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The Role of \textit{MAFB} in Reversing the Transformed Phenotype of Human Acute Myeloid Leukemia Cells

by

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The five-year survival rate for patients with acute myeloid leukemia is 27.4%, with most patients who achieve temporary remission relapsing, despite chemotherapeutic treatments (National Cancer Institute, 2014). With the inadequacy of current drug therapies for human acute myeloid leukemia patients, the genetic changes in AML cells’ genomes and transcriptomes have been studied for insights as to more effective targeted therapies. Data obtained by the Roberts lab demonstrates that treatment of HL-60 cells with PMA, which leads to cell cycle arrest, differentiation and apoptosis, is accompanied by upregulation of approximately 100 transcription factor genes. For this project, the basic leucine zipper transcription factor, $MAFB$, was selected for further research exploration as a potential regulator of transcriptomic changes leading to reversal of the transformed phenotype of AML cells. $MAFB$ was successfully overexpressed in HL-60 cells by transient transfection, eliciting the consequential upregulation of the putative gene targets $TINAGLI1$ and $IL1A$. Quantified with RT-qPCR, the upregulation of $TINAGLI1$ and $IL1A$ from the overexpression of $MAFB$ supports the hypothesis that $MAFB$ elicits changes to the transcriptome that might drive differentiation. Additionally, $MAFB$ was observed to significantly slow the division of HL-60 cells over the course of 60 hours, supporting the hypothesis that it plays a key role in inciting hematopoietic cell differentiation at the expense of cell cycle progression. These results justify further research on the transcriptional responses to $MAFB$ overexpression, which are currently being explored by RNA-sequence analyses. It is the hope that the transcriptional changes mediated by $MAFB$ will identify new targets of therapeutic consequence that could promote differentiation and/or cell cycle arrest.
Introduction

Acute Myeloid Leukemia

Characterized by its fast progression and high rate of relapse, acute myeloid leukemia (AML) is the most common form of leukemia in adults. Drug therapies for AML patients have induced remission, but many patients relapse, and this relapse proves fatal in most cases. There is an increasing incidence and mortality rate of AML in the United States, with the 5-year survival rate being only 27.4%, according to the National Cancer Institute (2014). This low survival rate and high rate of relapse mandates further research to develop a lasting treatment and drives the research we are performing to search for potential gene therapy targets. AML is a form of blood cancer where the myeloid progenitor cells fail to commit to cell differentiation during hematopoiesis, creating an accumulation and uncontrolled cell division of undifferentiated cells in the bone marrow (Stone et al., 2004). Undifferentiated pluripotent stem cells, like AML cells, maintain a genetic program allowing for continual division, but blocks differentiation, while differentiation requires cell cycle exit (Aziz et al., 2009). In AML, the accumulation of undifferentiated cells hinders the proliferation of normal, differentiating cells and the cancerous cells can accumulate and enter into the blood stream, at which point they can circulate throughout the body. If the tumorigenic cells could be reprogrammed to commit differentiation, it is expected that they would concurrently commit to cell cycle arrest.

A cell line of human leukemia cells (HL-60) was isolated from a patient with acute myeloid leukemia in 1977 and grown in culture for laboratory research (Birnie, 1988). The cells were obtained from the peripheral blood of a 36 year old Caucasian female (ATCC, 2016). The HL-60 cell line was used in this research, as it has been established and studied
extensively regarding the ability of phorbol ester treatment to initiate macrophage-like cell differentiation and eventual apoptosis (Rovera et al., 1979). Preliminary data from previous experiments in the Roberts lab have genotypically characterized the transcriptome after PMA treatment. Other AML cell lines may be of further research interest in the lab, to compare keystone genetic commonalities among different AML subtypes. There are 11 known genomic subgroups of AML, distinguished by their driver mutations (Papaemmanuil, 2016).

*Phorbol Myristate Acetate Induction of Differentiation and Apoptosis*

With treatment by Phorbol Myristate Acetate (PMA), a mimic of the signaling molecule Diacyl Glycerol (DAG), HL-60 cells have been shown to display the phenotypes of cell cycle arrest, differentiation into macrophage-like cells, and eventual apoptosis (Martin et al., 1990). This experiment was performed in the Roberts lab previously, and in collaboration with Ahkeem Simmons and Professor John Henson, cells were visualized by scanning electron microscopy over the course of 48 hours (Fig. 1). In whole genome DNA microarray experiments over a 24-hour time course of PMA treatment, approximately 1150 genes show significant changes in gene expression. Approximately 100 transcription factor genes display altered expression in response to PMA, as quantified by real-time PCR. The PMA response would be desirable for cancer treatment, however PMA is too toxic to administer in humans (Strair, 2002). Since PMA is too toxic to administer to human patients, our research has aimed to identify how the transcriptome changes in response to PMA in the hope of identifying key transcription factor genes and their targets that mediate either cell cycle arrest, differentiation and/or apoptosis. This information may provide insights into a more directed way to elicit the desirable phenotypic changes of the PMA response.
Cytotoxic Chemotherapies

The current primary treatment for AML is cytotoxic chemotherapy, which frequently involves the combination drug therapy of cytarabine and an anthracycline drug (American Cancer Society, 2018). Cladribine may also be administered in this initial chemotherapy phase termed *induction*, where the goal of the therapy is to destroy and kill as many cancer cells as possible, without significantly harming the patient (American Cancer Society, 2018). This initial treatment lasts approximately one week, and a targeted therapy for patients with *FLT3* gene mutations is available. *FLT3* gene mutations are one of the most common among AML patients, with *FLT3* mutations being the oncogenic driver in approximately 25%-30% of AML subtypes (Kindler et al., 2010). However, successful targeted therapies are not as widely available for mutations found in the less common AML subtypes. Subsequent treatments depend upon numerous bone marrow biopsies, which may result in the treatment with rounds of high dose chemotherapy and stem cell transplants (American Cancer Society, 2018). Unfortunately, these treatments are extremely taxing on AML patients, and bring most to extremely low blood cell counts with sickening side effects. The most widely used treatment remains the high-dose rounds of chemotherapy and stem cell transplants in attempts to wipe out the cancerous AML cells and replace them with new, non-cancerous cells. The genetic programming of stem cells has added an additional hurdle in providing therapy designed to kill tumor cells, as research has suggested that cancer stem cells are chemo- and radioresistant, enabling them to survive cytotoxic therapies and continue growth (Prieto-Vila et al., 2017). This is characteristic of relapse, as the tumors recur after treatment and metastasize (Prieto-Vila et al., 2017). Even a minor remaining population of tumor cells
resistant to chemotherapy may escape detection and later metastasize, leading to a patient’s relapse.

Selection of MAFB as Gene of Interest

Previous research, performed by the Roberts’ lab using DNA microarray analysis, has identified approximately 100 transcription factor genes that change in expression in response to PMA, including MAFB. MAFB is a basic leucine zipper transcription factor that shows up-regulation in HL-60 cells treated with PMA at 3 hours post-treatment, and remains upregulated for 24 hours (Fig. 2). In addition, previous research in the Roberts lab has found that MAFB shows upregulation in response to PMA treatment in multiple cell lines. MAFB is comprised of a basic DNA-binding domain, transactivation domain and a leucine zipper structure (Zhang et al., 2016). MAFB is an essential transcription factor for differentiation of monocytes, macrophages, islet beta cells and others, and regulates ETS1-mediated transcription of erythroid-specific genes in myeloid cells (GeneCards, 2018). MAFB can act as both a transcriptional activator and a repressor, depending on the cell line (GeneCards, 2018). MAFB is closely related to c-Maf, as they are members of the Maf subgroup, and literature has indicated that overexpression of c-Maf and MAFB are often found in the cells of multiple myeloma patients (Zhang et al., 2016). It was hypothesized that the overexpression of MAFB in HL-60 cells creates transcriptional changes in downstream genes, which induce observable phenotypic changes in HL-60 cells, characteristic of differentiation and apoptosis.

A variety of targeted drug therapies exist for treatment of cancers, but targeted changes to the transcriptome (genetic reprogramming) is not yet in clinical practice. Using a
bioinformatics approach for mapping the interconnectivity of transcription factors with PMA-responsive gene promoters, *MAFB* was selected as a potential gene that could create changes in the expression of downstream genes that mediate desirable phenotypic effects. Should the results of this research indicate that *MAFB* contributes crucial phenotypic changes in HL-60 cells, therapies might be designed representing a less toxic treatment option for acute myeloid leukemia patients.

*MAFB* has been found to be a regulator of several genes necessary for angiogenesis, immune response and differentiation. Data has shown that *MAFB* is up-regulated in myeloid differentiation from progenitor cells to macrophages (Kelly et al., 2000). This data points to the differentiation properties of *MAFB* overexpression, which this research aims to confirm. *MAFB* has also been shown to act as a direct binding partner and repressor of Ets-1, which can act to transactivate and trans repress target genes (Sieweke et al., 1996). Ets-1 has also been found to be highly expressed in conditions requiring angiogenesis, such as the menstrual cycle and wound healing (Wernert et al., 1994; Maroulakou and Bowe, 2000). Several matrix metalloproteases are also target genes of Ets-1, so the overexpression of *MAFB*, as an inhibitor of Ets-1 could be expected to suppress angiogenesis, among other downstream effects with the lack of transactivation and transrepression of Ets-1 target genes. From the biological interactions of *MAFB* with downstream target genes, it can be understood that *MAFB* functions as a critical gene in the differentiation, and growth of myelocytes.

When considering the feasibility of utilizing the observed transcriptional changes initiated by *MAFB*, it is not being suggested that upregulation is a therapeutic goal since *MAFB* is a transcription factor, whose activity occurs in the nucleus of the cell and causing
transcriptional upregulation is not generally practiced. However, by understanding the interactions and transcriptome changes as a result of MAFB overexpression, a piece of the larger genetic profile of a reprogrammed cancer cell may be painted. The results of this project aim to inform the growing genetic profile of a reprogrammed AML cell, with the ultimate goal of discovering ways in which a continuously dividing leukemia cell could be induced to undergo cell cycle arrest, differentiation, and/or apoptosis.
Methods

Creation of the MAFB cDNA Expression Clone

A human cDNA clone was purchased from the GenScript GenEZ™ ORF cDNA collection, with the MAFB gene engineered into the pcDNA3.1+/C-(K)-DYK expression vector (containing the cytomegalovirus, CMV, promoter) with both Amp<sup>+</sup> and Neo<sup>+</sup> gene selectable markers (Fig. 3). The Roberts lab protocol for bacteria transformation with plasmid DNA was followed to transform 25µl of competent E. coli [JM109, Promega, L1001] with 5ng of MAFB plasmid DNA (Roberts, 2017). The plates were grown up overnight at 37°C and overnight culture was made from the transformed E. coli colonies. The QIAGEN QIAprep protocol was followed for plasmid DNA isolation, using the ZymoPURE™ Plasmid Miniprep Kit [D4211]. The concentration of the isolated plasmid DNA was measured with Nanodrop.

A restriction enzyme digest was performed to confirm the isolation of the desired plasmid DNA. The isolated plasmid DNA was added to 10X NEB Buffer and NcoI restriction enzyme for 1 hour at 37°C. The restriction enzyme digest product was run on a 1.0% TBE agarose gel with TBE buffer. Loading dye (6x) was added to each sample and 20 µl of the sample was loaded in the gel. The gel was run at 100V for 45 minutes, using a 20log DNA ladder. The vector was mapped in New England Biolabs cutter and found to have 4 fragments, expected to be visible in the gel.

Plasmid DNA was then used in a transfection by nucleofection of HL-60 cells in triplicate, with an empty vector plasmid as a control. The protocol for the Roberts lab was followed to perform the transfection with 1 million HL-60 cells as follows:
1) Transfection solution made in the Roberts lab was combined in a 5:1 ratio with mannitol supplement to create the transfection solution master mix.

2) HL-60 cells were pelleted by centrifugation at 2.5k rpm for 8 minutes, with 1 million cells per sample (4 samples were made to be transfected with MAFB DNA and 4 samples were made to be transfected with the vector DNA).

3) The pelleted cells were resuspended in 100 µl of transfection master mix per reaction (1 million cells) and 6 µg of plasmid DNA per 4 samples.

4) The resuspended cells were pipetted into cuvettes in 100μl aliquots.

5) Cuvettes were inserted into the LONZA 4D NucleofectorTM X unit and electroporated using the HL-60 Nucleofector Program.

6) The samples were allowed to sit in the cuvette for 10 minutes at room temperature.

7) A transfer pipette was used to transfer the electroporated cells from the cuvettes to a 24 well plate with 1 ml pre-warmed complete HL-60 medium added to the wells. An additional 500 µl of pre-warmed medium was added into the cuvette, then transferred into the corresponding well on the 24 well plate.

8) The 24 well plate was incubated at 37°C.

At 8 hours post-transfection, RNA was purified from three of the samples transfected with the vector DNA and three samples transfected with MAFB DNA following the protocol for the QIAGEN RNeasy® Plus Mini Kit. RNA was eluted using RNase-free H2O. Protein was purified from the fourth transfection reaction with MAFB and the vector using Buffer RLT from the RNeasy® Plus Mini Kit and Bio-Rad 2x Laemmli Sample Buffer + β-mercaptoethanol.
The Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Cat. # 4368814) was used according to the kit’s protocol to synthesize cDNA from the purified RNA samples. The Applied Biosystems protocol was used to set up the RT-qPCR plate. The Applied biosystems TaqMan® Gene Expression Master Mix, appropriate TaqMan® Probe, sample cDNA and H2O was combined in quantities defined by the protocol, and added in 20µl aliquots to each well of the 96 well RT-qPCR plate. The samples were loaded in triplicate, and the QuantStudio™ 7 Flex System was used to analyze the sample reactions with comparative Ct detecting TaqMan® reagents. MAFB and vector RNA samples from the transfection, isolated at 8 hours post-transfection, were sent for RNA-sequence analysis. The RNA-sequence analysis will be used to detect any changes to the transcriptome mediated by MAFB overexpression.

The RT-qPCR was performed once with a high fidelity TaqMan® probe for MAFB and no change in MAFB was observed in the comparative Ct analysis. After contacting life technologies™, it was found that the TaqMan® probe bound to the 3’ untranslated region of the MAFB gene, which was not included in the cDNA plasmid transfected. Therefore, only the endogenous MAFB gene was detected. The RT-qPCR was repeated with a custom designed MAFB probe, with homology to the protein coding region, using the primers defined below:

Forward Primer:

5′ – ATGGCCGCGGAGCTGAGCAT – 3′

Tm = 2(A+T) + 4(G+C) = 2(7) + 4(13) = 66°C
Reverse Primer:

5’ – CAGAAAGAACTCGGGAGAGG – 3’

T_m = 2(9) + 4(11) = 62°C

The isolated protein was used in a western blot to confirm the overexpression of MAFB protein. The transfected cells were lysed with Tris-HCL, SDS, glycerol, bromophenol blue and β-mercaptoethanol. In each well of a 10% SDS-PAGE gel, 25µl of protein was loaded and run with electrophoresis. The resolved protein was then transferred to a polyvinylidene fluoride membrane. The membrane was blocked with GenScript® rabbit anti-DYKDDDDK-tag primary antibody, to the TAG sequence of the inserted MAFB clone, in a 1:1000 dilution in 5% non-fat dry milk and 0.1%Tween, in 15 mL of Tris-Buffered Saline at 4°C. This was followed by three consecutive washes with 10mL of TBST at room temperature. Secondary antibody (R&D Systems, HRP conjugated anti-rabbit IgG) in a 1:10,000 dilution was added and blocked at room temperature, followed by three consecutive 10 mL washes of TBST. Enhanced chemiluminescence with the FluorChem E imaging system was used to image the membrane. The protein samples were loaded in duplicate, using protein isolated from the vector control transfected HL-60 cells as a negative control. The membrane was stained with Coomassie Blue (BioRad, 1610436) to measure loading accuracy, and it was confirmed that equal quantities of protein were loaded for each sample.
Construction of an Inducible Plasmid

The GenScript® plasmid with MAFB was used following the protocol for a Phusion® High-Fidelity DNA Polymerase PCR (M0530) to amplify the MAFB cDNA from the plasmid. The primers used for this reaction were to the restriction sites Age I and BstZ17I and are listed below:

Forward Primer:
5’ – TTGAGTATACATGGCCGCGGAGCTGAGCAT – 3’

\( T_M = 65.9°C \)

Reverse Primer:
5’ – TTAGACCCGGTCAGAAAGAACTCGGGAGAG – 3’

\( T_M = 63.5°C \)

The primers were 25 nmole DNA oligos obtained from Integrated DNA Technologies (IDT®).

The Phusion PCR product was purified with 1.5% agarose gel electrophoresis with a 6X LB marker and a 100 bp DNA ladder was used. The bands representing the MAFB cDNA were cut from the gel. The Zymoclean™ Gel DNA Recovery Kit was used and its protocol was followed to extract the MAFB cDNA from the gel. The cDNA product concentration was 67 ng/µl.

Following the New England BioLabs® Inc. protocol for Ligation with T4 DNA Ligase (M0202), the MAFB cDNA product was ligated into the pLVX-TetOne™-Puro vector and transformed into competent E. coli. To date, this cloning has not been successful, but is being continued with a new strategy.
Phenotypic Observation and Characterization using Light Microscopy

HL-60 cells were transfected with either the MAFB GenEZ™ ORF cDNA clone (GenScript®) or the empty vector control and observed using light microscopy over a time course of 48 hours. Images of the MAFB transfected and vector control transfected cells were taken and observed for morphological changes indicative of cell differentiation, such as adhesion and spreading. Cell counts were also taken for the time points of 6 hours, 12 hours, 24 hours and 48 hours post-transfection. Cell counts of Trypan Blue excluding (live) cells were made using a hemocytometer. Growth curves were generated to quantitatively evaluate cell cycle arrest as an indicator of possible differentiation.

Quantification of Putative MAFB Target Gene Overexpression

The RNA isolated 8 hours post-transfection from the MAFB transfected HL-60 cells was used to make cDNA, which was used for RT-qPCR. RT-qPCR was performed to measure the expression levels of putative target genes selected from literature review and promoter analysis of PMA upregulated genes (Labott & Lopez-Pajares, 2016, Lopez-Pajares, 2015 and Stralen et al., 2009). The RT-qPCR consisted of a gene panel with RPS28 as the endogenous reference gene, MAFB, TINAGL1, PRDM1, NEIL3, KIT, IL1RN, CCND1, BCL6, CENPM, IGFBP7, and IL1A. The probes for these target genes were used in the RT-qPCR, with the empty vector transfected RNA sample as the control reference sample, measuring endogenous gene expression in the absence of MAFB overexpression.

From the results of this RT-qPCR, it was decided to perform a similar reaction, using samples of MAFB-transfected HL-60 cells that were harvested for RNA isolation over a time course of 16 hours, with samples collected at 4 hours, 8 hours, and 16 hours post-
transfection. The samples were then used in a RT-qPCR experiment with probes for the target genes that reflected upregulation in the first RT-qPCR of the gene panel (RPS28 control, MAFB, TINAGLI, IL1A and KIT). The results were quantified and compared with the previous data.
Results

MAFB was successfully transfected into HL-60 cells using the GenScript® GenEZ™ ORF cDNA clone, and overexpression of MAFB was observed (Fig. 5). In the first RT-qPCR, the TaqMan probe used detected only the endogenous MAFB expression of the samples, showing no upregulation with transfection (Fig. 4). The probe was engineered to the 3’ untranslated region of MAFB, therefore not detecting the exogenous MAFB in the plasmid, and only reported the endogenous MAFB, rather than the overexpressed exogenous MAFB. In the subsequent RT-qPCR with a custom MAFB probe, designed to match the sequence of the MAFB inserted in the vector, overexpression of the MAFB transfected cells was quantified. Measured in triplicate with the empty vector and RPS28 probe as the control reference for the RT-qPCR, the transfection of HL-60 cells with the transient vector with MAFB inserted successfully elicited a 150-200 fold overexpression of MAFB (Fig. 5). Western blotting of protein isolated from the transfection of MAFB displayed a prominent signal at 36 kD, confirming the overexpression of MAFB on the protein level as well as the RNA level (Fig. 7). Additional bands were observed in lanes 1 and 2 at approximately 45-50 kD (Fig. 7). No bands were visible in lanes 3 and 4 with the vector control protein samples (Fig. 7).

The results from the RT-qPCR measuring the expression of the genes MAFB, TINAGL1, PRDM1, NEIL3, KIT, IL1RN, CCND1, BCL6, CENPM, IGFBP7, and IL1A displayed reproducible overexpression of several genes. These selected genes were found in a literature review of genes that were upregulated with the overexpression of MAFB, with the resulting phenotype of cell differentiation (Stralen, 2009). Of the overexpressed genes, TINAGL1 was overexpressed 2.78 fold, KIT was overexpressed 1.5 fold, PRDM1 was overexpressed 1.43 fold, IL1A was overexpressed 1.53 fold, while the transfected MAFB
gene was overexpressed 234.6 fold (Fig. 8 & 9). In the follow up experiment with the time course harvested RNA from MAFB transfected cells, the RT-qPCR results with probes for TINAGLI, MAFB, KIT and IL1A displayed overexpression in all of the sample, over the course of 16 hours. MAFB showed 161.50-fold overexpression at 4 hours post-transfection, 113.17-fold overexpression at 8 hours post-transfection and 114.50-fold overexpression at 16 hours post-transfection (Fig. 10). IL1A showed 31.33-fold overexpression at 4 hours post-transfection, 37.22-fold overexpression at 8 hours post-transfection and 36.67-fold overexpression at 16 hours post-transfection (Fig. 10). KIT showed 0.13-fold underexpression at 4 hours, 1.06-fold expression at 8 hours and 2.43-fold expression at 16 hours (Fig. 10). TINAGLI showed 17.42-fold overexpression at 4 hours post-transfection, 88.92-fold overexpression at 8 hours post-transfection and 32.29-fold overexpression at 16 hours post-transfection (Fig. 10). It should be noted that transfection of a GFP gene under the control of the CMV promoter reveals through fluorescence microscopy that about 50% of the cells are transfected by nucleofection in a typical experiment (Crossland, unpublished data). Therefore, the fold increases reported are in a background of non-transfected cells suggesting that the actual upregulation is approximately twice that observed.

The cell counts of MAFB transfected HL-60 cells over a 60-hour time course were increasingly lower than those of the vector-transfected HL-60 cells. Performed in triplicate, the cell counts are displayed in Table 1. After performing a Chi-squared goodness of fit test, it was determined that the MAFB transfected cells had a significantly different count than the vector-control transfected cells, as the p-value was less than 0.0001.

The images collected of the MAFB transfected and empty vector control transfected cells were not discernably different with any morphological changes over the course of 48
hours (Fig. 11). The $MAFB$ transfected HL-60 cells did not display noticeable phenotypic changes of adhesion, contact inhibition or morphological differentiation when compared to the vector control transfected HL-60 cells, at all time points.
Discussion

Overexpression of MAFB in Transfected HL-60 Cells

Given the overexpression observed in the RT-qPCR from the first transfection, and the corresponding western blot confirming the overexpressed MAFB protein, the transfection was deemed successful and it was concluded that MAFB was transiently overexpressed at both the mRNA and protein level in the transfected HL-60 cells. The three visible bands in lanes 1 and 2 with protein from the MAFB transfected cells is MAFB (36 kD) and possibly modified (phosphorylated) forms of MAFB. The absence of bands in lanes 3 and 4 with the protein from the vector transfected cells confirms the specific binding of the primary and secondary antibodies, as well as the presence of MAFB protein overexpressed in the MAFB transfected HL-60 cells. According to Tanahashi (2010), phosphorylation of MAFB by JNK has been found to signal degradation of MAFB by the proteasome, so these phosphorylated forms may be the result of regulation by JNK (Tanahashi et al., 2010).

RNA samples, from the transfection of MAFB into HL-60 cells, were sent for sequencing, but the results have not yet been returned for analysis. The inability to create an inducible vector raised an obstacle for measuring any long-term impacts of MAFB overexpression on differentiation or apoptosis. Although it was the initial plan to create this inducible vector with the purpose of utilizing it to control the gene expression of the HL-60 cells to observe phenotypic changes, after trouble shooting, alternative tactics were pursued.

Putative Target Gene Panel Analysis

In devising a way to analyze the potential target genes of MAFB and their levels of regulation after the overexpression of MAFB, a gene panel was composed, based on evidence
from literature review as to potential downstream targets of MAFB as well as a promoter analysis provided by collaboration by Ashir Borah and Professor Jeffrey Forrester (Labott & Lopez-Pajares, 2016, Lopez-Pajares, 2015 and Stralen et al., 2009). The promoter analysis identified a list of genes that were upregulated with the treatment of PMA and had MAFB binding sites in their promoters. From this review and promoter analysis, the TaqMan probes were selected for the candidate genes that could be putative targets of MAFB, and the comparative RT-qPCR revealed overexpression of several of the target genes in response to the overexpression of MAFB in HL-60 cells. The change in expression for the putative target genes using the RNA harvested 8 hours post-transfection of the HL-60 cells was observed for PDRM1, TINAGL1, IL1A and KIT, but not for CCND1, CENPM or NEIL3 (Fig. 9). The disparity in the level of overexpression of MAFB observed compared to the level of upregulation of other genes could in part be due to the nature of the CMV promoter in the MAFB plasmid, causing MAFB to be constitutively and highly transcriptionally activated, compared to the endogenous promoters of the downstream putative target genes.

Approaching the next phase of the research, experiments were designed as a time course to observe three different criteria: morphological changes, gene expression changes, and cell count changes in MAFB transfected HL-60 cells over time. In further investigation of the RT-qPCR results from the gene panel, a time course was designed where HL-60 cells were transfected with MAFB, then cells were harvested and RNA was isolated at the time points 4 hours post-transfection, 8 hours post-transfection, and 16 hours post-transfection. The RNA from these time points, along with the control vector-transfected HL-60 cells’ RNA was used in another gene panel RT-qPCR, measuring the expression of selected genes that were upregulated in the first gene panel RT-qPCR experiment. The results indicated that
the genes *TINAGL1* and *IL1A* were significantly upregulated at all three time points, while *KIT* was significantly upregulated at the 16 hour time point. As *MAFB* overexpression peaked at 4 hours, then was steadily at a level above 100-fold overexpression, the transfection was deemed successful and the upregulation of the target genes *TINAGL1* and *IL1A* was validated as a downstream result of the *MAFB* overexpression. This result is both significant and informative as to the role of *MAFB* in initiating differentiation. *IL1A* encodes a protein that is involved in both hematopoiesis with differentiation and apoptosis, as well as the inflammatory response (GeneCards, 2018). *MAFB* has been shown to be an essential transcription factor for other immune regulators including IL-10 in macrophages (Cao et al., 2005). Data supporting *MAFB* as an upregulator of *IL1A* provides additional evidence suggesting the role of *MAFB* in initiating differentiation.

*TINAGL1* is similar to the secreted glycoprotein, tubulointerstitial nephritis antigen, which can be recognized by antibodies (Wex et al., 2001). In non-small cell lung cancer, *TINAGL1* was found to be a candidate therapeutic target, as it functions as a tumor suppressor (Umeyama, Iwadate and Taguchi, 2014). It should be noted that *TINAGL1* has been shown to be up-regulated in highly metastatic tumors, contrary to its generalized categorization as a tumor suppressor (Naba et al., 2014). This variance in the activity of *TINAGL1* recorded in literature to date make the observed interaction with *MAFB* interesting and provides an avenue for further research into the exact impacts of *MAFB*-induced upregulation of *TINAGL1* in HL-60 cells. The data from this research suggests that overexpressed *MAFB* acts to up-regulate *TINAGL1* in HL-60 cells, yet the implications of this expression has not been phenotypically qualified with the subsequent behavior of the reprogrammed HL-60 cells. In certain human breast cancer cell lines, *TINAGL1* appears to
show anti-metastatic effects, passably through the inhibition of EGFR signaling (Roberts, unpublished results). The role of EGFR signaling in HL-60 cells has not yet been determined.

Time Course Morphological Changes and Cell Cycle Arrest

In addition to analyzing the genetic changes in regulation in MAFB transfected cells over a time course, Light microscopy was used to observe morphological and phenotypic changes elicited by the overexpression of MAFB. In imaging the transfected HL-60 cells with light microscopy over a course of 48 hours, along with cell counts taken at the various time points over a course of 60 hours, it was determined that cell proliferation was slowed in the MAFB transfected HL-60 cells. Although no significant morphological changes in the cells could be observed, changes on the genetic and cell count level are consistent with initiation of a differentiation program. This can be quantified in Table 1, displaying the cell counts over time from 0 hours to 60 hours. The cell counts of the MAFB transfected HL-60 cells over the course of 60 hours were significantly lower than those of the control vector-transfected HL-60 cells over the same time course. When analyzed with a Chi-squared test for goodness of fit, the null hypothesis was rejected with high confidence and a p-value of less than 0.0001. The null hypothesis stated that the cell counts of the MAFB transfected HL-60 cells did not differ significantly from the cell counts of the control vector transfected HL-60 cells. As the data was extremely significant, the null hypothesis was rejected and the MAFB transfected HL-60 cells exhibited slowed growth over 60 hours, compared to the control. This data further supported the hypothesis that MAFB overexpression partially reverts the transformed phenotype of HL-60 cells.
Future Directions

In future research, an inducible vector with MAFB will be created, so that stable MAFB cell lines can be isolated. The creation of an inducible vector and stable cell line production was the next step in this research, had time permitted, and it would enable more definitive phenotypic characterization through imaging over a longer time course, on a uniformly MAFB overexpressing cell line.

It was possible that no morphological changes were observed because MAFB was only transiently overexpressed, with expression peaking at 4 hours. Even with downstream protein overexpression at 8 hours, this was likely to have diminished impacts at the 24 hour, 48 hour and 60 hour timepoints that would be desirable for observing morphological changes.

Another explanation for the lack of observed morphological changes observed in the differentiation HL-60 cells would be that MAFB regulates only a subset of the genes, which would not as visibly impact the morphology of the cell in vitro.

Furthermore, MAFB has been observed to form both homodimers and heterodimers with other proteins, such as c-Fos and c-Jun to control apoptosis (Suda et al., 2014). Additional experiments might explore the construction of a double clone with c-Fos or c-Jun with MAFB to explore the impacts of these heterodimer formations on differentiation and/or apoptosis of HL-60 cells. Since both c-Fos and c-Jun are induced in PMA treated cells, it may be that MAFB/JUN and/or MAFB/FOS heterodimers are critical in driving transcriptional changes mediating some of the phenotypic effects.
Conclusions

The results provided by this research contributed further insights to the functional pathway of *MAFB* in signaling putative target genes to reprogram HL-60 cells. The data support the hypothesis that the gene *MAFB* has downstream effects, which may be integral for cell differentiation and cell cycle arrest. It can be confirmed that the overexpression of *MAFB* in HL-60 cells contributes to slowed cell division (Table 1 and Fig. 12), potentially through the induction of cell differentiation through putative target genes. Following the successful construction of the transient *MAFB* clone, the RT-qPCR with a gene panel performed to measure changes in expression of putative *MAFB* targets reflects significant increased expression of *TINAGL1* and *IL1A* with response to the overexpression of *MAFB*.

From these observed upregulations in downstream gene expression, it is suggested that *MAFB* protein is being activated and transported into the nucleus to act upon the promoters of the downstream target genes. To further validate the hypothesis that *MAFB* is responsible for the observed downstream changes in gene expression, it would be beneficial to perform experiments to qualify the nuclear activity of *MAFB*. These experiments might include staining for cell surface markers or tagging and staining *MAFB*, then visualizing the transfected cells for the presence of *MAFB* in the nucleus. This additional data would provide qualitative confirmation of *MAFB* activity in the nucleus, adding plausibility to the mechanism of downstream gene upregulation through the selective binding of *MAFB* protein to the promoters of these genes.

Further future research should aim to confirm this preliminary data, while expanding the gene panel to include more differentiation, cell cycle regulatory and apoptosis putative *MAFB* target genes. As mentioned previously, this would best be achieved through the RNA
sequencing data. Total gene expression changes can be analyzed to assess the full extent of *MAFB*’s impact on the transcriptome. The putative target genes of *MAFB* could then be categorized by functionality, to fully assess whether *MAFB* plays a significant role in regulating the immune response or angiogenesis.

In vivo experiments would also be interesting to pursue in the future, as any effect on angiogenesis may be fully observed phenotypically, whereas in vitro experiments do not supply the necessary tumor microenvironment for such analysis. With current research being performed on tumor cells in a variety of cancers, more evidence is pointing to the importance of the combination of tumor microenvironment, immune suppression and genetic composition of cancer cells as the contributors to tumor progression and metastasis. The research performed in the Roberts lab on the changes to the transcriptome of HL-60 cells in response to overexpression of genes serves to further complete the understanding of the cancer cell’s genetic profile, which is necessary for the progression of effective drug therapies.
References

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GeneCards. 2018. IL1A Gene. Retrieved April, 2018 from GeneCards: http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL1A


Figures

Figure 1. Electron micrograph images of an HL-60 cell after 0, 12, 24 and 48 hours post treatment with PMA. After 12 hours, the cell cycle arrest has occurred in the HL-60 cell. After 24 hours, the HL-60 cell has begun differentiation, and after 48 hours, the cell is undergoing apoptosis.

<table>
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<th>Gene</th>
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<th>6</th>
<th>12</th>
<th>24</th>
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<td>5.65</td>
<td>6.28</td>
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Figure 2. Microarray-measured changes in the gene expression of MAFB in response to PMA over the course of 24 hours. MAFB was significantly upregulated, from its baseline level of expression, as a response to the PMA treatment. From this time course sampling, the peak upregulation was at 12 hours post-treatment with PMA.
Figure 3. Map of GenScript® GenEZ™ ORF cDNA clone with \textit{MAFB} under the control of the CMV promoter (A). Map of empty GenScript® GenEZ™ ORF expression vector (B).
Figure 4. Histogram of RT-qPCR results from overexpression at 8 hours post-transfection of MAFB in the transient plasmid using the standard TaqMan probe with primers for the 3’ untranslated region of the transfected DNA. The transfection by nucleofection of MAFB was performed in triplicate, the results of which are displayed in the columns marked MAFB1, MAFB2 and MAFB3. The overexpression is measured in reference to the empty vector, shown in the column marked Vector. The histogram displays under-expression of MAFB, as the TaqMan probe contained primers for the 3’ untranslated region, rather than the transfected MAFB. The quantitation of MAFB measured in these samples is the endogenous MAFB, indicating potential synergistic regulation of the gene.
Figure 5. Histogram of RT-qPCR results from overexpression at 8 hours post-transfection of MAFB in the transient plasmid using the custom TaqMan probe. The transfection by nucleofection of MAFB was performed in triplicate, the results of which are displayed in the columns marked MAFB1, MAFB2 and MAFB3. The overexpression is measured in reference to the empty vector, shown in the column marked Vector. MAFB1 had 197 fold overexpression, MAFB2 had 147 fold overexpression, and MAFB3 had 183 fold overexpression.
Figure 6. Map of the pLVX-TetOne™-Puro vector to be used for the inducible expression of MAFB. This vector is a construct that is highly induced by doxycycline, but is turned off in its absence. The MAFB gene is to be inserted between the AgeI and EcoRI restriction sites.
Figure 7. Western blot confirming overexpression of MAFB on the protein level at 8 hours post-transfection. A western blot was performed in duplicate with the protein isolated from the transfection of MAFB into the transient vector. Protein from the MAFB transfected HL-60 cells was loaded into lanes 1 and 2 of the 10% SDS-PAGE gel. Protein from the vector control transfected HL-60 cells was loaded in lanes 3 and 4. The Precision Plus Protein Standards™ ladder was loaded in the far right lane. Bright bands were visualized in lanes 1 and 2 at 36 kD with the MAFB transfected cell protein, and no bands were visualized in lanes 3 and 4 with the vector control transfected cell protein.
Figure 8. *MAFB* overexpression in the targeted gene RT-qPCR at 8 hours post-transfection.

In a RT-qPCR performed with the probes for eight different potential putative target genes of *MAFB*, *MAFB* overexpression was 234.62 fold, compared to the endogenous control of RPS-28.
Figure 9. RT-qPCR probing MAFB-transfected cells for select putative target gene expression at 8 hours post-transfection. TaqMan probes for the genes BCL6, IGFBP7, IL1A, KIT, CCND1, CENPM, NEIL3, PRDM1 and TINAGL1 were used in this RT-qPCR experiment, measuring the gene expression of selected potential putative gene targets of MAFB. Both IL1A and KIT displayed 1.5-fold upregulation in MAFB-transfected cells, compared to the empty vector control transfected cells. PRDM1 showed a 1.4-fold upregulation in MAFB-transfected cells, compared to the empty vector control transfected cells. TINAGL1 showed a 2.78-fold upregulation in MAFB-transfected cells, when compared to the empty vector control transfected cells. Given the small error bars, this upregulation is significant and likely due to the overexpression of MAFB, indicative of changes to the transcriptome, induced by MAFB overexpression.
Figure 10. RT-qPCR results from *MAFB* transfected HL-60 cells, over a 16 hour time course. RNA was isolated at timepoints 4 hours post-transfection, 8 hours post-transfection, and 16 hours post-transfection. *MAFB* was 161.50-fold overexpressed, compared to the reference sample of empty vector-transfected HL-60 cell RNA measured with the RPS28 probe at 4 hours post-transfection. At 8 hours post-transfection, *MAFB* was 113.17-fold overexpressed. At 16 hours post-transfection, *MAFB* was 114.50-fold overexpressed.

*IL1A* was 31.33-fold overexpressed at 4 hours post-transfection, compared to the reference sample of empty vector transfected HL-60 cell RNA measured with the RPS28 probe at 4 hours post-transfection. *TINAGL1* was 17.42-fold overexpressed, and *KIT* showed no overexpression at 4 hours post-transfection. At 8 hours post-transfection, *IL1A* was 37.22-fold overexpressed, *TINAGL1* was 88.92-fold overexpressed and *KIT* showed no overexpression. At 16 hours post-transfection, *IL1A* was 36.67-fold overexpressed, *TINAGL1* was 32.29-fold overexpressed and *KIT* was 2.43-fold overexpressed.
Figure 11. Images of HL-60 cells over 48 hour time course post-transfection with $MAFB$-inserted vector or an empty vector control. Light microscopy was used to visualize HL-60 cells at 6 hours, 12 hours, 24 hours and 48 hours post-transfection. Images A, C, E and G visualize HL-60 cells transfected with an empty vector control at 6 hours, 11 hours, 24 hours, and 48 hours respectively. Images B, D, F and H visualize HL-60 cells transfected with $MAFB$ genomic DNA at 6 hours, 11 hours, 24 hours and 48 hours respectively. No significant morphological changes were observed between the empty vector control-transfected cells and the $MAFB$-inserted vector transfected cells at any time point.
Figure 12. Graphical representation of cell count assay comparing *MAFB* transfected cells with vector transfected cells. The cell count of *MAFB* transfected cells is significantly different from that of the vector transfected cells overall, as displayed by the increasing variance between the orange columns, representing *MAFB* transfected cells, and blue columns, representing vector transfected cells, over time.

Table 1. Cell counts of *MAFB* transfected HL-60 cells over 60 hours.

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