

EZH2 Knockdown Upregulates Expression of the Genes Involved in T-ALL Cell Differentiation

Sahar Safaei^{1,2}, Behzad Baradaran¹, Behzad Mansoori¹, Masoumeh Fardi^{1,3}, Elham Baghbani^{1,2}, Mohammad Amini¹, Nima Hemmat¹, Elham Safarzadeh¹, Mahdi Abdoli Shadbad^{2,1}, Dariush Shanehbandi¹, Saeed Solali^{1,3*}

¹Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

²Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.

³Department of Immunology, Division of Hematology and Transfusion Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.

Article Info

Article History:

Received: 7 August 2021

Accepted: 3 September 2021

ePublished: 10 September 2021

Keywords:

-EZH2
-Leukemia
-MOLT-4
-T-ALL
-Transcription Factor

Abstract

Background: EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit), as one of the polycyclic group proteins (PcGs), is an epigenetic regulator that plays a crucial role in the pathophysiology of hematologic malignancies through regulating cell differentiation. Also, it is well known that aberrant expression of specific transcription factors can be involved in the pathogenesis of various cancers. Herein, we aimed to suppress EZH2 expression in MOLT-4 cells, T-ALL (T cell acute lymphoblastic leukemia) cell line, and evaluate the role of EZH2 on the expression of transcription factors that regulate T cell maturation, differentiation, and apoptosis.

Methods: EZH2-siRNA was transfected into MOLT-4 cells, and the expression levels of EZH2, NOTCH1, TCF1, IKZF1, and NFATC1 were measured using real-time PCR. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was performed to study the effect of EZH2 knockdown on MOLT-4 cell viability. The apoptosis rate of EZH2-siRNA transfected cells was assessed by flow cytometry. The interaction of mentioned genes was investigated using STRING and GO (gene ontology).

Results: Our results have shown that EZH2-siRNA transfection can substantially decrease EZH2 expression in MOLT-4 cells. Besides, EZH2 suppression can upregulate NOTCH1, TCF1, IKZF1, and NFATC1 expression levels. EZH2 knockdown does not affect the viability and apoptosis of MOLT-4 cells. The most remarkable protein-protein interaction of EZH2 has been with NOTCH1. Besides, GO analysis has demonstrated that EZH2, NOTCH1, TCF1, IKZF1, and NFATC1 were located within nucleoplasm and can regulate RNA polymerase II-mediated transcription.

Conclusion: MOLT-4 cells harbor increased expression of EZH2 in comparison with normal human T cells. EZH2 knockdown can upregulate the expression of the transcription factors involved in T cell differentiation. Thus, EZH2 can halt the differentiation of immature lymphoblastic T cells.

Introduction

T-ALL is one of the aggressive malignancies of T lymphocytes, which is responsible for about 25% of T cell-associated malignancy cases in adults and 15% in children.¹ In T-ALL, the maturation of the precursor T cells is dysregulated, and the bone marrow is filled with T lymphoblasts. The abnormal proliferation of immune cells results in the infiltration of immature cells into other organs,² like the central nervous system.³ Besides, tumoral lymphoblasts can pave the way for the development of severe infectious diseases.⁴

TFs (transcription factors) can substantially regulate the growth, differentiation, and maturation of hematopoietic stem cells; therefore, their dysregulation can facilitate

the development of hematological malignancies, like T-ALL. NOTCH1, TCF1, IKZF1, and NFATC1 are among the critical TFs involved in T cell maturation. Growing evidence has indicated that aberrant expression of TFs and chromosomal translocation are the common cause of leukemias.⁵⁻⁹

Epigenetic changes are among the factors involved in regulating the expression of TFs. Unlike chromosomal mutations, epigenetic changes do not affect the original DNA sequence but alter gene expression via the structural modification of DNA.^{10,11} EZH2, as one of the PcGs, is an epigenetic regulator that plays a remarkable role in the formation of the polycomb repressive complex 2 (PRC2). Recent findings have suggested that PRCs

*Corresponding Author: Saeed Solali, E-mail: ssolali@gmail.com

©2022 The Author(s). This is an open access article and applies the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>). Non-commercial uses of the work are permitted, provided the original work is properly cited.

dysregulation can lead to cancer development. PRC1 and PRC2, as members of the PRCs family, are involved in epigenetic modifications by altering the methylation of histones. As a subunit of PRC2, EZH2 can result in trimethylating of lysine 27 of histone number 3; therefore, it can suppress target genes expression. Tri-methylation of histone in this position recruits PRC1 and leads to the formation of heterochromatin. Studies have shown that EZH2 can regulate the transcription of its target genes, which are involved in the differentiation of cells. Besides, accumulating evidence indicates that EZH2 expression only increases in malignant conditions; therefore, EZH2 can be considered a potential diagnostic biomarker and a therapeutic target for cancer treatment.¹²⁻¹⁴

In the current study, we evaluated the effect of EZH2 knockdown on the expression of NOTCH1, TCF1, IKZF1, and NFATC1, which regulate the maturation and differentiation of the MOLT-4 cells. Besides, we assessed the effect of EZH2 knockdown on the apoptosis and viability of MOLT-4 cells.

Materials and Methods

Cell line

The MOLT-4 T-ALL cell line was purchased from Pasteur Institute, Tehran, Iran. The cells were cultured in RPMI-1640 medium complemented with 10% fetal bovine serum (FBS), and streptomycin (100 mg/mL), penicillin (100 U/mL) (Sigma- Aldrich, St. Louis, MO, USA), and incubated at 37°C in a humidified 5% CO₂ condition. The cells were sub-cultured 48–72 hours later with a primary concentration of 5×10⁴ cells/ml. All the tests were performed using cells in the logarithmic phase.

T lymphocyte isolation

Using negative selection, the isolation of autologous and analogous CD3⁺ T cells were done from PBMC (peripheral blood mononuclear cells) samples according to the protocol provided by the human Pan T cell Isolation Kit II (MiltenyiBiotec, Bergisch Gladbach, Germany). The purity of the isolated T cells and MDSCs (myeloid-derived suppressor cells) were determined after using flow cytometry. The populations with >70% purity were used in the research.

The transfection of siRNA

The EZH2 and negative control siRNAs were synthesized by Santa Cruz Biotechnology (Table 1). All the transfections were performed when the cells reached 60% confluence according to the manufacturer's instructions. Before siRNA transfection, a density of 2×10⁴ cells/well was cultivated in 6-well plates and grown in antibiotic and FBS-free RPMI-1640 medium. Scrambled siRNA was used as a negative control. Briefly, at a final concentration of 80 pmol, transfection components (6 μL/mL of the transfection reagent, which was Lipofectamine) and siRNA were diluted in siRNA transfection medium (Santa Cruz Biotechnology) independently and mixed. 20 minutes

Table 1. EZH2-siRNA sequences (Note: All the sequences are provided in 5' → 3' orientation).

Cat. Number	Strand	Sequence (5'–3')
sc-35312A	Sense	GGAAAGAACGGAAAUCUUAtt
	Antisense	UAAGAUUUCCGUUCUUUCctt
sc-35312B	Sense	GAAGCCAAAUUAUGAACCUtt
	Antisense	AGGUUCAAUUUUGGCUUCtt
sc-35312C	Sense	GAAGAGGGAAAGUGUAUGAtt
	Antisense	UCAUACACUUUCCUCUUCtt

after incubating at 37°C, the diluted solutions were mixed and incubated for an extra 30 minutes at 37°C. Then, the blends were added to each well containing medium and cells, and incubation was done for 6 hours at 37°C in a humidified CO₂ incubator. Afterward, they were added to an RPMI-1640 medium containing FBS 20%.¹⁵ The cells were incubated as described earlier. At 48 hours after the transfection, the cells were gathered, and qRT-PCR (quantitative real-time PCR) was employed to evaluate the EZH2 expression.

RNA isolation and qRT-PCR

Using TRIzol reagent, the total RNA of cells was isolated according to the manufacturer's instruction (Roche Diagnostics, Mannheim, Germany). cDNA (complementary DNA) synthesis was done using the TAKARA cDNA synthesis kit (Japan). Then, the target gene expression was investigated by qRT-PCR using SYBR Premix Ex Taq II (TAKARA, Japan) and the Roche Diagnostics light cycler 96 system (Mannheim, Germany). The primers were blasted using the primer-blast section of the NCBI website (<http://www.ncbi.nlm.nih.gov>) (Table 2). Then the primers were synthesized by SinaGene (Tehran, Iran). Relative mRNA expression was interpreted with the 2^{-(ΔΔct)} method using GAPDH as an internal control.

MTT assay

The MTT assay (Sigma, Germany) was used to study the potential cytotoxic effect of the EZH2-siRNA transfection on the tumoral cells. Briefly, cells were seeded in 96-well culture plates and incubated for 24 hours in a humidified 5% CO₂ incubator until they reached 75–80% confluency. Then, they were transfected with scrambled siRNA, paclitaxel (as the negative and positive control groups), transfection reagent, pure siRNA, and various concentrations of EZH2-siRNA. 48 hours after transfection, 100 μL of MTT reagent (with the concentration of 0.5 mg/mL in phosphate-buffered saline) was added to the wells, and the incubation was done for 4 hours. Afterward, 200 μL of solubilization mix, i.e., DMSO+Sorensen buffer, was added to each well to dissolve the formazan crystals. A half-hour after incubation, in the aforementioned condition, the optical density of each plate well was evaluated at 570 nm using an ELISA reader (Awareness Technology, FL).

Apoptosis assay

Annexin V/PI (propidium iodide) staining was performed

Table 2. Primer sequences used in this study (Note: All the sequences are provided in 5' → 3' orientation).

Gene/Accession Number	FW/RV	Sequence 5'à	Product size
EZH2/NM_004456.5	FW	CCTGAAGTATGTCGGCATCGAAAGAG	252
EZH2/NM_004456.5	RV	TGCAAAAATTCACCTGGTACAAAACACT	252
TCF-1(TCF7)/NM_001366502.2	FW	AGCACCAAGAATCCACCACA	151
TCF-1(TCF7)/NM_001366502.2	RV	CAGCAGATGGTATGAGGGTGA	151
IKZF1/NM_001291837.2	FW	ACAGAGTCGTGGCCAGTAATG	172
IKZF1/NM_001291837.2	RV	ACTCCCGACAAAGCCGAGC	172
NOTCH1/NM_017617.5	FW	AGTGAGGGACGTCAGACTTG	167
NOTCH1/NM_017617.5	RV	AACATCTTGGGACGCATCTGG	167
NFATC1/NM_001278675.2	FW	CTGTCTGGCCACAACCTCC	287
NFATC1/NM_001278675.2	RV	CGCTCATGTTACGGCTTAC	287
CD44/NM_001202555.2	FW	CAAGCCACTCCAGGACAAGG	216
CD44/NM_001202555.2	RV	ATCCAAGTGAGGGACTACAACAG	216
GAPDH/NM_001357943.2	FW	CAAGATCATCACC AATGCCT	166
GAPDH/NM_001357943.2	RV	CCCATCAGCCACAGTTTCC	166

to investigate the effect of EZH2 knockdown on apoptosis. Briefly, MOLT-4 cells at the density of 1×10^6 were transfected with EZH2-siRNA and seeded into 6-well plates. After 48 hours of incubation, the cells were harvested and washed two times with cold phosphate-buffered saline. The cells were resuspended in PI and FITC-labeled Annexin V solution (100 μ L) and incubated in the dark at 25 °C for 10 minutes. Afterward, the cells were washed and suspended in cold phosphate-buffered saline and subjected to flow cytometry (BD FACSCalibur system, BD Biosciences, San Diego, CA, USA). Data analysis was performed using Cell Quest software (FLOW JO).¹⁵

Differentiation analysis

Flow cytometry was used to examine the effect of EZH2 knockdown on T cell differentiation. For this purpose, the cells were transfected with EZH2-siRNA and incubated for 48 hours. Then, the transfected cells were harvested and washed with phosphate-buffered saline. Afterward, the cells were resuspended in 100 μ L of FITC-labeled antibody targeting CD44 protein. Following incubating for 45 minutes, the cells were subjected to flow cytometry to measure CD44 surface expression.

Statistical analysis

Data were presented as mean \pm standard deviation (SD). T-test and ANOVA, followed by Dunnett's test, were used to study the statistical significance of differences between groups. A P-value below 0.05 was considered significant. All the statistical analyses for qRT-PCR were performed

using R software statistical analysis, and other graphs were analyzed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA).

Bioinformatic analysis

GeneMANIA (<http://genemania.org>) is a bioinformatics database for predicting gene function and their protein level interactions. The website also searches for genes with similar functions and represents them based on the functional value and the type of network between genes.¹⁶ STRING database is an online tool for presenting networks between genes.¹⁷ This tool was used to detect expression analyzed genes and protein-protein interaction. The analyzed genes were uploaded, and the cut-off criteria were set to >0.700 as high confidence, 0.400-0.700 as moderate confidence, and <0.400 as low confidence for each node. GO enrichment analysis is a bioinformatics tool used to determine the location of gene products and the most likely function in the cells.^{18,19} The biological process, cellular function, and cellular component of the studied genes were studied, and P-value < 0.05 ($-\log_{10}$ P-value > 1.30) was set as the cut-off.

Results

Higher expression levels of EZH2 in the MOLT-4 cells compared to normal T cells

To better understand EZH2 expression in the MOLT-4 and normal T cells, EZH2 expression was evaluated using qRT-PCR. Quantitative data from each sample were normalized using GAPDH as a reference gene. Our results have shown

that EZH2 is significantly upregulated in MOLT-4 cells compared to normal T cells (P-value<0.0001) (Figure 1). This suggests that EZH2 upregulation might be involved in T-ALL development.

Downregulation of EZH2 mRNA expression by EZH2-siRNA in MOLT-4 cells

We evaluated the effect of EZH2-siRNA transfection on the mRNA expression of EZH2 in MOLT-4 cells. The relative gene expression was measured using qRT-PCR. Normalization of the quantitative data from all the samples was done using GAPDH as a reference gene, and relative gene expression was evaluated in relation to the control group (untreated cells). The relative expression of EZH2 in different concentrations of EZH2-siRNA, i.e., 20, 40, 60, and 80 pmol, and at different times, i.e.,

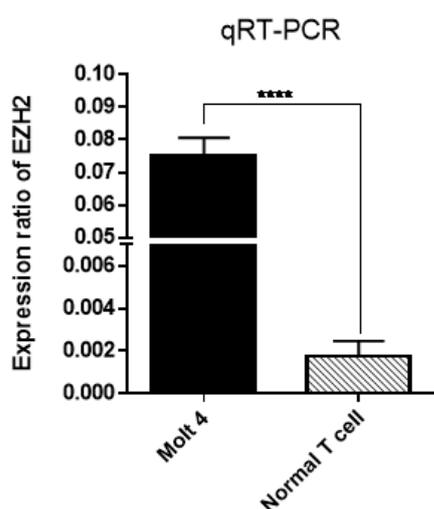


Figure 1. Comparison of the expression of EZH2 in MOLT-4 cells and normal T cells. EZH2 expression levels in normal T cells and MOLT-4 cells were investigated using qRT-PCR. (****P<0.0001).

24, 48, and 72 hours, are demonstrated in Figure 2. Our results have shown that 80 pmol of EZH2-siRNA at the 48 hours after transfection are the optimal dose and time for downregulating the expression of EZH2 in MOLT-4 cells (both P-values<0.0001)

Upregulation of NOTCH1, TCF1, IKZF1, and NFATC1 mRNA expression through EZH2 knockdown in MOLT-4 cells

NOTCH1, TCF1, IKZF1, and NFATC1 are essential TFs for T cells differentiation. We investigate their mRNA expression levels following transfecting MOLT-4 cells with EZH2-siRNA. Our results have indicated that EZH2 silencing can significantly upregulate the mRNA expression of NOTCH1, TCF1, IKZF1, and NFATC1 in MOLT-4 cells (P-value<0.0001, P-value<0.001, and P-value<0.01, respectively) (Figure 3).

EZH2 ineffectiveness on the regulation of pro-apoptotic factors

To investigate the possible relation of EZH2 with the pro-apoptotic factors, like BAX, BAD, BID, BOK, BIK, BAK, BCLx, HRK, we used the GeneMANIA database. We have found no strong relation between EZH2 and pro-apoptotic factors (Figure 4).

Induction of no significant effects on MOLT-4 cell viability by EZH2 silencing

We used MTT assay to assess the effect of EZH2 knockdown on the viability of MOLT-4 cells. Our results have demonstrated that different doses of EZH2-siRNA, i.e., 20, 40, 60, 80 pmol, have not decreased the viability of MOLT-4 cells (Figure 5).

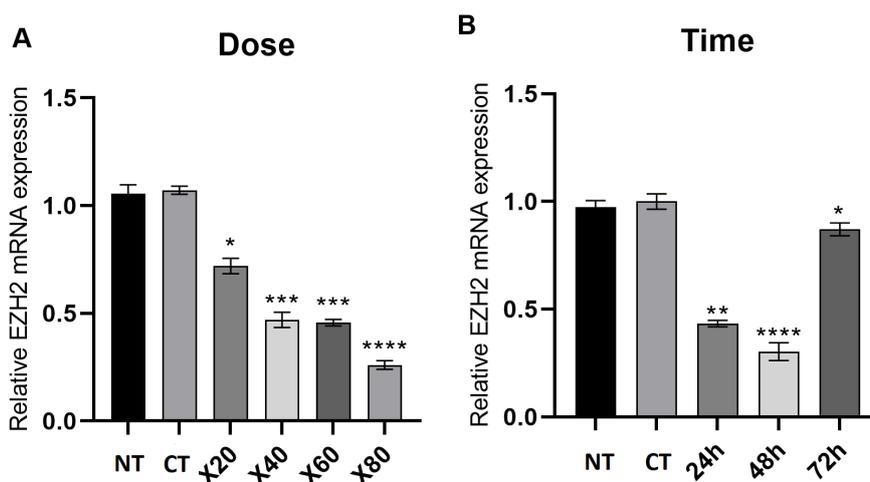


Figure 2. EZH2 knockdown using EZH2-siRNA in MOLT-4 cells. **A.** MOLT-4 cells were transfected with EZH2-siRNA with doses of 20, 40, 60, and 80 pmol. Transfection of MOLT-4 cells with 80 pmol EZH2-siRNA significantly decreased EZH2 expression **B.** The mRNA expression level of EZH2 was evaluated after 24, 48, 72 hours of EZH2-mRNA transfection. 48 hours after transfecting MOLT-4 cells with EZH2-siRNA was the optimal time for downregulating EZH2 mRNA expression in MOLT-4 cells. The data were represented as mean \pm SD. qRT-PCR was used to assess the relative EZH2 mRNA expression using $2^{-\Delta\Delta Ct}$ method. (n = 3); (*P<0.05, **P<0.01, ***P=0.001, ****P<0.0001 versus control) (NT: negative control, CT: control transfected).

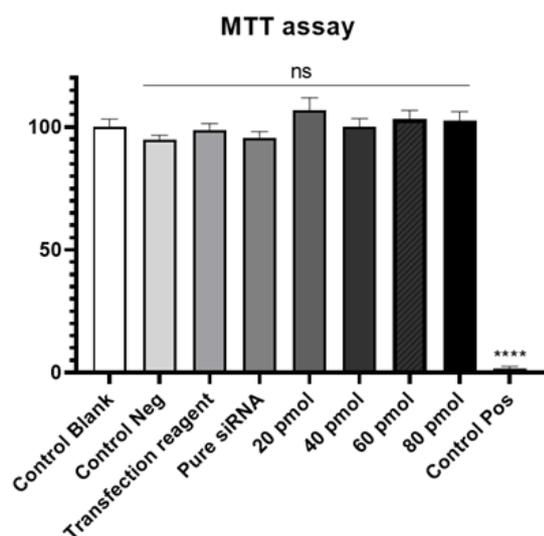


Figure 5. The effect of EZH2-siRNA on the viability of MOLT-4 cells. The MOLT-4 cells were transfected with 20 to 80 pmol EZH2-siRNA after 48 hours of EZH2-siRNA transfection. Then the potential cytotoxicity of EZH2-siRNA was investigated using the MTT assay. The results were expressed as mean \pm SD. ****: $p < 0.0001$

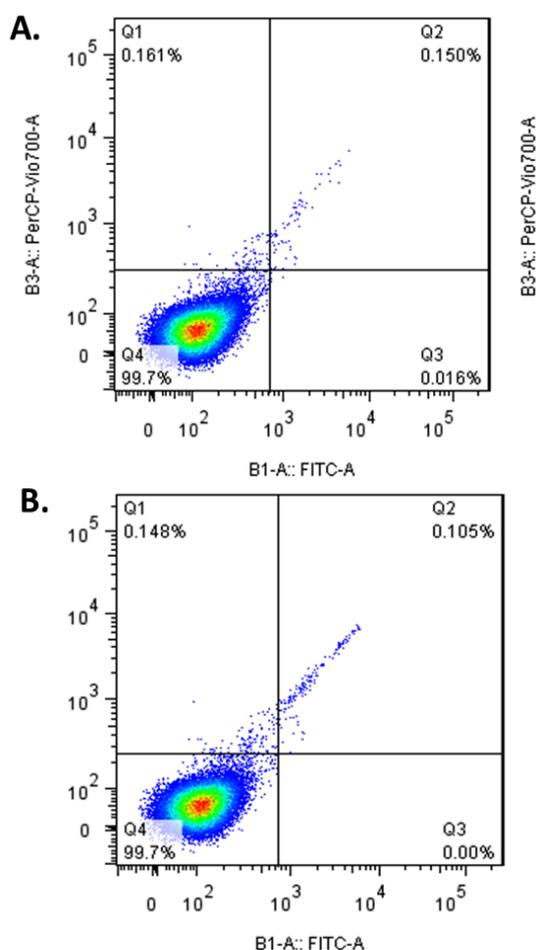


Figure 6. Investigating apoptosis following EZH2-siRNA transfection in MOLT-4 cells using flow cytometry. 48 hours after transfection with 80 pmol EZH2-siRNA, the cells were stained with Annexin V-FITC and PI. (A) Apoptosis in MOLT-4 cells transfected with EZH2-siRNA. (B) Apoptosis in MOLT-4 cells transfected with scrambled siRNA.

EZH2 interaction with NOTCH1 in the protein-protein interaction network

We used the STRING tool to study the protein-protein interaction network between EZH2, IKZF1, NOTCH1, TCF1, and NFATC1. The interaction highest confidence was between EZH2 and NOTCH1. Moreover, EZH2 was shown to be located upstream of NOTCH1 (Figure 9).

Regulation of the RNA polymerase II-mediated transcription by the given module

The analyzed genes were set to the GO enrichment analysis tool. It has been shown that the most likely location of these genes' production is nucleoplasm (P -value= 4.73×10^{-5} or $-\log_{10}$ P -value= 4.325) and they regulate RNA polymerase II-mediated transcription (P -value= 1.72×10^{-6} or $-\log_{10}$ P -value= 5.764) (Figure 10).

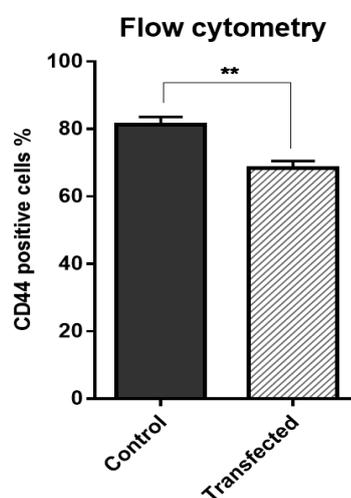


Figure 7. The flow cytometry analysis of cellular differentiation in MOLT-4 cells transfected with EZH2-siRNA. EZH2 silencing down-regulated CD44 surface expression in MOLT-4 cells (** $P < 0.01$).

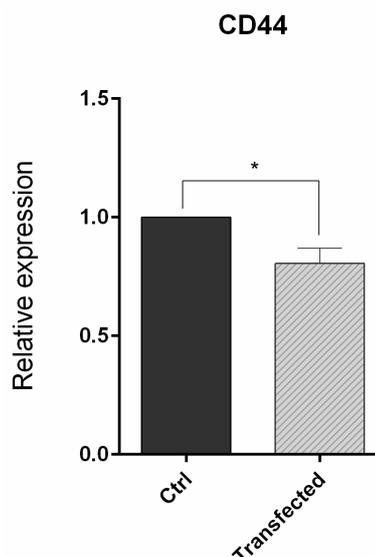


Figure 8. Decreased mRNA expression of CD44 after transfection of MOLT-4 cells with EZH2-siRNA (* $P < 0.05$).

Discussion

Epigenetic modifications substantially regulate gene expression. One of the most critical epigenetic modifiers is histone-modifying enzymes. These enzymes play an essential role in modifying the binding of different groups to histones, which ultimately regulate the expression of specific genes.¹⁰ EZH2 plays such a role as the catalytic element of the PRC2. PRC2 is the second member of the polycomb complexes class that uses EZH2 in its structure and triggers tri-methylation of lysine 27 on histone H3 (H3K27me3); thus, it can silence its target gene. Studies have shown that EZH2-mediated H3K27me3 can recruit PRC1 to the mentioned methylation location and cause ubiquitination of lysine 119 from the H2A histone (H2AK119ub1), leading to the stabilization of the chromatin inhibition.²⁰

Growing evidence indicates that PRC1 and PRC2 can maintain the pluripotency and self-renewability of hematopoietic cells.^{20,21} It has been reported that EZH2 can physiologically regulate the cell cycle of hematopoietic stem cells and control the expression of genes involved in inhibiting the differentiation of these cells.^{22,23} During lymphopoiesis, EZH2 is also extensively expressed in proliferating cells, including B cells, T cells, and B lymphoblasts of the germinal centers, indicating its central role in regulating the cell cycle and lymphocytes division. On the other hand, EZH2 decreases during the differentiation and maturation of B lymphocytes; thus,

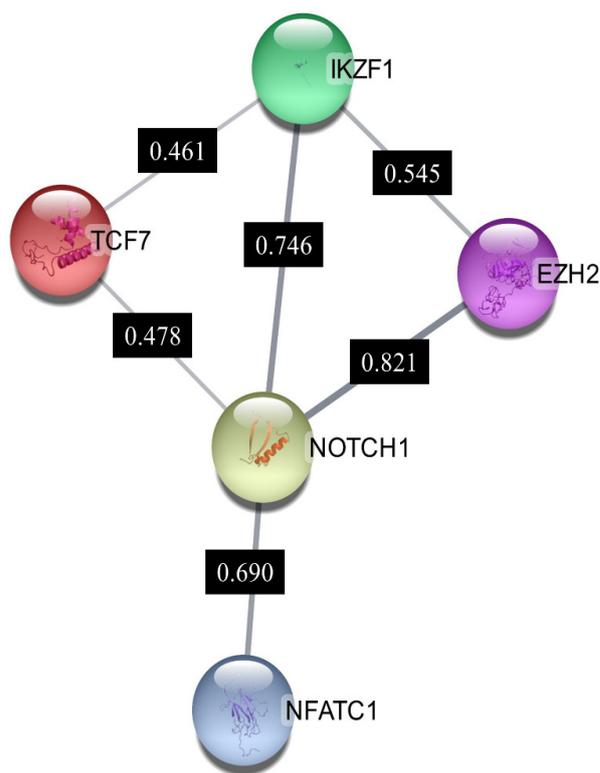
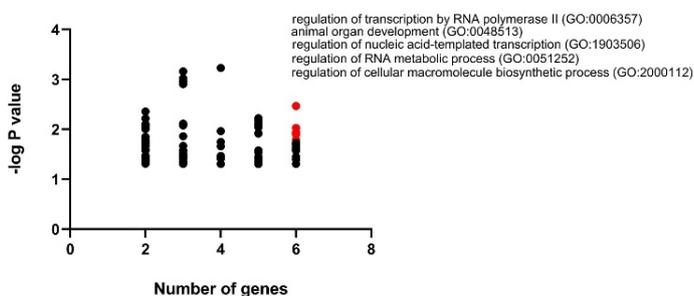
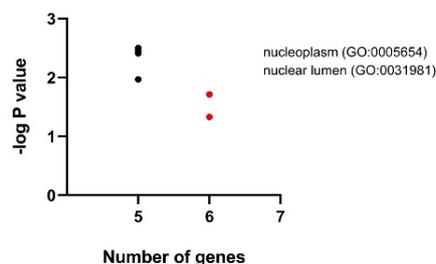


Figure 9. Protein-protein interaction network of the studied gene using STRING. EZH2-NOTCH1 node has the highest confidence score; TCF1-IKZF1 node has the lowest confidence score.

Gene Ontology (Biological processes)



Gene Ontology (Cellular component)



Gene Ontology (Molecular functions)

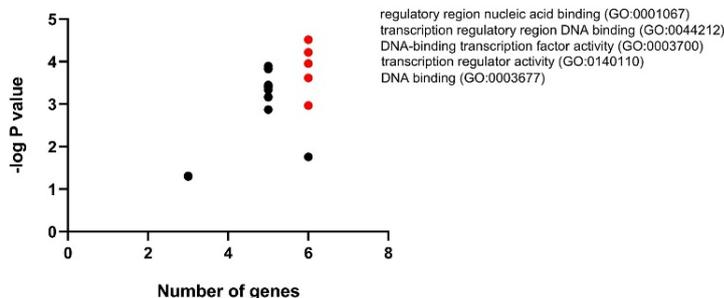


Figure 10. GO analysis of each gene was done in the cellular components, biological process, and molecular function levels. In the x-axis of the graphs, the number of genes analyzed in this study was illustrated as well as the -log P-value of the functions showing the significance of the process on the y-axis. The process, cell component, or molecular function with the largest -log p-value and gene number was distinguished as the most involved pathways during the knockdown of EZH2. Nucleoplasm and regulation of transcription by RNA polymerase II were the most likely location and functions of EZH2, IKZF1, NOTCH1, TCF1, NFATC1 productions.

EZH2 can inhibit the differentiation of B lymphocytes. Indeed, EZH2 can inhibit the differentiation of the pro-B cells to pre-B cells through interaction with STAT5 (signal transducer and activator of transcription 5).²⁴

T-ALL is developed following the arrest of the differentiation of lymphoblastic T cells, the maintenance of the cells in the blastic stage, and the abnormal proliferation of the cells. Proliferated cells can infiltrate from the blood into other organs, resulting in secondary malignancies.²⁵

We have hypothesized that the increased EZH2 expression in T cells can inhibit differentiation. Therefore, we have examined EZH2 expression in MOLT-4. Besides, the protein atlas has indicated that the MOLT-4 cells can express the highest expression level of EZH2 among all the malignant cell lines.²⁵⁻²⁷ Our results have indicated that EZH2 mRNA expression is substantially upregulated in MOLT-4 cells compared to normal T cells. It has been shown that EZH2 is a critical factor in the development of various cancers, e.g., prostate,²⁷ breast,^{28,29} bladder,^{30,31} and colon cancers.^{32,33} Also, among the hematologic malignancies, elevated expression of EZH2 is a risk factor for MDS (myelodysplastic syndrome) development.³⁴

Despite the extensive research on EZH2, there have been controversies about its function. For example, it has been shown that a somatic gain-of-function mutation in the EZH2 gene can reflect the role of increased expression of EZH2 in B-cell lymphoma development.³⁵ In contrast, Ernst *et al.*³⁶ have suggested that the inactivation of EZH2 due to loss-of-function mutations plays a critical role in developing myeloid disorders. Another study has also linked EZH2 downregulation to the onset of MDS.³⁷ These results indicate that the role of EZH2 varies among different malignancies. The present study has indicated that EZH2 has an oncogenic role in T-ALL cells.

In the next step, we have hypothesized that the increased expression of EZH2 might affect the expression of specific TFs involved in the differentiation and maturation of T cells. Therefore, we transfected MOLT-4 cells with EZH2-siRNA. Our results have demonstrated that 48 hours after EZH2-siRNA transfection is the optimal time for downregulating EZH2 expression in MOLT-4 cells. Since after 48 hours of transfection, EZH2-siRNA gradually starts to be degraded, EZH2 mRNA expression begins to increase. Thus, the expression levels of TFs involved in the induction of T cell differentiation, i.e., NOTCH1, TCF1, IKZF1, and NFATC1, have been studied using qRT-PCR after 48 hours of EZH2-siRNA transfection. Although it is necessary to investigate the expression of the specific T cell maturation markers, we did not study these factors before and after EZH2-siRNA transfection; because this study has aimed to investigate the effect of EZH2 suppression on the expression of differentiation-inducing factors.

Also, the data obtained from the GeneMANIA database have shown that EZH2 had no remarkable relation with pro-apoptotic factors, like BAD, BAK, BID, and HRK. Consistent with this, our results have indicated that EZH2 knockdown has not altered apoptosis and viability of

MOLT-4 cells.

Various studies have investigated the role of EZH2 overexpression in the differentiation of different cells. Tanaka *et al.*³⁸ have reported that EZH2 overexpression can halt the differentiation of AML (acute myeloid leukemia) cells via the inhibition of Egr1; thus, EZH2 silencing can result in the differentiation of AML cells into chronic myelomonocytic leukemia-like cells. Yin *et al.*³⁹ have indicated that the enzymatic activity of EZH2 is essential for determining the fate of hematopoietic progenitor cells differentiation. EZH2 inhibition can upregulate IL-15 receptor (IL-15R) on the surface of hematopoietic progenitor cells, resulting in increased differentiation of these cells into NK (natural killer) cells.

Other studies have also investigated the role of EZH2 in non-hematopoietic cells. For instance, Jin *et al.*⁴⁰ have shown that EZH2, through direct suppression of NEUROG3 and KIT, can provide a balance between self-renewability and differentiation in spermatogonia cells. EZH2 can also inhibit apoptosis in spermatid cells; thus, EZH2 inhibition can stimulate apoptosis of these tumoral cells. Fujimura *et al.*⁴¹ have demonstrated that EZH2 depletion can cause a partial loss of PRC2 function and accelerate the differentiation of the glial cells. They have shown that EZH2 partial depletion can upregulate the expression of several TFs that regulate cell differentiation. Yu *et al.*⁴² have shown that specific EZH2 inhibition can increase the differentiation of human embryonic stem cells into mesenchymal stem cells and increase the expression of mesenchymal markers by suppressing H3K27me3. Collectively, EZH2 plays an essential role in regulating the differentiation of various cells. The current study has shown that suppressing EZH2 can upregulate the TFs involved in T cell differentiation and maturation.

Some studies have specifically focused on the increased expression of NOTCH1 under the influence of EZH2 knockdown.⁴³⁻⁴⁵ Chen *et al.*⁴⁶ have shown that EZH2 can be involved in the activating and silencing of NFATC1 expression. Although EZH2 can inhibit NFATC1 expression in the terminal stages of regenerative-associated pancreatic plasticity conditions, NFATC1 can be expressed in 79% of the pancreatic ductal adenocarcinoma cells expressing EZH2.⁴⁶ Collectively, these findings indicate that the effect of EZH2 on the expression of NFATC1 is context-dependent. Our results have shown that EZH2 knockdown can upregulate NFATC1 expression in MOLT-4 cells. Also, our study has demonstrated that EZH2 suppression can downregulate CD44, which is the marker of early T cells, and CD44 expression decreases along with lymphocyte differentiation.⁴⁷

Epigenetic machinery, which depends on the interaction between various stimulators, shapes a very controlled gene-expression network and regulates the expression of different genes. Overall, our study has indicated that the EZH2 knockdown can upregulate the expression of T cell-differentiating TFs, i.e., TCF1, IKZF1, NOTCH-1, and NFATC1. Also, EZH2 silencing can downregulate CD44,

which is an immature marker of lymphoid cells. Indeed, our results provide new insight into the role of EZH2 as an epigenetic modifier in T-ALL biology.

Conclusion

As a transcription factor, EZH2 regulates the expression of many genes in physiological states. However, its aberrant expression is involved in the pathogenesis of various cancers. Our results have indicated that EZH2 is upregulated in MOLT-4 cells, and its knockdown increases the expression of T cell differentiation factors, including NOTCH1, TCF1, IKZF1, and NFATC1. Also, EZH2 silencing substantially downregulates CD44 expression in MOLT-4 cells. Therefore, EZH2 can substantially inhibit T cell differentiation in T-ALL.

Author Contributions

Conceptualization: SS and BB; methodology and formal analysis: BM; bioinformatics: NM; writing and reviewing the article: MF; editing: MAS and DS. Investigation: SS and EB, ES, and MA. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors report no conflicts of interest.

References

- Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Rev.* 2018;32(1):36-51. doi:10.1016/j.blre.2017.08.006
- Attarbaschi A, Mann G, Dworzak M, Wiesbauer P, Schrappe M, Gadner H. Mediastinal mass in childhood t-cell acute lymphoblastic leukemia: Significance and therapy response. *Med Pediatr Oncol.* 2002;39(6):558-65. doi:10.1002/mpo.10164
- Oruganti SR, Torres DJ, Krebsbach S, Asperti-Boursin F, Winters J, Matlawska-Wasowska K, et al. Carma1 is a novel regulator of t-all disease and leukemic cell migration to the CNS. *Leukemia* 2016;31:255. doi:10.1038/leu.2016.272
- Chong CY, Tan AM, Lou J. Infections in acute lymphoblastic leukaemia. *Ann Acad Med Singap.* 1998;27(4):491-5.
- Sundaresh A, Williams O. Mechanism of etv6-runx1 leukemia. *Adv Exp Med.* 2017;962:201-16. doi:10.1007/978-981-10-3233-2_13
- Churchman ML, Mullighan CG. Ikaros: Exploiting and targeting the hematopoietic stem cell niche in b-progenitor acute lymphoblastic leukemia. *Exp Hematol.* 2017;46:1-8. doi:10.1016/j.exphem.2016.11.002
- Olsson L, Johansson B. Ikaros and leukaemia. *Br J Haematol.* 2015;169(4):479-91. doi:10.1111/bjh.13342
- Potluri S, Coleman D, Bonifer C. Pharmacological inhibition of aberrant transcription factor complexes in inversion 16 acute myeloid leukemia. *Stem Cell Investig.* 2018;5:30. doi:10.21037/sci.2018.09.03
- Cardenas H, Zhao J, Vieth E, Nephew KP, Matei D. Ezh2 inhibition promotes epithelial-to-mesenchymal transition in ovarian cancer cells. *Oncotarget.* 2016;7(51):84453-67. doi:10.18632/oncotarget.11497
- Fardi M, Solali S, Farshdousti Hagh M. Epigenetic mechanisms as a new approach in cancer treatment: An updated review. *Genes Dis.* 2018;5(4):304-11. doi:10.1016/j.gendis.2018.06.003
- Asadi M, Shanebandi D, Zarintan A, Pedram N, Baradaran B, Zafari V, et al. Tp53 gene pro72arg (rs1042522) single nucleotide polymorphism as not a risk factor for colorectal cancer in the iranian azari population. *Asian Pac J Cancer Prev.* 2017;18(12):3423-7. doi:10.22034/apjcp.2017.18.12.3423
- Safaei S, Baradaran B, Hagh MF, Alivand MR, Talebi M, Gharibi T, et al. Double sword role of ezh2 in leukemia. *Biomed. Pharmacother.* 2018;98:626-35. doi:10.1016/j.biopha.2017.12.059
- Lund K, Adams PD, Copland M. Ezh2 in normal and malignant hematopoiesis. *Leukemia.* 2014;28(1):44-9. doi:10.1038/leu.2013.288
- Sashida G, Iwama A. Multifaceted role of the polycomb-group gene ezh2 in hematological malignancies. *Int J Hematol.* 2017;105(1):23-30. doi:10.1007/s12185-016-2124-x
- Shirafkan N, Shomali N, Kazemi T, Shanebandi D, Ghasabi M, Baghbani E, et al. MicroRNA-193a-5p inhibits migration of human ht-29 colon cancer cells via suppression of metastasis pathway. *J Cell Biochem.* 2018;120(5):8775-83. doi:10.1002/jcb.28164
- Franz M, Rodriguez H, Lopes C, Zuberi K, Montojo J, Bader GD, et al. Genemania update 2018. *Nucleic Acids Res.* 2018;46(W1):W60-W4. doi:10.1093/nar/gky311
- Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. String v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2018;47(D1):D607-D13. doi:10.1093/nar/gky1131
- Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: Tool for the unification of biology. The gene ontology consortium. *Nat Genet.* 2000;25(1):25-9. doi:10.1038/75556
- Consortium GO. The gene ontology resource: 20 years and still going strong. *Nucleic Acids Res.* 2018;47(D1):D330-D8.
- Margueron R, Reinberg D. The polycomb complex prc2 and its mark in life. *Nature* 2011;469(7330):343-9. doi:10.1038/nature09784
- Di Croce L, Helin K. Transcriptional regulation by polycomb group proteins. *Nat Struct Mol Biol.* 2013;20(10):1147-55. doi:10.1038/nsmb.2669
- Xie H, Xu J, Hsu JH, Nguyen M, Fujiwara Y, Peng C, et al. Polycomb repressive complex 2 regulates normal

- hematopoietic stem cell function in a developmental-stage-specific manner. *Cell Stem Cell*. 2014;14(1):68-80. doi:10.1016/j.stem.2013.10.001
23. Good-Jacobson KL. Regulation of germinal center, b-cell memory, and plasma cell formation by histone modifiers. *Front. Immunol*. 2014;5:596. doi:10.3389/fimmu.2014.00596
 24. Mandal M, Powers SE, Maienschein-Cline M, Bartom ET, Hamel KM, Kee BL, et al. Epigenetic repression of the igk locus by stat5-mediated recruitment of the histone methyltransferase ezh2. *Nat Immunol*. 2011;12(12):1212-20. doi:10.1038/ni.2136
 25. Chiaretti S, Foa R. T-cell acute lymphoblastic leukemia. *Haematologica*. 2009;94(2):160-2. doi:10.3324/haematol.2008.004150
 26. Soen B, Vandamme N, Berx G, Schwaller J, Van Vlierberghe P, Goossens S. Zeb proteins in leukemia: Friends, foes, or friendly foes? *Hemasphere*. 2018;2(3):e43. doi:10.1097/hs9.0000000000000043
 27. Yang YA, Yu J. EZH2, an epigenetic driver of prostate cancer. *Protein Cell* 2013;4(5):331-41. doi:10.1007/s13238-013-2093-2
 28. Pourakbar S, Pluard TJ, Accurso AD, Farassati F. EZH2, a novel target in detection and therapy of breast cancer. *Onco Targets Ther*. 2017;10:2685-7. doi:10.2147/ott.s138777
 29. Klee CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proc Natl Acad Sci U S A*. 2003;100(20):11606-11. doi:10.1073/pnas.1933744100
 30. Arisan S, Buyuktuncer ED, Palavan-Unsal N, Caskurlu T, Cakir OO, Ergenekon E. Increased expression of EZH2, a polycomb group protein, in bladder carcinoma. *Urol Int*. 2005;75(3):252-7. doi:10.1159/000087804
 31. Martinez-Fernandez M, Rubio C, Segovia C, Lopez-Calderon FF, Duenas M, Paramio JM. EZH2 in bladder cancer, a promising therapeutic target. *Int J Mol Sci*. 2015;16(11):27107-32. doi:10.3390/ijms161126000
 32. Chen Z, Yang P, Li W, He F, Wei J, Zhang T, et al. Expression of EZH2 is associated with poor outcome in colorectal cancer. *Oncol Lett*. 2018;15(3):2953-61. doi:10.3892/ol.2017.7647
 33. Ramaglia M, D'Angelo V, Iannotta A, Di Pinto D, Pota E, Affinita MC, et al. High EZH2 expression is correlated to metastatic disease in pediatric soft tissue sarcomas. *Cancer Cell Int*. 2016;16(1):59. doi:10.1186/s12935-016-0338-x
 34. Xu F, Li X, Wu L, Zhang Q, Yang R, Yang Y, et al. Overexpression of the EZH2, RING1 and BMI1 genes is common in myelodysplastic syndromes: Relation to adverse epigenetic alteration and poor prognostic scoring. *Ann Hematol*. 2011;90(6):643-53. doi:10.1007/s00277-010-1128-5
 35. Morin RD, Johnson NA, Severson TM, Mungall AJ, An J, Goya R, et al. Somatic mutations altering EZH2 (tyr641) in follicular and diffuse large b-cell lymphomas of germinal-center origin. *Nat Genet*. 2010;42(2):181-5. doi:10.1038/ng.518
 36. Ernst T, Chase AJ, Score J, Hidalgo-Curtis CE, Bryant C, Jones AV, et al. Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nat Genet*. 2010;42(8):722-6. doi:10.1038/ng.621
 37. Nikoloski G, Langemeijer SMC, Kuiper RP, Knops R, Massop M, Tönnissen ERLTM, et al. Somatic mutations of the histone methyltransferase gene ezh2 in myelodysplastic syndromes. *Nat Genet*. 2010;42:665. doi:10.1038/ng.620
 38. Tanaka S, Miyagi S, Sashida G, Chiba T, Yuan J, Mochizuki-Kashio M, et al. Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia. *Blood*. 2012;120(5):1107-17. doi: 10.1182/blood-2011-11-394932
 39. Yin J, Leavenworth JW, Li Y, Luo Q, Xie H, Liu X, et al. Ezh2 regulates differentiation and function of natural killer cells through histone methyltransferase activity. *Proc Natl Acad Sci U S A*. 2015;112(52):15988-93. doi:10.1073/pnas.1521740112
 40. Jin C, Zhang Y, Wang ZP, Wang XX, Sun TC, Li XY, et al. EZH2 deletion promotes spermatogonial differentiation and apoptosis. *Reproduction*. 2017;154(5):615-25. doi:10.1530/rep-17-0302
 41. Fujimura N, Kuzelova A, Ebert A, Strnad H, Lachova J, Machon O, et al. Polycomb repression complex 2 is required for the maintenance of retinal progenitor cells and balanced retinal differentiation. *Dev Biol*. 2018;433(1):47-60. doi:10.1016/j.ydbio.2017.11.004
 42. Yu Y, Deng P, Yu B, Szymanski JM, Aghaloo T, Hong C, et al. Inhibition of EZH2 promotes human embryonic stem cell differentiation into mesoderm by reducing h3k27me3. *Stem Cell Reports*. 2017;9(3):752-61. doi:10.1016/j.stemcr.2017.07.016
 43. Simon C, Chagraoui J, Kros J, Gendron P, Wilhelm B, Lemieux S, et al. A key role for EZH2 and associated genes in mouse and human adult t-cell acute leukemia. *Genes Dev*. 2012;26(7):651-6. doi:10.1101/gad.186411.111
 44. Acharyya S, Sharma SM, Cheng AS, Ladner KJ, He W, Kline W, et al. TNF inhibits Notch-1 in skeletal muscle cells by Ezh2 and DNA methylation mediated repression: implications in duchenne muscular dystrophy. *PLoS One*. 2010;5(8):e12479. doi: 10.1371/journal.pone.0012479
 45. Wang S, Cai L, Zhang F, Shang X, Xiao R, Zhou H. Inhibition of ezh2 attenuates sorafenib resistance by targeting notch1 activation-dependent liver cancer stem cells via notch1-related micrnas in hepatocellular carcinoma. *Transl Oncol*. 2020;13(3):100741. doi:10.1016/j.tranon.2020.01.002
 46. Chen NM, Neesse A, Dyck ML, Steuber B, Koenig AO, Lubeseder-Martellato C, et al. Context-dependent epigenetic regulation of nuclear factor of activated t cells 1 in pancreatic plasticity. *Gastroenterology*. 2017;152(6):1507-20. doi:10.1053/j.gastro.2017.01.043

47. Schumann J, Stanko K, Schliesser U, Appelt C, Sawitzki B. Differences in CD44 surface expression levels and function discriminates IL-17 and IFN- γ producing helper T cells. *PLoS One*. 2015;10(7):e0132479. doi:10.1371/journal.pone.0132479