RESEARCH ARTICLE

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Knockdown of Stromal Interaction Molecule 1 (*STIM1*) Suppresses Acute Myeloblastic Leukemia-M5 Cell Line Survival Through Inhibition of Reactive Oxygen Species Activities

Stromal Etkileşim Molekülü 1'in (*STIM1*) Bozulması Reaktif Oksijen Türevleri Aktivitesinin İnhibisyonu Aracılığı ile Akut Myeloblastik Lösemi-M5 Hücre Dizilerinin Sağkalımını Baskılar

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Abstract

Objective: This study aimed to investigate the role of the stromal interaction molecule 1 (*STIM1*) gene in the survival of the acute myeloblastic leukemia (AML)-M5 cell line (THP-1).

Materials and Methods: The *STIM1* effect was assessed via dicer-substrate siRNA-mediated *STIM1* knockdown. The effect of *STIM1* knockdown on the expression of *AKT* and *MAPK* pathway-related genes and reactive oxygen species (ROS) generation-related genes was tested using real-time polymerase chain reaction. Cellular functions, including ROS generation, cell proliferation, and colony formation, were also evaluated following *STIM1* knockdown.

Results: The findings revealed that *STIM1* knockdown reduced intracellular ROS levels via downregulation of *NOX2* and *PKC*. These findings were associated with the downregulation of *AKT*, *KRAS*, *MAPK*, and *CMYC*. *BCL2* was also downregulated, while *BAX* was upregulated following *STIM1* knockdown. Furthermore, *STIM1* knockdown reduced THP-1 cell proliferation and colony formation.

Conclusion: This study has demonstrated the role of *STIM1* in promoting AML cell proliferation and survival through enhanced ROS generation and regulation of AKT/MAPK-related pathways. These findings may help establish *STIM1* as a potential therapeutic target for AML treatment.

Keywords: *STIM1*, Reactive oxygen species, Survival, Proliferation, Therapeutic targets in acute myeloid leukemia

Öz

Amaç: Bu çalışmada stromal etkileşim molekülü 1 (STIM1) geninin akut myeloblastik lösemi (AML)-M5 hücre dizisinin (THP-1) sağkalımındaki rolü araştırıldı.

Gereç ve Yöntemler: *STIM1* etkisi, yarıcı substrat siRNA aracılı *STIM1* bozulması yoluyla değerlendirildi. *STIM1* bozulmasının AKT ve MAPK yolakları ve reaktif oksijen türevleri (ROS) üretimi ile ilişkili genlerin ekspresyonu üzerindeki etkisi RT-qPCR ile test edildi. Hücresel fonksiyonlar, ROS üretimi, hücre proliferasyonu ve koloni oluşumu dahil, *STIM1* bozulmasını takiben değerlendirildi.

Bulgular: Sonuçlar *STIM1* bozulmasının, *NOX2* ve *PKC* aşağı düzenlemesi ile hücre içi ROS seviyesini düşürdüğünü ortaya koydu. Bu bulgular *AKT, KRAS, MAPK* aşağı düzenlemesi ile birlikteydi ve ayrıca CMYC, BCL2'de aşağı düzenlenmişti, BAX ise *STIM1* bozulmasını takiben yukarı düzenlenmişti. Ek olarak *STIM*1 bozulması THP-1 hücre proliferasyonu ve koloni oluşumunu da azaltmıştı.

Sonuç: Bu çalışma, *STIM1*'in artmış ROS üretimi ve AKT/MAPK ile ilişkili yolların düzenlenmesi yoluyla AML hücre proliferasyonunu ve sağkalımını desteklemedeki rolünü göstermiştir. Bu bulgular, *STIM1*'in AML tedavisi için potansiyel bir terapötik hedef oluşturulmasına yardımcı olabilir.

Anahtar Sözcükler: *STIM1*, Reaktif oksijen türevleri, Sağkalım, Proliferasyon, Akut Myeloid lösemide terapötik hedefler

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Introduction

Acute myeloid leukemia (AML) is a highly aggressive hematological malignancy that is more common in adults, with a median patient age of 67 years [1]. Childhood AML is the fifth most frequent childhood cancer and its prognosis is still poor compared to other types of leukemia [1]. AML in infants is a unique subset of AML with specific clinical and molecular characteristics. Infants with AML have been classified as high-risk patients because of the high incidence of unfavorable prognostic features and their increased susceptibility to treatment-related toxicity. Advances in the molecular understanding of and therapeutic strategies for AML resulted in clear improvement of the 5-year survival rate of childhood AML, which has reached 70% [2]. Despite that, relapse after remission remains a critical challenge in AML. Therefore, many molecular and biochemical disturbances associated with AML still require more investigation.

Growing evidence in cancer research supports the contribution of disrupted calcium homeostasis to tumor initiation and progression [3,4]. Stromal interaction molecule 1 (*STIM1*) is located on the endoplasmic reticulum (ER) membrane and it acts as a sensor for calcium storage and regulates calcium influx through store-operated calcium entry (SOCE). Recently, *STIM1* was found to play a critical role in the development and metastasis of a variety of cancers, such as brain, prostate, and colorectal cancers and multiple myeloma [5,6,7]. The Human Protein Atlas and Expression Atlas show that *STIM1* has an elevated level of expression in many AML cells, including THP-1 cells [8,9], but the role of *STIM1* in AML survival is still not fully understood. Furthermore, most of the myeloid leukemia subtypes are associated with increased production of reactive oxygen species (ROS), which has been observed to promote leukemic cell proliferation and survival [10].

There is increasing evidence suggesting an interplay between *STIM1* and ROS in cancer cell biology [11,12]. ROS stimulates *STIM1* and activates SOCE directly, regardless of calcium ER store depletion [11]. Upregulation of hypoxia inducible factor 1 (*HIF1*) in response to hypoxia leads to upregulation of *STIM1* in hepatocarcinoma cells [13]. In multiple myeloma, *STIM1* suppression reduced calcium influx into the cells [14]. Reducing mitochondrial calcium uptake in breast cancer cells resulted in reduced mitochondrial ROS production and inhibition of cell growth and migration [12]. *NOX2* is a significant source of intracellular ROS, and its activity was linked to oncogenic signals in AML [15]. Depletion of *NOX2* reduced ROS levels and suppressed self-renewal of leukemic stem cells [15]. *AKT* and *MAPK* signaling pathways are critical for many cellular functions such as cell proliferation, survival, and differentiation, and their significant roles in carcinogenesis have been reported for a variety of cancers [16]. At certain levels, ROS activate the *AKT* and *MAPK* signaling pathways [17].

The effect of *STIM1* on ROS generation and *AKT* and *MAPK* signaling pathways in AML is still unexplored. Therefore, this study was designed to explore the effect of *STIM1* on ROS generation in THP-1 cells and to investigate its involvement in the regulation of cell proliferation and survival. To assist with that, *STIM1* was knocked down in THP-1 cells using dicer-substrate siRNA (dsiRNA). ROS levels were subsequently measured and the expression levels of related genes were assessed. Cell proliferation and survival and the expression levels of certain genes related to the *AKT* and *MAPK* signaling pathways were evaluated. This study suggests that *STIM1* may be involved in the regulation of ROS generation, as well as the proliferation and survival of AML cells through the regulation of *AKT* and *MAPK* signaling pathway-relate genes. The findings may provide novel insights into AML pathogenesis and may be useful in improving therapeutic targets for AML in the future.

Materials and Methods

Cell Culture

THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Roswell Park Memorial Institute Medium (RPMI-1640, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, Life Technologies, USA) at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection with dsiRNA

dsiRNA (TriFECTa, Integrated DNA Technologies, Coralville, IA, USA) was transfected into THP-1 cells ($2x10^6$/mL) at doses of 10 nM for 24 h. STIM1 dsiRNA was transfected to the cells using the Bio-Rad Gene Pulser Xcell Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA) at a pulse of 300 V for 7 ms. The transfected cells were diluted 20-fold with culture medium and incubated at 37 °C and 5% CO_2. All experiments were compared against a dsiRNA-negative control.

qRT-PCR Analysis

Total RNA was extracted from cells using the Monarch Total RNA Miniprep Kit (New England BioLabs, Hitchin, UK) 24 h after dsiRNA transfection. The cDNA was synthesized by reverse transcription using Rever Tra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) following the manufacturer's protocol. Luna Universal qPCR Master Mix (New England BioLabs, UK) and the StepOnePlus Real-Time PCR System (Applied Bioscience, Wilmington, NC, USA) were used to measure gene expression levels after *STIM1* knockdown. The $2^{-(\Delta\Delta CT)}$ comparative threshold cycle (Ct) method was used to analyze the data, where $\Delta\Delta CT = (Ct^{\text{Target sample}} - Ct^{\text{Reference gene sample}}) - (Ct^{\text{Target control}} - Ct^{\text{Reference gene control}})$. All targeted gene primers used in the present study are listed in Table 1. *GAPDH* was used as the endogenous control.

Western Blotting

Western blotting was performed to confirm the suppression of STIM1 protein after dsiSTIM1 transfection. Protein samples (30 µg) were analyzed by SDS-PAGE on 12% gel. Following electro-blotting to polyvinylidene difluoride membranes, the membranes were blocked in 5% non-fat dry milk or 3% bovine serum albumin (BSA) in 0.1% Tris-buffered saline with Tween-20 (TBST) for 1 h at room temperature. Membranes were rinsed in 1X TBST three times and incubated in primary antibody solutions overnight at 4 °C with gentle rocking. The primary antibodies included rabbit monoclonal anti-human STIM1 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:500 dilution in 5% non-fat dry milk in 0.1% TBST and rabbit monoclonal anti-human β-actin antibody (Cell Signaling Technology, USA) at 1:2000 dilution in 3% BSA in 0.1% TBST. The membranes were washed the next day with 1X TBST three times and incubated in HRP-conjugated polyclonal anti-rabbit secondary antibody (Cell Signaling Technology, USA) at 1:500 dilution in 0.1% TBST including non-fat dry milk or BSA for 1 h at room temperature. After washing with TBST, the membranes were incubated in ECL substrate (Bio-Rad, USA) according to the manufacturer's directions. After incubation, membranes were imaged using the VersaDoc imaging system (Bio-Rad, USA). Band intensity was measured using Image Lab software version 6.1 (Bio-Rad, USA).

Table 1. List of primers.

No.	Gene name	Primer sequence
1	*STIM1*	F 5'-AGAAACACACTCTTTGGCACC-3' R 5'-AATGCTGCTGTCACCTCG-3'
2	*GAPDH*	F 5'-AACGGATTTGGTCGTATTG-3' R 5'-GCTCCTGGAAGATGGTGAT-3'
3	*KRAS*	F 5'-TCCAACAATAGAGGTGTTATTAAGC-3' R 5'-ACTCGGGGATTTCCTCTTGA-3'
4	*MAPK*	F 5'-GTACGACTCACTATAGGGAATTATGCATCCCACTGACCA-3' R 5'-AGGTGACACTATAGAATACTGGCTCGGCACACAGAT-3'
5	*CMYC*	F 5'-TGAGGAGACACCGCCCAC-3' R 5'-CAACATCGATTTCTTCCTCATCTTC-3'
6	*PI3K*	F 5'-ACGACTTTGTGACCTTCGGC-3'R 5'-CCGATAGCAAAACCAATTTCTCGAT-3'
7	*AKT*	F 5'-CAAAGAAGTCAAAGGGGCTGC-3' R 5'-ATGTACTCCCCTCGTTTGTGC-3'
8	*NFKB*	F 5'-TAGGAAAGGACTGCCGGGAT-3'R 5'-CACGCTGCTCTTCTTGGAAGG-3'
9	*BAX*	F 5'-TCAGGATGCGTCCACCAAGAAG-3' R 5'-TGTGTCCACGGCGGCAATCATC-3'
10	*BCL2*	F 5'-ATCGCCCTGTGGATGACTGAGT-3' R 5'-GCCAGGAGAAATCAAACAGAGGC-3'
11	*RAC1*	F 5'-GCCAATGTTATGGTAGAT-3'R 5'-GACTCACAAGGGAAAAGC-3'
12	*NOX2*	F 5'-CTTCATTGGCCTTGCCATCC-3'R 5'-GGGTTTCCAGCAAACTGAGG-3'
13	*PKC*	F 5'-CTTTCATCCACTGGCCTCGT-3'R 5'-GTTGGGCTGCATGAACCTTG-3'

Measurement of Intracellular ROS Levels

Cells were seeded in triplicate at $2x10^5$/mL in a 96-well flat-bottom plate. After 24 h of transfection, the cells were washed with PBS, suspended in 100 µL of PBS loaded with 5 µM CM-H2DCFDA (Invitrogen, Waltham, MA, USA), and incubated for 30 min. Subsequently, cells were washed and suspended in PBS for 1 h. ROS levels were measured using the FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) at 485 nm excitation and 520 nm emission. An Olympus IX71 fluorescence microscope (Olympus Corp., Tokyo, Japan) was used to visualize fluorescent dye rising from the cells.

Proliferation Assay

THP-1 cells were seeded at $2x10^5$ cells/mL in triplicate in a 96-well flat-bottom plate after dsiRNA transfection. Cells were incubated for 3 time points of 24, 48, and 72 h. At each time point, cell proliferation was assessed by adding 10 µL of SF cell count reagent (Nacalai Tesque, Kyoto, Japan) with incubation for 2 h. Absorbance was measured at 450 nm using a microplate reader (Bio-Tek, US).

Colony Formation Assay

THP-1 cells were seeded in triplicate after dsiRNA transfection at $2x10^3$ cells/mL in methylcellulose medium in a 24-well plate and incubated at 37 °C for 8 days. The colonies were counted under an Olympus CKX 41 light microscope (Olympus Corp., Japan) at magnifications of 40^x and 200^x. The selected colonies were those that consisted of 50 cells or more.

Statistical Analysis

All statistical analysis was carried out using IBM SPSS Statistics 26 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were carried out using the paired-samples Student t-test. Data were considered significant at $p<0.05$ (*), highly significant at $p<0.01$ (**), and very highly significant at $p<0.001$ (***).

Results

STIM1 Knockdown Reduced ROS Generation

STIM1 was efficiently knocked down in THP-1 cells at both mRNA ($p=0.001$) and protein ($p<0.05$) levels (Figure 1a). Intracellular ROS levels were measured 24 h after *STIM1* knockdown. The results exhibited a significant reduction by 55% ($p<0.01$) in ROS levels in the dsiSTIM1 transfected group of THP-1 cells compared to the control group, dsiCtrl (Figure 1b). Fluorescence microscopy revealed a reduction of ROS-derived fluorescent

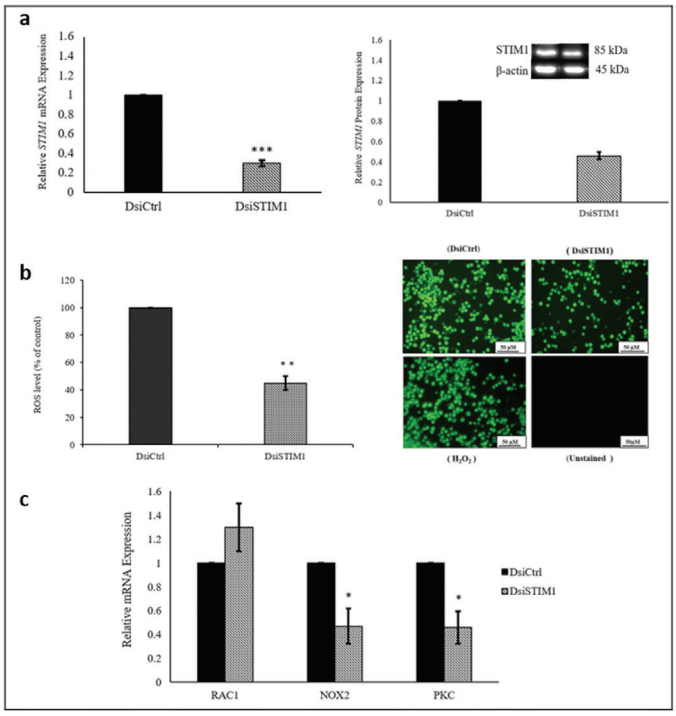

Figure 1. Reactive oxygen species (ROS) levels after *STIM1* knockdown. **a)** *STIM1* knockdown at mRNA and protein levels. **b)** Percentage of ROS level normalized to control after *STIM1* knockdown. Fluorescence microscopy image of cells after incubation with 5 µM CM-H2DCFDA, except unstained group, for 30 min. H_2O_2 was used as positive control. **c)** Effect of *STIM1* knockdown on the expression of NADPH oxidase-derived ROS pathway-related genes. Data are representative of the mean ± standard deviation of three independent experiments. All experiments were carried out in triplicate. Comparisons were made between the control (dsiCtrl) and transfected (dsiSTIM1) groups. *, **, and *** indicate p<0.05, p<0.01, and p<0.001, respectively, based on paired-samples t-tests.

signals in the dsiSTIM1 transfected group compared to the signals from the control groups, namely dsiCtrl and the H_2O_2-positive control (Figure 1b). The expression profiles of targeted genes (*RAC1*, *NOX2*, and *PKC*) included in the NADPH oxidase-derived ROS pathway were tested in THP-1 cells after *STIM1* knockdown. The results showed significant downregulation of *NOX2* and *PKC* (p<0.05) following *STIM1* knockdown (Figure 1c). No significant change was observed (p>0.05) in the expression of *RAC1* in response to *STIM1* knockdown (Figure 1c).

STIM1 Knockdown Downregulated *KRAS*/*MAPK* and AKT

The effect of *STIM1* on the targeted genes involved in the *KRAS*/*MAPK* and *PI3K*/*AKT* proliferative and survival pathways was evaluated after suppression of *STIM1*. Significant downregulation of *KRAS* (p<0.05), *MAPK* (p<0.01), and *CMYC* (p<0.05) was observed following *STIM1* knockdown (Figure 2a). A very significant downregulation of *AKT* by 71% (p<0.01) was detected in THP-1 cells in association with significant

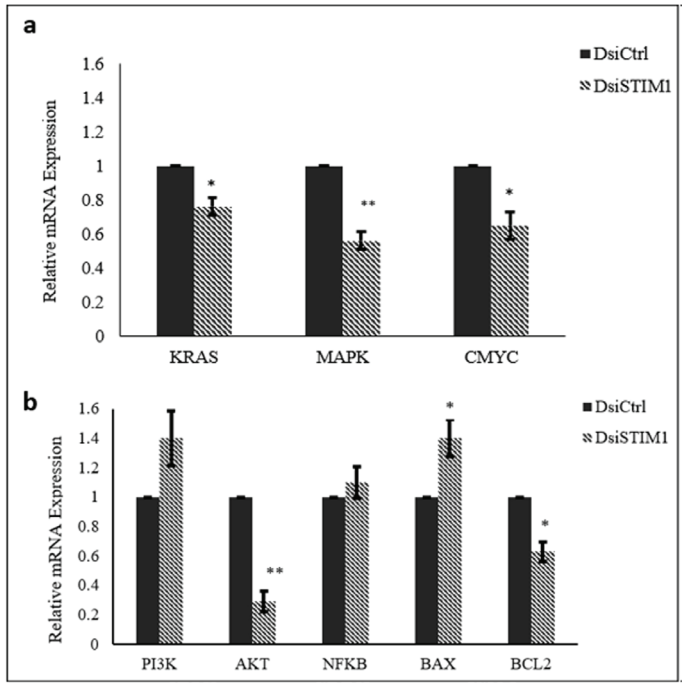

Figure 2. Effect of *STIM1* knockdown on the expression of proliferative and survival pathway-related genes. The expression of **(a)** proliferative and **(b)** survival pathway-related genes was tested 24 h after *STIM1* knockdown. Data are representative of the mean ± standard deviation of three independent experiments. All experiments were carried out in triplicate. Comparisons were made between the control (dsiCtrl) and transfected (dsiSTIM1) groups. * and ** indicate p<0.05 and p<0.01, respectively, based on paired-samples t-tests.

upregulation of *BAX* (p<0.05) and significant downregulation of *BCL2* (p<0.05) (Figure 2b). No significant changes were observed in *PI3K* or *NFKB* levels (Figure 2b).

STIM1 Knockdown Reduced THP-1 Cell Proliferation and Colony Formation

The proliferation rate of THP-1 cells was tested over a period from 24 to 72 h after dsiRNA transfection. Knockdown of *STIM1* in THP-1 cells resulted in suppression of cell proliferation (p<0.05) at 24-48 h after knockdown compared to the control, dsiCtrl (Figure 3a). The colony formation ability of THP-1 cells was tested under bright-field microscopy by counting the number of colonies formed by cells transfected with dsiSTIM1 compared to the control (dsiCtrl). The results revealed a significant reduction in the number of colonies formed by THP-1 cells transfected with dsiSTIM1, reaching 35% (p<0.05) compared to the dsiCtrl cells (Figure 3b). In addition, a decrease in the size of colonies formed by cells transfected with dsiSTIM1 compared to the control was observed (Figure 3b).

Discussion

The current investigation involved the suppression of *STIM1* expression in THP-1 cells to determine its role in AML. Given

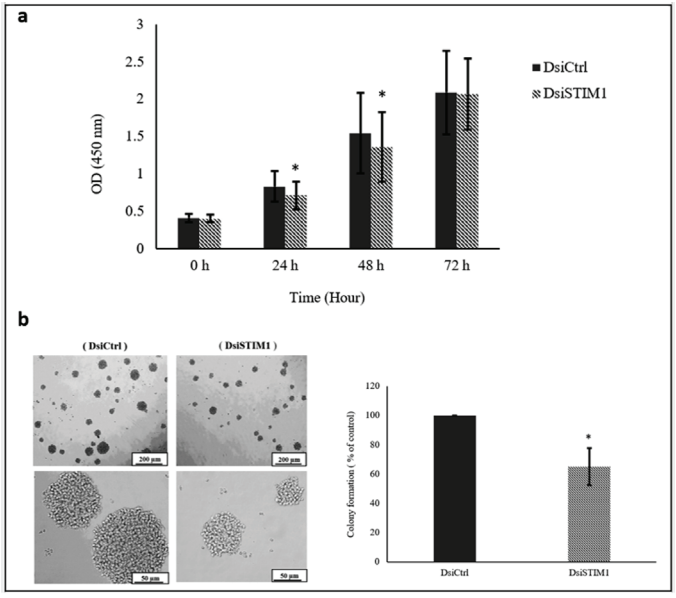

Figure 3. THP-1 cell proliferation and colony formation after *STIM1* knockdown. **a)** THP-1 cell proliferation rate was tested over time from 24 to 72 h after *STIM1* knockdown. **b)** Bright-field microscopy showed reduced colony number and size of THP-1 cells transfected with dsiSTIM1, which was supported by statistical results. Data are representative of the mean ± standard deviation of three independent experiments. All experiments were carried out in triplicate. Comparisons were made between the control (dsiCtrl) and transfected (dsiSTIM1) groups at each time point. * indicates $p<0.05$ based on paired-samples t-tests.

that a certain level of ROS is critical for cancer cell survival [4,18,19], this study evaluated whether *STIM1* plays a role in the regulation of ROS levels in AML cells. The study's findings showed a decline in intracellular ROS levels in response to *STIM1* knockdown. This supports the significance of *STIM1* in maintaining pro-survival levels of ROS in THP-1 cells. In addition, *STIM1* knockdown downregulated *NOX2* and *PKC*, which are ROS generation-related genes. These findings may help explain the mechanism through which *STIM1* knockdown reduces ROS levels in AML cells, which could be through the suppression of the expression of *NOX2*, a ROS-generating membrane-bound enzyme complex, and the suppression of the expression of *PKC*, the *NOX2* cytosolic activator [20]. *PKC* is important for the initiation of *NOX2*-mediated ROS generation, where it is crucial for activation of *p47phox*, which is responsible for activation and translocation of its other subunits (*p67phox*, *p40phox*, and a GTPase *RAC1* or *RAC2*) to the plasma membrane to complete the process of *NOX2* activation and ROS generation [20,21].

Previous evidence revealed that ROS production through NADPH oxidases is influenced by calcium ions, which are essential for the activation of *PKC* [20,22]. In addition, numerous recent studies have highlighted the interplay between calcium and ROS in a variety of cancers such as breast, thyroid, and hepatocellular carcinoma [12,23,24]. *STIM1* has been shown to

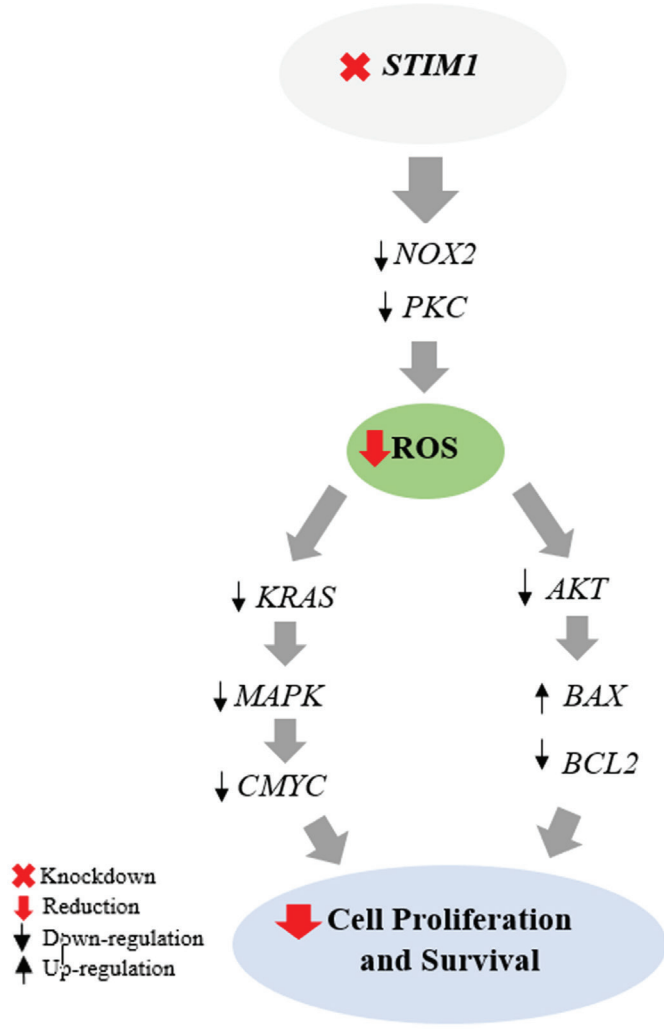

Figure 4. Schematic diagram of the mechanism through which *STIM1* knockdown reduces THP-1 cell proliferation and survival by reducing reactive oxygen species (ROS) production via downregulation of *NOX2* and *PKC*, and by controlling the proliferation and survival-related genes *KRAS, MAPK, CMYC, AKT, BAX*, and *BCL2*.

have a regulatory effect on calcium influx in acute lymphoblastic leukemia and multiple myeloma cells, with knockdown of *STIM1* in these cells associated with a reduction in calcium influx [14,25]. Therefore, *STIM1* appears to play a role in regulating ROS generation in THP-1 cells, most likely through regulation of the NADPH oxidase source of ROS. This regularity effect appears to arise through the regulation of *NOX2* and *PKC* expression and could be indirect via calcium-mediated activation of *PCK*. Despite the importance of *RAC1* for NADPH-mediated ROS production, *RAC1* was not affected by *STIM1* knockdown in the present study, which could support the non-involvement of *RAC1* in *STIM1*-mediated ROS production in THP-1 cells.

The *RAS/MAPK* signaling pathway is a vital proliferative signaling pathway that has been found to be hyperactive in many cancers, including leukemia [26,27]. The present work evaluated the

effect of *STIM1* knockdown on the expression of *KRAS*, *MAPK*, and *CMYC*, critical genes involved in the *RAS/MAPK* signaling pathway. The findings revealed downregulation of *KRAS*, *MAPK*, and *CMYC* following *STIM1* knockdown. *STIM1*'s regularity effect on *MAPK* signaling activity has previously been reported in acute lymphoblastic leukemia and other cancer cells [28,29]. *PI3K/AKT* is a crucial survival pathway, and the oncogenic activity of *AKT* has been reported in many cancers [30]. Interestingly, this study discovered the influence of *STIM1* in the regulation of *AKT* and its downstream genes. Suppression of *STIM1* resulted in a significant downregulation of *AKT* expression. Downregulation of *AKT* led to upregulation of proapoptotic *BAX* and downregulation of the antiapoptotic *BCL2* genes. These findings indicate that *STIM1* knockdown may initiate certain apoptotic effects in THP-1 cells following *AKT* suppression. This supports previous findings in hepatocellular carcinoma and malignant melanoma cells, where *STIM1* knockdown caused *AKT* inactivation and increased apoptosis susceptibility [31,32]. Even though *PI3K* and *NFKB* are known to play roles in cancer cell survival [33], there were no clear changes in their expression levels observed after *STIM1* knockdown in the present study.

Knockdown of *STIM1* eventually resulted in a reduction of THP-1 cell proliferation and colony formation. Colony size also decreased following *STIM1* knockdown, indicating impairment in the ability of cells to survive and maintain their growth. Similar findings were reported in other cancers, such as colorectal cancer, where *STIM1* knockdown resulted in inhibition of cancer cell proliferation and colony formation [34]. *STIM1* was found to be abundantly expressed in multiple myeloma tissues and cell lines, and the silencing of *STIM1* produced a reduction in cell viability and caused cell cycle arrest [14]. The present study may support the role of *STIM1* in promoting THP-1 cell proliferation and survival, which occur through maintained pro-survival ROS levels and via ROS-mediated regulation of proliferative and survival pathway-related genes (Figure 4). Recent cancer research revealed an interaction between ROS and the *RAS/MAPK* and *PI3K/AKT* proliferative and survival pathways to maintain cancer cell proliferation and survival [18,19,20,35,36]. Further work is still needed to elucidate *STIM1*/ROS interactions and their roles in AML pathogenesis and to confirm the potential of *STIM1* as a therapeutic target for AML treatment.

Conclusion

The present study showed an important finding regarding the role of *STIM1* in maintaining THP-1 cell proliferation and survival via regulation of ROS generation and control of the expression of *KRAS* and *AKT* pathway-related genes. Further comprehensive work is still needed to support the regularity role of *STIM1* in other AML cell lines, which could suggest *STIM1* as a potential therapeutic target for AML.

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Ethics

Ethics Committee Approval: Not applicable since no patients or animals were included in this study.

Authorship Contributions

Surgical and Medical Practices: E.J.M., S.I.O., N.A.A.R., N.M.; Concept: E.S.A., R.B.S.M.N.M.; Design: E.S.A., R.B.S.M.N.M.; Data Collection or Processing: E.S.A., R.B.S.M.N.M., E.J.M., S.I.O., N.A.A.R., N.M.; Analysis or Interpretation: E.S.A., R.B.S.M.N.M., E.J.M., S.I.O., N.A.A.R., N.M.; Literature Search: E.S.A, R.B.S.M.N.M.; Writing: E.S.A, R.B.S.M.N.M.

Conflict of Interest: No conflict of interest was declared by the authors.

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