

# Rsf-1 regulates malignant melanoma cell viability and chemoresistance via NF- $\kappa$ B/Bcl-2 signaling

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**Abstract.** Remodeling and spacing factor 1 (Rsf-1) has been reported as overexpressed in numerous cancers; however, its expression, biological functions and mechanisms in malignant melanoma remain unknown. In the present study, the expression of Rsf-1 was investigated in 50 cases of malignant melanoma samples using immunohistochemistry. The results revealed that Rsf-1 expression was elevated in 38% of specimens. MTT, colony formation, Transwell and flow cytometry assays were performed to investigate the functions of Rsf-1. Knockdown of Rsf-1 in the MV3 and A375 melanoma cell lines decreased the viability, invasion and cell cycle transition of cells. Conversely, overexpression of Rsf-1 in M14 cells with low endogenous Rsf-1 expression induced opposing effects. Further analysis revealed that Rsf-1 knockdown decreased matrix metalloproteinase-2, cyclin E and phosphorylated-I $\kappa$ B expression. Additionally, Rsf-1 depletion reduced cisplatin resistance and significantly increased the cisplatin-associated apoptotic rate, whereas Rsf-1 overexpression exhibited opposing effects. Rsf-1 also maintained the mitochondrial membrane potential following cisplatin treatment. Analysis of apoptosis-associated proteins revealed that Rsf-1 positively regulated B-cell lymphoma 2 (Bcl-2), cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2, and downregulated Bcl-2-associated X protein expression. Nuclear factor  $\kappa$ -light-chain-enhancer of activated B-cells (NF- $\kappa$ B) inhibition reversed the effects of Rsf-1 on Bcl-2. In conclusion, Rsf-1 was overexpressed in malignant melanoma and may contribute to the malignant behaviors of melanoma cells, possibly via the regulation of NF- $\kappa$ B signaling. Therefore, Rsf-1 may be a potential therapeutic target in the treatment of malignant melanoma.

## Introduction

Malignant melanoma arises from melanocytes, which are responsible for pigment production (1-3). The incidence of melanoma has increased at an alarming rate and patients with advanced malignancies exhibit poor prognoses, with an average survival time of 3-11 months (4-8). Melanoma can be removed via surgical resection in patients with early diagnosis; however, melanoma has high metastatic potential and treatment options for metastatic melanoma are limited (9-12). Therefore, novel targets against melanoma are urgently required for the identification of effective therapies.

Remodeling and spacing factor 1 (Rsf-1), also known as hepatitis B X-antigen associated protein, is a subunit of RSF (13,14). Rsf-1 protein is located in the nucleus and binds to human sucrose nonfermenting protein 2 homolog (hSNF2H), forming a chromatin remodeling complex (15). The Rsf-1/hSNF2H complex regulates adenosine 5'-triphosphate-dependent chromatin remodeling and alters the chromatin structure of nucleosomes (15,16), which are required for biological processes, including activation or repression of transcription, DNA replication and cell cycle progression (17,18).

Rsf-1 overexpression has been reported in a number of solid tumors, including breast cancer, ovarian cancer and oral squamous cell carcinoma (19-25); increased Rsf-1 expression was associated with poor prognosis in bladder cancer (15) and nasopharyngeal cancer (26). Additionally, ectopic expression of Rsf-1 promoted cell and tumor growth in a mouse xenograft model (27). Furthermore, Rsf-1 was associated with paclitaxel resistance in ovarian cancer (28); however, there are no reports concerning the expression profile of Rsf-1 in malignant melanoma. The aim of the present study was to determine the status of Rsf-1 in malignant melanoma tissues, and the effects of Rsf-1 on the biological behavior of melanoma cell lines.

## Materials and methods

**Patients and specimens.** The present study was approved by the Ethics Committee of China Medical University (Shenyang, China). Informed consent was obtained from all patients. Melanoma and adjacent normal specimens were obtained from 50 patients diagnosed with malignant melanoma who underwent resection at The First Affiliated Hospital of China Medical University (Shenyang, China)

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between November 2009 and March 2012. Patients did not receive chemotherapy or radiation therapy prior to surgical resection. Histological classification was performed according to the American Joint Committee on Cancer (29). There were 20 female and 30 male patients, with an age range of 25-82 years (mean, 53.2±8.67 years).

**Immunohistochemical staining.** Tumor samples were obtained from The First Affiliated Hospital of China Medical University. The samples were fixed in 37% formaldehyde at room temperature for 18 h and embedded in paraffin. Immunostaining was performed using the Elivision Plus method (Fuzhou Maixin Biotech. Co., Ltd., Fuzhou, China). Sections of 4- $\mu$ m thickness were deparaffinized in xylene and rehydrated with a graded alcohol series (100, 95, 80 and 70%). Sections were permeabilized with Triton X-100 and then boiled in citrate buffer. Sections were blocked with goat serum (Fuzhou Maixin Biotech Co., Ltd.) at room temperature for 20 min. Hydrogen peroxide (0.3%) was used to block peroxidase activity. Sections were incubated with rabbit anti-Rsf-1 polyclonal antibody (1:1,000; HPA046129, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C overnight. Sections were then incubated for 2 h at 37°C with a biotinylated anti-rabbit horseradish peroxidase (HRP) polymer (KIT-9902, Fuzhou Maixin Biotech. Co., Ltd.). Sections were developed with 3,3'-diaminobenzidine plus from Fuzhou Maixin Biotech. Co., Ltd. Sections were counterstained with hematoxylin at room temperature for 2 min.

All tumor slides were analyzed by two independent investigators randomly under a light microscope (magnification, x400; BX53; Olympus Corporation, Tokyo, Japan). Immunostaining of Rsf-1 was scored using a semi-quantitative scale by evaluating the intensity and percentage of tumor cells. Nuclear immunostaining was considered positive. The intensity of Rsf-1 staining was scored as 0 (no signal), 1 (moderate) or 2 (strong). Percentage scores were assigned as 1 (1-25%), 2 (26-50%), 3 (51-75%) or 4 (76-100%) (30). The scores of each tumor sample were multiplied to provide a final score of 0-8; tumor samples that scored 4-8 were considered to demonstrate Rsf-1 overexpression.

**Cell culture and reagents.** M14 cells with low Rsf-1 expression, and MV3 and A375 cells with high Rsf-1 expression were purchased from the American Type Culture Collection (Manassas, VA, USA). M14 and A375 cells were cultivated in Dulbecco's Modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.). MV3 cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. Cells were cultured under conditions of 37°C and 5% CO<sub>2</sub>, and seeded at a density of 1x10<sup>6</sup> cells/ml. Then, cells were treated with cisplatin (final concentration, 5  $\mu$ M) following attachment of cells to plates at 37°C for 24 h. Additionally, M14 cells were treated with NF- $\kappa$ B inhibitor (Bay11-7082; cat. no. S2913, Selleck Chemicals, Houston, TX, USA) at a concentration of 10  $\mu$ M for 12 h at 37°C.

**Small interfering RNA (siRNA) and plasmid transfection.** Oligonucleotide pools of siRNA targeting Rsf-1 and non-targeting siRNA (control siRNA) were purchased from

GE Healthcare Dharmacon, Inc. (Lafayette, CO, USA), and MV3 and A375 cells were transfected with 50 nM siRNA using Lipofectamine<sup>®</sup> 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The targeting sequences were as follows: Rsf-1 siRNA, 5'-GGA AAGACAUCUCUACUAU-3'; and control siRNA, 5'-GCG CGATAGCGCGAATATA-3'. pCMV6-Rsf-1 and control empty plasmids were purchased from OriGene Technologies, Inc. (Rockville, MD, USA), and M14 cells were transfected with 1  $\mu$ g plasmid using Lipofectamine 3000 according to the manufacturer's protocols. Subsequent experiments were performed 48-72 h following transfection.

**Western blotting.** Total protein from cells was extracted using Pierce<sup>™</sup> Universal Nuclease for Cell Lysis (Pierce; Thermo Fisher Scientific, Inc.) and quantified by the Bradford method. A total of 40  $\mu$ g protein was separated by 8-12% SDS-PAGE. Samples were transferred to polyvinylidene difluoride membranes (EMD Millipore), blocked at room temperature for 1 h in 3% bovine serum albumin (BioSharp Co., Hefei, China), and incubated overnight at 4°C with antibodies against: Rsf-1 (1:2,000; cat. no. HPA046129, Sigma-Aldrich; Merck KGaA), cyclin E (1:700; cat. no. 4129, Cell Signaling Technology, Inc., Danvers, MA, USA), matrix metalloproteinase-2 (MMP2; 1:1,000; cat. no. 4022, Cell Signaling Technology, Inc.), I $\kappa$ B (1:1,000; cat. no. 9242, Cell Signaling Technology, Inc.), phosphorylated (p)-I $\kappa$ B (1:1,000; cat. no. 9246, Cell Signaling Technology, Inc.), nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B; 1:1,000; cat. no. 4764, Cell Signaling Technology, Inc.), B-cell lymphoma (Bcl-2; 1:1,000; cat. no. 15071, Cell Signaling Technology, Inc.), Bcl-2-associated X protein (Bax; 1:1,000; cat. no. 2774, Cell Signaling Technology, Inc.), cellular inhibitor of apoptosis protein 1 (cIAP1; 1:1,000; cat. no. 7065, Cell Signaling Technology, Inc.), cIAP2 (1:1,000; cat. no. 3130, Cell Signaling Technology, Inc.) and  $\beta$ -actin (1:2,000; cat. no. 4970, Cell Signaling Technology, Inc.). Following incubation with HRP-conjugated anti-mouse/rabbit IgG (1:1,000; cat nos. 7076/7074, Cell Signaling Technology, Inc.) at 37°C for 2 h, proteins were visualized using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.) and detected using a DNR Bio-Imaging System (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel). Relative protein levels were quantified using ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from MV3, M14 and A375 cells using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific, Inc.). Total RNA (500 ng) was then reverse-transcribed using PrimeScript RT Master Mix (10X; Takara Biotechnology Co., Ltd., Dalian, China) at 85°C for 2 min and 37°C for 30 min. qPCR was conducted using the Reverse Transcription System kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols. An ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for gene amplification, under the conditions of: 95°C for 2 min, and 40 cycles of 95°C for 2 sec and annealing/extension at 60°C for 30 sec. A dissociation step was performed at 60-95°C for 6 sec to generate a melting curve.  $\beta$ -actin was used

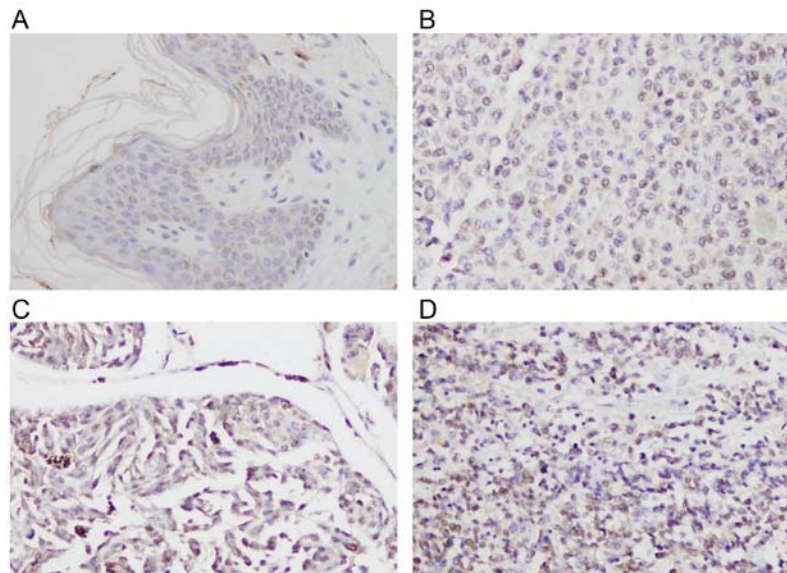


Figure 1. Expression of Rsf-1 in melanoma tissue. (A) Negative Rsf-1 staining observed in normal skin tissue. Moderate Rsf-1 staining was detected in cases of melanoma tissue of stages (B) II and (C) III. (D) Strong Rsf-1 staining was detected in a case of melanoma tissue in stage IV. Magnification, x400. Rsf-1, remodeling and spacing factor 1.

as the reference gene, and relative levels of gene expression were represented as:  $\Delta Cq = Cq_{\text{gene}} - Cq_{\text{reference}}$ . The fold change in gene expression was calculated using the  $2^{-\Delta\Delta Cq}$  method (31). The experiment was performed in triplicate. The primers were as follows: Rsf-1, forward 5'-GATACTATGCGTCTCCAGC CAA-3', reverse, 5'-CAACTCGTTTCGATTTCTGACAA-3'; and  $\beta$ -actin, forward 5'-CCAACCGCGAGAAGATGACC-3' and reverse, 5'-GATAGCACAGCCTGGATAGCAAC-3'.

**MTT assay.** A total of 5,000 cells were plated in 96-well plates and cultured overnight, followed by the addition of 20  $\mu$ l of 5 mg/ml MTT solution to each well; cells were incubated for 4 h at 37°C. The supernatant was removed from each well, and dimethyl sulfoxide (150  $\mu$ l) was added to dissolve the formazan crystals. The absorbance was detected at 490 nm using a microplate reader (Infinite F50; Tecan Group, Ltd., Mannedorf, Switzerland).

**Colony formation assay.** For colony formation, cells were seeded into three 6-cm cell culture dishes (~800 cells/dish) 48 h following transfection. Cells were incubated for 14 days at 37°C. Plates were washed with PBS and then stained with Giemsa at room temperature for 10 min. The number of colonies with >50 cells was manually counted under a light microscope (magnification, x200; BX53).

**Transwell invasion assay.** A Transwell invasion assay was performed using a 24-well Transwell chamber with a pore size of 8  $\mu$ m (Costar; Corning Inc., Corning, NY, USA), and the inserts were coated with 20  $\mu$ l Matrigel (1:3; BD Biosciences, San Jose, CA, USA). After 48 h following transfection, cells were trypsinized (0.25% trypsin) at 37°C for 30 sec and then transferred to the upper Matrigel-coated chamber in 100  $\mu$ l serum-free medium ( $1 \times 10^5$  cells/ml). Medium (DMEM for M14 and A375 cells, RPMI-1640 for MV3 cells) supplemented with 10% FBS was added to the lower chamber as the chemoattractant. Cells

were incubated for 18 h at 37°C. Non-invading cells on the upper membrane surface were then removed with a cotton tip, and the cells that passed via the filter were fixed in 4% paraformaldehyde at room temperature for 20 min. Cells were stained with hematoxylin at room temperature for 5 min. Cells were observed under a light microscope (magnification, x200; BX53). The experiments were performed in triplicate.

**Flow cytometry for cell cycle and apoptosis analyses.** Cells in 6-well plates were collected using tryptase 48 h following transfection. Cells were washed twice with PBS, followed by resuspension in 250  $\mu$ l binding buffer (BD Pharmingen; BD Biosciences). Cells were fixed in 1% paraformaldehyde at 4°C overnight and then stained with 5 mg/ml propidium iodide (PI) alone or together with Annexin V/fluorescein isothiocyanate (BD Pharmingen; BD Biosciences) at room temperature for 15 min for cell cycle or apoptosis analysis, respectively. Incubation was performed in the dark for 15 min. Flow cytometry was performed using flow cytometer and analyzed using NovoExpress 1.2.5 software (ACEA Biosciences, Inc.; Agilent Technologies, Inc., Santa Clara, CA, USA). The apoptotic rate was calculated by adding the percentage of early apoptotic (Annexin V-positive, PI-negative) and late apoptotic cells (Annexin V-positive, PI-positive).

**Detection of the mitochondrial membrane potential (MMP).** The MMP was detected via the JC-1 staining method. Briefly, cells (300 cells/ $\mu$ l) were harvested, washed with PBS and incubated with 5  $\mu$ M JC-1 (Cell Signaling Technology, Inc.) at 37°C for 30 min in an incubator. Cells were then washed and analyzed using a flow cytometer. Data were analyzed using NovoExpress 1.2.5 software.

**Statistical analysis.** SPSS version 16 for Windows (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. A  $\chi^2$  test was used to examine potential associations between Rsf-1

Table I. Distribution of Rsf-1 status in melanoma according to the clinicopathological characteristics of patients.

Clinicopathological characteristics	Number of patients	Rsf-1 low expression	Rsf-1 high expression	$\chi^2$	P-value
Age (years)				0.3302	0.5655
<60	34	22	12		
≥60	16	9	7		
Sex				2.3911	0.1220
Female	20	15	5		
Male	30	16	14		
TNM stage				3.8606	0.0494
II	43	29	14		
III+IV	7	2	5		
T stage				0.0212	0.8842
T1-3	10	6	4		
T4	40	25	15		

Rsf-1, remodeling and spacing factor 1; TNM, tumor, node, and metastasis.

expression and the clinicopathological features of patients with melanoma. A Student's t-test was used to compare differences between the control and treatment groups. Data were presented as the mean  $\pm$  standard deviation of at least three experiments.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of Rsf-1 in human malignant melanoma.** Rsf-1 expression in 50 cases of malignant melanoma was determined by immunohistochemistry (Fig. 1). Normal skin tissue exhibited weak or negative staining (Fig. 1A). In total, 19/50 (38%) cases of skin melanoma demonstrated high Rsf-1 immunoreactivity (Rsf-1 overexpression, or an immunostaining score of  $\geq 4$ ), which was localized to the nuclear compartment of tumor cells (Fig. 1B-D). The association between Rsf-1 expression and the clinicopathological characteristics of patients with melanoma was analyzed (Table I). The frequency of Rsf-1 overexpression was increased in melanomas of advanced tumor, node and metastasis (TNM) stages (III+IV vs. II,  $P = 0.0494$ ). The results revealed that no significant association was observed between Rsf-1 expression and patient age ( $P = 0.5655$ ) and gender ( $P = 0.122$ ), or T stage ( $P = 0.8842$ ).

**Rsf-1 promotes malignant melanoma cell viability and invasion.** Rsf-1 expression levels in malignant melanoma cell lines (MV3, M14 and A375) were investigated using western blotting and RT-qPCR. It was revealed that the Rsf-1 expression levels were low in M14 cells, and high in MV3 and A375 cell lines (Fig. 2A). To investigate the biological roles of Rsf-1 in malignant melanoma, Rsf-1 siRNA-mediated interference was performed in the MV3 and A375 melanoma cell lines, whilst Rsf-1-encoding plasmid transfection was performed in the M14 cell line. As presented in Fig. 2B, Rsf-1 siRNA significantly downregulated Rsf-1 protein and mRNA expression, whereas the Rsf-1 plasmid significantly upregulated Rsf-1 expression. An MTT assay was performed to investigate

cell viability, which demonstrated that Rsf-1 depletion in MV3 and A375 cells notably decreased cell viability compared with the controls, whereas Rsf-1 overexpression in M14 cells markedly promoted cell viability (Fig. 3A). A colony formation assay also revealed that Rsf-1 depletion significantly decreased colony number in the MV3 and A375 cell lines, while Rsf-1 overexpression significantly increased the colony number in the M14 cell line compared with the control (Fig. 3B). To determine the effects of Rsf-1 on cell invasion, a Transwell invasion assay was performed. The results revealed that the number of invasive cells was significantly reduced following Rsf-1 depletion in the MV3 (control siRNA vs. Rsf-1 siRNA,  $243 \pm 20$  vs.  $52 \pm 12$  cells;  $P < 0.05$ ; Fig. 3C) and A375 cell lines (control siRNA vs. Rsf-1 siRNA,  $214 \pm 15$  vs.  $90 \pm 8$  cells;  $P < 0.05$ ) compared with the control. Conversely, Rsf-1 overexpression significantly increased the invasive ability of M14 cells compared with the control (empty plasmid vs. Rsf-1 plasmid,  $100 \pm 7$  vs.  $221 \pm 15$  cells;  $P < 0.05$ ).

**Rsf-1 regulates cell cycle progression and associated protein expression.** Cell cycle analysis was performed in melanoma cell lines. Rsf-1 depletion in MV3 and A375 cells significantly increased the percentage of cells in G1 phase and decreased that in S phase compared with the control (Fig. 4A). Rsf-1 overexpression in M14 cells had opposing effects; the percentage of cells in G1 phase was significantly reduced, while the percentage of cells in S phase increased compared with the control, suggesting that Rsf-1 depletion suppresses G1/S cell cycle transition (Fig. 4A). To analyze the potential molecular mechanisms underlying the effects of Rsf-1 on the cell cycle, the expression of associated proteins was examined by western blotting. As presented in Fig. 4B, the expression levels of MMP2, cyclin E and p-I $\kappa$ B were decreased in Rsf-1-depleted MV3 and A375 cells compared with control cells. Conversely, Rsf-1 overexpression upregulated MMP2, cyclin E and p-I $\kappa$ B expression in M14 cells.

**Rsf-1 enhances cisplatin resistance and MMP.** To investigate the role of Rsf-1 in the chemoresistance of malignant melanoma

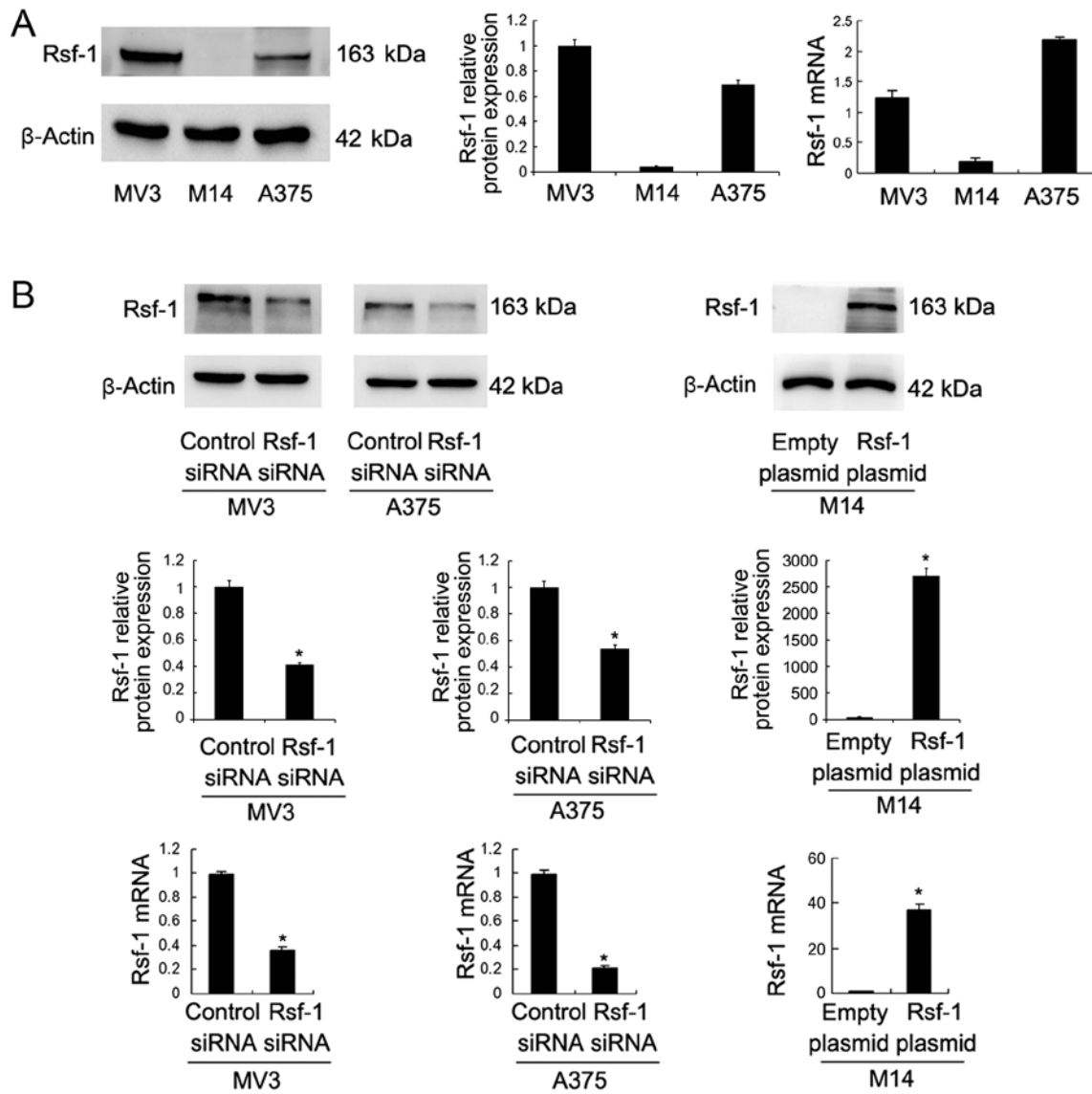


Figure 2. Rsf-1 expression in melanoma cell lines and Rsf-1 knockdown efficiency. (A) Western blotting and RT-qPCR analysis revealed the endogenous expression levels of Rsf-1 in three melanoma cell lines (MV3, M14 and A375). (B) Western blotting and RT-qPCR analysis demonstrated that Rsf-1 siRNA transfection significantly decreased Rsf-1 expression levels in MV3 and A375 cells, while Rsf-1 plasmid transfection upregulated the protein and mRNA expression of Rsf-1 in M14 cells. Data were presented as the mean  $\pm$  standard deviation of at least three experiments. \* $P < 0.05$  vs. control. Rsf-1, remodeling and spacing factor 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

cells, Rsf-1 depleted and control cells were treated with cisplatin (5  $\mu$ M). The results of the MTT assay revealed that Rsf-1 siRNA significantly decreased cell survival rate following 3 days of cisplatin treatment in MV3 and A375 cells compared with the control. Rsf-1 overexpression significantly increased cell viability in M14 cells treated with cisplatin (Fig. 5A). Furthermore, apoptosis analysis revealed that the cell apoptotic rate was significantly increased following Rsf-1 depletion in MV3 and A375 cells treated with cisplatin, and reduced in Rsf-1-overexpressing M14 cells treated with cisplatin compared with the control (Fig. 5B). Collectively, the results demonstrated that Rsf-1 expression promotes cisplatin resistance in melanoma cells.

As resistance to chemotherapeutic drugs is closely associated with mitochondrial function, whether Rsf-1 affected the MMP was investigated. JC-1 staining was used to monitor alterations in MMP following cisplatin treatment. JC-1 staining exhibits

red fluorescence under normal conditions; however, green fluorescence is observed when the MMP is depolarized following cisplatin treatment. As presented in Fig. 5C, in M14 cells treated with cisplatin, Rsf-1 overexpression notably decreased the percentage of cells exhibiting green fluorescence, suggesting that Rsf-1 promoted mitochondrial membrane polarization. Conversely, Rsf-1 depletion led to notable depolarization of the MMP in MV3 and A375 cells treated with cisplatin.

*Rsf-1 regulates Bcl-2 expression via NF- $\kappa$ B signaling.* Furthermore, Rsf-1-induced alterations in apoptosis-associated protein expression were investigated via western blot analysis (Fig. 6A). Rsf-1 depletion downregulated Bcl-2, cIAP1 and cIAP2 expression levels, and upregulated Bax expression in MV3 and A375 cell lines compared with the control; opposing effects were observed in Rsf-1-overexpressing M14 cells. As

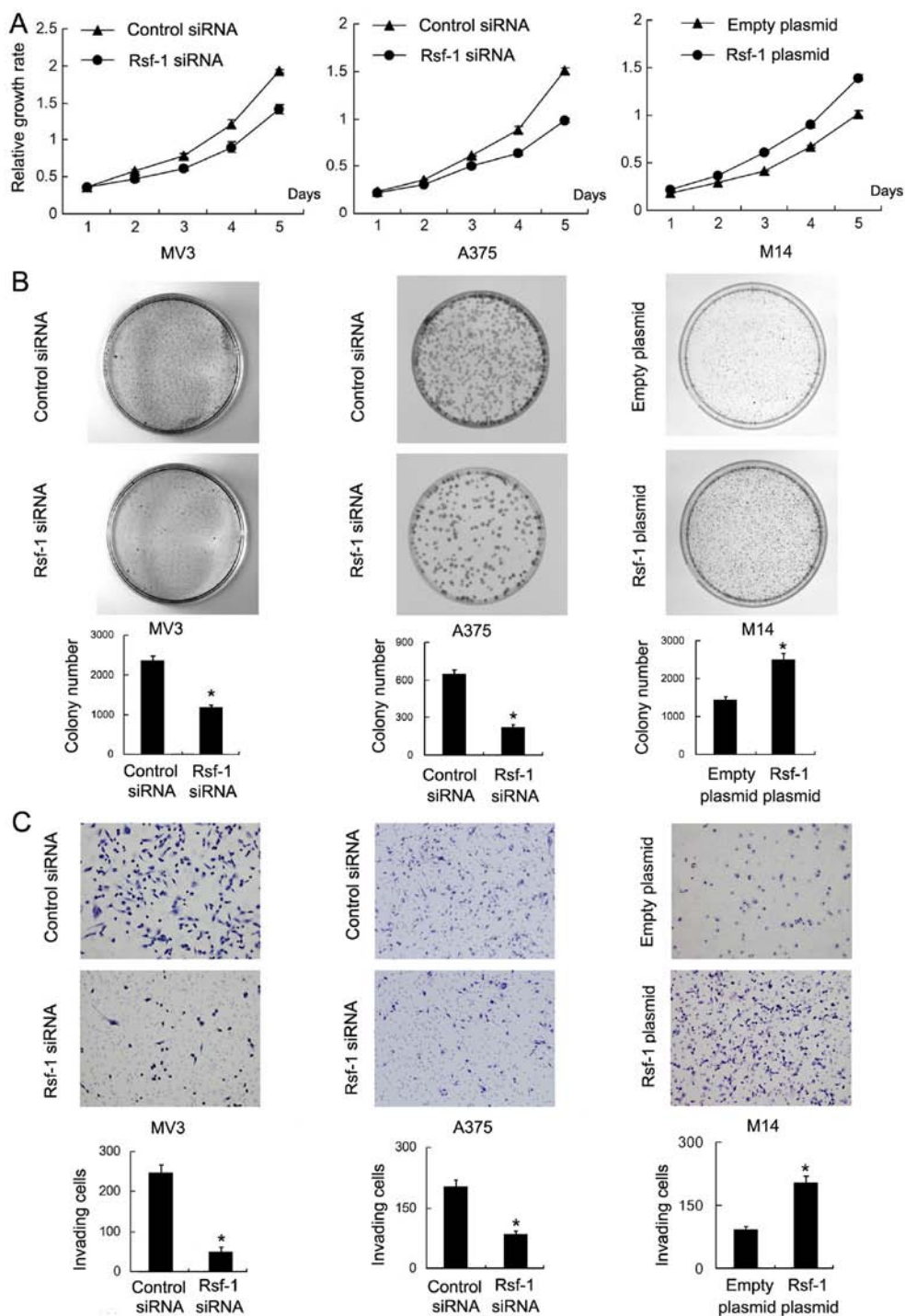


Figure 3. Rsf-1 regulates melanoma cell viability and invasion. (A) An MTT assay (96-well plate) revealed that Rsf-1 depletion decreased the viability of MV3 and A375 cells; conversely, Rsf-1 overexpression increased M14 cell viability. (B) A colony formation assay (culture dish diameter, 6 cm) demonstrated that the colony number was reduced in MV3 and A375 cells transfected with Rsf-1 siRNA, while Rsf-1 overexpression promoted colony formation ability in M14 cells. (C) A Transwell invasion assay (24-well plate) revealed that the number of invading cells decreased following Rsf-1 depletion in MV3 and A375, and increased following Rsf-1 overexpression in M14 cells. Data were presented as the mean  $\pm$  standard deviation of at least three experiments. \* $P < 0.05$  vs. control. Magnification,  $\times 200$ . Rsf-1, remodeling and spacing factor 1; siRNA, small interfering RNA.

Rsf-1 positively regulates p-I $\kappa$ B, and Bcl-2 expression was reported as associated with NF- $\kappa$ B signaling (32), whether Rsf-1 regulated the activity of Bcl-2 via its effects on NF- $\kappa$ B was investigated. To validate this, M14 cells were treated with NF- $\kappa$ B inhibitor (10  $\mu$ M). As presented in Fig. 6B, NF- $\kappa$ B inhibition significantly downregulated p-I $\kappa$ B and NF- $\kappa$ B p65 protein levels in control and Rsf-1 plasmid-transfected

M14 cells. Furthermore, treatment with the NF- $\kappa$ B inhibitor eliminated the effects of Rsf-1 upregulation on Bcl-2 expression.

## Discussion

Previous studies have reported that Rsf-1 overexpression occurs in numerous cancers, including ovarian cancer, breast cancer,

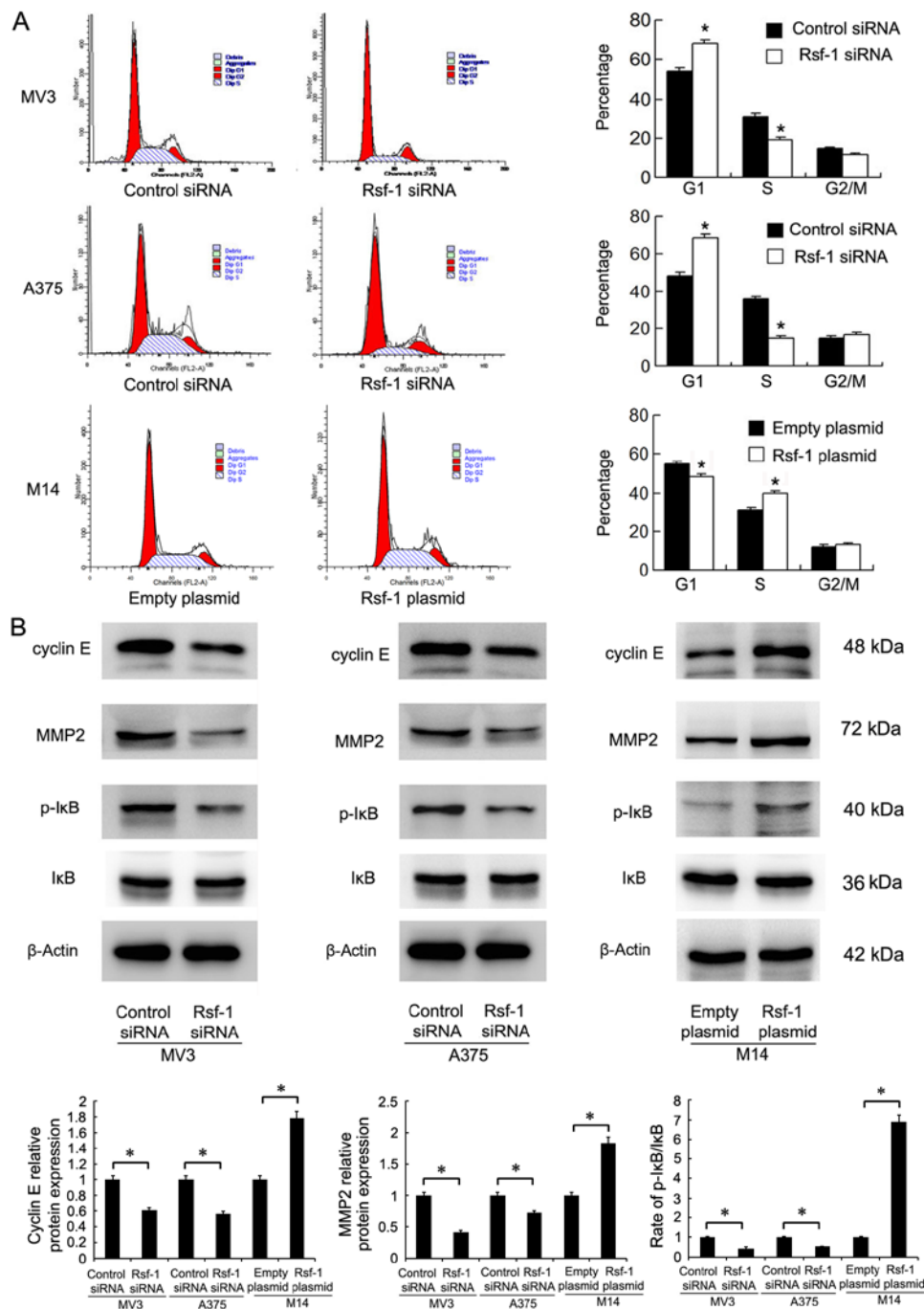


Figure 4. Rsf-1 regulates cell cycle progression of melanoma and expression of MMP2, cyclin E and p-IκB. (A) Cell cycle analysis revealed that Rsf-1 depletion increased the percentage of G1 phase cells and decreased that of S phase cells in MV3 and A375 cell groups; Rsf-1 overexpression in M14 cells exhibited opposing effects. (B) Western blotting demonstrated that Rsf-1 depletion decreased the levels of MMP2, cyclin E and p-IκB in MV3 and A375 cell lines. Rsf-1 overexpression upregulated expression of MMP2, cyclin E and p-IκB in M14 cells. Data were presented as the mean ± standard deviation of at least three experiments. \*P<0.05 vs. control. MMP2, matrix metalloproteinase-2; p, phosphorylated; Rsf-1, remodeling and spacing factor 1; siRNA, small interfering RNA.

nasopharyngeal carcinoma, non-small cell lung cancer, gastric adenocarcinoma and colon cancer (20-23,25,26,28,33-35); however, its involvement in melanoma has not been investigated. In the present study, the expression of Rsf-1 was analyzed in 50 malignant melanoma specimens via immunohistochemistry. Overexpression of Rsf-1 was detected in 19 cases, which was positively associated with advanced TNM stage. As surgical therapy is not the preferred treatment for patients with advanced melanomas, particularly

stage IV melanoma, the number of stage III and IV melanoma specimens analyzed was markedly lower than that for stage II melanoma. It was observed that the incidence of Rsf-1 overexpression was notably higher in melanomas with advanced TNM stage (III and IV vs. II). Accordingly, overexpression of Rsf-1 was reported to be associated with advanced TNM stage, nodal metastasis and poorly differentiated tumor cells in other cancers (20,22,23,33,34). Thus, Rsf-1 tends to be overexpressed in advanced stage melanomas, suggesting its

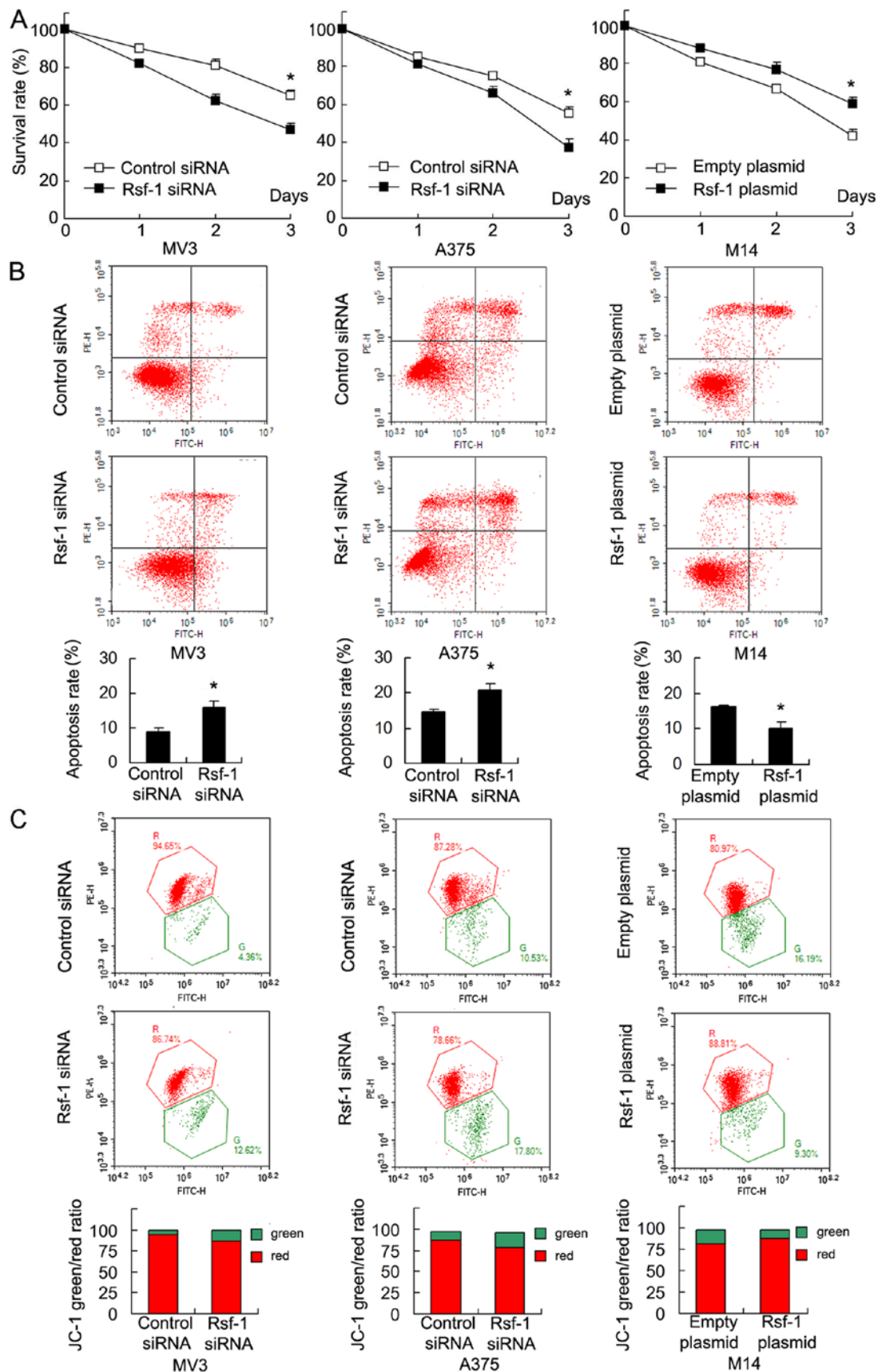


Figure 5. Rsf-1 regulates chemoresistance and the MMP of melanoma cells. (A) An MTT assay revealed that cell viability was decreased following Rsf-1 depletion in MV3 and A375 cells treated with cisplatin. Rsf-1 overexpression promoted cell viability in M14 cells treated with cisplatin. (B) Annexin V/propidium iodide analysis revealed that the percentage of apoptotic cells was significantly increased in Rsf-1-depleted MV3 and A375 cells compared with controls. Rsf-1 overexpression downregulated cisplatin-induced apoptosis in M14 cells. (C) Rsf-1 overexpression reduced MMP depolarization in M14 cells, while Rsf-1 depletion increased depolarization in MV3 and A375 cells treated with cisplatin. Data were presented as the mean  $\pm$  standard deviation of at least three experiments. \* $P < 0.05$  vs. control. FITC, fluorescein isothiocyanate; MMP, mitochondrial membrane potential; Rsf-1, remodeling and spacing factor 1; siRNA, small interfering RNA.

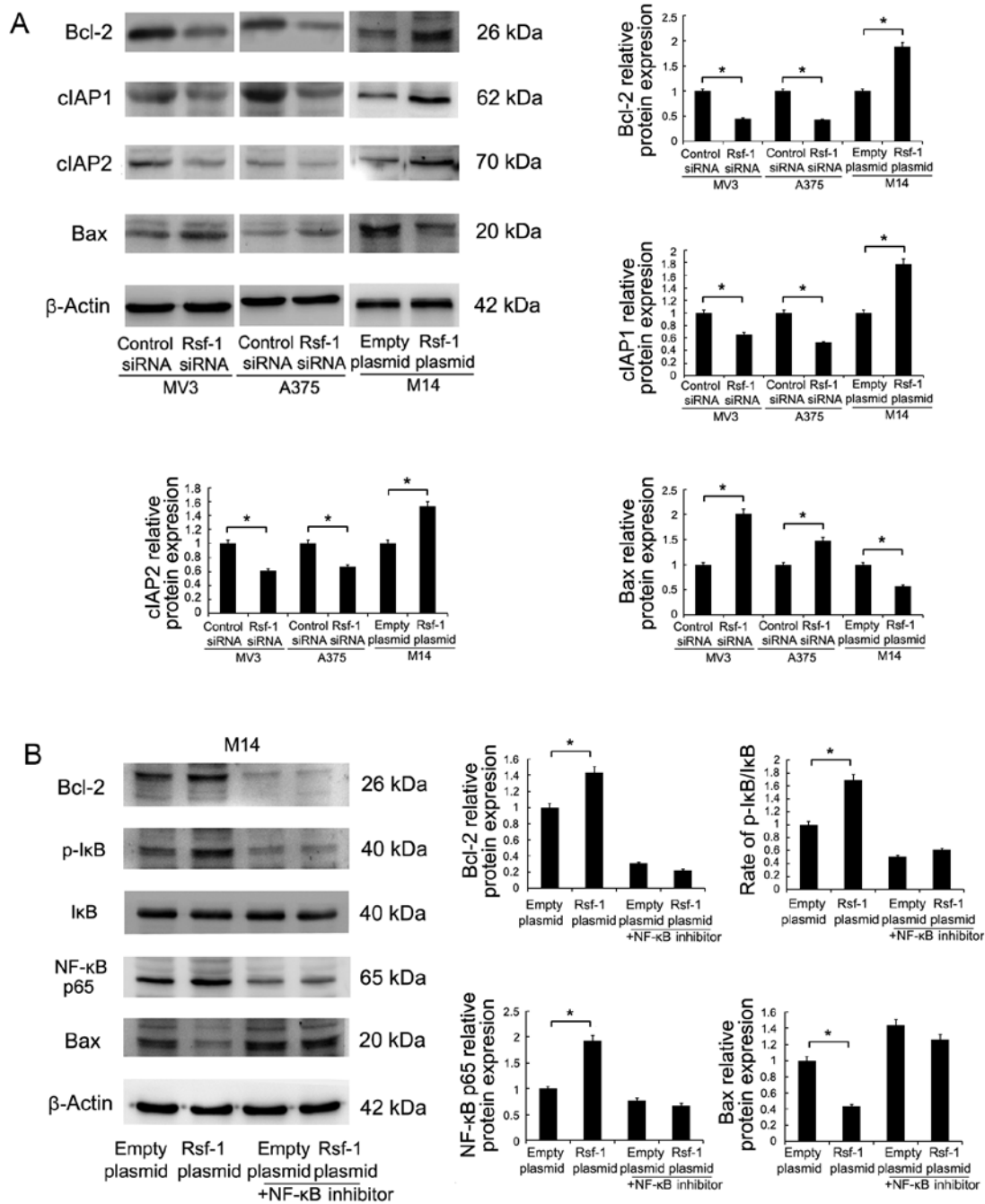


Figure 6. Rsf-1 regulates Bcl-2 expression via NF-κB signaling. (A) Western blotting revealed that Bax expression levels increased, whereas cIAP1, cIAP2 and Bcl-2 expression decreased significantly following Rsf-1 depletion in MV3 and A375 cells. Rsf-1 overexpression in M14 cells exhibited opposing effects. (B) NF-κB inhibition significantly downregulated p-IκB and NF-κB p65 protein levels in M14 cells. NF-κB inhibition also eradicated the effects of Rsf-1 overexpression on Bcl-2 upregulation. Total IκB expression was markedly altered. Data were presented as the mean ± standard deviation of at least three experiments. \*P<0.05 vs. control. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; cIAP1, cellular inhibitor of apoptosis protein 1; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; p, phosphorylated; Rsf-1, remodeling and spacing factor 1; siRNA, small interfering RNA.

association with the malignant progression of melanoma cells. To the best of our knowledge, the present study is the first to demonstrate the clinical significance of Rsf-1 in melanoma.

Rsf-1 has been reported to regulate cell behaviors including proliferation, invasion and cell cycle progression (16,26,36-39). A previous study reported that Rsf-1 depletion significantly decreased the proliferation rate and colony formation ability in colon cancer cell lines HT29 and HCT116 (23). Rsf-1 depletion also inhibited proliferation in lung cancer cells (21). The

present findings support these previous reports, demonstrating that Rsf-1 depletion decreased cell viability and colony number, while its overexpression promoted viability. For cell invasion, it was observed that the number of invading cells decreased significantly following Rsf-1 depletion, but markedly increased following overexpression of Rsf-1, which was consistent with previous reports demonstrating that Rsf-1 depletion inhibited invasiveness in the prostate cancer cell line DU145 (20) and lung cancer cells (30).

The regulatory effect of Rsf-1 on cell viability suggested that Rsf-1 may serve an important role in cell cycle progression. The present study revealed that Rsf-1 depletion enhanced the percentage of G1 phase cells and downregulated that of S phase, demonstrating that Rsf-1 can facilitate G1/S transition. Western blotting revealed that Rsf-1 depletion decreased the levels of MMP2, cyclin E and p-I $\kappa$ B, consistent with previous reports of MMP2 downregulation following Rsf-1 depletion in lung cancer cells (30). MMP2 is a member of the matrix metalloproteinase family, the members of which are involved in various pathological and physiological processes, including cancer cell growth, invasion and metastasis, suggesting that Rsf-1 regulates melanoma invasion via MMP2 (40). However, the possibility that other effects regulated by Rsf-1 may also be responsible for its effects on invasion cannot be excluded.

Transfection of cell lines exhibiting high Rsf-1 expression with siRNA targeted against Rsf-1 also increased the rate of apoptosis, which may also contribute to the decreased invasive ability of cells following Rsf-1 depletion. Cyclin E serves an essential role in fundamental biological processes, including cell cycle control and DNA replication (41-43). Sheu *et al* (13) revealed that cyclin E1 interacts with the first 441 amino acids of Rsf-1, and that their interaction promotes G1-S transition. Additionally, Rsf-1 depletion downregulated cyclin E in hepatocellular carcinoma (25). These reports further support the findings of the present study.

Furthermore, the present study proposed that Rsf-1 positively regulated the chemoresistance of melanoma cells, which has not been previously reported, to the best of our knowledge. In cells treated with cisplatin, MTT and Annexin V/PI analysis were performed to examine the effects of Rsf-1. The cell survival rate decreased, while the apoptotic rate increased significantly following Rsf-1 depletion. The role of Rsf-1 in chemoresistance has been indicated in various cancers including ovarian cancer (28), lung cancer (44) and glioma (36); however, the association between Rsf-1 and mitochondrial regulation has not yet been reported. Mitochondrial function serves an important role in the development of chemoresistance. Depolarization of the MMP induces apoptosis via the mitochondria-dependent pathway (45). It was demonstrated that Rsf-1 depletion depolarized the MMP, with opposing effects observed following Rsf-1 overexpression in M14 cells. To the best of our knowledge, the present study is the first to report of the association between the role of Rsf-1 in chemoresistance and the regulation of mitochondrial function.

It was revealed that expression of the pro-apoptotic protein Bax increased, while the levels of anti-apoptotic proteins, including cIAP1, cIAP2 and Bcl-2 decreased significantly following Rsf-1 depletion, as reported in previous studies (46-48); Rsf-1 overexpression induced opposing effects. cIAP1 and cIAP2 are members of the IAP family, which regulate apoptosis and chemoresistance (49).

The NF- $\kappa$ B signaling pathway is induced via activation of I $\kappa$ B, and is involved in numerous biological processes, including cell growth, tumorigenesis and apoptosis (50). Bcl-2 is a downstream effector of NF- $\kappa$ B, and serves as an important anti-apoptotic mediator in melanoma (51,52). The present study proposed that Rsf-1 could positively regulate the NF- $\kappa$ B pathway via upregulation of p-I $\kappa$ B. NF- $\kappa$ B signaling

was considered particularly noteworthy for two reasons. A previous study using Ingenuity Pathways Analysis Systems revealed that various molecular hubs including NF- $\kappa$ B, extracellular signal-regulated kinase (ERK) and protein kinase B (Akt) were identified in an Rsf-1-regulated gene network (28). In addition, analysis of numerous other signaling pathways was conducted, including p-ERK and p-Akt (data not shown); however, significant alterations were not observed in the expression profile of these proteins (data not shown). Notable alterations in p-I $\kappa$ B expression were observed. Thus, the NF- $\kappa$ B pathway was selected for further study, and its importance was confirmed via the use of an NF- $\kappa$ B inhibitor. Rsf-1 overexpression failed to induce Bcl-2 upregulation in cells treated by NF- $\kappa$ B inhibitor, supporting the association between Rsf-1 and Bcl-2 in melanoma cells.

There are two novel points to highlight based upon the findings of the present study. The clinical significance of Rsf-1, which has not been previously reported in melanoma, was demonstrated in this study. Additionally, the role of Rsf-1 in chemosensitivity was associated with mitochondrial function. In conclusion, the present study demonstrated that Rsf-1 is overexpressed in malignant melanoma, and may contribute to the proliferation, invasion and cell cycle progression of malignant cells by modulating the expression of MMP2, cyclin E and NF- $\kappa$ B. Furthermore, Rsf-1 may regulate chemoresistance and MMP in melanoma cells, with concomitant alterations in cIAP1, cIAP2, Bax and Bcl-2 protein expression. Thus, Rsf-1 may serve as a potential therapeutic target in the treatment of malignant melanoma.

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#### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### **Authors' contributions**

JH and LF performed the experiments, evaluated the data, drafted the manuscript and prepared the figures. QL made significant contributions towards the design of the study, evaluated the data and drafted the manuscript. All authors reviewed the manuscript.

#### **Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of China Medical University (Shenyang, China). Informed consent was obtained from all patients.

#### **Patient consent for publication**

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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