The Characterization of Acute Myeloblastic Leukemia-M2 Cell Lines with Suppressed Stromal Interaction Molecule 1 (*STIM1*) and its Impact on Calcium/Reactive Oxygen Species Profiles

Eman Salem Algariri [1,2], Rabiatul Basria S. M. N. Mydin [1*], Emmanuel Jairaj Moses [1], Nur Arzuar Abdul Rahim [3], Narazah Mohd Yusoff [3]

Abstract

Introduction: Acute myeloid leukemia-M2 subtype (AML-M2) is a severe type of blood cancer that has a high rate of recurrence and death. Recent cancer research has linked stromal interaction molecule 1 (STIM1) and calcium/reactive oxygen species (ROS) interactions to cancer progression, drug resistance, and cancer cell self-renewal. However, the involvement of STIM1 in the modulation of calcium and ROS activities and AML-M2 cell survival is still unclear. Methods: The current study uses dicer-substrate siRNA (dsiRNA) knockdown of STIM1 to assess its functional activity in the AML-M2 cell line. Following STIM1 knockdown, the expression levels of genes involved in cell survival and ROS generation were measured by RT-qPCR. Calcium influx, ROS generation, cell proliferation, and colony formation were all evaluated. Results: Knocking down STIM1 exhibited a reduction in calcium influx and ROS generation. Kasumi-1 cell proliferation and colony formation were inhibited following STIM1 knockdown. Further transcriptomic profiling in this knockdown model revealed downregulation of KRAS, MAPK, C-MYC, Akt, NOX2, and PKC. Conclusion: The findings point to STIM1's potential role in promoting AML-M2 cell survival through calcium/ROS interplay-mediated control of KRAS and Akt-related pathways. Furthermore, it might recommend STIM1 and/or ROS for targeted therapy, which may contribute to regression of disease and improve the AML therapeutic strategy.

Keywords: Kasumi-1, STIM1, Acute Myeloblastic Leukemia-M2, cancer, disease

Significance | STIM1's potential role in promoting AML-M2 cell survival by regulating calcium/ROS interactions, as well as KRAS/MAPK and Akt-related pathways

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Editor Md Asaduzzaman Shishir And accepted by the Editorial Board Jan 23, 2024 (received for review Nov 30, 2023)

Introduction

Acute myeloid leukemia (AML) is an extremely malignant hematologic cancer and is responsible for 1.9% of all cancer deaths (National Cancer Institute, 2021). Among leukemia subtypes, AML had the highest incidence and mortality rate (National Cancer Institute, 2021). Despite the remarkable improvement in the AML survival rate in the last decade, especially among children, which reached 65-70% (Sasaki et al., 2021), relapse is still the biggest challenge in AML, which occurs in 40-50% of the younger patients and the great majority of elderly patients

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Please cite this article.
Eman Salem Algariri, Rabiatul Basria S. M. N. Mydin, Emmanuel Jairaj Moses et al., (2024). The Characterization of Acute Myeloblastic Leukemia-M2 Cell Lines with Suppressed Stromal Interaction Molecule 1 (STIM1) and its Impact on Calcium/Reactive Oxygen Species Profiles, Journal of Angiotherapy, 8(1), 1-9, 9435

(Thol and Ganser, 2020). Kasumi-1 cells are an acute myeloblastic leukemia cell line (AML-M2) established by a child in the second relapse following bone marrow transplantation. Despite numerous molecular and cytogenetic studies on Kasumi-1 cells, the correlation of calcium and ROS homeostasis with the relapsed model of AML remains not fully understood.

Stromal interaction molecule 1 (*STIM1*) is a store-dependent intracellular calcium regulator implicated in the development of several cancers (Liu et al., 2020, Zhao et al., 2020, Lunz et al., 2019, Chen et al., 2019, Ge et al., 2019, Yang et al., 2017, Vashisht et al., 2015, Li et al., 2013). Furthermore, calcium/ROS interplay has been linked to drug resistance and relapse in many cancers (Reczek and Chandel, 2018, Hempel and Trebak, 2017, Görlach et al., 2015, Feno et al., 2015, Wei et al., 2014). Induced dysregulation of this interplay in cancer cells results in inhibiting tumor growth, enhancing cell apoptosis, and increasing sensitivity to chemotherapy (Liu et al., 2020, Wei et al., 2014). The influence of *STIM1* on ROS generation and AML cell survival has yet to be established. Therefore, via dicer-substrate siRNA (dsiRNA)-mediated *STIM1* knockdown, the current study assesses the impact of *STIM1* on calcium influx and ROS generation, as well as its effect on Kasumi-1 cell proliferation and survival. It is expected that this study could unveil new knowledge about Kasumi-1 cells' leukemogenesis and, with further comprehensive work, could contribute to discovering a promising therapeutic target for AML.

Materials and Methods

Cell culture and transfection condition

Kasumi-1 cells (AML-M2) were purchased from the American Type Culture Collection (ATCC) (Virginia, USA) and grown at 37 ºC in a humidified atmosphere in Roswell Park Memorial Institute Medium (RPMI-1640) (Sigma-Aldrich, US), with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, US) and 1% penicillin/streptomycin (Gibco, Life Technologies, US). *STIM1* dicer-substrate siRNA (DsiSTIM1) (TriFECTa, Integrated DNA Technologies, US) was transfected into Kasumi-1 cells (2 x 10^6/ml) at 20 nM for 48 hours. All data were compared versus a dsiRNA negative control (DsiCtrl). Both DsiRNA (DsiSTIM1 and DsiCtrl) were transfected to the cells using a Bio-Rad Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, USA) at a pulse of 330 V for 10 microseconds. Before incubation, the transfected cells were diluted in the culture medium 20 times.

qRT- PCR Analysis

Monarch® Total RNA Miniprep Kit (New England BioLabs, UK) was used to extract the total RNA from the cells 24 - 72 hours post dsiRNA transfection. The cDNA was produced using Rever Tra Ace® qPCR RT Master Mix (Toyobo, Japan) following the manufacturer's protocol. Gene expression profiles after *STIM1* knockdown were assessed through Luna® Universal qPCR Master Mix (New England BioLabs, UK) by using the Step One Plus Real-Time PCR System (Applied Bioscience, US). The gene-specific primers were as follows: STIM1 primers (F 5'-AGAAACACACTCTTTGGCACC-3'and R 5'-AATGCTGCTGTCACCTCG-3'), Akt primers (F 5'-CAAAGAAGTCAAAGGGGCTGC -3' and R 5'-ATGTACTCCCCTCGTTTGTGC -3'), KRAS primers (F 5'-TCCAACAATAGAGGTGTTATTAAGC-3 and R 5'-ACTCGGGGATTTCCTCTTGA -3), PIK3CA primers (F 5'-ACGACTTTGTGACCTTCGGC -3' and R 5'-CCGATAGCAAAACCAATTTCTCGAT- 3'), MAPK primers (F 5'-GTACGACTCACTATAGGGAATTATGCATCCCACTGACCA-3' and R 5'-AGGTGACACTATAGAATACTGGCTCGGCACACAGAT-3'), C-MYC primers (F 5'-TGAGGAGACACCGCCCAC -3' and R 5'-CAACATCGATTTCTTCCTCATCTTC-3'), NF-kB primers (F 5'-TAG GAA AGG ACT GCC GGG AT -3' and R 5'- CAC GCT GCT CTT CTT GGA AGG -3'), Bcl-2 primers (F 5'-ATCGCCCTGTGGATGACTGAGT-3' and R 5'-GCCAGGAGAAATCAAACAGAGGC-3'), BAX primers (F 5'-TCAGGATGCGTCCACCAAGAAG-3' and R 5'-TGTGTCCACGGCGGCAATCATC-3'), NOX2 primers (F 5'-CTT CAT TGG CCT TGC CAT CC -3' and R 5'- GGG TTT CCA GCA AAC TGA GG -3'), Rac1 primers (F 5'-GCCAATGTTATGGTAGAT-3' and R 5'-GACTCACAAGGGAAAAGC-3'), FLT3 primers (F 5'-TTTCACAGGACTTGGACAGAGATTT-3' and R 5'-GAGTCCGGGTGTATCTGAACTTCT-3') and PKC primers (F 5'- CTT TCA TCC ACT GGC CTC GT -3' and R 5'- GTT GGG CTG CAT GAA CCT TG -3') . GAPDH was used as the endogenous control with primers F 5'-AACGGATTTGGTCGTATTG-3' and R 5'-GCTCCTGGAAGATGGTGAT-3'.

Western Blot

Western blot was carried out to verify the inhibition of STIM1 protein following dsiSTIM1 transfection. SDS-PAGE on a 12% gel was used to analyze protein samples (30 µg). Following electro-blotting to the Polyvinylidene Difluoride (PVDF) membrane, membrane blocking was performed for 1 hour in 5 % non-fat dry milk or 3 % bovine serum albumin (BSA) in 0.1 % TBST at room temperature. After washing, the primary antibody solutions were incubated with the membranes overnight at 4 ˚C. The primary antibodies were Rabbit monoclonal anti-human STIM1 antibody (Cell Signalling Technology, USA) at 1:500 dilution in 5 % non-fat dry milk in 0.1 % TBST and Rabbit monoclonal anti-human β-actin antibody (Cell Signalling Technology, USA) at 1:2000 dilution in 3 % BSA in 0.1 % TBST. At room temperature, and after washing, the HRP-conjugated polyclonal anti-rabbit secondary antibody (Cell Signalling Technology, USA) at 1:500

dilution in 0.1 % TBST included non-fat dry milk or BSA was incubated with the membranes for 1 hour. Next, the membranes were set up in the ECL substrate (Bio-Rad, USA) based on the manufacturer's instructions. Lastly, the VersaDoc imaging equipment (Bio-Rad, USA) was used to capture images of the membranes. The software Image Lab version 6.1 (Bio-Rad, USA) was used to estimate band intensity.

Measuring intracellular calcium level

Kasumi-1 cells were seeded in triplicates at $5x10^5$ cells /ml in 96-well flat bottom plates for 48 hours. After washing with PBS, the cells were suspended in 100 µl HEPES buffer saline loaded with 3 µM Fura-2AM (EMD Millipore, USA) and incubated for 30 minutes. After washing, the cells were incubated in 100 µl calcium-free HEPES buffer for 1 hour at 25 ˚C. Next, calcium stores were depleted using 200 nM thapsigargin (TG) (EMD Millipore, USA), followed by adding 2 mM CaCl2. Fluorescence intensity was measured at alternating excitation of 340 and 380 nm and emission at 510 nm, using the Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA).

Measuring intracellular ROS level

Cells were seeded in triplicates at 4 x 105/ml in a 96-well flat bottom plate. 48 hours later, the cells were incubated in 100 µl PBS loaded with 5 µM CM-H2DCFDA (Invitrogen, US) for 30 minutes. After washing, the cells were suspended in PBS for 1 hour. The ROS level was measured using a FLUOstar Omega microplate reader (BMG LABTECH, Germany) at a wavelength of 485 nm for excitation and 520 nm for emission. A Fluorescent microscope (Olympus IX71, Japan) was used to visualize the fluorescent dye rising from the cells.

Proliferation Assay

Kasumi-1 cells were seeded at 4 x 10^5 cells/ml in triplicate in a 96-well flat bottom plate after dsiRNA transfection. Cells were incubated for three time points: 24, 48, and 72 hours. The proliferation rate was measured by incubating the cells for 2 hours with 10 µl of cell count reagent SF (Nacalai Tesque, Japan). A microplate reader (Bio-Tek, US) was used to measure the absorbance at 450 nm.

Colony formation Assay

After dsiRNA transfection, Kasumi-1 cells were seeded in triplicate at 8 x 10^3 cells/ml in methylcellulose medium in a 24-well plate and incubated at 37 ºC for 8 days. Under a light microscope (Olympus CKX 41), colony numbers have been counted at magnifications of 40x and 200x. The selected colonies consisted of 50 cells or more.

Statistical analysis

Data were analyzed using SPSS version 26. A comparison between the two groups was carried out using a paired sample student t-test.

Results

***STIM1* knockdown reduced calcium influx in Kasumi-1 cells**

Efficient *STIM1* knockdown in Kasumi-1 cells was achieved using 20 nM dsiSTIM1 for 48 hours with a 69% suppression in *STIM1* mRNA expression and a 47% reduction in STIM1 protein level compared to control (Figure 1a and b). After knocking down *STIM1* and inducing SOCE via diminishing calcium reserves in the endoplasmic reticulum (ER) with thapsigargin (TG), the effect of *STIM1* on calcium influx in Kasumi-1 cells was examined. The calcium influx was reduced by 20% in the dsiSTIM1 group compared to the control group after *STIM1* knockdown (Figure 1c).

***STIM1* knockdown reduced intracellular ROS levels**

The intracellular ROS levels showed a significant reduction of 40% in the dsiSTIM1 transfected group compared to the control group following *STIM1* knockdown (Figure 2a). The dsiSTIM1 group showed a reduction of ROS-derived fluorescent signals compared to controls, DsiCtrl, and H2O2 positive controls (Figure 2b). The results were supported by the expression levels of ROS production-related genes *FLT3*, *Rac1*, *NOX2*, and *PKC*. Knockdown of *STIM1* revealed down-regulation of *NOX2* and *PKC* by 41% and 30%, respectively (Fig. 2c). *FLT3* and *Rac1* showed non-significant changes (Figure 2c).

***STIM1* knockdown inhibited Kasumi-1 cells proliferation and colony formation**

The impact of *STIM1* knockdown on Kasumi-1 cell proliferation was examined over 24-72 hours. The proliferation rate of Kasumi-1 cells was reduced by 22-24% compared to the control at 24-48 hours after *STIM1* knockdown (Figure 3a). Furthermore, the colony numbers were significantly reduced by 46% compared to the control when *STIM1* was knocked down (Figure 3b and c). The size of colonies also showed a clear reduction in the cell group transfected with dsiSTIM1 compared to the group transfected with dsiCtrl (Figure 3b). Moreover, the expression of selected genes implicated in *KRAS/MAPK* and *PI3K/Akt* was investigated following the knockdown. The findings exhibited a clear down-regulation of *KRAS, MAPK, C-MYC,* and *Akt* (Figure 4a and b). *PI3K, NF-kB, BAX,* and *Bcl-2* showed non-significant changes following *STIM1* knockdown (Figure 4b).

Discussion

STIM1 and calcium signal dysregulation have been linked to tumor initiation, progression, and relapse in a variety of malignancies (Liu et al., 2020, Cheng et al., 2016, Chen et al., 2015, Umemura et al., 2014, Liu et al., 2011). The understanding of the link between *STIM1* and calcium signalling in AML remains limited. Therefore, the present study evaluates the functional role of *STIM1* in AML and its interaction with calcium signals. Initially, *STIM1* expression was effectively suppressed at both the

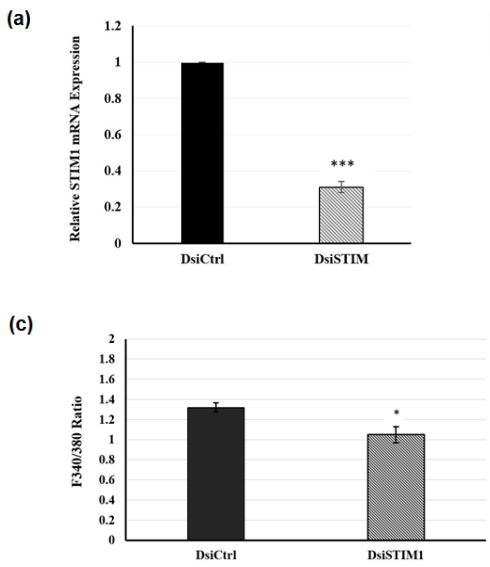

Figure 1. *STIM1* knockdown and the effect on calcium influx. (a and b) STIM1 expression at the mRNA and protein levels after knockdown (c) Calcium influx were measured as F340/380 ratio. Data are representative of mean ± SD of three independent experiments. All experiments were carried out in triplicates. A comparison was made between dsiCtrl and dsiSTIM1. * and *** indicate $p < 0.05$ and $p < 0.001$ respectively, based on paired sample t-test*Rhinacanthus nasutus*

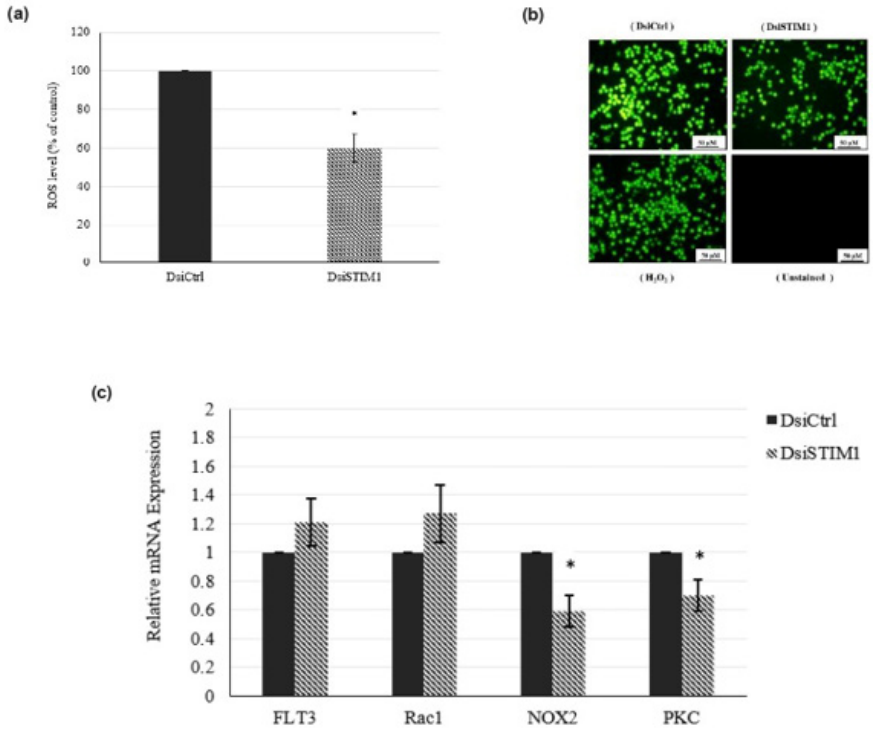

Figure 2. ROS levels after *STIM1* knockdown. (a) Percent of ROS level normalized to control. (b) Fluorescent microscope image of cells after incubation with 5μM CM-H2DCFDA, except unstained group, for 30 minutes. H2O2 used as positive control. (c) Expression of ROS production-related genes. Data are representative of mean ± SD of three independent experiments. All experiments carried out in triplicates. Comparison was made between dsiCtrl and dsiSTIM1. * indicates $p < 0.05$ based on paired sample t-test

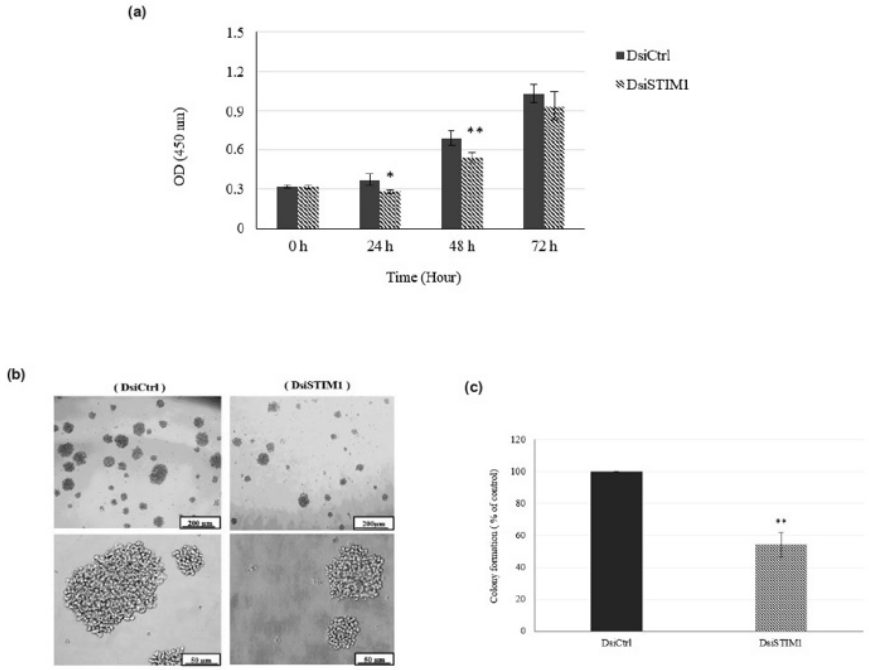

Figure 3. Cells proliferation and colony formation after *STIM1* knockdown. (a) The proliferation rate over the period from 24 - 72 hours after *STIM1* knockdown. (b) Bright field microscope shows reduced colony number and size in cells transfected with dsiSTIM1 compared to control 8 days after transfection. (c) Percent of colony formation normalized to control. Data are representative of mean ± SD of three independent experiments. All experiments carried out in triplicates. Comparison was made between dsiCtrl and dsiSTIM1. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, based on paired sample t-test

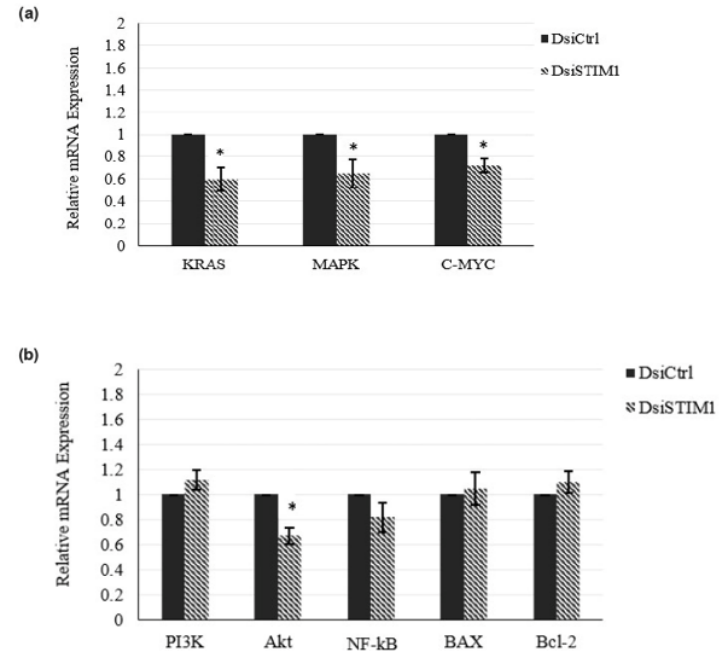

Figure 4. Expression of proliferative and survival pathway related genes after *STIM1* knockdown. (a and b) The expression profile was tested 48 hours after *STIM1* knockdown. Data are representative of mean ± SD of three independent experiments. All experiments carried out in triplicates. Comparison was made between dsiCtrl and dsiSTIM1. * indicate $p < 0.05$, respectively, based on paired sample *t*-test

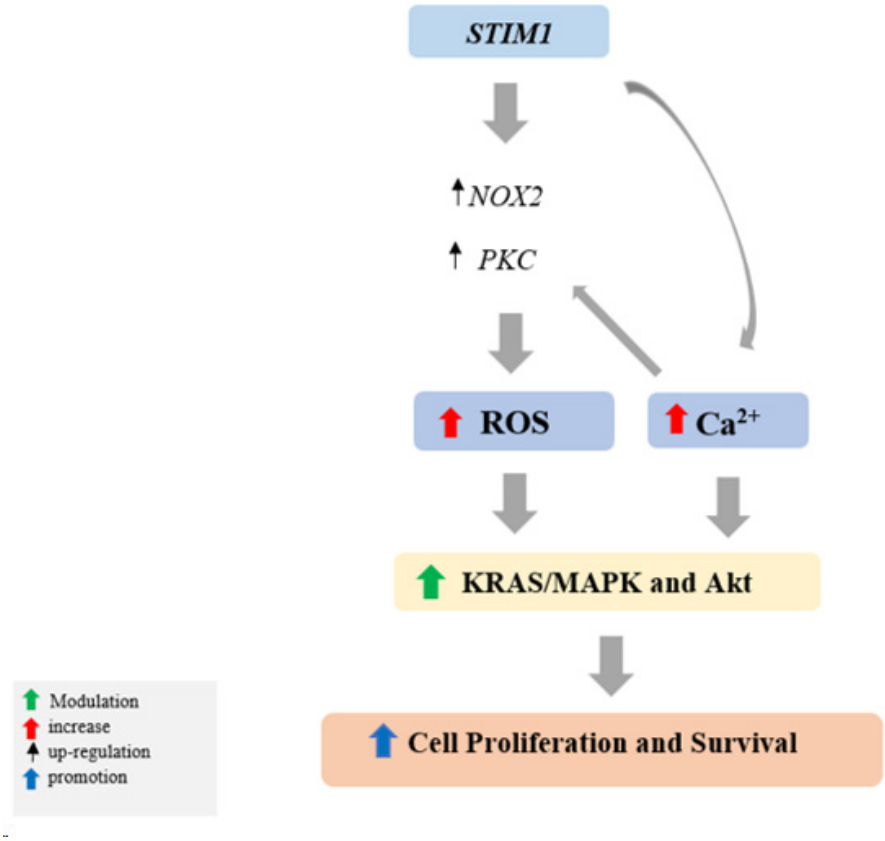

Figure 5. A schematic diagram illustrating the influence of *STIM1* on AML-M2 cell proliferation and survival. The diagram demonstrates the mechanism through which *STIM1* promotes AML-M2 cell proliferation and survival via enhancing the intracellular calcium/ROS interplay and modulating the expression of the proliferative and survival pathway-related genes *KRAS*/*MAPK* and *Akt*

mRNA and protein levels in the AML-M2 cell model. The findings revealed a reduction in the calcium influx following *STIM1* knockdown. Furthermore, *STIM1* knockdown and calcium reduction resulted in suppression of AML-M2 cell growth and the ability to survive and expand to form colonies. The regularity effect of *STIM1* on calcium influx was previously reported in other hematological malignancies, and both were strongly correlated with cancer progression, burden, and survival. In multiple myeloma (MM), *STIM1* was found to be highly expressed in MM tissue and cell lines, and *STIM1* silencing reduced calcium influx and arrested the cell cycle and viability (Wang at al., 2018). *STIM1* deletion in T cell acute lymphoblastic leukemia (T-ALL) was associated with a complete lack of calcium influx and prolonged the survival of mice with T-ALL by reducing the necroinflammatory response in leukemia-infiltrated organs and down-regulating pro-inflammatory pathways in leukemic T lymphoblasts (Saint Fleur-Lominy et al., 2018). In chronic lymphocytic leukemia (CLL), cases with high *STIM1* expression were associated with a poor survival rate, and suppression of *STIM1* in B-CLL cells resulted in reduced calcium influx and inhibited cell viability (Debant et al., 2019). Collectively, all these data represent the significance of *STIM1* and calcium signals in the pathogenesis of different blood cancers and may guide researchers toward the importance of selecting them for targeted therapy.

Further investigation of the AML-M2 cells revealed a reduction in intracellular ROS levels following *STIM1* knockdown and calcium reduction. This is explained by the downregulation of NADPH oxidase 2 (*NOX2*) and *PKC* after *STIM1* knockdown (Parascandolo and Laukkanen, 2019, Eun et al., 2017). Calcium is essential for the activation of *PKC,* which initiates the activation process of NOX2 to generate ROS. These findings can support the *STIM1* regularity effect on ROS generation via the NADPH oxidase 2. Numerous studies have highlighted the interplay between calcium and ROS in a variety of cancers, such as breast, liver, pancreatic, and cervical cancers (Delierneux et al., 2020, Feno et al., 2019). Furthermore, other cancer studies revealed an interaction between ROS and the RAS/MAPK and PI3K/AKT proliferative and survival pathways to maintain cancer cell proliferation and survival (Perillo et al., 2020, Grauers Wiktorin et al., 2020, Sillar et al., 2019, Parascandolo and Laukkanen, 2019, Kumari et al., 2018, Reczek and Chandel, 2018, Prasad et al., 2017, Moloney et al., 2017, Eun et al., 2017). Therefore, this can be linked to suppression of AML-M2 cell growth and survival in the current study following knockdown of *STIM1* and reduction of calcium and ROS, which was associated with downregulation of KRAS, *MAPK, C-MYC,* and *Akt* expression. Calcium and ROS have contributed to the control of the *MAPK* and *Akt* pathways in several cancers, including acute lymphoblastic leukemia, breast, ovarian, and brain cancers (Wang et al., 2021, Terrié et al., 2021, Liu et al., 2019, Zheng et al., 2018). All these data express the importance of *STIM1* to maintain growth and survival of AML-M2 cells through: 1) enhancing ROS production by NOX2; 2) maintaining the critical intracellular calcium/ROS interplay; and 3) modulating the *MAPK* and *Akt* survival pathways (Figure 5). The other survival-related genes PI3K, NF-kB, BAX, and BCL2 and ROS-related genes RAC1 and FLT3, were not affected by STIM1 knockdown in the present study, which could support their non-involvement in STIM1-mediated pathogenesis in AML-M2 cells.

Currently, the therapeutic strategy of AML is subjected to modification in some cases by adding certain approved targeted therapies, such as midostaurin, against mutated FLT3, and Ivosidenib, against mutated IDH1, to the standard therapy, and this modification has expressed a clear improvement in the survival rate of AML patients, particularly for the elderly and those with poor prognosis (Puccini et al., 2021, Wu et al., 2018). The important findings in the present study may nominate *STIM1* and ROS for targeted therapy. The trial of separated or combined inhibition of *STIM1* and/or ROS could help in the regression of disease, and together with standard AML treatment, it may improve the outcome among AML cases, especially the refractory or relapsed cases.

Conclusion

The present work supports STIM1's potential role in promoting AML-M2 cell survival by regulating calcium/ROS interactions, as well as KRAS/MAPK and Akt-related pathways. Further comprehensive research into the potential impact of *STIM1* inhibition on disease regression may recommend *STIM1* for targeted therapy and contribute to the improvement of AML therapeutic strategies.

Author contribution

A.E.S contributed to the experimental work, analyzing results, and writing process. M.R.B.S.M.N is the principal investigator who contributed to the concept idea, experimental design, writing process and gave final approval of this paper for publication. M.E.J, A.R.N.A, and Y.N.M assisted in the procedures. All authors have given approval to the final version of the manuscript.

Acknowledgment

The authors would like to acknowledge the funding from the Ministry of Higher Education for Fundamental Research Grant Scheme with project Code: FRGS/1/2021/SKK03/USM/02/3 and Universiti Sains Malaysia for all the support and facilities.

Competing financial interests

The authors have no conflict of interest.

Abbreviations

Akt: Protein kinase B
AML: Acute myeloid leukemia
BAX: BCL2 Associated X
Bcl2: B-cell lymphoma 2
C-MYC: Avian Myelocytomatosis Viral Oncogene Homolog
DsiRNA: Dicer-substrate small interfering RNA
ER: Endoplasmic reticulum
FLT3: FMS-like tyrosine kinase 3
GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase
KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog
MAPK: Mitogen-activated protein kinase
NF-kB: Nuclear factor kappa B
NOX2: NADPH oxidase 2
PI3K: Phosphatidylinositol-3-kinase
PKC: Protein kinase C
qRT-PCR: Quantitative reverse transcription-polymerase chain reaction
Rac1: Ras-related C3 botulinum toxin substrate 1
ROS: Reactive oxygen species
SOCE: Store- operated calcium entry
***STIM1*:** Stromal interaction molecule 1

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