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

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Research Article

Keywords: STIM1, ROS, calcium, survival, proliferation, acute myeloid leukemia

Posted Date: September 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-873613/v1

Abstract

Stromal interaction molecule 1 (*STIM1*) is a critical regulator of calcium homeostasis through store-operated calcium entry (SOCE) and recently considered a potential therapeutic target for cancer. However, the role of *STIM1* in acute myeloid leukemia (AML) remains unclear. The present study investigates the role of *STIM1* in AML cell line (THP-1) proliferation and survival and its effect on reactive oxygen species (ROS) activities. Dicer-substrate siRNA (dsiRNA) - mediated *STIM1* knockdown inhibited the THP-1 cells proliferation and colony formation ability. Further observation on ROS profile showed a significant reduction in the ROS level, which was associated with a significant down-regulation of *NOX2* and protein kinase C (PKC). Furthermore, *STIM1* knockdown exhibited significant down-regulation of *Akt*, *KRAS*, and *MAPK* which are critical proliferative and survival pathway-related genes. This study unveiled the importance of *STIM1* in the regulation of AML cells proliferation and survival which could be through maintaining ROS at level keeping the proliferative and survival pathways at an active state. These findings represent *STIM1* as a potential therapeutic target for AML treatment.

Introduction

Acute myeloid leukemia (AML) is a highly aggressive hematological malignancy. Despite it affects all age groups, AML is more common in adults with the median age is 67 years and around 30 % of AML patients above 75 years [1]. Advances in the molecular characterization of AML and targeted therapy combined with conventional chemotherapy improved the 5-year overall survival which raised from less than 10% to 50% in patients who were less than 40 years in age and reached more than 60% in childhood AML, but did not show any change in elderly AML, which was still less than 10% [1, 2]. In contrast to improving the remission rate among younger AML which was 70%–80% after induction chemotherapy, at least half of these patients showed relapse and eventually died [2]. Many molecular and biochemical disturbances associated with AML are still unclear, and these changes may be the key factors for improving therapeutic strategies for AML and treatment outcomes.

Stromal interaction molecule 1 (*STIM1*) protein, the main player of store-operated calcium entry (SOCE), acts as a sensor for calcium storage in the endoplasmic reticulum (ER). Recently, *STIM1* was found to play a critical role in the development and metastasis of a variety of cancers, such as brain, prostate, colorectal cancers, and multiple myeloma [3]. *STIM1* knockdown in glioblastoma cell lines exhibited a reduction of cell proliferation and survival [4]. Furthermore, there is increasing evidence that suggests the presence of an interplay between STIM1/calcium and reactive oxygen species (ROS) signaling pathways in the cancer cells biology [5, 18]. ROS directly stimulate *STIM1* and activate SOCE, independent of calcium ER store depletion [5]. Up-regulated HIF-1(Hypoxia Inducible Factor) in response to hypoxia lead to up-regulation of *STIM1* in hepatocarcinoma cells [6]. On other hand, calcium enhances and regulates ROS production by mitochondria which essential for cancer survival signaling pathways [7].

These findings support the role of *STIM1* in cancer pathogenesis and the cross-talk between calcium and ROS in cancer. Thus, determining whether *STIM1* plays an important role in AML and whether

STIM1/ROS interaction is critical to AML cell survival becomes important to investigate. So, this study aims to investigate the role of *STIM1* in AML after knocking down *STIM1* with dicer-substrate siRNA (dsiRNA). *STIM1* was suppressed in the AML cell lines (THP-1 cells), and changes in the cellular functions, biochemical, and also the expression level of pathways-related genes were evaluated. The results of the study may provide novel insights into AML pathogenesis and may be useful in improving therapeutic strategies for AML in the future.

Materials And Methods

Cell culture

THP-1 cells were purchased from the American Type Culture Collection (ATCC) (Virginia, USA) and cultured in Roswell Park Memorial Institute Medium (RPMI-1640) (Sigma-Aldrich, US), supplemented with 10 % fetal bovine serum (FBS) (Gibco, Life Technologies, US) and 1 % penicillin/streptomycin (Gibco, Life Technologies, US) at 37 ºC in a humidified atmosphere containing 5 % CO_2 and 95 % air.

Transfection with dsiRNA

STIM1 dicer-substrate siRNA (dsiRNA) (TriFECTa, Integrated DNA Technologies, US) was transfected in to THP-1 cells (2 x 10^6/ ml) at doses of 10, 20, and 50 nM for 24 to 72 h. STIM1 dsiRNA transfected to the cells using a Bio-Rad Gene Pulser Xcell electroporation system (Bio-Rad laboratories, USA) at pulse of 300 V for 7 microseconds. The transfected cells were diluted 20 - fold with culture medium and incubated at 37 ºC and 5 % CO_2. All experiments were compared against a dsiRNA negative control.

qRT- PCR Analysis

Total RNA was extracted from the cells using Monarch® Total RNA Miniprep Kit (New England BioLabs, UK) 24 - 72 h post dsiRNA transfection. The cDNA was synthesized by reverse transcription using Rever Tra Ace® qPCR RT Master Mix (Toyobo, Japan) following the manufacture's protocol. Luna® Universal qPCR Master Mix (New England BioLabs, UK) and Step One Plus Real-Time PCR System (Applied Bioscience, US) were used to measure gene expression levels after *STIM1* knockdown. The gene specific primers that used were as the following: *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 performed to confirm the suppression of STIM1 protein after dsiSTIM1 transfection. Protein samples (30 µg) were analysed by SDS-PAGE on 12 % gel. Following electro-blotting to Polyvinylidene Difluoride (PVDF) membrane, membranes were blocked in 5 % non-fat dry milk or 3 % bovine serum albumin (BSA) in 0.1 % TBST for 1 hour at room temperature. The membrane was rinsed in 1X TBST three times and incubated in the primary antibody solutions overnight at 4 ˚C with gentle rocking. The primary antibodies included 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 -actin antibody (Cell Signalling Technology, USA) at 1:2000 dilution in 3 % BSA in 0.1 % TBST. The membranes next day were washed with 1X TBST three times and incubated in 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, for 1 hour at room temperature. After washing with TBST, the membranes were incubated in the ECL substrate (Bio-Rad, USA) according to manufacturer's directions. After incubation, the membranes were imaged using VersaDoc imaging system (Bio-Rad, USA). Band intensity was measured using Image Lab software version 6.1 (Bio-Rad, USA).

Proliferation Assay

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

Colony formation Assay

THP-1 cells were seeded, after dsiRNA transfection, in triplicate at 2 x 10^3 cells/ml in methylcellulose medium in 24 - well plate and incubated at 37 ºC for 8 days. The colonies were counted under light microscope (Olympus CKX 41) at magnification of 40x and 200x. The selected colonies were those consist of 50 cells or more.

Measurement of intracellular calcium level

THP-1 cells were seeded in triplicates at 5x10^5 cells /ml in 96 - well flat bottom plates for 24 h. 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. Cells were washed with HEPES buffer saline and suspended in 100 µl calcium-free HEPES buffer saline and incubated for 1 hour at 25 C to permit dye de-esterification. After that, depletion of calcium stores was triggered by adding 200 nM thapsigargin (TG) (EMD Millipore, USA) to the cells followed by adding 2 mM $CaCl_2$. Fluorescence intensity was measured at alternating 340 and 380 nm excitation and 510 nm emission using Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA).

Measurement of intracellular ROS level

Cells were seeded in triplicates at 2 x 10^5/ml in 96 - well flat bottom plate. After 24 hour of transfection, the cells were washed with PBS, suspended in 100 µl PBS loaded with 5 µM CM-H2DCFDA (Invitrogen, US) and incubated for 30 minutes. After that, cells were washed and suspended in PBS for 1 hour. ROS level was measured using FLUOstar Omega microplate reader (BMG LABTECH, Germany) at 485 nm excitation and 520 nm emission. Fluorescent microscope (Olympus IX71, Japan) was used to visualize the fluorescent dye rising from the cells.

Statistical analysis

All statistical analysis was carried out using SPSS version 26. Comparison between the two groups was carried out using a paired sample student *t*-test. Data was considered significant when $p \leq 0.05$ which presented as (*), very significant when $p \leq 0.01$ (**), and extremely significant when $p \leq 0.001$ (***).

Results

STIM1 expression was efficiently suppressed by dsiSTIM1

The efficient *STIM1* knockdown profile on THP-1 cells was studied at three different concentrations of dsiSTIM1 (10, 20, and 50 nM) and three transfection periods (24, 48, and 72 h). *STIM1* expression in THP-1 cells were significantly suppressed by 70 % (*p*- value = 0.001) 24 h after transfection of cells with 10 nM dsiSTIM1 (Fig. 1b). At the protein level, STIM1 protein expression was reduced by 54 % after 24 h of transfection (Fig. 1c).

STIM1 knockdown inhibits THP-1 cells proliferation and colony formation

The proliferation rate of THP-1 cells was tested over the period from 24 – 72 h after dsiRNA transfection. Knockdown of *STIM1* in THP-1 cells resulted in 12 - 14 % suppression of cell proliferation ($p < 0.05$) at 24 – 48 h after knockdown compared to control, dsiCtrl (Fig. 2a). The colony formation ability of THP-1 cells was tested under a bright-field microscope by counting the number of colonies formed by the cells transfected with dsiSTIM1 compared to control (dsiCtrl). The results revealed a significant reduction in the number of colonies formed by THP-1 cells transfected with dsiSTIM1 reached 35 % ($p < 0.05$) compared to cells transfected with dsiCtrl (Fig. 2b). In addition to that, the study findings also revealed a decrease in the size of colonies formed by cells transfected with dsiSTIM1 compared to control (Fig. 2b).

STIM1 knockdown suppresses calcium influx in THP1- cells

The role of *STIM1* in controlling calcium influx in THP-1 cells was assessed after *STIM1* knockdown and after stimulation of SOCE through depletion of calcium store in ER using TG. Calcium influx curve was reduced in dsiSTIM1 transfected group compared to control groups, transfected with dsiCtrl and an untransfected group of THP-1 cells (Fig. 3a). Calcium influx peak was reduced by 25 % ($p < 0.01$) after *STIM1* knockdown compared to control (disCtrl) (Fig. 3b).

STIM1 knockdown suppresses ROS level in THP-1 cells

Intracellular ROS levels were measured 24 h after *STIM1* knockdown. The results exhibited a very significant reduction by 55 % ($p < 0.01$) in ROS levels of dsiSTIM1 transfected group of THP-1 cells compared to the control group, dsiCtrl (Fig. 4a). Fluorescent microscope study revealed a reduction of ROS – derived fluorescent signals in dsiSTIM1 transfected group compared to the signals from control groups, dsiCtrl and H_2O_2 positive control (Fig. 4b).

STIM1 Regulates *KRAS/ MAPK* and *PI3K/ Akt* pathways

The effect of *STIM1* on the targeted genes involved in the proliferative and survival pathways, *KRAS/ MAPK* and *PI3K/ Akt*, were evaluated after the suppression of *STIM1*. The expression profile in THP-1 cells showed a significant down-regulation pattern of *KRAS* and *MAPK*, $p < 0.01$ and $p < 0.05$ respectively (Fig. 5a). *STIM1* knockdown significantly suppressed the expression of *Akt* ($p < 0.01$), one of the main survival pathway regulators (Fig. 5b). *C-MYC* expression levels were down-regulated too, whereas *BAX* expression was slightly up-regulated after *STIM1* knockdown (Fig. 5a and b). No significant changes in *NF-kB* levels were found while *Bcl-2* was slightly down-regulated. *PI3K* exhibited an up-regulation pattern which may be a compensatory mechanism due to a disturbed survival pathway after Akt suppression.

STIM1 regulates NADPH oxidase-derived ROS pathway -related genes

The expression profile of targeted genes (*FLT3*, *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 down-regulation of *NOX2* and *PKC* ($p < 0.05$ for both) 24 h after *STIM1* knockdown (Fig. 5c). *FLT3* and *Rac1* revealed up-regulation pattern ($p > 0.05$) in response to *STIM1* knockdown (Fig. 5c).

Discussion

STIM1 was targeted in numerous recent studies where discovered to be highly expressed in many cancer cells and tissues and this elevated level was associated with enhancement of many cellular functions such as proliferation, survival, invasion and metastasis [3]. In breast cancer, *STIM1* was found highly expressed in malignant tissues compared to non-tumor tissues [8]. According to the Human Protein Atlas and Expression Atlas, *STIM1* is highly expressed in many AML cells, including THP-1 cells [9, 10], but

STIM1 effect on AML cells proliferation and survival remains unclear. In the present study, knockdown of *STIM1* in THP-1 cells revealed significant reduction in the cell proliferation and colony formation. Colony size also reduced following *STIM1* knockdown in THP-1 cells. In colorectal cancer, *STIM1* knockdown resulted in significant inhibition of cancer cell proliferation and colony formation ability [11]. *STIM1* was found abundantly expressed in multiple myeloma (MM) tissues and cell lines and silencing of *STIM1* produced reduction in cell viability and caused cell cycle arrest [12]. These findings indicate the critical role of *STIM1* in controlling the regularity mechanisms of cell proliferation and survival.

Furthermore, the regularity role of *STIM1* on the intracellular calcium level was evaluated. The results revealed significant reduction in the calcium influx levels in THP-1 cells following *STIM1* knockdown which indicates the importance of *STIM1* in the controlling SOCE and regulation of intracellular calcium level in AML cells. Given that a certain level of ROS is critical for cancer cell survival [7, 13, 15], this study evaluated whether *STIM1* plays a role in the regulation of ROS level in AML cells. The results exhibited that suppression of *STIM1* markedly reduced ROS level in the THP-1 cells and supports the significance of *STIM1* in maintaining a pro-survival ROS level. The transient receptor potential ankyrin 1 prevents ROS-induced cancer cell death by mediating the up-regulation of calcium-dependent anti-apoptotic pathways [13, 16]. Calcium is essential for the activation of *PKC* which plays role in activation of NADPH oxidase-derived ROS production [16]. Consequently, *STIM1* was suspected to control NADPH oxidase - derived ROS production.

The expression level of *NOX2* and *PKC,* genes involved in NADPH oxidase-derived ROS production, was assessed. Considerable reduction in *NOX2* and *PKC* levels were observed after *STIM1* knockdown. *FLT3* and *Rac1* exhibited increases in their expression levels which may be a compensatory mechanisms for decreasing level of ROS. Reduction of cells proliferation and survival rate following *STIM1* knockdown was explained through assessing the expression profile of proliferation and survival pathways related gene. *KRAS* and *MAPK* was significantly suppressed after *STIM1* knockdown. *Akt* which is one of the important survival genes was also significantly suppressed. These findings support the results of previous studies which exhibited presence interaction between ROS and proliferative and survival pathways, RAS/MAPK and PI3K/Akt, to maintain cell proliferation and survival [14, 17]. This study highlighted the important role of *STIM1* in maintaining this interaction (Fig. 6).

The present study showed an important findings regarding AML proliferation and survival and highlighted the critical role of *STIM1* in maintaining AML cell survival. *STIM1* ensures AML cell survival by increasing cellular level of ROS and up-regulation of proliferative and survival pathways. Further work is still needed to elucidate the *STIM1*-ROS interaction and its role in AML pathogenesis and confirm the potential of *STIM1* as a therapeutic target for AML treatment.

Conclusions

This study highlighted the importance of *STIM1* for AML cell proliferation and survival which may be facilitated by controlling of ROS level and activation of certain targeted proliferative and survival

pathways. *STIM1* and the calcium pathway are potential therapeutic targets for AML and thus should be subjected to molecular and functional studies using different AML cell lines.

List Of 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
CRC
Colorectal cancer
DsiRNA
Dicer-substrate siRNA
ER
Endoplasmic reticulum
FLT3
FMS-like tyrosine kinase 3
GAPDH
Glyceraldehyde-3-Phosphate Dehydrogenase
HIF-1
Hypoxia Inducible Factor 1
KRAS
Kirsten Rat Sarcoma Viral Oncogene Homolog
MAPK
Mitogen-activated protein kinase
MM
Multiple myeloma
NF-kB
Nuclear factor kappa B
NOX2
NADPH oxidase 2
OS
Overall survival
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

Declarations

Funding

This work is financially supported by The Ministry of Higher Education Malaysia under Fundamental Research Grant Scheme (FRGS) with Project Code: FRGS/1/2019/SKK15/USM/02/2.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data of this study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

All authors shared in performing this work and all approved the final manuscript.

Ethics approval and consent to participate

Not applicable since no patients or animals were used in this study.

Consent for publication

Not applicable.

Acknowledgements

The authors would like to thank The Ministry of Higher Education Malaysia for sponsoring this work under the Fundamental Research Grant Scheme (FRGS) with Project Code: FRGS/1/2019/SKK15/USM/02/2, and Universiti Sains Malaysia for all the support and facilitation. Also, we would like to thank Adam Azlan for his honourable cooperation to success this work.

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Figures

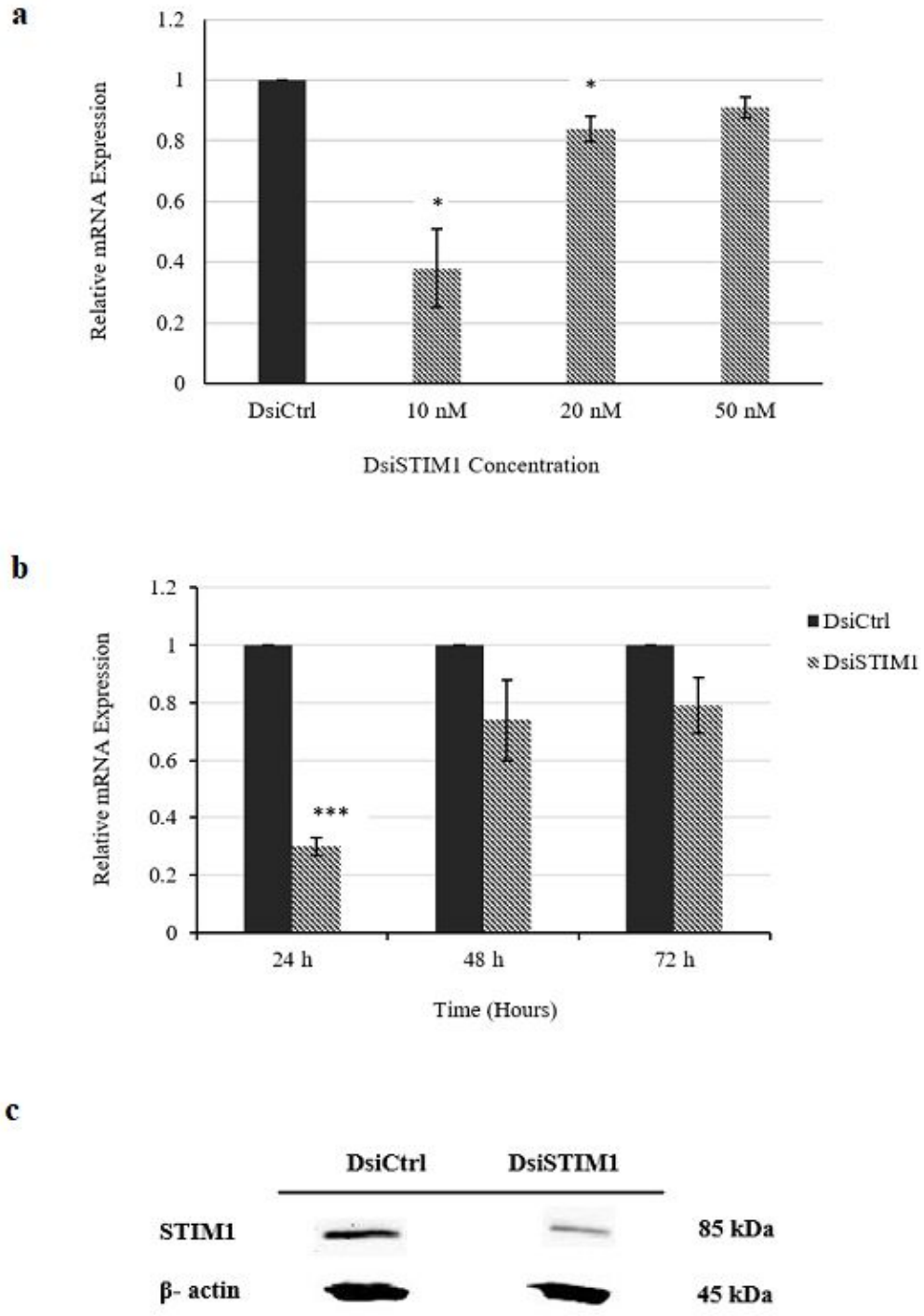

Figure 1

STIM1 knockdown in THP-1 cells. STIM1 expression after transfection of THP-1 cells with a different concentration of dsiSTIM1 and b after incubation of cells for different periods of time after transfection with 10 nM dsiSTIM1. 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 0.001, respectively, based on paired sample t-test. c STIM1 protein expression 24

h after transfection. The figure represents one of two independent experiments with close results. β - actin was used as a loading control

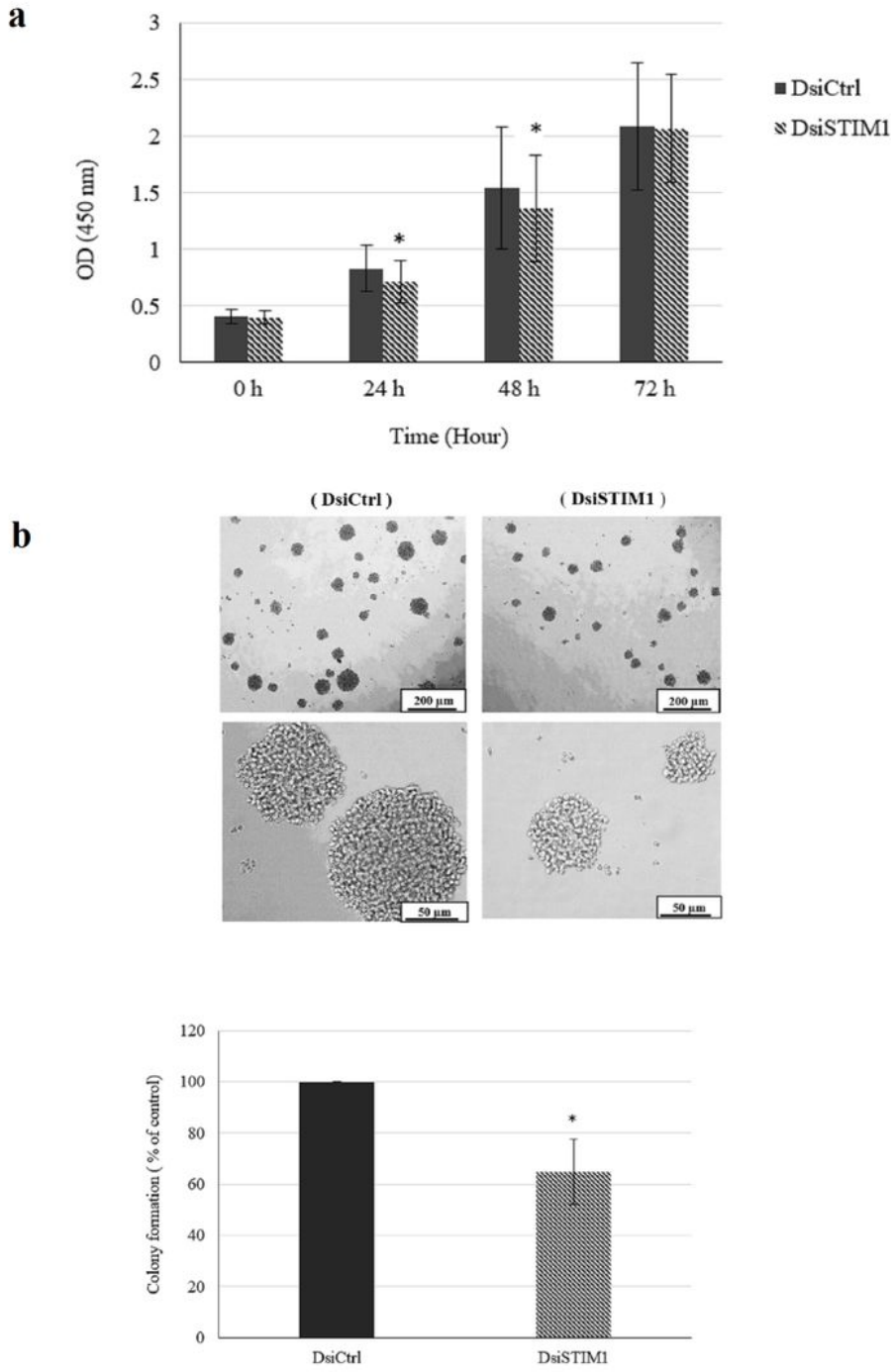

Figure 2

THP-1 cells proliferation and colony formation after STIM1 knockdown. a THP-1 cells proliferation rate was tested over time from 24 - 72 hours after STIM1 knockdown. b Bright field microscope shows reduced colony number and size of THP-1 cells transfected with dsiSTIM1 which supported with

statistical result. Data are representative of mean ± SD of three independent experiments. All experiments carried out in triplicates. Comparison was made between dsiCtrl and dsiSTIM1 at each time point. * indicates p ≤ 0.05 based on paired sample t-test

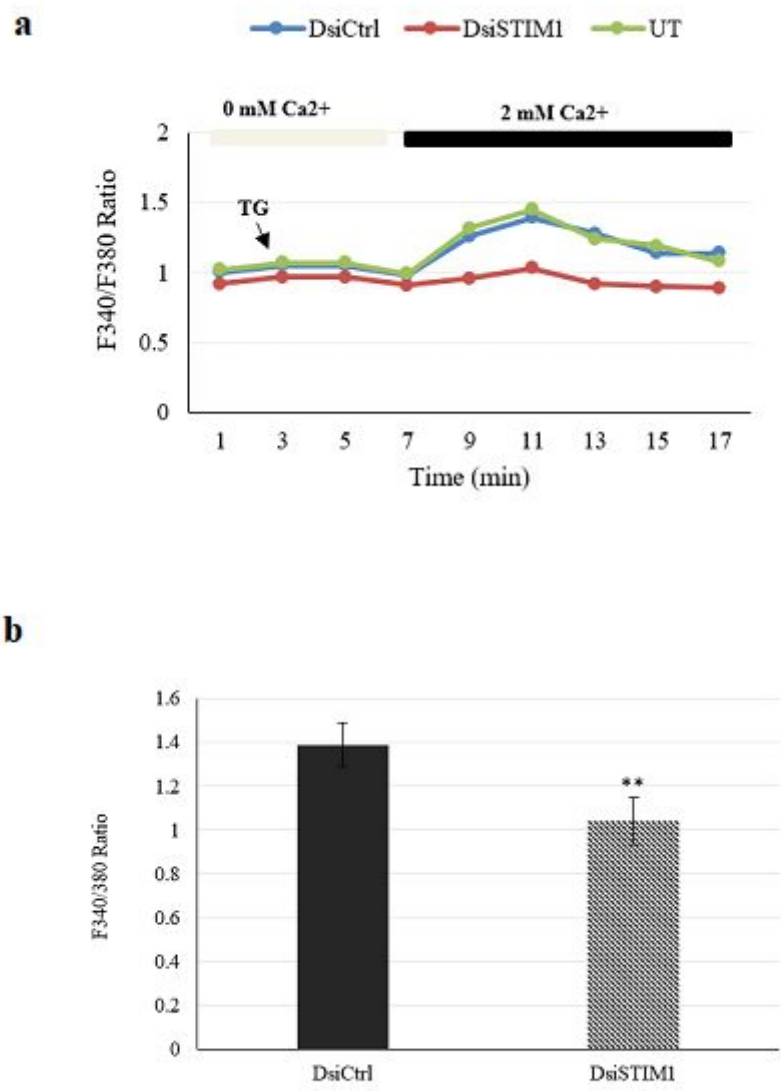

Figure 3

Effect of STIM1 knockdown on calcium influx in THP-1 cells. ER calcium store were depleted using 200 nM TG which added to calcium – free buffer followed by adding 2 mM calcium to induce SOCE. a Change in calcium influx level over time were measured as F340/380 ratio. b Quantification of calcium influx peak amplitudes. 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 ≤ 0.01 based on paired sample t-test

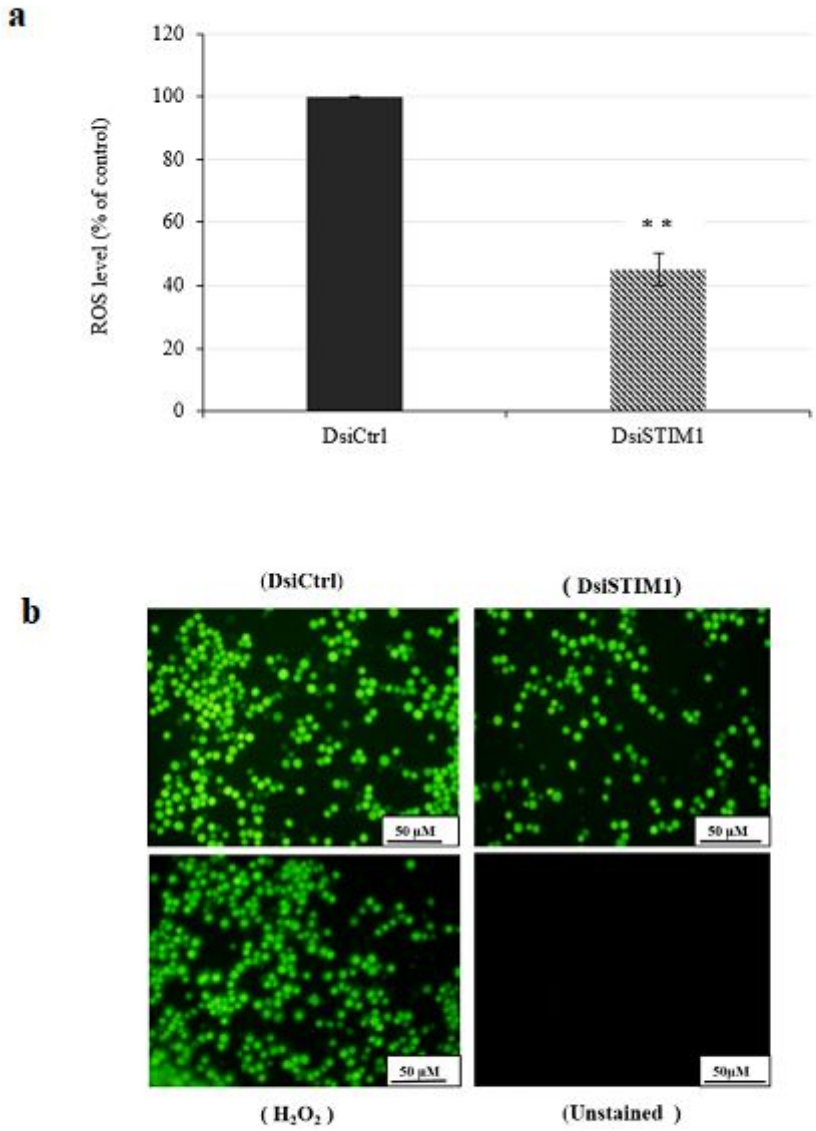

Figure 4

ROS level in THP-1 cells after STIM1 knockdown. a Percent of ROS level normalized to control 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. ** indicates p 0.01 based on paired sample t-test. b Fluorescent microscope image of cells after incubation with 5µM CM-H2DCFDA, except unstained group, for 30 minutes. H2O2 used as positive control

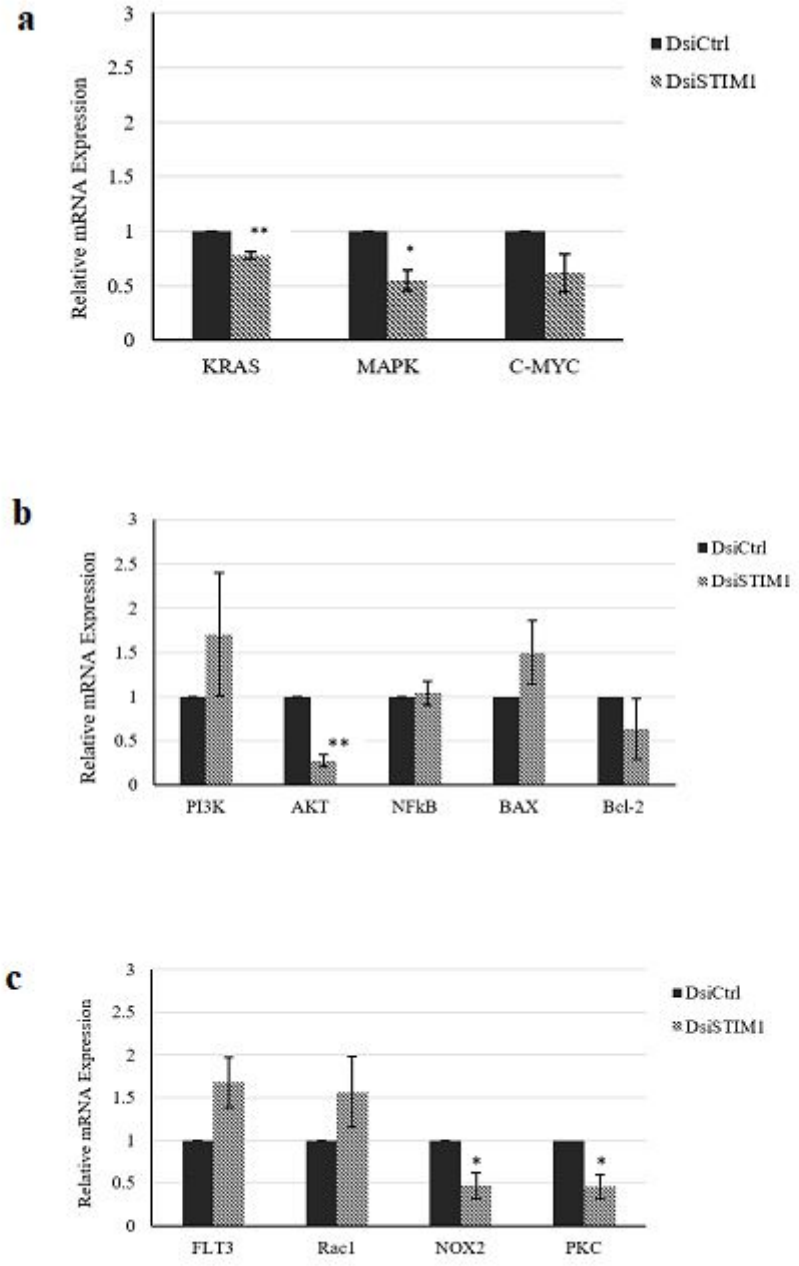

Figure 5

Effect of STIM1 knockdown on the expression of proliferative, survival, and NADPH oxidase–derived ROS pathways-related genes in THP-1 cells. The expression of a Proliferative b survival and c NADPH oxidase–derived ROS pathways-related genes was tested 24 h 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. * and ** indicate p □ 0.05 and p □ 0.01, respectively, based on paired sample t-test

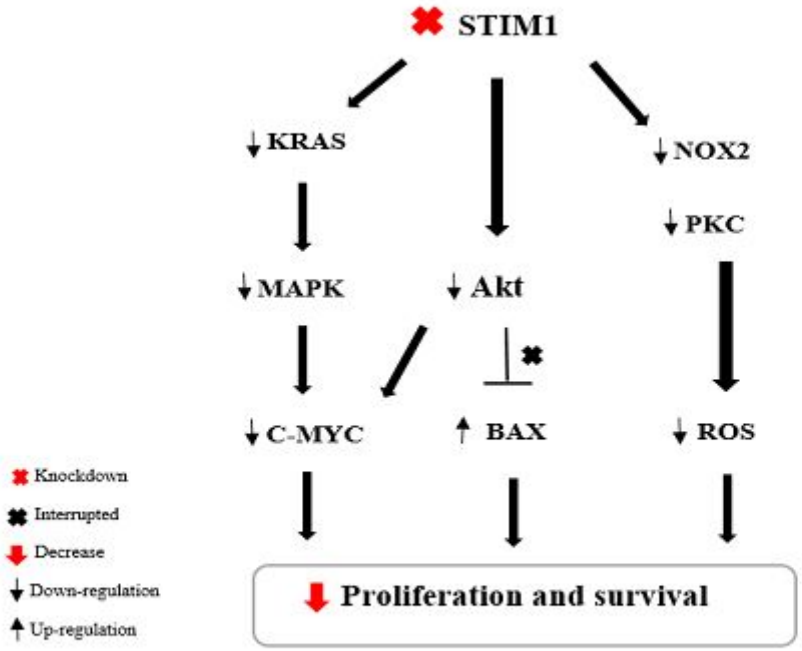

Figure 6

Schematic diagram of the mechanism through which STIM1 regulates THP-1 cells proliferation and survival. The diagram illustrates the mechanism through which STIM1 regulates THP-1 cells proliferation and survival via regulation of certain proliferative, survival related genes, KRAS, MAPK, C-MYC and Akt. Also the mechanism includes the control of ROS production by down-regulation of NOX2 and PKC. BAX, pro-apoptotic, slightly up-regulated in response to STIM1 knockdown