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Original Article

Exogenous SARI interacts with c-JUN and triggers the intrinsic apoptotic pathway in MG-63 osteosarcoma cells

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Abstract: The *SARI* (suppressor of AP-1, regulated by IFN) gene has been demonstrated to be deregulated in a variety of malignancies including osteosarcoma (OS). However, the biological feature of SARI in OS remains unknown. This study sought to characterize the effects of SARI overexpression on the biological properties of MG-63 OS cells and trace the underlying mechanisms. The pCMV-SARI-Flag vector connected with full length *SARI* cDNA was constructed and transfected into MG-63 OS cell line which lacks of endogenous SARI expression. The biological features were correspondingly assessed by proliferation and apoptosis assays. Potential interactions between SARI and c-JUN were detected by co-immunoprecipitation and immunoblotting. RT-PCR and immunoblotting both indicated an increased SARI expression in MG-63 OS cells as compared with the mock and no-treatment controls. Ectopic expression of SARI obviously attenuated cell viability and migration, and induced apoptosis in MG-63 cell line. Co-immunoprecipitation assay revealed a directly binding effect between SARI and c-JUN in pCMV-SARI-Flag-transfected MG-63 cells. Further immunoblotting analysis demonstrated that SARI overexpression could activate the intrinsic apoptotic pathway characterized by a repression of Bcl-2, up-regulation of Bax, and activation of the caspase cascades. Our data manifested that SARI behaves as a potent tumor suppressor in MG-63 OS cells and enhances apoptosis through binding with c-JUN and triggering the intrinsic apoptotic singling pathway.

Keywords: SARI, osteosarcoma, apoptosis, c-JUN, mechanism

Introduction

Osteosarcoma (OS) remains the most commonly occurring bone cancer seen in children and adolescents [1]. Current treatment regime of surgery and intensive chemotherapy only cure 15% to 30% OS cases in advanced stages [2]. Novel precise and effective OS-targeting treatment approaches are being developed on the condition that the biology of osteosarcoma as well as the molecular mechanisms could constantly be investigated and uncovered.

The *SARI* (suppressor of AP-1, regulated by IFN) gene, also known as *BATF2* or *ATF-like2*, is a putative cancer-suppressor gene that located at 11q12-11q13 [3]. The *SARI* gene possesses three coding exons and encodes a protein (SARI) with molecular mass of 29.4 kDa. Since its discovery, the SARI has been demonstrated to be associated with the occurrence of several types of carcinomas, such as lung adenocarcinoma, prostate cancer, hepatocellular carcinoma, colorectal cancer, esophageal squamous cell carcinoma, chronic myelogenous leukemia, melanoma, and so forth [4-18]. It has been shown that SARI was down-regulated in prostate cancer cells as compared with normal prostate tissues and cells, hinting that SARI might play an important role in the tumorigenesis and development of prostate cancer [5, 6]. In vitro study has demonstrated that silencing of SARI expression could initiate epithelial mesenchymal transition and promote lymph node metastases in lung adenocarcinoma [4]. Similarly, down-regulation of SARI partially decreased imatinib mesylate-induced apoptosis in human K562 leukemia cells [12]. Of note,

decreased expression of SARI is associated with a worse clinical outcome in hepatocellular carcinoma [13] and oral tongue squamous cell carcinoma [14]. All of the above-mentioned studies have in common that SARI may be served as a potential candidate biomarker or target for diagnosis, prognosis and therapies in cancers.

In the present study, we focused on verifying the gene function of *SARI* in OS. For this purpose, we selected the MG-63 OS cell line which lacks of endogenous SARI expression for our in vitro studies. The full length *SARI* coding DNA sequences were cloned and connected into the pCMV-C-Flag eukaryotic vector. The combined pCMV-SARI-Flag plasmid was then transfected into MG-63 cells to establish SARI-Flag stable cells. Our data showed that SARI may act in a tumor suppress manner in MG-63 cells mainly by interacting with c-JUN and activating the intrinsic apoptotic pathway. This study may provide novel insights into the functional role of SARI in OS.

Materials and methods

Main reagents

High glucose DMEM medium (Hyclone, USA); Fetal Bovine Serum (PAN-Biotech GmbH, Germany); pCMV-C-Flag vector, Lipo6000™ Transfection Reagent, Cell lysis buffer for Western and IP, Enhanced BCA Protein Assay Kit, CCK-8 Assay Kit, Caspase-3 Activity Assay Kit, DNA Fragmentation Kit, mouse anti-human Flag IgG_1 (for WB and IP), rabbit anti-human Bcl-2 IgG, mouse anti-human Bax IgG_1, rabbit anti-human cleaved caspase-3 (Asp175) IgG, mouse anti-human β-actin IgG_{2a}, were all purchased from Beyotime Institute of Biotechnology (Haimen, China); mouse anti-human full length caspase-9 IgG_1, mouse anti-human c-JUN IgG_1, horseradish peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were from Santa Cruz Biotechnology (Dallas, TX, USA). Protein A Agarose (EMD Millipore Corporation, USA).

Cell culture

Human MG-63 osteosarcoma cells were maintained using high glucose DMEM medium plus 10% FBS with 1% penicillin/streptomycin. Cells were cultured in 37°C atmosphere containing 5% CO_2.

Semi-quantitative RT-PCR

Total RNA was extracted using a commercial RNApure Tissue&Cell Kit (DNase I) (CWBIO, Beijing, China) and was reverse-transcribed into double strand cDNAs. Detection of the mRNA level of *SARI* in the treated cells was enabled by using the following primers: *SARI* forward, 5'-CACCAGCAGCACGAGTCTC-3', SARI reverse, 5'-TGTGCGAGGCAAACAGGAG-3', product length 406 bp. *GAPDH* were utilized as the internal reference gene and the primers were: *GAPDH* forward, 5'-GGAGCGAGATCCCTCCAAAAT-3', *GAPDH* reverse 5'-GGCTGTTGTCATACTTCTCATGG-3', product length 197 bp. Amplification of the DNA sequences was performed based on the platform of Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific). Identification of the PCR products was carried out using 1.5% agarose gel electrophoresis.

Vector construction and transfection

The full length *SARI* cDNA sequence (825 bp) was obtained from the UCSC database (http://genome.ucsc.edu/index.html), and was cloned to the basic plasmid, pCMV-C-Flag, utilizing the following primers with Hind III, Xba I double enzyme sites: forward, 5'-CCGAAGCTTATGCACCTCTGTGGGGGC-3'; reverse, 5'-CCGTCTAGAGAAGTGGACTTGAGCAG-3', product length 840-bp. The MG-63 cells were transfected using Lipo6000™ Transfection Reagent according to its protocol: concisely, cells were seeded at a density of 2.5×10^5 cells/well in 6-well plate, and 4 μg plasmids plus 5 μl Lipo6000™ were supplemented. Expression of the SARI-Flag fusion protein was measured via immunoblotting at 24 h following transfection. Other than the no-treatment cells, the pCMV-C-Flag-transfected cells were treated as a mock control.

Cell viability assay

MG-63 cells were seeded into 96-well plates at a density of 5000 cells per well. Then, 10 μl/well CCK-8 reagent was added to the medium of the pre-seeded cells. The plates were incubated for 3 h at 37°C plus 5% CO_2. The absorbance (OD value) was periodically detected at 450 nm by a microplate reader. Proliferation inhibition ratio (%) = (1-OD value of pCMV-SARI-Flag-transfected cells/OD value of non-treatment cells) × 100%.

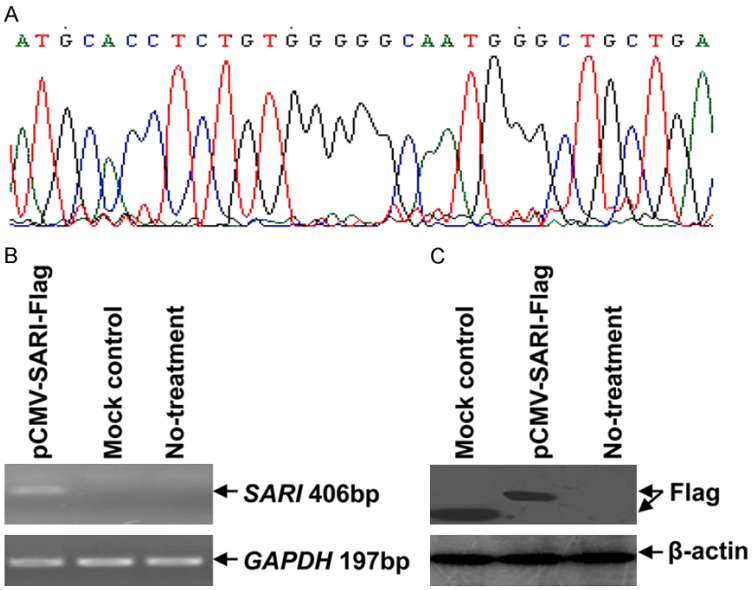

Figure 1. Construction and transfection of the combined pCMV-SARI-Flag vector. (A) Cloning of *SARI* cDNA into pCMV-C-Flag plasmid was confirmed by Sanger sequencing. Expression level of *SARI* mRNA and SARI-Flag fusion protein in MG-63 cells at 24 h during transfection were detected by (B) RT-PCR and (C) immunoblotting.

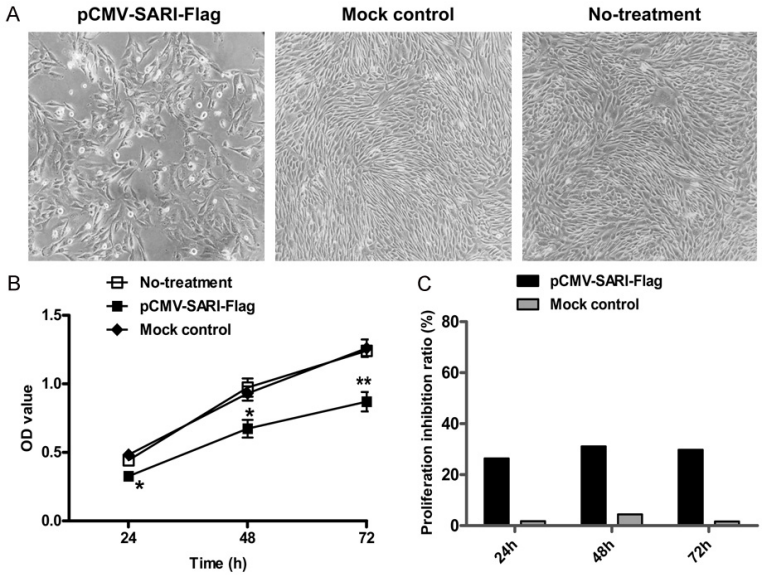

Figure 2. Up-regulation of SARI suppresses cell proliferation in MG-63 cell cline. A. Cell morphologies in ordinary light at 48 h after transfection (× 200); B. Cell viability assessed by CCK-8 assay; C. The proliferation inhibition ratio (%) at 72 h followed transfection. *P<0.05, **P<0.01 vs. the mock control.

Wound healing assay

A total of (5-8) × 10^5 cells (per well) were seeded into the 6-well culture plate to obtain the monolayer. When the confluence of seeded MG-63 cells reached approximately to 100%, a sterile 200 μl pipette tip (Eppendorf Lab Technologies, Shanghai, China) was applied to create an artificial wound, followed by washing 2 times with PBS or serum-free DMEM medium. Cells were then maintained using 2 ml serum-free DMEM medium and photographed at different time points.

Apoptosis analysis

Evaluation of cell apoptosis was enabled by performing the caspase-3 activity and "DNA ladder" analyses. The enzyme activity was determined by recording the absorbance changes of caspase-3 at 405 nm produced by a catalyzation of the substrate, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) [19]. The specific activity of caspase-3 was expressed as fold of the baseline caspase-3 activity of the control group. "DNA ladder" analysis was performed with a DNA Fragmentation Kit according to its instructions. Total DNA was extracted at 72 h after transfection, and a total of 2 μg extracted DNA for each lane was subjected to the agarose gel electrophoresis (1.0%) with a constant voltage of 20 V for 4 h.

Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation assay was enabled by using the Protein A agarose beads according to its protocol. Cell lysates were diluted with PBS to a roughly concentration of 1 μg/μl and 5 μg of mouse anti-human Flag IgG_1 (conjuncted with Protein A agarose beads) were used to hook SARI-Flag fusion protein. The reaction mixture was cultured at 4°C for 12 to 48 h and was finally detected by immunoblot-

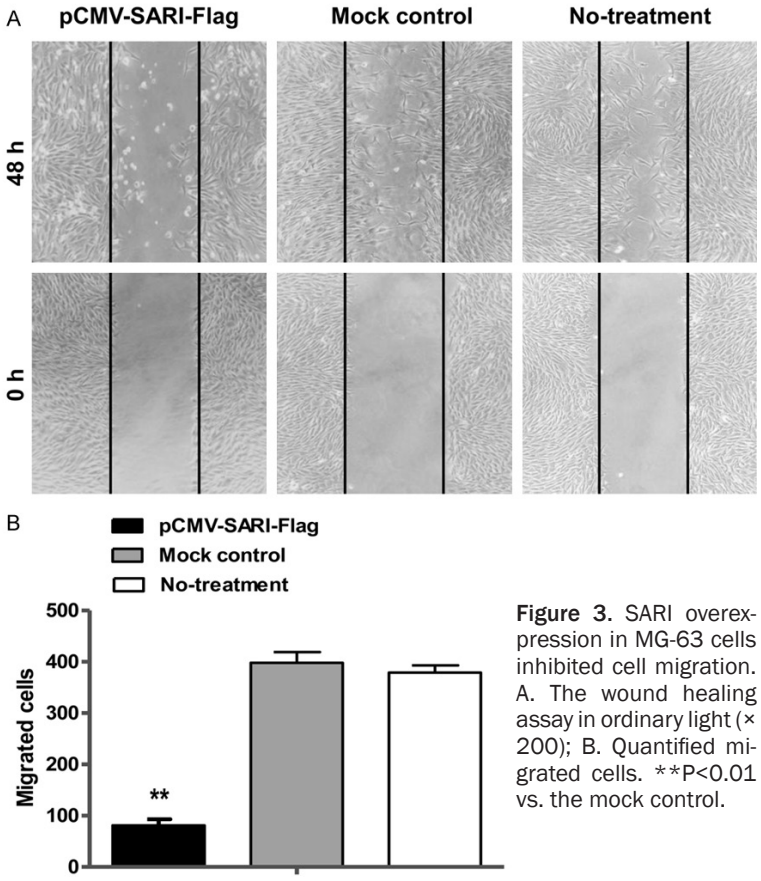

Figure 3. SARI overexpression in MG-63 cells inhibited cell migration. A. The wound healing assay in ordinary light (× 200); B. Quantified migrated cells. **P<0.01 vs. the mock control.

ting utilizing mouse anti-human Flag IgG_1 and mouse anti-human c-JUN IgG_1 antibodies. For immunoblotting, cells were lysed in lysis buffer without inhibitors for 5 min and spun at × 13000 rpm for 15 min. Total protein concentration was measured using the Enhanced BCA Protein Assay Kit. Then, proteins (25 μg/lane) were separated on 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% milk in TBST buffer at room temperature for 2 h and incubated with the primary antibodies at 4°C overnight: mouse anti-human Flag IgG_1 (1:600), mouse anti-human c-JUN IgG_1 (1:600), rabbit anti-human Bcl-2 IgG (1:500), mouse anti-human Bax IgG_1 (1:300), rabbit anti-human cleaved caspase-3 (Asp175) IgG (1:200), and mouse anti-human full length caspase-9 IgG_1 (1:500). Mouse anti-human β-actin IgG_{2a} were treated as the control protein and diluted in 1:1000.

Statistical analysis

Data are presented as the means ± standard deviation (SD), and analyzed by Student's *t*-test with the platform of SPSS 16.0 program (SPSS Inc., Chicago, USA). Significant level between studies or groups was set at a *P* value miner than 0.05.

Results

pCMV-SARI-Flag vector was successfully constructed and transfected into MG-63 cells

Sanger sequencing showed that the full length *SARI* cDNA were successfully connected into the pCMV-C-Flag vector without base mutation (**Figure 1A**). Additionally, RT-PCR exhibited that levels of exdogenous *SARI* mRNA were markedly elevated in pCMV-SARI-Flag treated MG-63 OS cells at 24 h following transfection (**Figure 1B**). Correspondingly, immunoblotting assay showed that SARI-Flag fusion protein were detectable in pCMV-SARI-Flag-transfected MG-63 cells, suggesting the pCMV-SARI-Flag vector was successfully transfected into MG-63 cells and encoded the SARI-Flag fusion protein (**Figure 1C**).

Up-regulation of SARI suppresses proliferation and migration in MG-63 cells

The proliferation assay showed that up-regulation of SARI could inhibit cell viability and growth in MG-63 cells: the OD values of pCMV-SARI-Flag and pCMV-C-Flag-transfected cells (mock control) were 0.87 ± 0.71 versus 1.26 ± 0.64 at 72 h during transfection (*P*<0.05, **Figure 2A**, **2B**). Correspondingly, the proliferation inhibition rate of pCMV-SARI-Flag-transfected cells elevated to 31.1% at 48 h after transfection, whereas the inhibition rate of the mock control was slightly attenuated as time increases (**Figure 2C**). Moreover, the wound healing assay showed that SARI overexpression decreased the migration ability of MG-63 OS cells (*P*<0.05, **Figure 3**).

Overexpression of SARI promoted apoptosis in MG-63 cells

We further conducted caspase-3 activity analysis and DNA degradative fragments assay in

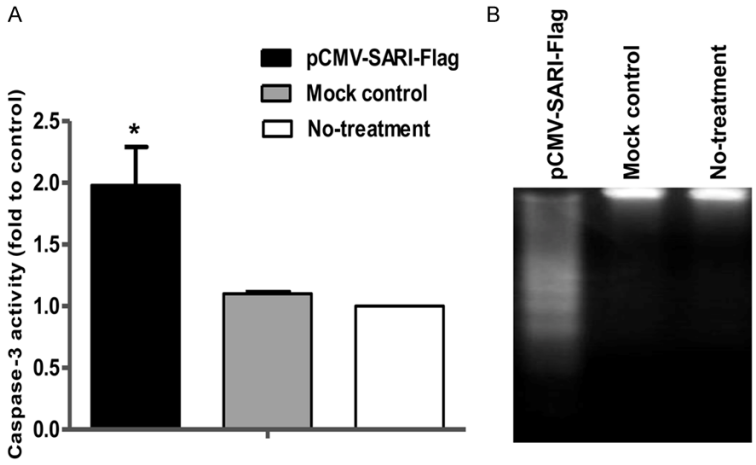

Figure 4. Evaluation of apoptosis by (A) caspase-3 activity and (B) "DNA ladder" analyses. For the DNA degradative fragments assay, total DNA was extracted at 72 h after transfection. *P<0.05 vs. the mock control.

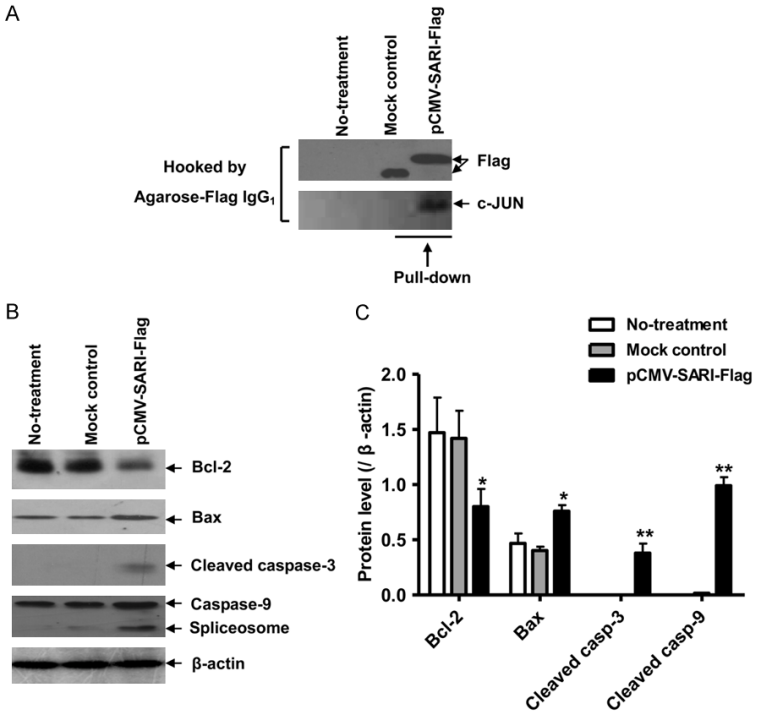

Figure 5. Mechanism study by co-immunoprecipitation and immunoblotting assays. A. Potential interactions between SARI and c-JUN in MG-63 cells detected by co-immunoprecipitation and immunoblotting. In co-immunoprecipitation, Protein A agarose-Flag IgG_1 was employed to pull-down the presumptive "SARI-Flag-c-JUN" complex, and the mixture was cultured at 4°C for 48 h; in immunoblotting, the harvested protein mixture from co-immunoprecipitation procedure was respectively detected by mouse anti-human Flag IgG_1 and mouse anti-human c-JUN IgG_1; The original evidence of WB membrane with no modifications was attached in Supplementary Figure 1. B and C. Alteration of the apoptosis-related proteins during transfection by immunoblotting. *P<0.05, **P<0.01 vs. the mock control.

SARI restored MG-63 OS cells. As expected, the caspase-3 activity of pCMV-SARI-Flag-transfected cells were markedly increased when compared with the mock control (P<0.05, **Figure 4A**). DNA degradative fragments assay was further conducted to examine the apoptosis effects among SARI restored MG-63 cells. In comparison to the mock and no-treatment controls, pCMV-SARI-Flag-transfected MG-63 cells exhibited increased apoptotic 'DNA ladders' at 72 h followed transfection (**Figure 4B**). The data indicated that the apoptosis effect might be one of the reasons that suppressed cells growth in MG-63 cells.

SARI interacts with c-JUN and triggers the intrinsic apoptotic pathway in MG-63 cells

In order to further address the underlying mechanisms, we performed the co-immunoprecipitation and immunoblotting assays. As shown in **Figure 5A**, pCMV-SARI-Flag-transfected cell lysates hooked by agarose-anti-Flag IgG_1 indicated a direct binding effect between SARI and c-JUN. On the other hand, evaluation of the alterations of apoptosis-related proteins by immunoblotting showed that endogenous level of Bcl-2 in pCMV-SARI-Flag-transfected cells was decreased, whereas Bax were elevated, corresponding to an activation of caspase-3 and caspase-9 (**Figure 5B**, **5C**).

Discussion

The *SARI* (suppressor of AP-1, regulated by IFN) gene has been highlighted to be impli-

cated in the tumorigenesis of many types of malignancies [4-18]. The biological feature of SARI in osteosarcoma (OS) remains unclear. In the current study, we made a primary evaluation of the functional role of SARI overexpression in MG-63 OS cell line. Our data demonstrated that SARI may behave as a tumor suppressor in MG-63 OS cells and enhances apoptosis through binding with c-JUN and triggering the intrinsic apoptotic pathway.

Loss of SARI is associated with the risk of several kinds of carcinomas [4-6, 8, 9, 13]. In vitro study has been reported that up-regulation of SARI resulted in a prohibition of cell growth and migration in lung cancer A549 cells [4]. In leukemia cells, silencing of SARI level could partially decrease the imatinib mesylate-induced apoptosis in K562 cells [12]. In the present study, we up-regulated the SARI level in SARI-negative MG-63 OS cells, and found that SARI overexpression could inhibit MG-63 cell viability and growth, and decrease the migration ability. Moreover, our further study showed that exogenous SARI could induce cell apoptosis, which may be responsible for the causes of growth inhibiting effects in MG-63 cells. These observations indicated that *SARI* is likely to behave as a tumor-inhibiting gene in MG-63 OS cell line. Our findings are supported in fully by the previous studies [5-7, 10].

SARI is reported to have a close relationship with c-JUN [3, 20]. The SARI protein possesses a bZIP domain which could interact with c-JUN through its leucine zipper [3]. Herein, we also focused on identifying whether c-JUN is a likely partner of SARI and contributed to the growth inhibiting effects in MG-63 cells. Our co-immunoprecipitation and immunoblotting assays confirmed that SARI had a directly binging effect with c-JUN in MG-63 cells. On the other hand, we further evaluated the expression status of apoptosis related proteins involved in the intrinsic apoptotic pathway. Our results manifested that SARI overexpression could activate the intrinsic apoptotic proteins by repression of Bcl-2, up-regulation of Bax, as well as activation of the caspase cascades, hinting that SARI re-expression in MG-63 cells could activate the intrinsic apoptotic signaling. It has been reported that SARI is implicated in HGF/MET signaling pathway [8], and our findings suggested that SARI is involved in the intrinsic apoptotic signaling pathway in OS. Simultaneously, this observation also prompted us to imagine that an interaction between SARI and c-JUN might be a necessary approach in initiating the intrinsic apoptotic pathway. However, due to a lack of sufficient evidence-based literature support, whether SARI-c-JUN complex is a necessary factor in activating the intrinsic apoptotic pathway in OS still needed more evidence.

The current study still yields many limitations. Firstly, only one kind of OS cell line was enrolled for the final study and the functional character of SARI in other types of OS cell lines remains unclear. Secondly, the deduced conclusions of our study only based on the restricted in vitro experiments and further in vivo evidences are still warranted. Lastly, in the co-immunoprecipitation analysis, we only employed agarose-anti-Flag IgG_1 to pull-down SARI-Flag fusion protein, yet the mutual detection using agarose-anti-c-JUN antibody to inversely hook SARI-Flag should be performed as well. These experiments will be conducted in our future studies.

Collectively, data of the present study manifested that SARI is likely to act as a tumor suppressor in MG-63 OS cell line, which possibly by interacting with c-JUN and triggering the intrinsic apoptotic pathway. Our study offers new resource and insight into the functional role of SARI in OS.

Disclosure of conflict of interest

None.

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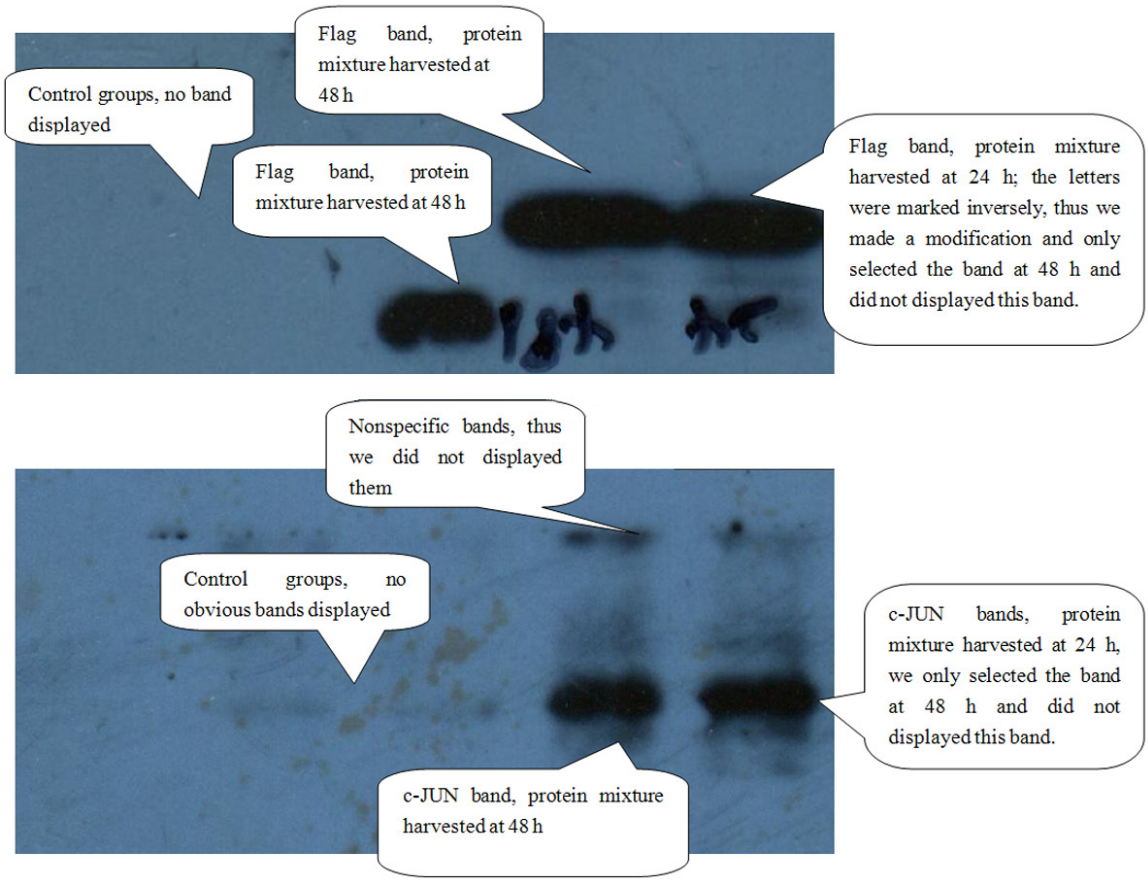

Supplementary Figure 1. The original evidence (WB membrane with no modifications) for **Figure 5A** was attached below.