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**EZH2 knockdown upregulates expression of the genes involved in T-ALL cell differentiation**

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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.

Objective: 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: Our results have shown that 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.

Keywords: EZH2, Leukemia, T-ALL, MOLT-4, Transcription Factor
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. 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, like the central nervous system. Besides, tumoral lymphoblasts can pave the way for the development of severe infectious diseases. 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.

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. 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 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 tri-methylating 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.
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 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%. 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.

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

| Cat. Number | Strand       | Sequence (3’–5’)          |
|-------------|--------------|---------------------------|
| sc-35312A   | Sense        | GGAAAGAACGGAAUCUUAtt      |
|             | Antisense    | UAAGAUUUCGUUCUUCtt        |
| sc-35312B   | Sense        | GAAGCCAAUAUUGAACCtt      |
|             | Antisense    | AGGUUCAAUUUUGCUUCtt      |
| sc-35312C   | Sense        | GAAGAGGGAAAGUGUAUGAtt    |
|             | Antisense    | UCAUACACUUUCCUCUUCtt    |

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.

Table 2. Primer sequences

| Gene/Accession Number | FW/RV | Sequence 5’→ 3’ | Product size |
|-----------------------|-------|-----------------|--------------|
| EZH2                  | FW    | CCTGAAGTGATGTCGCGCATCGAAAGAG | 252          |
| NM_004456.5            |       |                 |              |
| EZH2                  | RV    | TGCAAAAAATTCCTGGTACAAAAACACT | 252          |
| NM_004456.5            | FW    | AGCACCAAGAATCCACCACA | 151          |
| TCF-1(TCF7)            |       |                 |              |
| Gene     | Accession | Primer Direction | Primer Sequence                  | Length |
|----------|-----------|------------------|----------------------------------|--------|
| TCF-1(TCF7) | NM_001366502.2 | RV               | CAGCAGATGGGTATGGGTTA             | 151    |
| IKZF1    | NM_001291837.2 | RV               | ACTCCCGACAAAGCCGAGC             | 172    |
| NOTCH1   | NM_017617.5   | RV               | AACATCTTGGGACGCTCTGG            | 167    |
| NFATC1   | NM_001278675.2 | RV               | CGCTCATGTTACGGCTTAC             | 287    |
| CD44     | NM_001202555.2 | RV               | ATCCAAGTGAGGGACTACACAG          | 216    |
| GAPDH    | NM_001357943.2 | RV               | CCCATCAGGCCACAGTTTCC           | 166    |
|          |            |                  |                                  |        |
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 to investigate the effect of EZH2 knockdown on apoptosis. Briefly, MOLT-4 cells at the density of 1×10⁶ 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 µL) 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 µL 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 ± 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](http://genemania.org)) is a bioinformatics database for predicting genes 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 expressional 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 (-log₁₀ P-value >1.301029996) was set as the cut-off.

**Results:**

**EZH2 expression in the MOLT-4 cells is higher than 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) (Fig. 1). This suggests that EZH2 upregulation might be involved in T-ALL development.
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).

**EZH2-siRNA can downregulate EZH2 mRNA expression 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., 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) (Fig. 2).
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 ± 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).
EZH2 knockdown can upregulate the mRNA expression of NOTCH1, TCF1, IKZF1, and NFATC1 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) (Fig. 3).
Figure 3. The effect of EZH2 knockdown on the mRNA expression of NOTCH1, TCF1, IKZF1, and NFATC1 in MOLT-4 cells; 48 hours after transfection with 80 pmol of EZH2-siRNA, the mRNA expression of NOTCH1, TCF1, IKZF1, and NFATC1 genes were investigated by qRT-PCR using internal control of GAPDH. The results were presented as the mean ± SD of (n=3) (**P<0.01, ***P<0.001, and ****P<0.0001).

**EZH2 has no remarkable relation with 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 (Fig. 4).

![GeneMANIA network diagram](image)

**Figure 4. EZH2 had no remarkable relation with pro-apoptotic factors.**

**EZH2 silencing has not decreased the viability of MOLT-4 cells.**

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 (Fig. 5).

![MTT assay results](image)
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 ± SD.

**EZH2 knockdown has not altered apoptosis in MOLT-4 cells.**

Annexin V-FITC and PI double staining were used to investigate the effect of EZH2-siRNA on the apoptosis of MOLT-4 cells. Following 48 hours of transfecting MOLT-4 cells with 80 pmol EZH2-siRNA, EZH2 has not substantially altered the apoptosis of MOLT-4 cells (Fig. 6).
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 silencing can result in CD44 downregulation.

To investigate the rate of T cell differentiation after EZH2 knockdown, flow cytometry and qRT-PCR were used to analyze CD44 expression. Our results have indicated that EZH2 knockdown can significantly decrease surface CD44 and CD44 mRNA expression in MOLT-4 cells (P-value<0.01 and P-vlau<0.05, respectively) (Fig. 7 and Fig. 8, respectively). Thus, EZH2 silencing can increase cellular differentiation of MOLT-4 cells via upregulating CD44 expression.
Figure 7. The flow cytometry analysis of cellular differentiation in MOLT-4 cells transfected with EZH2-siRNA. EZH2 silencing downregulated CD44 surface expression in MOLT-4 cells (**P<0.01).
EZH2 has remarkable 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 (Fig. 8).
The studied genes are located in the nucleoplasm and regulate the RNA polymerase II-mediated transcription. 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 -log10 P-value= 4.32514) and they regulate RNA polymerase II-mediated transcription (P-value= 1.72×10^{-6} or -log10 P-value= 5.76447) (Figure 9).
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, IKFZ1, NOTCH1, TCF1, NFATC1 productions.

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 trimethylation 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.\textsuperscript{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, 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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \textsuperscript{27}, breast \textsuperscript{28,29}, bladder \textsuperscript{30,31}, and colon cancers.\textsuperscript{32,33} Also, among the hematologic malignancies, elevated expression of EZH2 is a risk factor for MDS (myelodysplastic syndrome) development.\textsuperscript{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.\textsuperscript{35} In contrast, Ernst et al. have suggested that the inactivation of EZH2 due to loss-of-function mutations plays a critical role in developing myeloid disorders.\textsuperscript{36} Another study has also linked EZH2 downregulation to the onset of MDS.\textsuperscript{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. 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.\textsuperscript{38} Yin et al. 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.\textsuperscript{39}

Other studies have also investigated the role of EZH2 in non-hematopoietic cells. For instance, Jin et al. 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.\textsuperscript{40} Fujimura et al. 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.\textsuperscript{41} Yu et al. 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.\textsuperscript{42} 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.\textsuperscript{43-45} Chen et al. 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.\textsuperscript{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.\textsuperscript{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.

**Ethics Approval and Consent to Participate**

Not applicable.
Human and Animal Rights
No animals/humans were used for studies that are the basis of this research.

Consent for Publication
Not applicable.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

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Authors' contributions
Conceptualization: Saeed Solali and Behzad Baradaran; methodology and formal analysis: Behzad Mansoori; bioinformatics: Nima Hemmat; writing and reviewing the article: Masoumeh Fardi; editing: Mahdi Abdoli Shadbad and Dariush Shanehbandi. Investigation: Sahar Safaee and Elham Baghbane, Elham Safar Zadeh, and Mohammad Amini. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest:
The authors declare that there are no conflicts of interest.

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