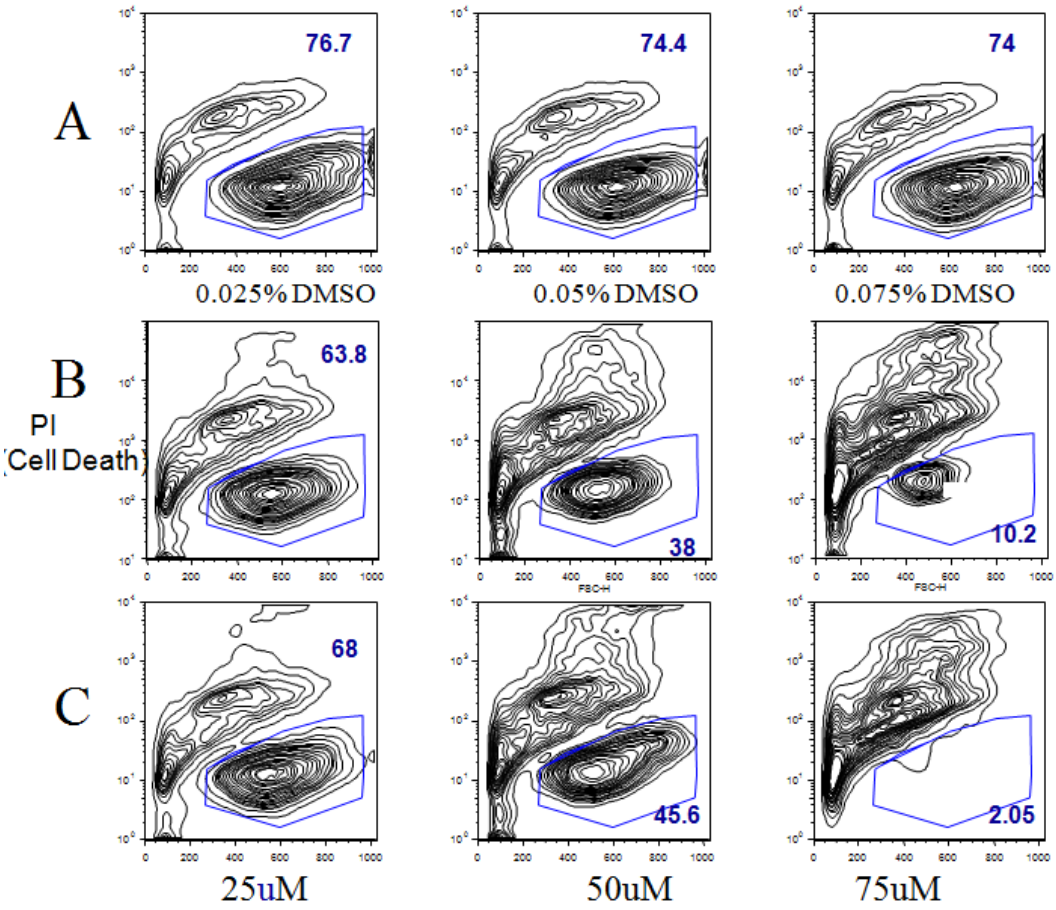


Figure 2. FACS Analysis of CBFβ-Inhibitor-Treated ME-1 Cells. ME-1 cells were treated with various concentrations of CBFβ inhibitor or equivalent DMSO, and were stained with PI after treatment. CBFβ-inhibitor-treated ME-1 cells showed decreased portion of live cells than control cells. The decreased viability was inhibitor-concentration-dependent.

1-8 *Erag*-reporter transfected cells were treated with 200 µM, 250 µM, 275 µM and 300 µM CBFβ inhibitor or equivalent concentration of DMSO in duplicates for 48 hours and with 200µM CBF inhibitor or equivalent concentration of DMSO duplicates for 24 hours. Cells were harvested and stained with PI before FACS analysis.

The numbers of live cells of 1-8 cells treated with CBFβ inhibitor for 48 hours decreased dramatically compared to DMSO-treated controls (**Fig. 3**; $p<0.00005$). #12- and #24-transfected 1-8 cells treated with 0.25% DMSO for 48 hours had 89.4% and 88.2% live cells respectively. Treatment of 200 µM CBFβ inhibitor for 48 hours decreased the live cells percentages of

transfected 1-8 cells to 12.9% (#12) and 11.835% (#24); 250 µM CBFβ inhibitor for 48 hours decreased the numbers to 1.6185% (#12) and 3.08% (#24); 275 µM CBFβ inhibitor for 48 hours decreased the viability of #24-transfected 1-8 cells to 3.08%, but the viability of identically treated #12-transfected 1-8 cells went up to 7.02%; 300 µM CBFβ inhibitor for 48 hour resulted in almost no detectable live cells (0.6445% for #12 and 0.2929% for #24). The results confirm that CBFβ inhibitor disrupted CFB function on which the growth of 1-8 cells depends on.

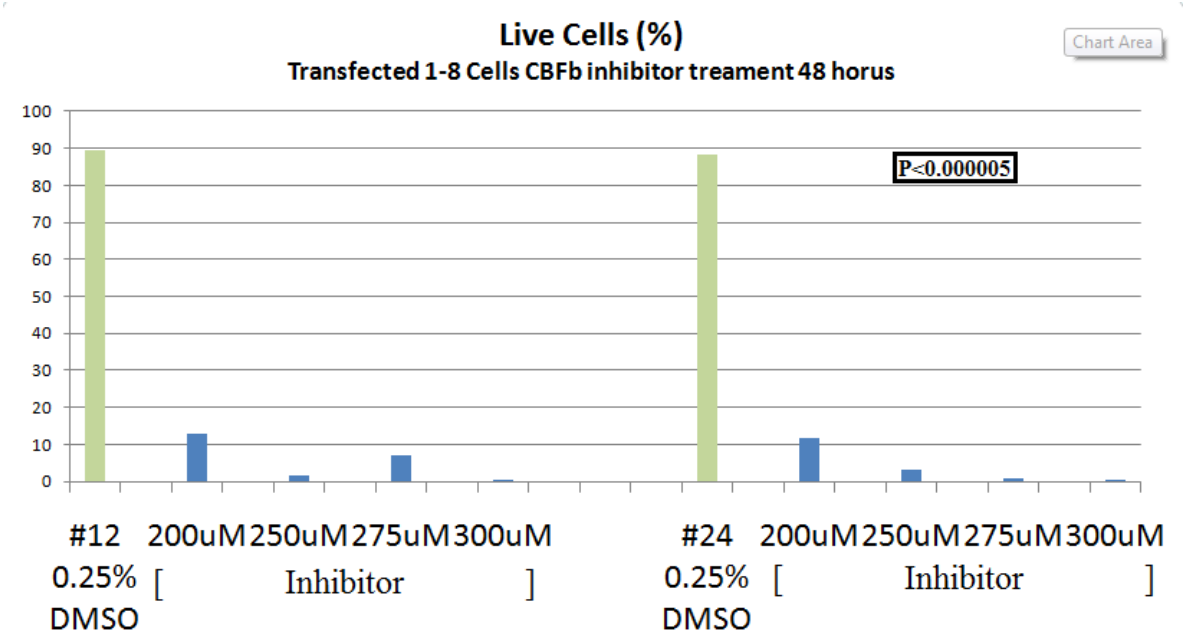


Figure 3. FACS Analysis of Live Cells of CBFβ-Inhibitor-Treated #12- or #24-Transfected 1-8 Cells for 48 Hours. Cell viability decreased dramatically after drug treatment and the decrease in viability is concentration dependent.

The GFP expressions of the drug-treated transfected cells were also analyzed. It was expected that the GFP expressions of the drug-treated transfected cells would as well decrease because the *Erag* reporter construct activity, in the absence of drug, was expected to be positively regulated by CBF function. The CBF function was proved to be disrupted by the inhibitor, so the GFP expression was expected to decrease upon drug treatment. However, the percentages of GFP+ cells did not share the same trend as the viability. Because the viabilities of

275 and 300 µM drug-treated cells were too low, their GFP+ percentages were not believed to be informative. Compared to the controls (**Fig. 4**, green bars) which have 33.9% (#12) and 19.8% (#24) GFP+ cells, the 200 and 250 µM drug-treated cells showed increased portions of GFP+ cells – 40.3% (#12 200 µM), 35.35% (#12 250 µM), 30.1% (#24 200 µM) and 32.85% (#24 250 µM) (**Fig.2**). The results suggested the increased portions of GFP+ cells likely resulted from the treatment (ANOVA, P<0.1).

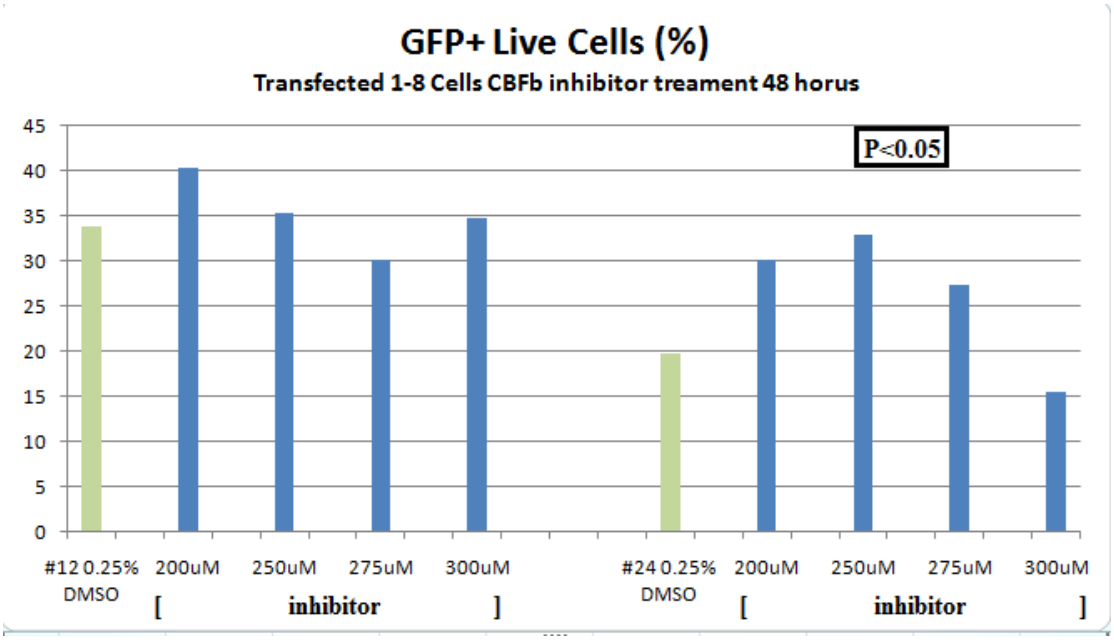


Figure 4. Percentages of GFP+ Cells of CBFβ-Inhibitor-Treated Transfected 1-8 Cells.
Percentages of treated cells seemed to have increased compared to the controls (green bars).

The FACS analysis also revealed increased amount of GFP expression per cell of CBFβ-inhibitor-treated cells compared to controls. The results showed the 200 and 250 µM CBFβ-inhibitor-treated cells had significantly increased GFP geometric means compared to the controls (**Fig. 5**, P<0.05). The DMSO-treated controls showed GFP geometrical means as 43.7 (#12) and 38.7 (#24), while the 200 µM drug-treated transfected cells had increased GFP geometric means as 83.4 (#12) and 59.15 (#24) (P<0.05), and the 250 µM drug-treated transfected cells had increased GFP geometric means as 68.45 (#12) and 55.35 (#24) (P<0.2). The results of portions

of GFP+ cells and GFP geometrical means showed a possible trend of increased GFP expression after treatment.

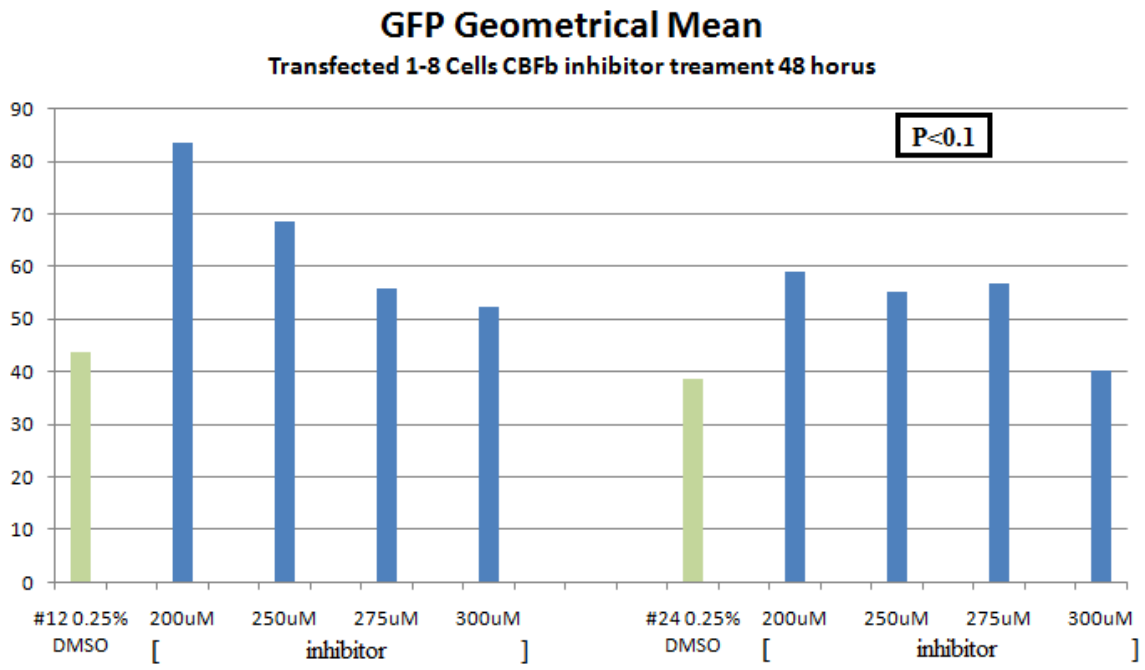


Figure 5. GFP Geometrical Means of CBFβ-Inhibitor-Treated Transfected 1-8 Cells. The GFP geometrical means of treated cells seemed to have increased compared to controls (green bars).

Transfected 1-8 cells were then treated with 200 µM CBFβ inhibitor for 24 hours to observe if there would be similar results with better viability in shorter treatment time. The viabilities of treated cells were about 90% which were not changed compared to the controls, but the portions of GFP+ cells and the geometrical means increased in similar trends (**Table 1**).

Table 1. Live cells, GFP+ portions and GFP geometrical means of 200 μM CBFβ-inhibitor-treated transfected 1-8 cells for 24 hours.

Sample	Live Cells (%)	GFP+ Cells (%)	GFP+ Geometrical Means
1: #12 0.2%DMSO 24hrs	92.6	34.8	44.1
2: #12 200uM CBFb inhi. 24hrs	92.3	38.5	51.4
3: #12 200uM CBFb inhi. 24hrs	94.1	37.9	50.4
4: #24 0.2%DMSO 24hrs	93.5	19.6	36.1
5: #24 200uM CBFb inhi. 24hrs	93	23	43.5
6: #24 200uM CBFb inhi. 24hrs	93.4	23.7	46.8

The viability didn't change in a 24-hour treatment, so the transfected 1-8 cells were then treated with CBFβ inhibitor or equivalent DMSO for 6 hours and were then harvested to make cDNA for qPCR. Two RUNX1-dependent genes were tested: transcript level of Igll (λ5), a protein specifically expressed in pro-B cells and required for B cell development, and mb-1 (CD79), a cluster differentiation expressed on B cells all throughout the B cell lifespan, were measured through qPCR. The results were normalized by β-actin controls. According to the qPCR results, the expression of Igll was significantly decreased with CBFβ inhibitor treatment compared to the controls (**Fig. 6,** $P<0.005$); the expression of CD79 also seemed to have decreased due to the treatment (**Fig. 7,** $P<0.2$). These experiments confirm that the CBF inhibitor decreases expression of RUNX-dependent genes in 1-8 cells.

Future experiments will determine whether transcript levels of RAG1 and RAG2 are decreased as a result of treatment with the inhibitor.

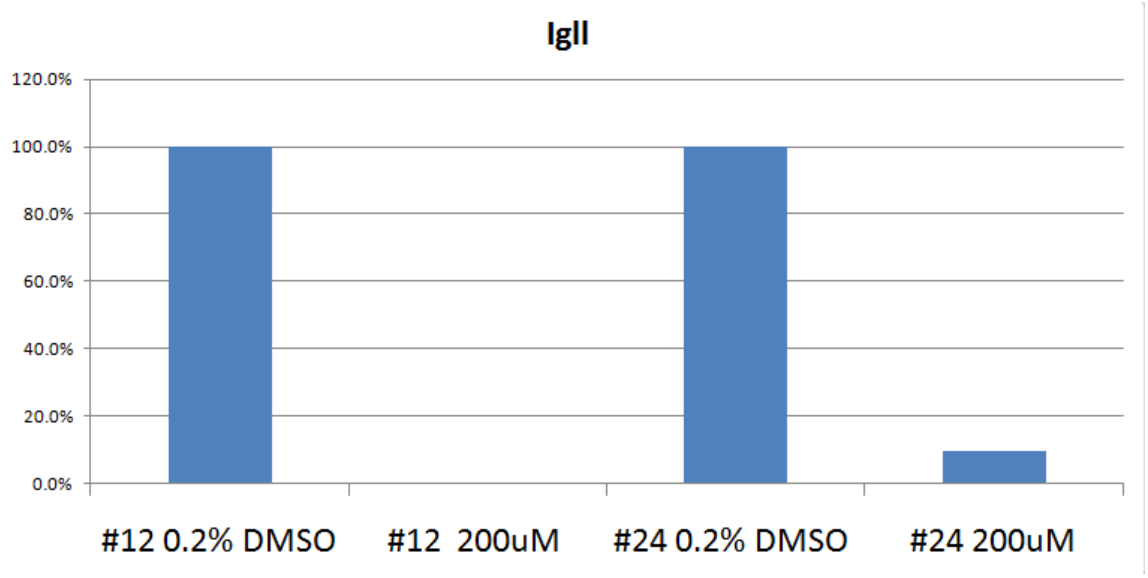


Figure 6. qPCR Results of Igll (λ5) of CBFβ-Inhibitor-Treated Transfected 1-8 Cells for 6 Hours. The transcript level of Igll was significantly decreased after the treatment (P<0.005).

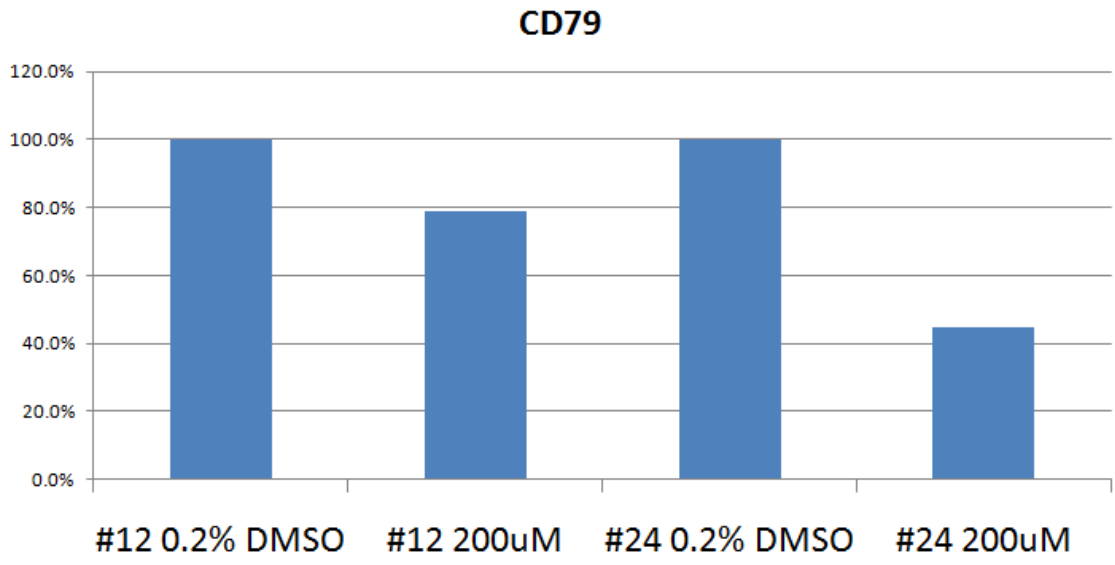


Figure 7. qPCR Results of CD79 of CBFβ-Inhibitor-Treated Transfected 1-8 Cells for 6 Hours. The transcript level of Igll was decreased after the treatment (P<0.2).

Two possible RUNX1 binding sites were found in *Erag* and it led to the question of whether CBF regulates RAG genes by binding to *Erag*. Therefore the two binding sites were mutated separately by first isolating and ligating *Erag* into pUC19 before mutagenesis PCR. Mutated *Erag* was isolated from pUC19 by restriction digestion and were then ligated back into

SpeI partially digested JL13xGFP vector (see Methodology). pUC19/mutated *Erag* were digested with XbaI and PvuI and were loaded on low melt gel after purification. The pUC19 was cut into two fragments and the Erag was separated from the vector (**Fig. 8**). The first four lanes were *Erag* Mutagenesis Site 1 while the next four lanes were *Erag* mutagenesis site 2. The last lane was the Lamda/HindIII ladder. Except for lane 2, all lanes showed a 2.3 kb fragment which corresponded to the size of *Erag*. The fragments were isolated, purified and stored for ligation.

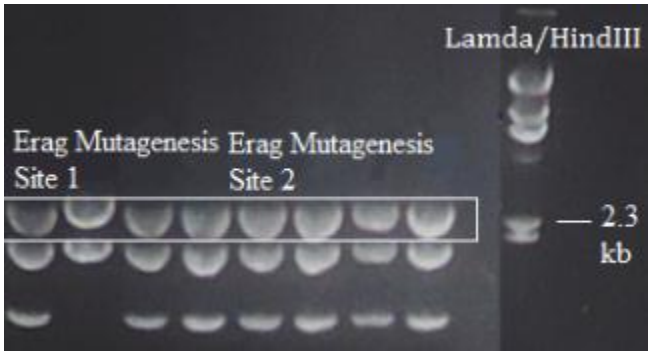


Figure 8. Mutated *Erag* Fragments on Low Melt Gel. Lanes 1-4, *Erag* mutagenesis site 1; lane 5-8 *Erag* mutagenesis site 2; lane 9 Lamda/HindIII ladder. Erag was a 2.3 kb fragment.

The JL13xGFP vector (construct without *Erag*) which has two SpeI sites was partially digested with SpeI. Digested plasmids were loaded on low melt gel. Four fragments were found on the gel. The two lower fragments were products of complete digestion. The upper fragment was the partially digested, and the second upper fragment was uncut plasmid in supercoiled form (**Fig. 9**). The partially digested plasmid was isolated, purified and used for ligation with mutated *Erag*.

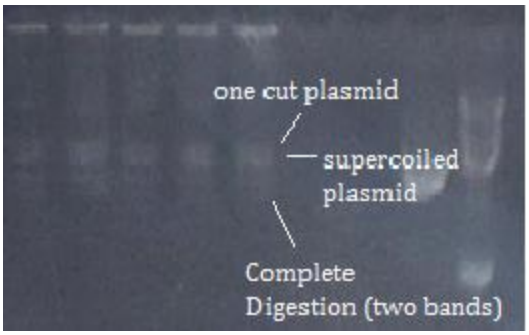


Figure 9. SpeI-Digested JL13xGFP Vector. There were four fragments found on the gel. The two lower fragments were products of complete digestion. The upper fragment was the partially digested, and the second upper fragment was uncut plasmid in supercoiled form.

The ligated mutated Erag and JL13xGFP vector were then transformed to competent *E.coli*. Single colonies were collected and used for minipreps. The miniprep products were digested with NotI to screen the clones. Successful ligation should produce one 5.8 kb fragment and one 5.9 kb fragment while vector only would produce one 3.5 kb fragment and one 5.9 kb fragment. One positive clone (lane 20) was found according to the gel (**Fig. 10**). The clone will be sequenced and transfected to obtain more results.

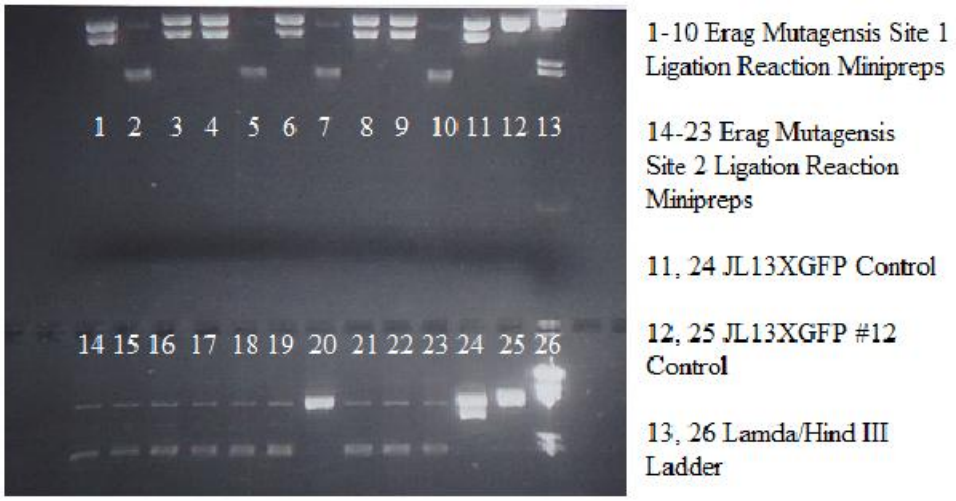


Figure 10. Ligation Clone Screening. Lane 20 was a successful ligation.

DISCUSSION

The FACS analysis of GFP expression of transfected 1-8 cells, a pro-B cell line, validated the use of the *Erag* GFP reporter construct as a useful tool to study *Erag* activity in pro-B cells. As shown in Fig.1, the transfected 1-8 cells had about 45% of GFP+ cells positive for the reporter for the *Erag* activity. The results were consistent to those of the study of Hsu et al. which indicated stably transfected pro-B cells had 30~40% GFP+ cells (2003). The conclusion was that the transfected 1-8 cells showed *Erag* activity, and they could be used to further test the Erag function.

ME-1, an Acute Myeloid Leukemia cell line, and 1-8 cells, were treated with different CBFβ inhibitor concentrations and were analyzed by FACS for their viability or viability and GFP expression. The FACS analysis showed that the viability of CBFβ-inhibitor-treated ME-1 cells decreased in an inhibitor-concentration-dependent manner (Fig. 2). The Gorcynski et al. study did the same experiment but measured the proliferation of treated ME-1 cells by MTT assay (2007). They as well observed a decrease in proliferation signal in treated ME-1 cells – compared to the DMSO-treated control, about 20% less of 25 μM inhibitor-treated cells, about 30% less of 50 μM inhibitor-treated cells, and about 40% less of 100 μM inhibitor-treated cells (Gorcynski et al., 2007). Both results shared a decrease in cell growth which was dependent on the inhibitor concentration. The results proved the CBFβ inhibitor was effective in disrupting the CBF function on which the growth of ME-1 cells was dependent on, and the effectiveness of the inhibitor was dependent on its concentration.

Transfected 1-8 cells were treated with different CBFβ inhibitor concentration for 48 hours and were analyzed by FACS for their viability and GFP expression. The viability of treated

1-8 cells were significantly decreased which was expected because CBF function was essential for pro-B cell growth (Fig. 3). Because only 200 and 250 μM CBFβ-inhibitor-treated cells were believed to have detectable live cells, only the GFP expression of these cells were believed to be valid. The portions of GFP+ cells were expected to decrease because the CBF was proved to be positive regulator of RAG genes, as well as the construct drive by RAG2 promoter (Kuo et al., 2008). CBF has been known to be essential for RAG expression because it positively regulates E2A which regulate the RAG locus, but whether CBF can regulate RAG genes via the RUNX1 binding sites in Erag is unknown (Borghesi et al., 2005; Kuo et al., 2008).

According to the FACS analysis, the portions of GFP+ cells did not decrease, and seemed to either stay the same or even increase (Fig. 4, $P<0.1$ for #12 and $P<0.2$ for #24). The results of GFP geometric means shared the same trend, as treated cells had increased GFP geometric means compared to the DMSO treated control (Fig. 5, $P<0.05$ for #12 and $P<0.2$ for #24). The transfected 1-8 cells were also treated with 200 μM of CBFβ inhibitor for 24 hours and were analyzed by flow cytometry. The treated cells showed no decrease in viability while the portions of GFP+ cells and the GFP geometrical means showed slight increases (Table 1). The results proved that the CBFβ inhibitor treatment disrupted the CBF function on which the growth of 1-8 cells was dependent on, and the disruption of CBF function either increased *Erag* activity or had no significant effect, suggesting CBF either doesn't regulate *Erag* activity directly or it negatively regulates it. If negative regulation is correct, one possible explanation is that RUNX1 binds to *Erag* and prevents other transcription factors from binding to *Erag*. The RUNX1 binding sites on *Erag* were close to the binding sites of Ikaros, E2A and Pax-5. Ikaros and E2A were also known to be regulators of RAG expression in B-cell development. Compared to other

transcription factors, CBF may serve as a relatively negative regulator. However, these claims require further proof some of which might be obtained by ensuing experiments of this project.

The effect of CBFβ inhibitor on the mRNA levels is expected to occur earlier over the time course of treatment than the effect on viability, so the transfected 1-8 cells were treated with 200 μ of CBFβ inhibitor for 6 hours and were harvested to make cDNA for qPCRs of key B cell proteins that are known to be regulated by RUNX1. Compared to the control, the transcript level of Igll (λ5), a protein specifically expressed in B cells and required for B-cell development, were significantly decreased (Fig. 6 $P<0.005$). The transcript level of CD79, a cluster differentiation expressed in pro-B cells and throughout the B cell lifespan, was also decreased (Fig. 7 $P<0.02$). The results proved the disruption of CBF function resulted in decreased expression of B-cell specific transcription factors which then resulted in decreased viability. More qPCRs measuring the transcript levels

As shown in Fig. 10, a clone of construct with *Erag* mutated on the second RUNX1 binding site was successfully obtained. There were several difficulties during the process of generating the clone and these difficulties are valuable learning experience for further experiments. Because the JL13xGFP vector contains two SpeI sites, and the large-size partially digested vectors were difficult to separate from the supercoiled ones, the efficiency of generating a clone was very low. A possible improvement could be to use a linker gene, or clone a larger part of the construct with *Erag* in middle by other sites. The clone will be sequenced to prove the success of mutagenesis and will be transfected into 1-8 cells.

Another experiment that should be done is to transfect 1-8 cells with JL13xGFP vector. The vector is believed to be able to render cells GFP expression which would be much lower than the #12- and #24-transfected 1-8 cells. Hsu et al. showed a similar experiment, and though

they did not reveal the actual data, they claim the pro-B cells transfected with *Erag* constructs had 30~40% more GFP+ cells than the cells transfected with empty vector.

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