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Original Article

Induction of Cell Death in U937 Myeloid Leukemia Cell Line Co-cultured with Genetically Engineered Adipose-derived Stem Cells Expressing a High Level of Bone Morphogenetic Protein 4 through miR-424-5p

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Abstract

Introduction: Acute myeloid leukemia (AML) is a heterogeneous and complex malignancy characterized by rapid cellular proliferation, an aggressive clinical course, and generally high mortality. Transforming growth factor-beta (TGF-β) plays a significant role in tumorigenesis. Bone morphogenetic protein 4 (BMP4) is a member of the TGF-β superfamily whose expression is mainly controlled by the Smad signaling pathway. Studies have shown that this protein can control and induce the expression of some microRNAs during the cancer treatment process. MiR-424-5p plays an essential role in various types of cancer at different stages of tumorigenesis, including the promotion and/or inhibition of tumorigenesis, regulation of tumor development in the tumor microenvironment, and influencing cancer chemotherapy outcomes. The aim of this study was to evaluate the induction of cell death in the U937 myeloid leukemia cell line co-cultured with genetically engineered adipose-derived stem cells (ADSCs) expressing a high level of BMP4 through miR-424-5p.

Materials and Methods: The induction of cell death and apoptosis in the U937 cell line was assessed using MTT and Annexin V/ PI assays, respectively. The expression of miR-424 and TGF-β was determined in the co-culture system using real-time PCR.

Results: The results of MTT and Annexin V/ PI assays showed that BMP4-expressing adipose-derived stem cells (ADSCs) induced cell death in the U937 cells in the co-culture system. Co-culture of engineered ADSCs with U937 cell line led to the downregulation of *miR-424* and *TGF-β* genes in U937 cells.

Conclusions: In the current study, a new strategy based on BMP4 induction was designed to significantly suppress the cell viability of the leukemia cell line U937. It appears that the use of engineered ADSCs could be useful for the treatment of hematological malignancies.

Keywords: Acute Myeloid Leukemia, Transforming Growth Factor-beta, Bone Morphogenetic Protein 4, MiR-424-5p, Adipose-derived Stem Cells

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Introduction

The type of treatment and the prognosis for leukemia vary depending on the stage and advancement of the disease.[1] Leukemia occurs in 35-40% of cases under the age of 60, while it affects 5-15% of individuals over the age of 60.[2] A majority of cases diagnosed with leukemia are resistant to conventional therapies or have a relapse after the treatment course. Treatment methods can include bone marrow transplantation, radiotherapy and the use of chemotherapy agents such as Daunorubicin.[3] In addition, new treatments are in the stages of clinical trials, which include the use of targeted drugs such as tyrosine kinase signaling inhibitors, as well as gene and cell-based therapies.[4] A new therapeutic approach in this field of treatment is based on genetic manipulation of normal cells to induce the secretion of specific protein factors, including growth factors that inhibit the growth and migration of cancer cells. Studies have shown that genetically engineered cells capable of high expression of bone morphogenetic protein 4 (BMP4) can be used to transform cancer cells into cells sensitive to chemotherapy drugs.[5] Acute myeloid leukemia (AML) is a heterogeneous and complex disease characterized by rapid cell reproduction, an invasive clinical course, and generally widespread mortality.[6] Changes in the expression level of TGF-β have been observed in different types of human cancer such as AML. In AML, the presence of two distinct mutations in the *Smad4* gene, a missense mutation and a frameshift mutation, have been shown to impair its ability to enhance TGF-β transcriptional activity. In previous studies, it has been found that the expression of TGF-β1 is decreased in AML patients compared to healthy individuals.[7] BMP4

belongs to the bone morphogenetic protein family and the TGF-β superfamily transporter, which was initially recognized as an osteogenesis-inducing factor. It factor induces the differentiation of human mesenchymal stromal cells (hMSCs) into the osteogenic lineage and enhances the activity of mature osteoblasts.[8] Similar to TGF-β, the expression of BMP4 is mainly induced by the Smad signaling pathway. It has been shown that this factor can effectively control the expression of some microRNAs that are effective in the cancer process. In addition, various studies have shown the role of this factor in controlling the behavior of hematopoietic stem cells (HSCs), especially in regulating their proliferation and differentiation.[9] Therefore, the reduction of BMP4 expression in HSCs-AML can affect the activity of these cells.[9] Accordingly, BMP4 can regulates the number and function of HSCs and directly affects hematopoiesis.[10]

MicroRNAs (miRNAs) are small (18-22 nucleotides) non-coding RNAs possessing critical functions in numerous natural processes, such as proliferation, development, apoptosis, and cell isolation.[11] More importantly, it is suggested that the development and progression of cancer be associated with the aberrant upregulation or downregulation of specific miRNAs and their targets in multiple types of cancer. In other words, certain histotypes of cancer could be classified based on the miRNA expression profile.[12,13] MiR-424 is a part of the family of miR15/107, which is involved in cell cycle regulation, epithelial-mesenchymal transition (EMT), differentiation, hypoxia, proliferation, apoptosis, angiogenesis, as well as drug resistance and sensitivity.[14] MiR-424 plays an important role in promoting monocyte differentiation in a number of leukemia cell lines.[15] Accordingly, the overexpression of miR-424 is capable of promoting monocytic differentiation in U937 cells as a human hematopoietic cell line established from a generalized histiocytic carcinoma, displaying several parcels of immature monocytic cells.[16] Since its establishment, the U937 cell line has been considerably employed as an important *in vitro* model for the study of hematopoietic cell isolation, blood cancer, and cancer therapeutics.[17]

On other hand, MiR-424-5p plays a critical role in different types of cancer at various stages of tumors, including the promoting and/or inhibition of tumorigenesis, the regulation of tumor development in the tumor microenvironment, and the effectiveness of cancer chemotherapy. The downregulation of miR-424-5p has been reported in cervical cancer,[18] epithelial ovarian cancer,[19] and liver cancer.[20] MiR-424-5p can function as a tumor repressor in these malignancies, while miR-424-5p is significantly upregulated in pancreatic cancer.[21] MiR-424-5p has been preliminarily reported to be dysregulated in colorectal cancer,[22] non-small cell lung cancer,[23] esophageal squamous cell carcinoma,[24] and diffusion of large B cells.[25] In hematological malignancies, miRNA-424-5p has been proven to enhance HOXA expression according to bioinformatics analysis. This effect shows that miRNA-424-5p can be oncogenic and promote the proliferation and migration of cancer cells in acute myeloid leukemia.[26]

In this study, which is based on cell therapy and changes in the process of cell proliferation, an attempt has been made to induce the cell death in AML-resistant cancer cells by using recombinant BMP4 protein secreted from genetically engineered adipose-derived stem cells (ADSCs).

Materials and Methods

Cell Isolation and Culture

In this experiment, male adipose tissues were obtained from nine men with an age range of 25-40 who underwent liposuction surgery in Imam Khomeini Hospital. Informed consent was obtained from all participants. The collected tissues were rinsed several times with phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin (Gibco, Germany). The isolated tissues were also sectioned into small pieces, treated with an equal volume of 0.075% type I collagenase (Sigma, Germany), and subjected to a constant agitation at 37 °C for 1 h. The enzyme inactivation was performed by Dulbecco's Modified Eagle Medium (DMEM) high glucose without glycerophosphate (Sigma, Belgium) supplemented with 10% fetal bovine serum (Gibco, UK). The resulting solution was centrifuged at 1200 g for 10 min to gather a high-viscosity cell pellet (Clinical Benchtop Centrifuges). The obtained supernatant was discarded, and the pellet was mixed with stromal vascular fraction (SVF) and 2 ml DMEM. The suspended cells were passed through a 100 µm nylon filter mesh (Falcon Company, USA) and also cultured in DMEM containing 10% fetal bovine serum (FBS) and incubated at 37 °C in a 5% CO_2 incubator. The cell culture medium was replaced with a fresh one every two days.

Characterization of Adipose-derived Stem Cells by Flow Cytometry

The isolated adipose-derived stem cells (ADSCs) were washed three times with ice-cold PBS. Then, the cells were fixed with fresh 4% paraformaldehyde solution (pH 7.2) at room temperature for 5 min. In order to block nonspecific bindings, cells were washed with 10% BSA/PBS for 30 min and rinsed three times again in PBS, and finally incubated with mouse anti-human CD73 (Abcam, Germany), Rabbit anti-human CD105 (Abcam, Germany), Rabbit anti-human CD34 (Abcam, Germany), and rabbit anti-human CD45 (Abcam, Germany) as primary antibodies at 4 °C for 1 h. Then, samples were rinsed three times with PBS and incubated with rabbit anti-mouse IgG conjugated with FITC as a secondary antibody (at a ratio of 1:100) at 37 °C for 30 min in the dark. Next, the cells were washed two times in PBS and analyzed by flow cytometry (Olympus, Japan).

Adipogenic Differentiation

The isolated ADSCs at passage 4 were exposed to the adipogenic conservation medium containing 50 µg/ml indomethacin, 50 µg/ml ascorbic acid, and 100 nM dexamethasone (all chemicals were from Sigma, Germany) for 21 days. The medium was changed every three days, and the adipogenic differentiation was examined using the Oil Red O (Sigma, Germany) staining method. Afterward, the adipogenic medium was removed, and the cells were washed three times in PBS, also fixed by immersing into 10% formalin for 30-60 min at room temperature. In addition, the cells were washed with distilled water and treated with 2 ml isopropanol (60%) for 5 min. Next, isopropanol was discarded, and the cells were stained with Oil Red O (2 ml to each well) at room temperature for 5 min. Finally, the cells were washed with tap water and visualized under an inverted microscope (Olympus, Japan).

Osteogenic Differentiation

The culture medium of ADSCs was changed to the osteogenic conservation medium containing 10 mM β-glycerophosphate, 0.2 mM ascorbic acid, and 7-10 M dexamethasone (all chemicals were from Sigma, UK), and the cells were maintained in this medium for 21 days. The cell culture medium was replaced with the fresh differentiating medium every three days. In order to confirm the differentiation of ADSCs into osteogenic cells, Alizarin Red S staining was conducted. In this assay, the osteogenic medium was discarded, and the cells were washed with PBS three times. The cells were also fixed in 70% ethanol at 4 °C for 1 h. Next, cells were washed with deionized water and air-dried. The fixed cells were stained with 2% Alizarin Red S (pH 7.2, Sigma) at 37 °C for 1 h, washed in deionized water, and examined under an inverted microscope (Olympus, Japan).

Preparation of BMP4-expressing Lentiviral Vectors

The Lenti-Pac™ HIV Expression Packaging Kit containing a lentiviral vector expressing BMP4 and a blank lentiviral vector as a control vector (abmGood. Co, Canada) were used. All vectors were independently transferred into *E. coli* DH5α to achieve high copies of vectors needed for cell transfection. The bacterial culture medium was changed with fresh overnight-grown DH5α bacteria according to standard protocols. The purified lentiviral vectors were kept at -70 °C until usage.

Protocol of Extraction of Vectors from E. coli

Bacterial cells were lysed using a French press, and inclusion bodies in the cell lysate were pelleted by low-speed centrifugation. The pellet fraction was washed (preextracted) with urea and Triton X-100 to remove *E. coli* membrane and cell wall material. Guanidine hydrochloride (8 M) and dithiothreitol (DTT) were used to solubilize the washed pellet protein. Extraction with the denaturant simultaneously dissociates protein-protein interactions and unfolds the protein. As a result, the extracted protein consists (ideally) of unfolded monomers, with sulfhydryl groups (if present) in the reduced state.

Study Design

In this study, three cell groups were employed to analyze the impact of the co-culture of ADSCs with the U937 cell line on apoptosis induction in cancer cells. These groups included (1) U937 cells, (2) ADSCs+U937 co-culture, and (3) B-ADSCs+U937 co-culture. B-ADSCs imply transfected ADSCs expressing BMP4 with the aid of lentiviral vectors.

Cell Cytotoxicity Assay

The cell viability was measured using the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2 and 5-diphenyl-tetrazolium bromide, Atocel, Austria) assay to determine the cytotoxicity of BMP4- expressing lentiviral vectors against ADSCs. In this assay, 1×10^4 cells were seeded on 96 well-plates and incubated at 37 °C overnight to allow the cells to adhere. ADSCs were transfected with various concentrations (0, 0.25, 0.5, and 1 µg) of the BMP4-expressing lentiviral vector. After the transfection procedure, ADSCs were incubated with the MTT solution (5 mg/ml) for 4 h at 37 °C. Afterward, the cell culture medium was removed, and 100 µl dimethyl sulfoxide (DMSO; Merck, Germany) was added to each well to solubilize formazan crystals. Next, the optical absorbance of samples was measured using an ELISA reader instrument (Bio-Rad Laboratories, USA) at a wavelength of 570 nm. The viability of ADSCs was estimated by comparing the absorbance of treated cells with control cells.

ADSC Transfection

For transfection of ADSCs with lentiviral vectors, 1×10^6 ADSCs were seeded on a 6-well plate containing DMEM medium supplemented with 10% FBS. In addition, the cells were incubated in a 5% CO_2 incubator at 37 °C for 24 h. The next day, the cell culture medium of the growing cells was replaced with a prepared DMEM medium supplemented with 2% FBS. The cell transfection was performed with lentiviral vectors using lipofectamine 3000 (Invitrogen, USA). After 24 h, the green fluorescent protein (GFP) activity was monitored in transfected ADSCs using fluorescent microscopy (Labo Med, USA). The GFP activity was considered an internal control.

Co-culture of U937 Cells with ADSCs

The U937 cell line was purchased from the Pasteur Institute of Iran and cultured in DMEM- medium (Sigma, Belgium) supplemented with 10% FBS (Gibco, UK). In order to perform the co-culture system, transfected ADSCs expressing BMP4 were seeded at a density of 5×10^4 cells/ well onto a 24-well plate and incubated at 37 °C for 24 h. The next day,

the U937 cells at a density of 1 × 10^5 were added to transfected ADSCs and allowed to grow in DMEM- medium supplemented with 10% FBS.

Annexin-V Staining

The U937 cell line, ADSCs, and B-ADSCs were collected after the co-culture process and washed once with a culture medium supplemented with 2 mM $CaCl_2$ in the absence of phenol red. After discarding the supernatant, 5 µl Annexin-V conjugate was added to the residual volume (roughly 130 µl). After incubation for 45 min at room temperature, the cells were washed two times and suspended in the same medium containing 1 µg/ ml phosphatidyl inositol (PI). The samples were assessed using FACS Caliber (BD Biosciences, San Jose, CA, USA).

RNA Isolation and RT-qPCR

The total RNA was extracted from the U937 cell line by Qiazol (Qiazol lysis reagent, USA) according to the manufacturer instruction. Next, the concentration and purity of the extracted RNA were determined using a NanoDrop ND-100 spectrophotometer (Thermo Scientific, Waltham, MA, USA). After that, RNA was reverse-transcribed into cDNA using the Revert-Aid cDNA Synthesis Kit (Fermentas, Germany; 25 µl). In this experiment, 500 ng of the newly synthesized cDNA was used to evaluate the relative gene expression. The PCR reactions were conducted in a total volume of 25 µl containing 12.5 µl SYBR Green Premix 2X (Takara, Shiga, Japan) and 10 pM mixed primers. Thermocycling conditions were 95 °C for 10 s, followed by 40 cycles of denaturation at 94 °C for 5 s, as well as the annealing at 60 °C and extension processes at 72 °C for 30 s. The Primer3 software was utilized to design the specific primers. The specificity of the designed primers was determined by the NCBI BLAST Tool, and the primer sequences are listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was applied to determine the relative expression of the *BMP4* gene, its cognate receptor, and the *TGF-β* gene. The presence of miR-424 was also detected using RT2 miRNA First-Strand Kit (SA Biosciences). The specific primers for miR-424 were purchased from QIAGEN

Table1. The Sequence of Primers

Genes	Sequences (5´- 3´)
BMP4	F: CGGGACAGGAAGAAGAATAAGAA
	R: GAATGGTTGGTTGAGTTGAGGTG
BMPR	F: AGATGACCAGGGAGAAACCAC
	R: CAACATTCTATTGTCCGGCGTA
TGF-β	F: CCCAGCATCTGCAAAGCTC
	R: GTCAATGTACAGCTGCCGCA
GAPDH	F: GCAGGGATGATGTTCTGG
	R: CTTTGGTATCGTGGAAGGAC
miR-424-5p	F: GGCCAGCAGCAATTCATGTT
	R: GTGCAGGGTCCGAGGT
	Stem Loop: GTC GTA TGC AGT GCA GGG TCC GAG GTA TTC GCA CTG CAT ACG ACT TCA

to carry out real-time PCR. The relative expression was assessed using the relative Ct method ($2^{-\Delta\Delta Ct}$). The Ct values of samples were compared with the Ct value of the internal control gene (*GAPDH* gene). The real-time PCR reactions were performed on the ABI 7500 FAST real-time PCR platform. All experiments were performed in triplicate. The specificity of PCR products was also examined by electrophoresis and melting curve analysis.

Statistical Analysis

All experiments were conducted in triplicate, and the results were represented as the means and standard deviation (mean ± SD). The difference between the experimental groups was analyzed using one- way analysis of variance (ANOVA) followed by Tukey's post hoc test. The level of the statistical significance was set at $p<0.05$.

Results

ADSCs and U937 Cell Culture

The morphology of the attached ADSCs is similar to that of bone marrow mesenchymal stem cells with rapid proliferation, as shown in Figure 1. In the early hours, the cells were floating, and the nucleus was visible. After 24 h, the floated cells adhered to the dish to form fibroblast-like colonies. The loaded ADSCs formed a spindle-like shape (fibroblast-like) with several lipid granules inside them. After the first passage, the cells showed extended proliferative capacity. After the fourth passage and 13 days of incubation, the ADSCs were used for the differentiation analysis (Figure 1A). In addition, the image on the right represents U937 cells exhibiting a round shape (Figure 1B).

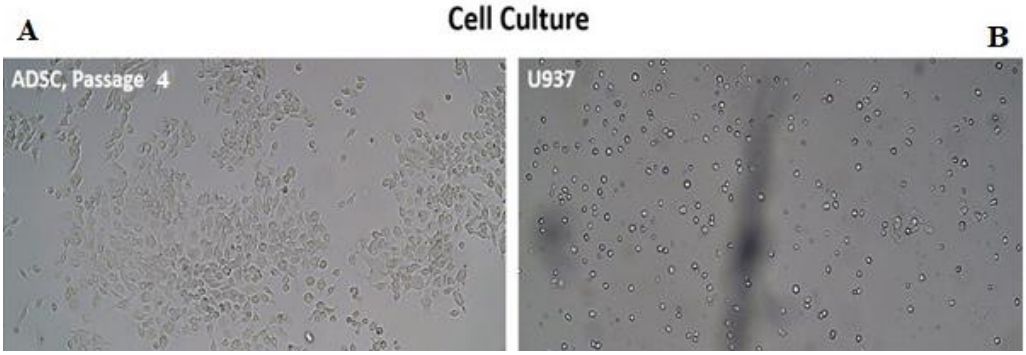

Figure 1. ADSCs and U937 Cell Culture. After the fourth passage and 13 days of incubation, the ADSCs were used for the differentiation analysis **(A)**; the image on the right represents U937 cells exhibiting a round shape **(B)**.

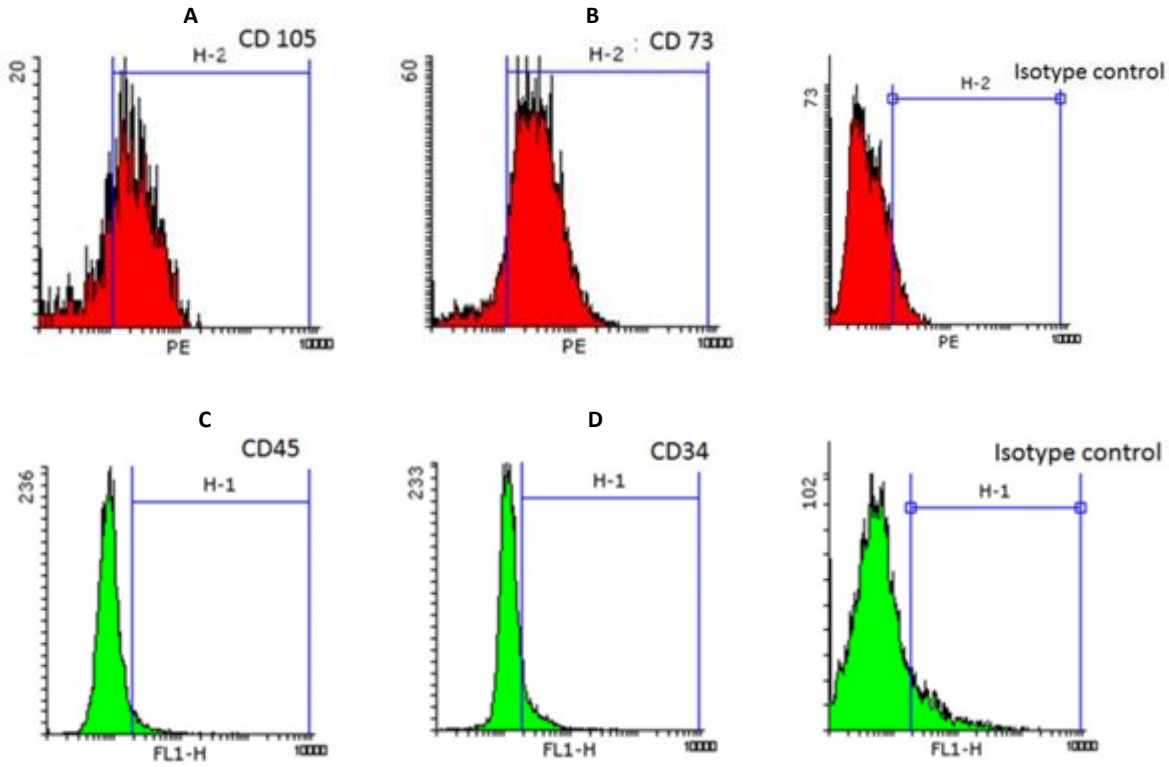

Figure 2. The *in vitro* Mesenchymal Stem Cells Identification. Analyses showed that about 90% (90.1 ± 3) of cells were positive for CD105 **(A)**, 76% (75.8 ± 3.61) of cells were positive for CD73 **(B)**, A low percent of cells were positive for CD34 (5.98 ± 1.64) **(C)**, and CD45 (7.15 ± 0.26) labels **(D)**, which are specific for hematopoietic stem cells, confirming the mesenchymal feature of the isolated cells. Scale bar = 100 µm. The data was Mean ± SD.

ADSCs Characterization

The surface marker analysis of the isolated ADSCs showed that about 90% of cells were positive for CD105 (90.1 ± 3) (Figure 2a), while 76% of cells were positive for CD73 (75.8 ± 3.61) (Figure 2b), confirming the mesenchymal feature of the isolated cells. A low percent of cells was positive for CD34 (5.98 ± 1.64) (Figure 2c) and CD45 (7.15 ± 0.26) (Figure 2d), which are specific for hematopoietic stem cells, indicating the high purity of the isolated and expanded ADSCs as mesenchymal stem cells.

Adipogenic and Osteogenic Differentiation

As displayed in Figure 3, ADSCs were successfully differentiated into adipogenic and osteogenic lineages. The Alizarin Red assay verified that ADSCs differentiated towards osteoblast under special differentiation conditions (Figure 3A). Additionally, the Oil Red O staining method showed differentiation of ADSCs into the adipogenic lineage through staining the lipid droplets in differentiated adipocytes (Figure 3B).

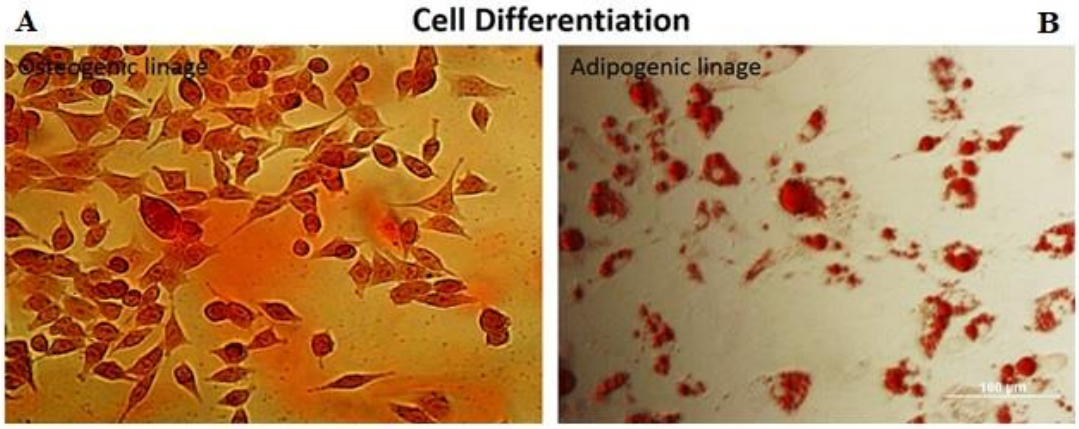

Figure 3. Differentiation Potential of Isolated Stem Cells into Osteocyte and Adipocyte Cells. The Alizarin Red assay verified that ADSCs differentiated towards osteoblast under special differentiation conditions **(A)**; the Oil Red O staining analysis showed ADSCs differentiation into the adipogenic lineage **(B)**.

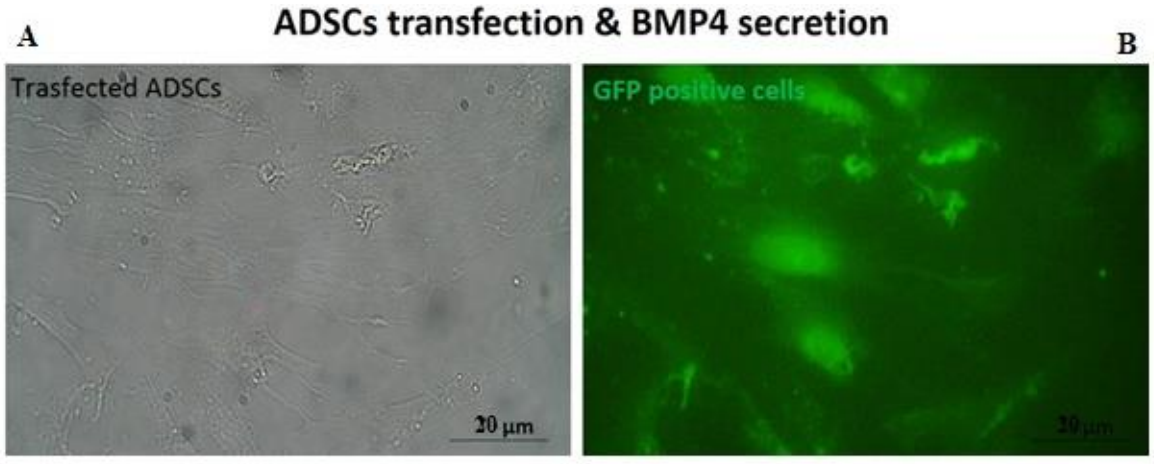

Figure 4. Transfection of ADSCs with BMP4-lentiviral Vector. Transfection with BMP4 containing lentiviral vector **(A)** exhibited a high exoression of GFP- expressing cells **(B)**, indicating the high effectiveness of transfection.

BMP4 Expression in ADSCs Transfected with BMP4-containing Lentiviral Vector

As depicted in Figure 4, ADSCs transfected with BMP4-expressing lentiviral vector (A) exhibited a high number of cells expressing GFP (B), indicating the high effectiveness of transfection. Therefore, it ensures that GFP-expressing cells also express BMP4 protein in transfected ADSCs.

Cell Viability Reduction in U937 Cells in the Co-culture System

To measure the inhibitory effect of BMP4 on the cell viability of U937 cells in 24, 48, and 72 h, the MTT assay method was used. Cells were treated with ADSCs engineered with 0, 0.25, 0.5, and 1 microliter concentrations of lentiviral vector for 24, 48, and 72 h. The best necessary concentration of BMP4 vector was 0.5 microliters. MTT measurement showed that in all three-time periods a decrease in U937 cells co-cultured with BMP4-expressing ADSCs (B-ADSC + U937) group, but this decrease was more in 72 h (Figure 5).

Apoptosis Assay

Consistent with the MTT results, the apoptosis assay demonstrated a significant (p<0.05) increase in the apoptotic U937 cells in the B-ADSC+U937 group at 72 h in comparison with the ADSC+U937 and U937 groups (Figure 6).

Relative Gene Expression Assay

The results of real-time PCR indicated the overexpression of BMP4 in ADSCs transfected with BMP4-expressing lentiviral vectors (Figure 7a). In addition, the upregulation of the BMP4 receptor (Figure 7b) was detected in U937 cells co-cultured with BMP4-expressing ADSCs (B- ADSCs). Notably, among the three studied cell groups, the expression levels of miR-424 (Figure 7c) and TGF-β (Figure 7d) genes were downregulated in the B-ADSC+U937 co-culture group

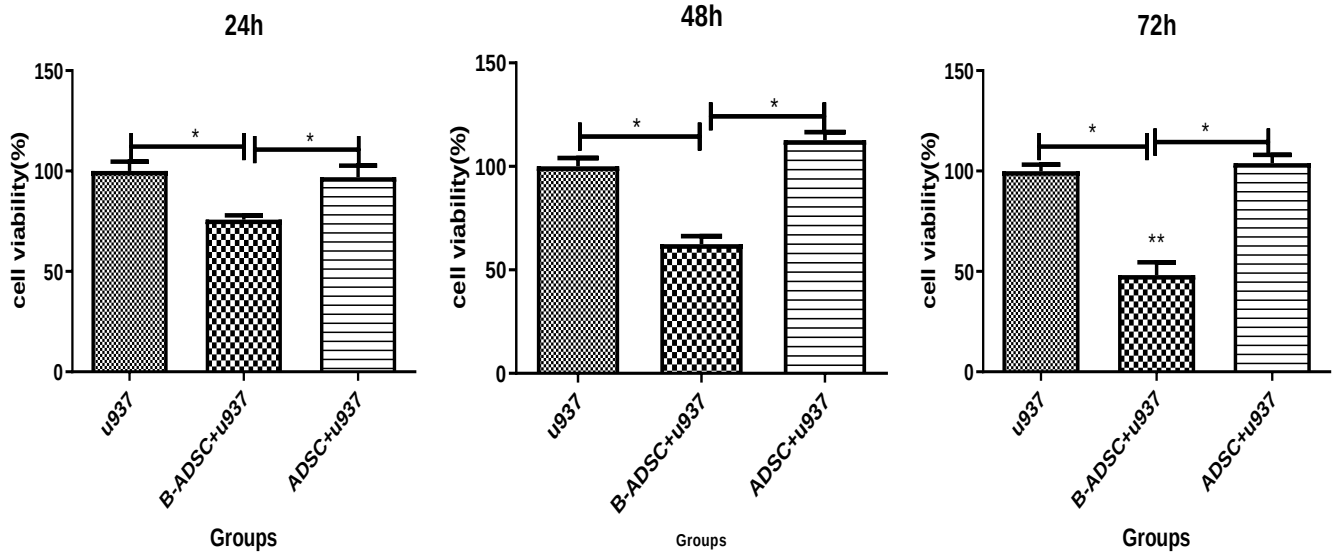

Figure 5. Assessment of Cell Viability of U937 Cells Co-cultured with ADSCs and Engineered ADSCs Using MTT Assay after 24, 48, and 72 h. In all times a decrease in cell viability was observed in the B-ADSC+U937 group, but in the 72 h period this decrease was more evident. The different signs above each graph show a significant difference (*p<0.5, **p<0.05).

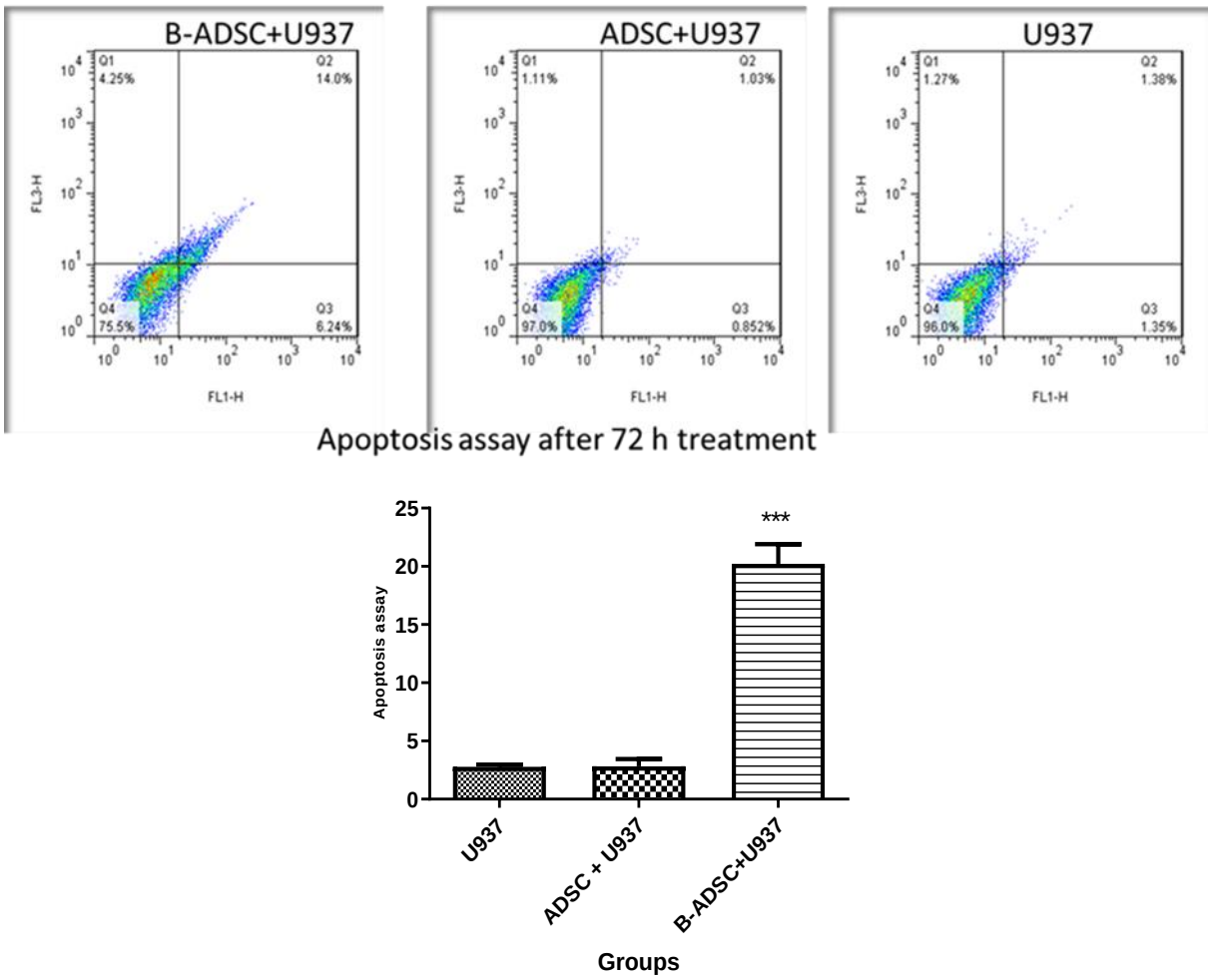

Figure 6. Apoptosis Analysis in U937 Cells Co-cultured with Enginnered ADSCs. Apoptosis analysis showed that the apoptosis of B-ADSC+U937 cells after 72h was significantly increased compared to ADSC+U937 and U937 groups. We show the result is in means + SD. The different signs above each graph show a significant difference (***p<0.005).

in comparison with the U937 and ADSC+U937 groups. However, a difference in the expression of TGF-β was not statistically significant.

Discussion

Mechanistic analyses have indicated that molecular signaling pathways are involved in the progression of AML in patients; thus, understanding the underlying mechanisms of AML is essential for efficient therapy in AML.[27]

Due to the fact that adipose tissue-derived mesenchymal stem cells can migrate toward tumor sites, a cell-based delivery system was designed by which genetically engineered mesenchymal stem cells derived from adipose tissue highly express BMP4 as a growth factor, belonging to the TGF-β family. Our findings showed significant expression of the BMP4 protein in transfected ADSCs secreting BMP4 into the extracellular space. A number of studies showed the anti-tumor activity of B-ADSCs in U937 cells in which the secreted form of BMP4 is suitable to induce apoptosis and necrosis in these cells under co-culture conditions. BMP4 is capable of reducing the expression of miR-424 and TGF-β in U937 cells under a co-culture system. Choi et al. established a stem cell-based gene therapy system for the treatment of brainstem glioma by inducing a lentiviral vector expressing the tumor necrosis factor-alpha gene in ADSCs. They administered the manipulated cells into the brainstem of murine models and found a potent anti-tumor activity.[5] Studies showed the *in vivo* effect of BMP4 on the maintenance of hematopoietic stem cells (HSC), especially in the regulation of the differentiation and proliferation of these cells.[9] Thus, diminished expression of BMP4 in hMSC-AML can alter the viability of HSCs and, accordingly, could be related to leukemic transformation. BMP4 regulates the proliferation and activity of HSCs, and, therefore, directly influences hematopoiesis[10] and induces osteogenic differentiation in hMSCs.[28]

Our results were in line with previous studies conducted in this regard, as BMP4 induces apoptosis and reduces cell survival in the leukemia cell line. At the gene level, the proliferative and inhibitory activity, as well as the differentiation potential of BMP4-ADSCs, were associated with the inhibition of miR-424 and TGF-β expression in U937 cells.

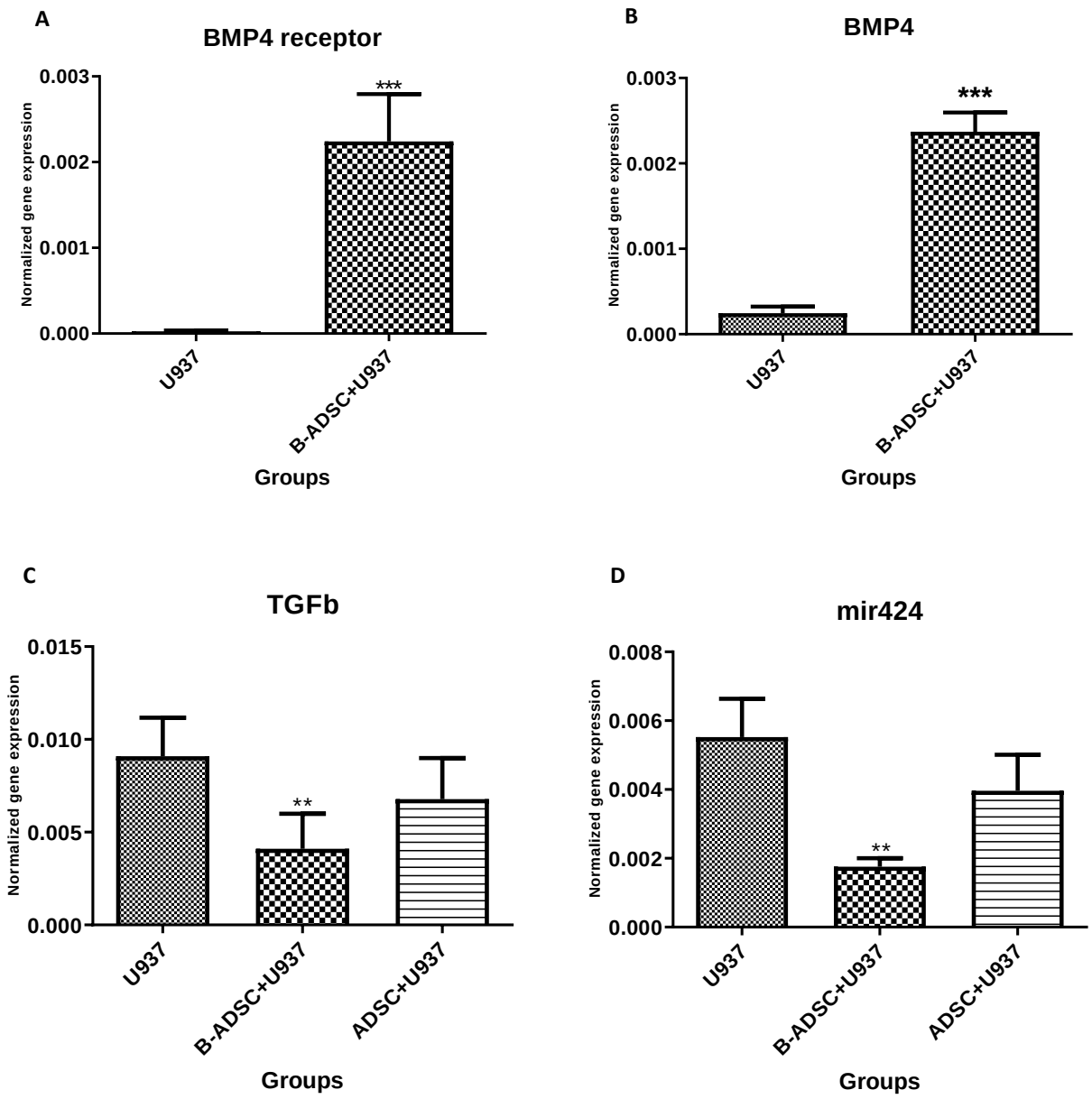

Figure 7. Expression Analysis of the *BMP4* Gene, BMP4 receptor, *TGF-β* Gene, and miR-424 in Related Groups Using real-time PCR. The expression of BMP4 in B-ADSC was higher than the ADSC **(A)**; The expression of BMP4 receptors in B-ADSC+U937 was higher than the U937 cells **(B)**; The expression of miR-424 in B-ADSC+U937 showed the highest decrease compared to the ADSC+ U937 group **(C)**; TGF-β showed the highest decrease in the B-ADSC + U937 group compared to the ADSC + U937 group **(D)**. The different signs above each graph show a significant difference (**p<0.05, ***p<0.005)

MicroRNAs (miRNAs) play a critical function in hematopoietic cell differentiation through post-transcriptional regulation.[29] MiR-424-5p has broad activity in different types of cancer and acts on various signaling pathways to regulate tumor progression. Studies indicated that miR-424 is abundantly expressed in breast cancer patients compared to healthy individuals and can distinguish early-stage breast cancer patients from healthy ones.[30] MiR-424-5p was found to be significantly upregulated in pancreatic cancer.[21] Zhang et al. found miR-424-5p expression is considerably upregulated in gastric cancer (GC) and correlated with pathological stage and poor prognosis of patients with GC.[31]

According to the bioinformatics analysis, in hematological malignancies, miRNA-424-5p has been demonstrated to improve HOXA expression. This event could be oncogenic and increase the proliferation and migration of cancer cells in AML.[26] Besides, Peng et al. showed that IL-8-induced miR-424-5p expression is increased in oral squamous cell carcinoma and can alter the signaling pathway of SOCS2/STAT5.[32] MiR-424-5p serves as an oncogene in colorectal cancer (CRC), and circulating exosomal miR-424-5p could be considered a novel possible diagnostic biomarker for monitoring CRC patients.[33] Liu et al. showed that the expression of miR-424-5p was considerably higher in thyroid

carcinoma than in normal tissues. It was confirmed that the upregulation of miR-424-5p suppresses several downstream genes of the Hippo pathway in thyroid cancer cells.[34] Increased expression of TGF-β can cause myelofibrosis, and its effects on the immune system and stroma may lead to the development of some blood malignancies.

Conclusion

The regulation of the TGF-β signaling pathway may pave the way for the treatment of hematological cancer. In the present study, a new BMP4-based induction strategy was designed to significantly suppress U937 leukemia cell line cell viability. It seems that this method can be used to treat hematologic cancer. However, further studies are needed to reveal the *in vivo* effect of this therapeutic strategy in improving blood malignancies.

Authors' Contributions

MGKT: Writing, Original Draft, Review, Editing, Validation, and Resources; MH: Writing, Validation, Resources, and Project administration; NB: Validation, Resources, Review, and Editing; AD: Statistical analysis and Editing; ZM: Validation, Resources, Editing, Supervision, and Project administration.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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