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Original Article

Endogenous SARI exerts oncogenic functions in human K562 leukemia cells by targeting the PI3K/Akt/mTOR and NF-κB signaling pathways

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Abstract: Suppressor of activator protein-1, regulated by interferon (SARI), is a novel basic leucine zipper containing type I IFN-inducible early response protein that plays an important regulatory role in a wide variety of tumors, including leukemia. However, the functional role of SARI in myeloid leukemia is not thoroughly understood. In this study, we discovered that knock-down of SARI expression suppressed cell growth and colony formation, inhibited invasion, enhanced imatinib (STI571)-mediated apoptosis, and induced G0/G1 and G2/M arrest in human K562 myeloid leukemia cells. Moreover, using immunoblotting, we provide evidence that silencing of SARI resulted in declined expression of cyclinD1 and cyclinA2, as well as down-regulation of mTOR, c-myc p-mTOR, p-PI3K (p85), p-Akt, p70-S6K, p-p70-S6K and NF-κB (p65) that involved in the PI3K/Akt/mTOR and NF-κB signaling pathways. Taken together, our results demonstrate that SARI functions as an oncogenic role in K562 myeloid leukemia cells through regulating the PI3K/Akt/mTOR and NF-κB signaling pathways.

Keywords: SARI, myeloid leukemia, K562 cell, mechanism

Introduction

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative hematologic neoplasm that is driven by the BCR-ABL fused oncogene developed from the Philadelphia chromosome (Ph) [1, 2]. Consequently, the genetic marker is represented by the presence of Ph in over 95% of patients with CML. Although the introduction of tyrosine kinase inhibitors has substantially changed the landscape of CML treatment, the problems of drug-resistance become more and more serious and challenging [3, 4]. In this respect, better understanding of the underlying mechanisms of myeloid leukemia biology will help to develop novel drug targets for therapies.

Genetic alterations have been regarded as critical factors participating in leukemia, which can inactivate genes involved in leukemia pathogenesis or progression [5]. Ample evidence has suggested that the functions of some genes could be directly inactivated by the BCR-ABL in CML [6-8]. Among these affected genes, the suppressor of activator protein-1, regulated by interferon (*SARI*) gene has been highlighted [8]. Human *SARI* gene, also called *mda-D-74, BATF-2* (basic leucine zipper transcription factor 2), or *ATF-like 2* (activating transcription factor like 2), is a novel basic leucine zipper containing gene that identified and reported in 2008 [9]. The *SARI* gene locates at 11q12-11q13, and possesses 3 exons in the coding region and codes for a putative protein (SARI) of 274 amino acid residues. Since its discovery, SARI has been reported to be associated with infectious diseases [10]. Of note, growing evidence suggests that SARI is a putative tumor suppressor and revels a tumor-suppressive role in many types of cancers, including lung adenocarcinoma [11], colorectal cancer [12, 13], esophageal

squamous cell carcinoma [14], hepatocellular carcinoma [15], prostate cancer [16, 17], as well as oral tongue squamous cell carcinoma [18], and so forth. In leukemia, Huang's study has demonstrated that *SARI* mRNA level could be suppressed by BCR-ABL through regulating the RAS/MAPK and JAK/STAT signaling in human K562 myeloid leukemia cells [8]. However, the function role of SARI in CML remains elusive. In our previous work, we found that the K562 cell line retains a high expression level of endogenous *SARI* in wild type. This article describes the effects of SARI-silencing on the biological features of K562 myeloid leukemia cells as well as the underlying mechanisms. Our data showed that silencing of SARI in K562 cells prohibited cell growth and colony formation, enhanced imatinib (STI571)-mediated apoptosis, induced G0/G1 and G2/M arrest, and inhibited invasion, possibly by regulating the PI3K/Akt/mTOR and NF-κB signaling pathways.

Materials and methods

Main reagents

RPMI-1640 medium, fetal bovine serum (FBS) (Hyclone, Logan, UT, USA); Trizol solution (Invitrogen; Thermo Fisher Scientific); Short hairpin RNA were designed and synthesized by the Genechem Company (Shanghai, China); Reverse Transcription Kit (Thermo Fisher Scientific, USA); MTS Assay Kit (Promega, USA); Annexin V-PE/7-AAD Apoptosis Assay Kit, Matrigel (Becton, Dickinson and Company, USA); STI571 (Selleck Chemicals, Houston, USA); RIPA lysis buffer, HRP-conjuncted goat anti-rabbit and anti-mouse secondary antibodies (all from KWBIO, Beijing, China); BSA (Bovine Serum Albumin) (Beyotime Institute of Biotechnology, Haimen, China); methyl cellulose (Sigma-Aldrich, St. Louis, MA, USA); rabbit anti-human SARI, anti-human c-myc, anti-human PI3K (p85), anti-human p-PI3K (p85), anti-human mTOR, anti-human p-mTOR, anti-human p-PTEN and mouse anti-human Bcl-2 (all from Abcam, Cambridge, MA, USA); mouse anti-human Caspase-3, anti-human CyclinD3, anti-human Akt, anti-human p-Akt, anti-human CyclinA2, anti-human CyclinE1, and rabbit anti-human Caspase-8, anti-human PARP, anti-human CyclinD, anti-human CyclinB1, anti-human CyclinD2, anti-human CyclinH1, anti-human CyclinE2, anti-human p70S6K, anti-human p-p70S6K were all from Cell Signaling Technology (Danvers, MA, USA); mouse anti-human β-actin IgG (Santa Cruz Biotechnology, Dallas, TX, USA); Cell Cycle Analysis Kit (KeyGEN Biotech, Nanjing, China); NC membrane (PALL Gelman laboratory, Kentucky, USA).

Cell culture and short hairpin RNA transfection

Human chronic myeloid leukemia cell line K562 was from Fujian Institute of Hematology and maintained using RPMI-1640 medium containing 10% FBS in 37°C, and 5% CO_2 atmosphere. The lentivirus-based short hairpin RNA (shRNA) specially targeting SARI were designed and synthesized with the antisense: 5'-CTCATGATTCTCCCAGCCT-3'. Three groups included shRNA-SARI-GFP infected group (KD), shRNA-negative-GFP infected group (NC) and blank control group (CON) were established. Cells were seeded and infected by the lentivirus vectors at a multiplicity of infection (MOI) of 100 according to the manufacturer's protocol.

Real-time quantitative PCR

Total RNA was extracted by Trizol solution, followed by transformed into double strand cDNAs using a commercial Reverse Transcription Kit. The cDNA was then amplified using the following primers: *SARI* forward: 5'-GCCTAAGCCATGCACCTCTGT-3', reverse: 5'-TCTTCAGCTGCCTTTGTTGCTC-3' (product length: 79 bp). *GAPDH* was utilized as a reference gene for endogenous normalization with the following sequence: forward: 5'-TCTCTGCTCCTCCTGTTC-3', reverse: 5'-GCCCAATACGACCAAATCC-3' (product length: 120 bp). The reaction system contains: cDNA template (1 μl), primers (1 μl), 2× SYBR Green Mix (10 μl), and RNase-free water (8 μl), with a total volume of 20 μl. PCR was performed based on the platform of ABI 7500 System (Life Technologies, USA). The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to calculate the relative expression level of *SARI* normalized to *GAPDH*. Each test was repeated in thrice.

Cell proliferative activity analysis

Cell proliferative activity analysis was enabled by performing the MTS assay according to the manufacturer's protocol. Cells in logarithmic growth phase were planted into the 96-well cell culture plates with a density of 0.25×10^5 cell/ml and total volume of 200 μl/well. Cells were

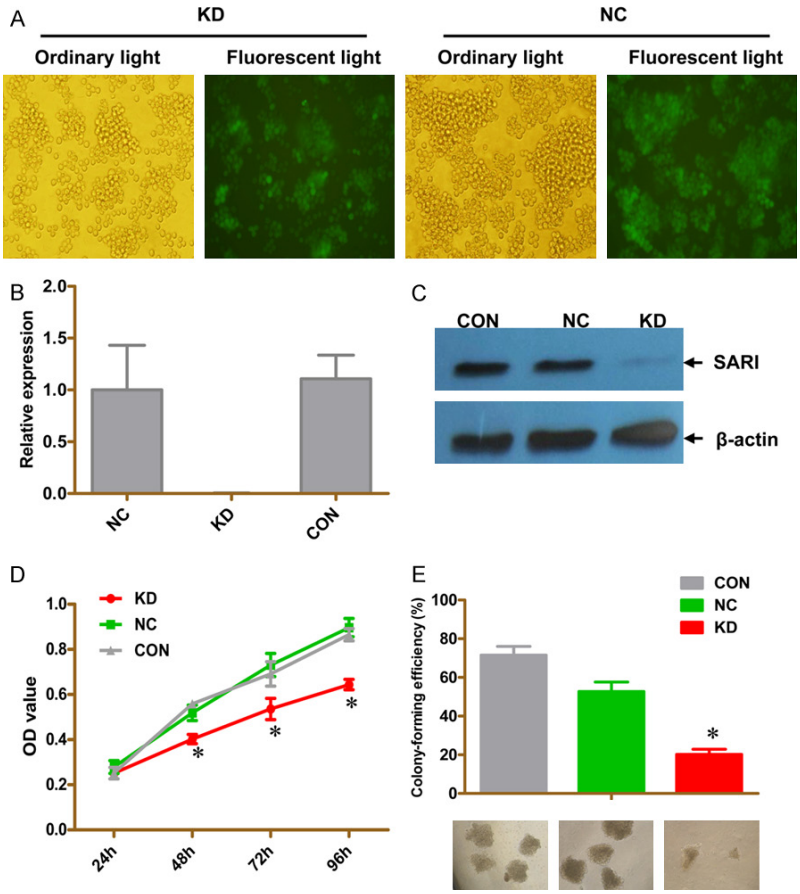

Figure 1. Effects of SARI-silencing on cell growth and colony formation of K562 cells. (A) Expression of GFP was imaged at 72 h following infection (×200), and the expression level of SARI measured by qRT-PCR (B) and immunoblotting (C). (D) Cell growth curve was plotted based on the OD value (proportional to cell numbers) obtained at different time points following infection. (E) Colony-forming assay (×100). *P<0.05, vs. NC group.

incubated at 37°C, and 5% CO_2 atmosphere for different time points (1 d, 2 d, 3 d, and 4 d). Before testing, 20 μl MTS regent was supplemented per well, followed by incubation at 37°C for another 3 h. The absorbance (OD value) at 490 nm and 630 nm was measured with the platform of STAT FAX-2100 spectrophotemeter (Awareness Technology, USA). Each test (per well) has at least three repeats.

Clone formation assay

Cells in logarithmic growth phase were plated into 24-well cell culture plates with a density of 300/well in RPMI-1640 culture medium containing 1.6% methyl cellulose. Cells were cultured at 37°C, 5% CO_2 atmosphere and the colonies containing >40 cells were counted on day 12-day 14 using an inverted microscope (Nikon, Tokyo, Japan). The clone forming rate was calculated as we previous described: (Cell colonies per well/300) ×100% [19].

Apoptosis and cell cycle analysis

Effects of SARI-silencing on apoptosis and cell cycle in K562 cells were assessed based on the Flow cytometry (FCM) analysis (BD FACS FCM). For the apoptosis analysis, cells following infection were labeled by employing Annexin V-PE/7-AAD double fluorescent following the protocols we previous described [20]. In the analysis of STI571-induced apoptosis, cells were treated with STI571 in a terminal concentration of 84.78 μM/L. The cell cycle analysis was conducted using a commercial Cell Cycle Analysis Kit according to its procedure. Results of apoptosis and cell cycle were respectively analyzed via FlowJo version 7.6 and Modfit LT version 3.2 programs.

Transwell assay

Briefly, 100 μl matrigel (300 μg/ml) per well was added in the pre-cooled transwell plates harbored in a 24-well plates, followed by incubating at 37°C for 2 h. Cells were suspended using FBS-free RPMI-1640 medium and seeded at a density of 5×10^5/ml/well in the upper chamber with a volume of 500 μl. The lower chamber contains 750 μl RPMI-1640 medium with 10% FBS. The chambers were incubated in 37°C, 5% CO_2 atmosphere for 24 h. Cell suspensions in the lower chamber were harvested and received further MTS assay following the protocols mentioned above. The invasion inhibition ratio (%) = [(Mean OD value of lentivirus-infected cells) - (Mean OD value of cells in CON group)]/(Mean OD value of cells in CON group) ×100%. The experiment was repeated in triplicate.

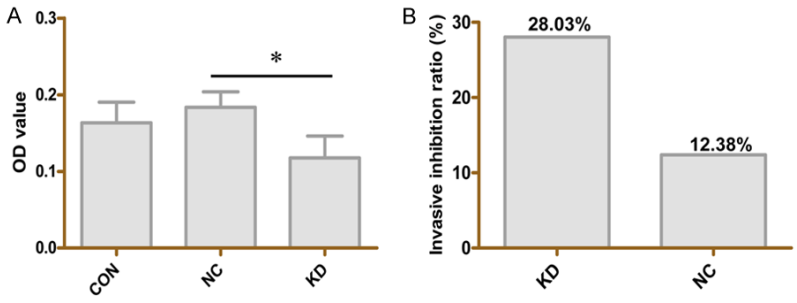

Figure 2. Effects of SARI-silencing on cell invasion of the K562 cell line. A. The OD values (proportional to cell numbers) of migrated cells between groups that measured by MTS assay. B. The migration inhibition ratio. *P<0.05, vs. NC group.

Immunoblotting analysis

Total protein was extracted using RIPA lysis buffer, followed by quantification with the spectrophotometer (Thermo Fisher Scientific, USA). Equal amounts of protein (10-15 μg) were then subjected to the 12% SDS-PAGE gel for electrophoresis. The separated proteins were then transferred onto the NC membranes and blocked in TBST buffer containing 5% BSA for 2 h. The primary antibodies were rabbit or mouse anti-human IgGs, involving anti-human SARI (1:2000), Bcl-2 (1:1000), Caspase-3 (1:1000), Caspase-8 (1:1000), PARP (1:1000), CyclinD (1:1000), CyclinA2 (1:1000), CyclinB1 (1:1000), CyclinD2 (1:1000), CyclinD3 (1:1000), CyclinH1 (1:1000), CyclinE1 (1:1000), CyclinE2 (1:1000), mTOR (1:1000), p-mTOR (1:1000), PI3K (p85) (1:1000), p-PI3K (p85) (1:500), Akt (1:1000), p-Akt (1:500), P70-S6K (1:1000), and p-P70-S6K (1:1000). β-actin (1:4000) was utilized as the internal reference protein. The HRP-conjuncted goat anti-rabbit or anti-mouse secondary antibodies were diluted in 1:10000. Developed images of proteins were captured and quantified to β-actin using ImageJ 1.43 software.

Statistical analysis

Data were expressed as mean ± SD (standard deviation) and analyzed base on SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software. Student's t-test, or non-parametric test was performed to compare differences between groups. The test level was set at $\alpha=0.05$, and $P<0.05$ was considered as study with statistically significant.

Results

SARI-silencing inhibited proliferation and colony formation in K562 cells

Via fluorescence microscope, expression of GFP in K562 cells was imaged at 72 h following lentivirus infection, and the positive rates were estimated to be 60% to 80% in both KD and NC groups (**Figure 1A**). Data from qRT-PCR and immunoblotting showed that SARI expression in the KD group was markedly decreased in both mRNA and protein levels (**Figure 1B** and **1C**). Our study further examined cell growth and colony-forming abilities in SARI-silencing K562 cells via MTS assay and colony-forming assay. As shown in **Figure 1D**, down-regulation of SARI induced a time-dependent, progressive decrease in cell viability, with the OD values of 0.54±0.05 versus that of 0.73±0.05 in NC group at 72 h following infection. Moreover, the colony-forming assay displayed similar results to the MTS assay: cells in KD group showed markedly reduced colony numbers and small sizes as compared with the NC and CON groups, corresponding to a decreased colony formation rate of (20.22±2.67)% ($P<0.05$) (**Figure 1E**).

SARI-silencing suppresses invasion in K562 cells

In **Figure 2A**, results of the transwell analysis showed that the OD value of migrated cells in KD was 0.12±0.03, which is lower than that in NC group (0.18±.020, $P<0.05$). Moreover, the migration inhibition ratio was estimated to be 28.03% in KD group, and 12.38% in NC group (**Figure 2B**), suggesting that SARI-silencing could suppress cell invasion ability in K562 cells.

Down-regulation of SARI sensitizes imatinib (STI571)-induced apoptosis in K562 cells

To confirm that whether SARI-silencing induced growth inhibition in K562 cells was due to apoptosis, we conducted Annexin V-PE/7-AAD double fluorescent labeling and FCM. Intriguingly, total apoptosis rates in KD, NC and CON groups were (2.25±1.37)%, (1.40±1.22)%,

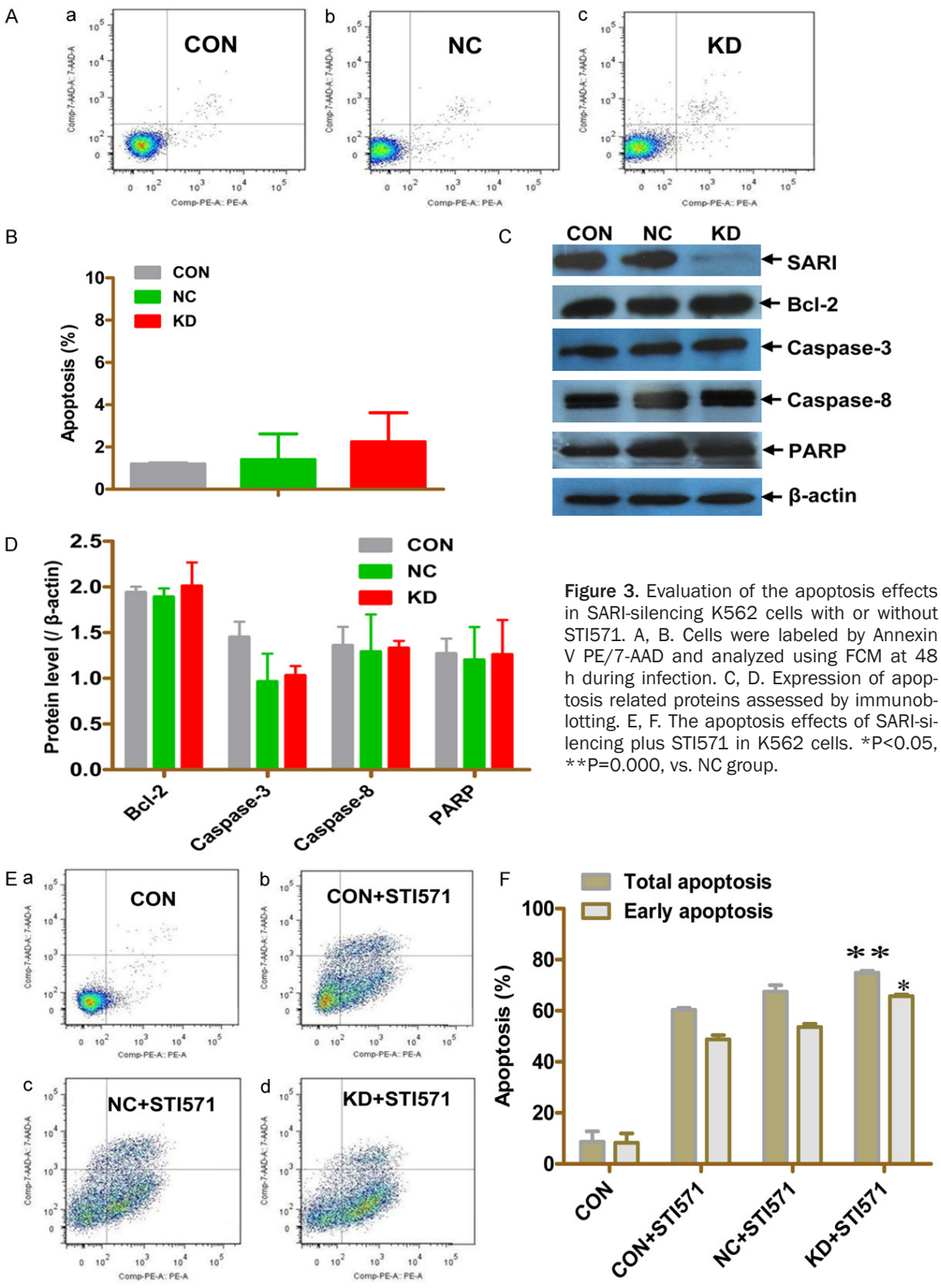

Figure 3. Evaluation of the apoptosis effects in SARI-silencing K562 cells with or without STI571. A, B. Cells were labeled by Annexin V PE/7-AAD and analyzed using FCM at 48 h during infection. C, D. Expression of apoptosis related proteins assessed by immunoblotting. E, F. The apoptosis effects of SARI-silencing plus STI571 in K562 cells. *P<0.05, **P=0.000, vs. NC group.

and (1.19±0.05)%, respectively (**Figure 3A** and **3B**). The KD group only revealed an increased apoptosis rate of 0.85% higher than the NC group, although P<0.05 was observed. Moreover, expression of the apoptotic related proteins as Bcl-2, Caspase-3, Caspase-8, and

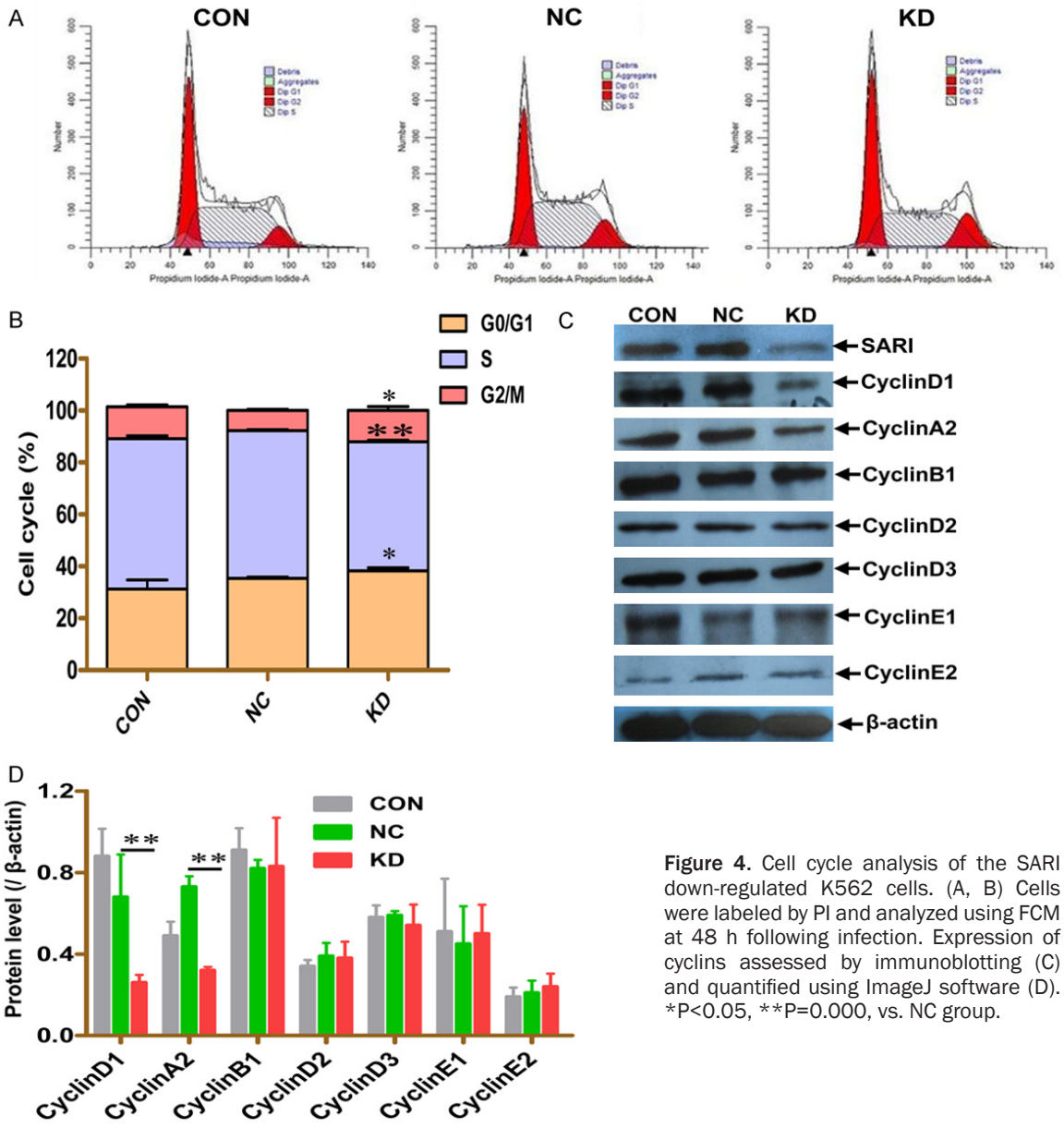

Figure 4. Cell cycle analysis of the SARI down-regulated K562 cells. (A, B) Cells were labeled by PI and analyzed using FCM at 48 h following infection. Expression of cyclins assessed by immunoblotting (C) and quantified using ImageJ software (D). *P<0.05, **P=0.000, vs. NC group.

PARP also revealed no significant alterations in the KD group as compared with the control groups (P>0.05) (**Figure 3C** and **3D**). The data indicated that SARI may not affect the growth of K562 cells through inducing apoptosis. When cells were treated with STI571, total apoptosis rate in KD+STI571 group reached to (74.90±0.76)%, which is higher than that of (67.45±2.58)% in NC+STI571 group (P<0.05) (**Figure 3E** and **3F**). Notably, the early apoptosis rate in KD+STI571 group was markedly evaluated when versus the NC+STI571 group (P=0.000) (**Figure 3E** and **3F**). The above data suggested that down-regulation of SARI could sensitize STI571-induced apoptosis in K562 cells.

SARI-silencing induced cell cycle arrest in K562 cells

We further conducted cell cycle analysis through PI staining by FCM. As shown in **Figure 4A** and **4B**, the percentage of cells in G0/G1 and G2/M phases in KD group was (38.21±1.27)% and (12.03±1.50)%, respectively, which is higher than that in the KD group (P<0.05). Additionally, the percentage in S phase was estimated to be (49.76±0.58)% in the KD group and is lower than that in the NC group (P=0.000), suggesting that down-regulation of SARI in K562 cells resulted in cell cycle arrest in G0/G1 and G2/M phases. Moreover, expres-

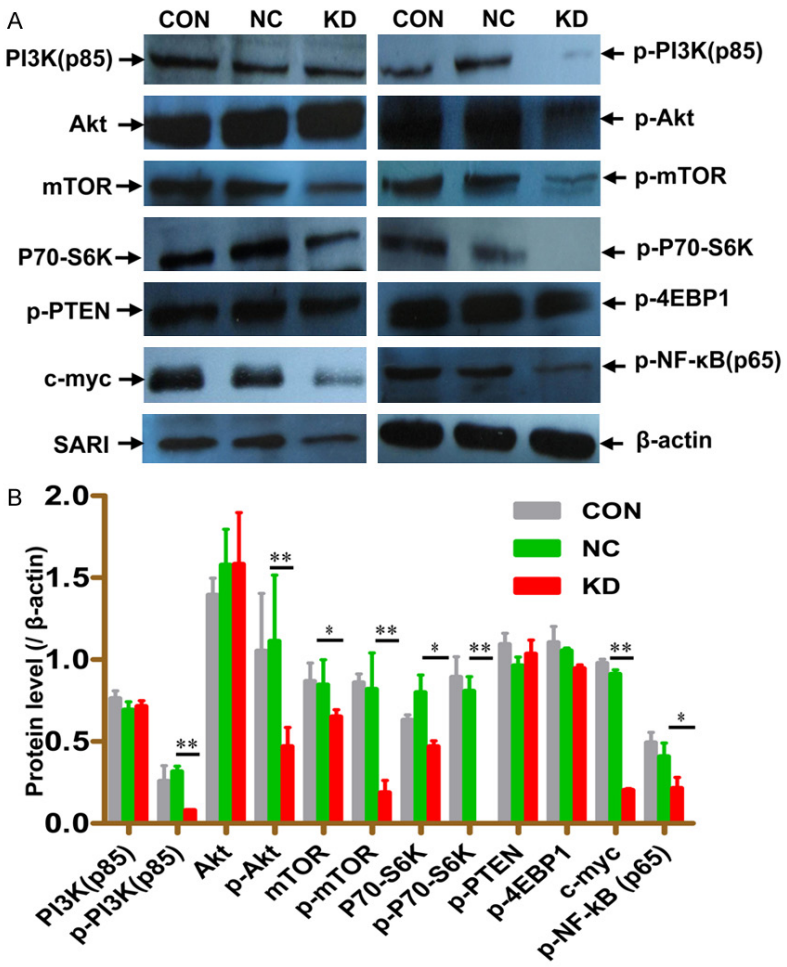

Figure 5. Expression of the proteins involved in the PI3K/Akt/mTOR and NF-κB signaling pathways. Protein levels were detected via immunoblotting (A) and quantified using ImageJ software (B). *P<0.05, **P=0.000, vs. NC group.

sion of the cyclins as CyclinD1 and CyclinA2 were dramatically declined in the KD group as compared with the NC group (P<0.05) (**Figure 4C** and **4D**), whereas other cyclins involved CyclinB1, CyclinD2, CyclinD3, CyclinH1, CyclinE1 and CyclinE2 showed no significant alterations in responding to the down-regulation of SARI (**Figure 4C** and **4D**).

SARI-silencing suppressed PI3K/Akt/mTOR and NF-κB signaling pathways

On the basis of the previous studies, we eventually assessed the expression levels of proteins involved in the PI3K/Akt/mTOR and NF-κB signaling. Although PI3K (p85), Akt, p-PTEN and p-4EBP1 showed no obvious changes in responding to SARI-silencing, levels of both basal and phosphorylated forms of such proteins as mTOR, c-myc p-mTOR, p-PI3K (p85), p-Akt, p70-S6K, p-p70-S6K were dramatically down-regulated in the KD group as compared with the ND groups (P<0.05 or P=0.000, **Figure 5**). Moreover, and phosphorylated NF-κB (p65) were significantly down-regulated in KD group as well (**Figure 5**). Collectively, it could be therefore speculated that SARI plays a role in regulating the PI3K/Akt/mTOR and NF-κB signaling pathways in K562 cells.

Discussion

In our previous work, we found that the K562 leukemia cell line retains a relatively high expression of levels endogenous *SARI* (wild type also confirmed) among several tested human myeloid leukemia cell lines. This prompted us to deeply elucidate the functional role of endogenous SARI in K562 cells. In the present study, we down-regulated the endogenous levels of *SARI* in K562 cells via short hairpin RNA transfection. Our data demonstrated that SARI-silencing inhibited proliferation and colony formation, suppressed metastasis, enhanced STI571-induced apoptosis, and resulted in G0/G1 and G2/M arrest in K562 leukemia cells, possibly by modulating the PI3K/AKT/mTOR and NF-κB signaling pathways.

SARI (suppressor of AP-1, regulated by IFN), as known as a member of the mda family, is associated with the risk of several types of human malignancies [10, 14, 15, 18]. There is increasing evidence that SARI may function as a putative tumor suppressor in many solid cancers [11-18]. Intriguingly, data form the current study showed that when knock-down the endogenous levels of SARI in K562 myeloid leukemia cells, the abilities of cells in proliferation, colony formation, and invasion were compromised. Moreover, it is interesting to find that SARI-

silencing did not led to effective apoptosis in K562 cells as the KD group only revealed an increased apoptosis rate of 0.85% higher than the NC group. Correspondingly, levels of apoptotic related proteins as Bcl-2, Caspase-3, Caspase-8, and PARP also revealed no obvious changes in responding to SARI inhibition, suggesting that SARI may not affect the viability of K562 cells through inducing apoptosis. Intriguingly, down-regulation of SARI could sensitize STI571-induced apoptosis in K562 cells. We speculated that SARI-silencing might exert synergistic effects that assisted STI571-mediated cytotoxicity in K562 cells. The emergence of drug resistance of tyrosine kinase inhibitors in treating leukemia is a big problem and demanding prompt solution thus far [3, 4]. Consequently, our findings indicated that SARI may be implicated in the mechanisms of drug resistance toward tyrosine kinase inhibitors.

Further cell cycle analysis manifested that SARI-silencing induced G0/G1 and G2/M arrest. To validate the underlying causes, we assessed the expression status of a series of crucial cyclins in cell cycle, with a result that CyclinD1 and CyclinA2 showed markedly alterations in SARI knock-down K562 cells. CyclinD1 acts as a key regulator in cell cycle and down-regulation of CyclinD1 was associated with both the cell cycle and apoptosis [21]. For instance, inhibition of CyclinD1 expression markedly suppressed cell proliferation and induced G1 phase arrest in gastric cancer [22, 23]. In line with the theories, our data showed that expression of CyclinD1 was reduced following SARI-silencing, which may be the reason for the blockage of transition from G0 to G1 phase in K562 cells. CyclinA2 is also an important component activated in cell cycle [24]. It is evident that CyclinA2 could activate two different CDK kinases: CDK1 and CDK2; CyclinA2 binds CDK1 during G2/M phase, and CDK2 during S phase [25]. The above evidence could well interpret the declined level of CyclinA2 in SARI-silencing K562 cells in responses to the G2/M arrest.

The PI3K/Akt/mTOR singling plays a critical role in control of a series of physiological activities included cell cycle, survival, and metabolism, etc [26, 27]. It is now well documented that the PI3K/Akt/mTOR singling is an established oncogenic driver in cancers [28]. Our study showed that expression of mTOR, p-mTOR, p-PI3K, p-Akt, p70-S6K, p-p70-S6K and c-myc were dramatically decreased in SARI knock-down K562 cells, suggesting that SARI may function as a crucial protein in modulating the PI3K/Akt/mTOR singling in K562 leukemia cells. The NF-κB signaling is also one of the well-known pathways that are implicated in cancer progression and angiogenesis [29]. NF-κB is an important regulator functions downstream of the PI3K/Akt/mTOR singling. Activation of NF-κB is characterized by the transfer of NF-κB (p65) to the nucleus [30]. In our study, we found that phosphorylated NF-κB (p65) was markedly down-regulated following SARI-silencing in K562 cells, and it could be speculated that the NF-κB singling is also implicated in this process. To our knowledge, we reported for the first time that the two singling pathways above are associated with SARI-mediated pathophysiological effects in leukemia. Based on the current literature, only one study demonstrated that SARI is linked to the RAS/MAPK and JAK/STAT signaling in K562 leukemia cells [8].

Our work still has several limitations. In the first place, all of the data obtained were only based on the investigations in one myeloid leukemia cell line, which the data may not be convincing enough. In the second place, we herein only provided *in vitro* evidence and further *in vivo* studies are still warranted to testify our findings. Once more, for the study of NF-κB activation, we only assessed the total expression level of p-NF-κB (p65) and the "cytoplasm-nucleus" shift effects should be confirmed as well. Last, our SARI-overexpression study conducted in other types of leukemia cell lines (*SARI* absent or low expression) like Kasumi-1, HL-60, and NB4 cell lines all showed opposite results against the findings of this study (unpublished data). As such, SARI may exert "double-edged sword" role in leukemia and thus its functional role in leukemia still needs deeply investigations.

In summary, our study reveals that endogenous SARI exerts oncogenic functions in human K562 leukemia cells and that knock-down of SARI resulted in an inhibition of cell proliferation, colony formation, and invasion, as well as G0/G1 and G2/M arrest in K562 cells. The mechanisms of action may be due to that SARI

functions as a critical protein in modulating the PI3K/Akt/mTOR and NF-κB signaling pathways in K562 cells.

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Disclosure of conflict of interest

None.

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