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Original Paper

Inhibiting ROS-TFEB-Dependent Autophagy Enhances Salidroside-Induced Apoptosis in Human Chondrosarcoma Cells

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Key Words
Salidroside • Autophagy • TFEB • Reactive Oxygen Species • Apoptosis • Chondrosarcoma

Abstract
Background/Aims: Autophagy modulation has been considered a potential therapeutic strategy for human chondrosarcoma, and a previous study indicated that salidroside exhibits significant anti-carcinogenic activity. However, the ability of salidroside to induce autophagy and its role in human chondrosarcoma cell death remains unclear. ***Methods:*** We exposed SW1353 cells to different concentrations of salidroside (0.5, 1 and 2 mM) for 24 h. RT-PCR, Western-blotting, Immunocytofluorescence, and Luciferase Reporter Assays were used to evaluate whether salidroside activated the TFEB-dependent autophagy. ***Results:*** We show that salidroside induced significant apoptosis in the human chondrosarcoma cell line SW1353. In addition, we demonstrate that salidroside-induced an autophagic response in SW1353 cells, as evidenced by the upregulation of LC3-II and downregulation of P62. Moreover, pharmacological or genetic blocking of autophagy enhanced salidroside -induced apoptosis, indicating the cytoprotective role of autophagy in salidroside-treated SW1353 cells. Salidroside also induced TFEB $^{(Ser142)}$ dephosphorylation, subsequently to activated TFEB nuclear translocation and increase of TFEB reporter activity, which contributed to lysosomal biogenesis and the expression of autophagy-related genes. Importantly, we found that salidroside triggered the generation of ROS in SW1353 cells. Furthermore, NAC, a ROS scavenger, abrogated the effects of salidroside on TFEB-dependent autophagy. ***Conclusions:*** These data demonstrate that salidroside increased TFEB-dependent autophagy by activating ROS signaling pathways in human chondrosarcoma cells. These data also suggest that blocking ROS-TFEB-dependent autophagy to enhance the activity of salidroside warrants further attention in treatment of human chondrosarcoma cells.

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Introduction

Chondrosarcoma is the second most prevalent (or frequent) general primary tumor in bone [1], and has been identified as an invasive and pathologically diverse malignant tumor with poor disease progression [2]. Although the long-term outcome of patients with high-grade chondrosarcoma who undergo surgery has been improved by the addition of systemic chemotherapy, the efficacy of most neoadjuvant chemotherapy drugs is uncertain, and these drugs are associated with severe side effects [3]. Recently, the application of small molecules or natural compounds isolated from plants has become an attractive strategy due to their high biological activity and safety; thus, these compounds have emerged as a vital type of complementary and alternative medicine. For instance, berberine [4], curcumin [5], and resveratrol [6], which are natural products derived from several medicinal plants abundant in southeast Asia, have been shown to inhibit chondrosarcoma cell growth and cause apoptosis by regulating of several cell cycle regulators and/or developmental signaling pathways.

Salidroside, one of the most potent compounds isolated from the plant Rhodiola rosea, has been suggested to possess anti-inflammatory, anti-proliferative, and anti-oxidant activities in various animal model systems and cell cultures [7, 8]. This compound has recently attracted considerable attention because of accumulating data demonstrating its strong inhibitory effect on bladder carcinoma, colorectal cancer [9], and breast cancer [10]. However, the potential anticancer properties of salidroside in chondrosarcoma are not well known.

Autophagy is a homeostatic, catabolic degradation process whereby cellular proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled to sustain cellular metabolism [11]. Autophagy can function as a cellular housekeeper by removing damaged organelles and recycling macromolecules; therefore, autophagy can protect cancer cells, particularly during malignant transformation and carcinogenesis [12]. A growing body of evidence indicates that autophagy can help human cancer cells survive by conferring apoptosis resistance, and inhibition of autophagy causes caspase-dependent apoptosis cell death, especially in the presence of other therapies [13, 14]. Understanding the interplay between apoptosis and autophagy in tumors is crucial to identify new targets for cancer therapy and improve the therapeutic efficiency. Thus, the ability of salidroside to increase autophagic activity in human chondrosarcoma cells should be determined because this effect has been hypothesized to significantly reduce treatment efficacy.

Recently, the activation of the basic helix-loop-helix leucine zipper transcription factor TFEB (transcription factor EB) has been shown to be involved in multiple aspects of oncogenesis, including inflammatory responses, cellular differentiation, proliferation, and survival in almost all multi cellular organisms [15]. TFEB is dysregulated in many forms of cancer, including breast cancer, lung cancer, and pancreatic cancer [16-18], and recent studies have suggested a correlation between autophagy and the TFEB pathway in human cancer. Specifically, TFEB initiates an autophagy–lysosomal biogenesis program, which stimulates the overall degradative capacity of cancer cells and plays an important role in cancer biology and clinical behavior [19, 20]. Importantly, under stress full conditions such as chemotherapy treatment, an intricate interplay between the homeostatic TFEB and autophagy pathways and the apoptotic executive process may occur in cancer cells that will ultimately dictate their fate between cell death or survival.

In the current study, we investigated whether the modulation of autophagy may be used as an adjuvant modality to improve the effects of chemotherapy during chondrosarcoma treatment. We demonstrated that salidroside exerts an antitumor effect and induces autophagy by targeting the ROS-TFEB signaling pathways in SW1353 cells. In addition, inhibiting autophagy enhanced the antitumor activity of salidroside. These findings provide important insights that may aid in the development of novel strategies to enhance the response of cancer cells to salidroside by exploiting the role of autophagy in chondrosarcoma therapy.

Materials and Methods:

Drugs and reagents

Bafilomycin A1, DCFH-DA, and N-acetyl cysteine (NAC) were purchased from Sigma–Aldrich (St. Louis, MO, USA), Salidroside (purity >99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Cell culture and salidroside treatment

The human chondrosarcoma cell line SW1353 was purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (P30-3302, PAN-Biotech, Germany) in a humidified incubator at 37˚C with 5% CO_2. The cells were passaged by dissociating them 0.25% trypsin-EDTA solution (Gibco) for 1-2 min after they had grown to 80-90% confluency. Salidroside was diluted in cell culture medium to a final concentration of 0.5, 1 or 2 mM, and cells were cultured in salidroside for 24 h.

The Sprague–Dawley rats used in this study were purchased from the Central South University. This study was approved by the Animal Care and Use Committee of Central South University and was carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the National Research Council. Cranial OBs were obtained from 18–19-day-old Sprague–Dawley rat fetuses. The calvarias were incubated with 0.25% (w/v) trypsin at 37 °C for 10 min and then cut into slices and incubated with 0.1% collagenase at 37 °C for 40 min. The isolated primary OBs were cultured with DMEM supplemented with 10% FBS (P30-3302, PAN-Biotech, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2 for 10–12 d. The phenotype was determined using an alkaline phosphatase (ALP) staining kit according to the manufacturer's protocol (Beyotime, China) [21]. Salidroside was diluted in cell culture medium to final concentrations of 0.5, 1 and 2 mM, and cells were cultured in salidroside for 24 h.

TUNEL assays

Briefly, chondrosarcoma cells were plated on sterile slides at a density of 5×10^4 cells/well in 24-well plates. After 24 h, the cells were treated as indicated concentrations of salidroside at 37 °C in 5% CO_2. The cells were then washed with TPBS twice, and subsequently fixed with 4% paraformaldehyde (PFA) for 30 min. Thereafter, the cells were washed three times with TPBS and incubated with 0.25% Triton X-100 in PBS sequentially. Cells were then incubated with 50 μl TdT-mediated dUTP nick-end labeling (TUNEL) reaction mixture (90% Label Solutions and 10% Enzyme) for 60 min at 37 °C in the dark. After washing with PBS and staining with DAPI, DNA fragmentation was examined using a Zeiss confocal laser scanning microscope (Zeiss, LSM780). Apoptotic cells with characteristic nuclear fragmentation (staining green) were counted in six randomly selected fields.

Apoptosis assay

An Annexin V-FITC/PI Detection Kit (BD Biosciences, San Diego, CA, USA) was used for the determination of cell apoptosis. OB cells were exposed to indicated concentrations of salidroside for 24 h, then harvested and washed twice with cold PBS, and re-suspended in f1 X binding buffer at a concentration of $1X\ 10^6$ cells/mL. Subsequently, according to the manufacturer's instructions, the cells were stained with Annexin VFITC and PI for 15 min at 37 C. Then, the cells were analysed immediately using a FACS Calibur flow cytometer.

Caspase-3 activity Assay

The caspase-3 activity in cell lysates was assessed using the Caspase-3 Colorimetric Assay Kit (C1116, Beyotime, China) based on the ability of caspase-3 to change acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into a yellow formazan product (p-nitroanilide, pNA). In brief, at the end of the incubation period, 5×10^6 cells were harvested in cell lysis buffer and incubated on ice for 30 min. After centrifugation at 10, 000× g for 15 min, the amount of protein in the supernatant was quantified. One hundred fifty micrograms of protein from each sample was placed in a 96-well plate and incubated with Ac-DEVD-pNA for 1 h at 37℃ in the dark, and the absorbance at 405 nm was then measured with an Infinite™ M200 Microplate Reader to detect the pNA released due to caspase-3 activity.

RFP-GFP-LC3 assay

To evaluate the numbers of autophagosomes and autolysosomes and to analyze autophagic flux, cells were infected with lentiviruses carrying expression cassettes that encode tandem fluorescence-tagged LC3B. Briefly, cells were grown on coverslips at 2×10^5 cells/well plated on coverslips and then using a Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B (HANBIO, China), in which GFP is more sensitive to acidic conditions than RFP. Twenty-four hours after infection, the cells were treated with 2 mM salidroside for 24 h. All samples were examined under a Zeiss confocal laser scanning microscope (LSM 780, Zeiss) equipped with a 63 x oil immersion objective [22].

Immunocytochemistry

Cells grown on cover slips were treated as indicated, washed twice with PBS, and then fixed with 4% PFA for 30 min. After a further three TBST washes, the fixed cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min at room temperature. The cells were then blocked in blocking buffer (1% BSA, 5% heat-inactivated donkey serum in PBS) for 1 h at room temperature, and subsequently incubated with TFEB primary antibodies overnight at 4°C. The cells were then rinsed and incubated with appropriate Alexa Fluor 568-conjugated secondary antibodies for 1 h, followed by an additional three 10 min TBST washes. The cells were then counterstained with DAPI Staining Solution (C1005, Beyotime, China) and mounted on glass slides using Antifade Mounting Medium (P0126, Beyotime, China). The stained samples were examined using a Zeiss confocal laser scanning microscope (LSM780, Zeiss) equipped with a 63×oil objective.

Luciferase Reporter Assays

To detect the activation of TFEB, SW1353 cells were transfected with plasmids encoding TFEB responsive firefly luciferase and Renilla luciferase using Lipofectamine 2000. After transfection for 24 h, the cells were washed with phosphate-buffered saline, serum starved in DMEM/F-12 overnight, and then assayed for luciferase activity using the a dual-luciferase reporter assay kit (Promega Corporation, WI, USA) according to the manufacturer's instructions [23].

Nuclear and Cytoplasmic Protein Extraction

After stimulation, cytoplasmic and nuclear proteins were extracted from SW1353 cells using nuclear and cytoplasmic extraction reagents obtained from ThermoFisher strictly according to the manufacturer's instructions.

Western blot analysis

SW1353 cells were treated with the indicated concentrations of salidroside. Then the cells were harvested and washed with cold phosphate-buffered saline (PBS). The proteins were extracted with RIPA Cell Lysis Buffer (Beyotime, China), and maintained on ice for at least 30 min. The lysates were centrifuged at 12, 000g and 4°C for 10 min, and the supernatant was subsequently transferred to a fresh tube. After the protein concentration was measured with the bicinchoninic acid (BCA) method, an equal quantity of total protein per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in 0.05% Tris-buffered saline and Tween 20 (TBST) for 1h at room temperature, and incubated overnight at 4°C with specialized antibodies. The primary anti-

Table 1. Sequences of primers used in quantitative RT-PCR

Target gene	Primer	Nucleotide sequence
MAP1LC3B	F	5'-AGCAGCATCCAACCAAAATC-3'
	R	5'-CTGTGTCCGTTCACCAACAG-3'
ATP6V0D1	F	5'-TTCCCGGAGCTTTACTTTAACG-3'
	R	5'-CAAGTCCTCTAGCGTCTCGC-3'
UVRAG	F	5'-GGCGTCTTCGACATCTTCGG-3'
	R	5'-GACGGTCTGGCATAATTCCAAA-3'
CTSB	F	5'-GAGCTGGTCAACTATGTCAACA-3'
	R	5'-GCTCATGTCCACGTTGTAGAAGT-3'
LAMP-1	F	5'-TCTCAGTGAACTACGACACCA-3'
	R	5'-AGTGTATGTCCTCTTCCAAAAGC-3'
TFEB	F	5'-ACCTGTCCGAGACCTATGGG-3'
	R	5'-CGTCCAGACGCATAATGTTGTC-3'
ACTB	F	5'-CATGTACGTTGCTATCCAGGC-3'
	R	5'-CTCCTTAATGTCACGCACGAT-3'

bodies used are listed in Table 1. After overnight incubation, the membranes were washed three times and then incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. The blots were then developed using the West Pico enhanced chemiluminescence detection kit (Thermo, USA).

Table 2. Antibodies used for the western blot experiments

Antigen	Dilution	Catalogue number	Supplier
LC3	1:1000	L7543	Sigma
ACTIN	1:5000	A5441	Sigma
TFEB	1:1000	PA1-31552	Thermo fisher
BCL2	1:1000	ab692	Abcam
BAX	1:1000	ab32503	Abcam
CL-PAPR	1:1000	5625 or 9848	Cell Signaling Technology
P62/SQSTM1	1:1000	ab91526	Abcam
H3	1:1000	SAB4500352	Sigma
p-TFEB$^{(Ser142)}$	1:200	ABE1971	Merck Millipore
P-70S6K	1:1000	9204	Cell Signaling Technology
70S6K	1:1000	2708	Cell Signaling Technology
p-4EBP1	1:1000	9456	Cell Signaling Technology
4EBP1	1:1000	ab2606	Abcam
anti-mouse (secondary antibody)	1:1000	A0208	Beyotime Company
anti-rabbit (secondary antibody)	1:1000	A0216	Beyotime Company
anti-goat (secondary antibody)	1:1000	A0181	Beyotime Company

RT-PCR

Total RNA was extracted using TRIzol reagent, and cDNA was synthesized using Super-Script II Reverse Transcriptase (Invitrogen, USA). qRT-PCR and data collection were performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, USA) system. The primers used to amplify the indicated genes are listed in Table 2. The mean fold change is shown as the natural logarithm of RQ values, and the error was estimated by evaluating the $2^{-\triangle\triangle Ct}$ equation using $\triangle\triangle$Ct plus the standard deviation and $\triangle\triangle$Ct minus the standard deviation.

Measurement of Reactive Oxygen Species (ROS)

Cultured Cells were treated with salidroside in the presence or absence of NAC for 2 h and then loaded with 25 mM DCFH-DA. After incubation for 30 min at 37°C in a 5% CO_2 incubator, the cells were washed twice with HBSS solution, and examined with an Infinite™ M200 Microplate Reader to detect the intracellular accumulation of ROS.

Gene knockdown using siRNA

The siRNAs to Beclin1, TFEB or control siRNA were all purchased from Shanghai GenePharma. Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were incubated for 48 h before further treatment.

Statistical analysis

The significance of differences between groups was assessed using a one-way ANOVA, followed by Tukey's post-hoc test. $P<0.05$ indicated statistical significance; all tests were two-sided and no corrections were applied for multiple significance testing. All experiments were repeated at least three times. Drug and biomarker distributions are represented as the mean values±s.e.m.

Results

Salidroside induces apoptosis in SW1353 cells

TUNEL staining was applied to detect the effects of salidroside treatment on apoptosis in SW1353 cells. Cultured SW1353 cells were treated with increasing concentrations of salidroside. As shown in Fig. 1A, cells were treated with 0.5, 1 and 2 mM salidroside for 24 h, which increased the proportion of apoptotic cells to 5.1 %, 24.7 %, and 37.8 % of total cells, respectively, compared to 2.8 % in the control. As key executors of cell apoptosis, both cleaved PARP and the ratio of Bax to Bcl-2 proteins increased in SW1353 cells treated with different concentration of salidroside for 24 h (Fig. 1B). We also found that treatment with salidroside increased caspase-3 activity in a dose-dependent manner (Fig. 1C). Taken together, these results demonstrate that apoptosis was involved in the response of SW1353 cells to salidroside treatment.

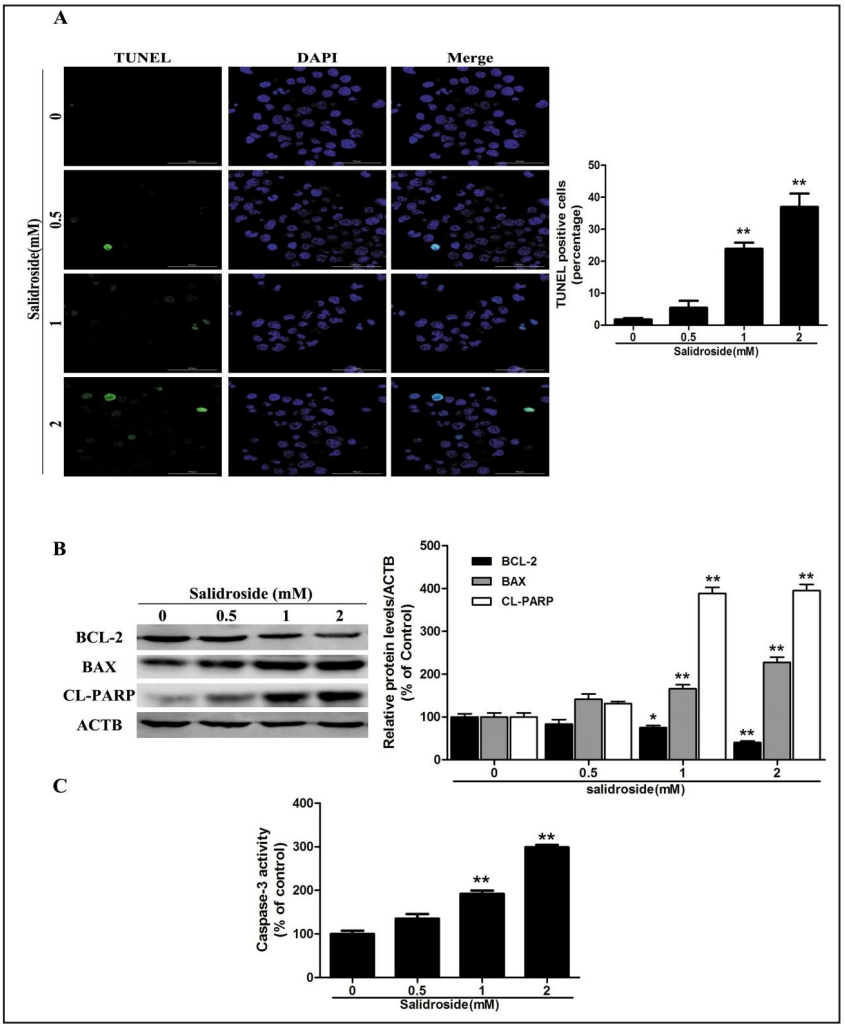

Fig. 1. Salidroside induces apoptosis in SW1353 cells. (A) SW1353 cells were treated with 0.5mM, 1mM, or 2mM salidroside for 24 h and stained with TUNEL. (B) SW1353 cells were treated with different concentrations of salidroside for 24 h, and the expression levels of cleaved-PARP (Cl-PARP), Bcl-2 and Bax were then assessed by western blotting; (C) Caspase-3 activity of SW1353 cells after 24 h of salidroside treatment at the indicated concentrations. The percentages of Caspase-3 activity are presented in bar charts; the data are presented as the mean ± SEM. *P<0.05, **P<0.01 versus the control group (n=6).

Salidroside induces autophagy in SW1353 cells

Several studies have suggested that autophagy may act as a protective mechanism in tumor cells and that autophagy induction is a common event in cancer cells in response to various chemotherapeutic treatments [24, 25]. Thus, we used the membrane-bound, autophagosome-associated form of microtubule- associated protein 1 light chain 3 (LC3-II), as a marker to monitor the effect of salidroside on autophagy induction [11]. First, the conversion of the cytoplasmic form of LC3 (LC3-I, 18 kDa) to the pre-autophagosomal and autophagosomal membrane-bound form of LC3 (LC3-II, 16 kDa) was biochemically demonstrated by western blotting in a dose-dependent manner(Fig. 2A). We next examined the change in the SQSTM1 protein levels. This protein is selectively incorporated into autophagosomes via direct binding to LC3 and is efficiently degraded by autophagy [26]. We observed an evident dose-dependent decrease in the SQSTM1 protein levels in SW1353 cells that were treated with salidroside, confirming that autophagic flux was intact in the salidroside-treated cell (Fig. 2A). Moreover, we transfected SW1353 cells with tandem fluorescent mRFP-GFP-LC3B (tf-LC3), a novel marker that allows for the assessment of autophagic flux, that is, the complete processing of autophagosomes after fusing with lysosomes, using fluorescence microscopy. Salidroside increased the number of red puncta in the merged image, which indicates the formation of autolysosomes(Fig. 2B). Taken together, our results indicate that salidroside treatment induced autophagic flux in human chondrosarcoma cells.

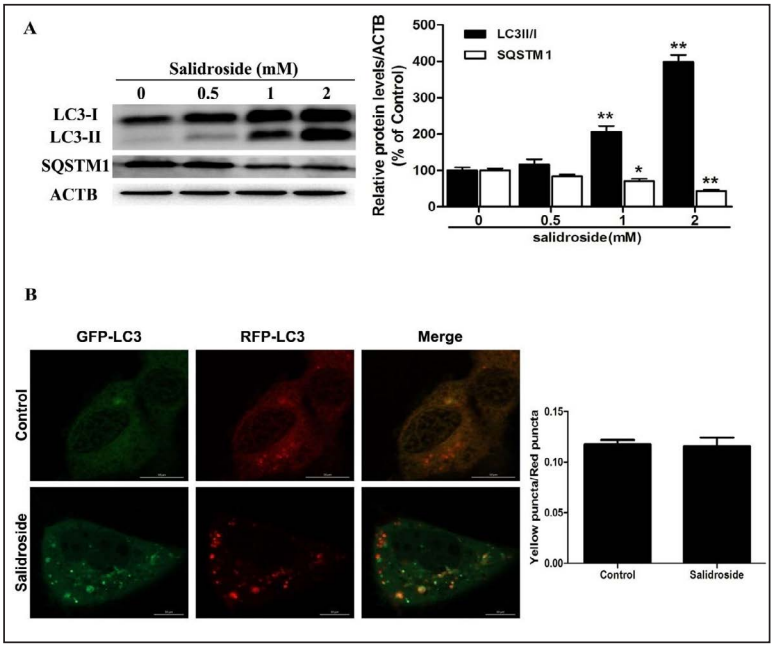

Fig. 2. Salidroside induces autophagy in SW1353 cells. (A) SW1353 cells were treated with different concentrations of salidroside for 24 h, and the expression levels of the autophagy-associated proteins LC3-I/II, and p62 were then assessed by western blotting. (B) The co-localization of GFP-LC3 puncta and RFP-LC3 puncta was examined by confocal microscopy in SW1353 cells infected using a Premo™ Autophagy Tandem Sensor RFP-GFP-LC3B Kit that were treated with 2 mM salidroside for 24 h; the data are presented as the means ± SEM. *P<0.05, **P<0.01 versus the control group (n=6).

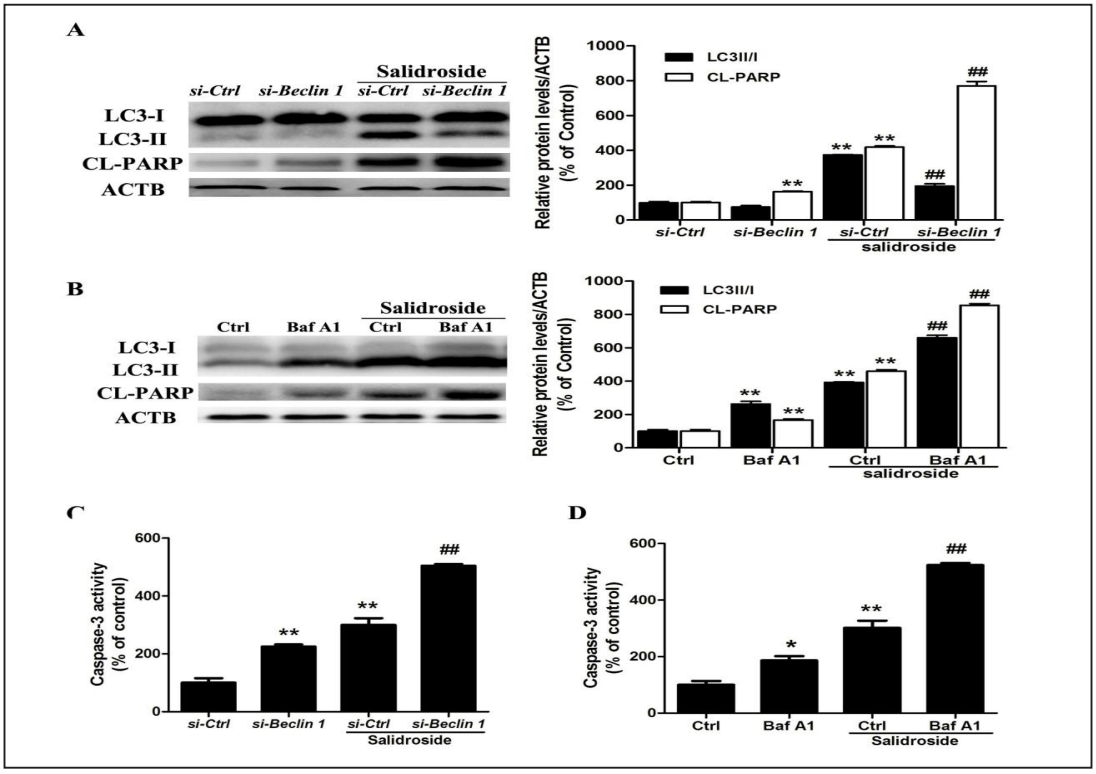

Fig. 3. Autophagy alleviates salidroside-induced apoptosis in SW1353 cells. SW1353 cells were treated with siRNA against Beclin1 and incubated with 2 mM salidroside for another 24 h. (A)The LC3 and Cl-PARP levels and (C)the caspase 3 activity were measured. The values are presented as the means ± SEM. **P<0.01versus the si-control group, ##P< 0.01 versus the si-control+ salidroside group. SW1353 cells were pretreated with 10 nM Bafilomycin A1 for 2 hand incubated with 2mM salidroside for another 24 h. (B)The LC3 and Cl-PARP level and (D) the caspase 3 activity were measured; the values are presented as the means ± SEM. *P<0.05, **P<0.01versus the control group, ##P< 0.01 versus the salidroside group (n=6).

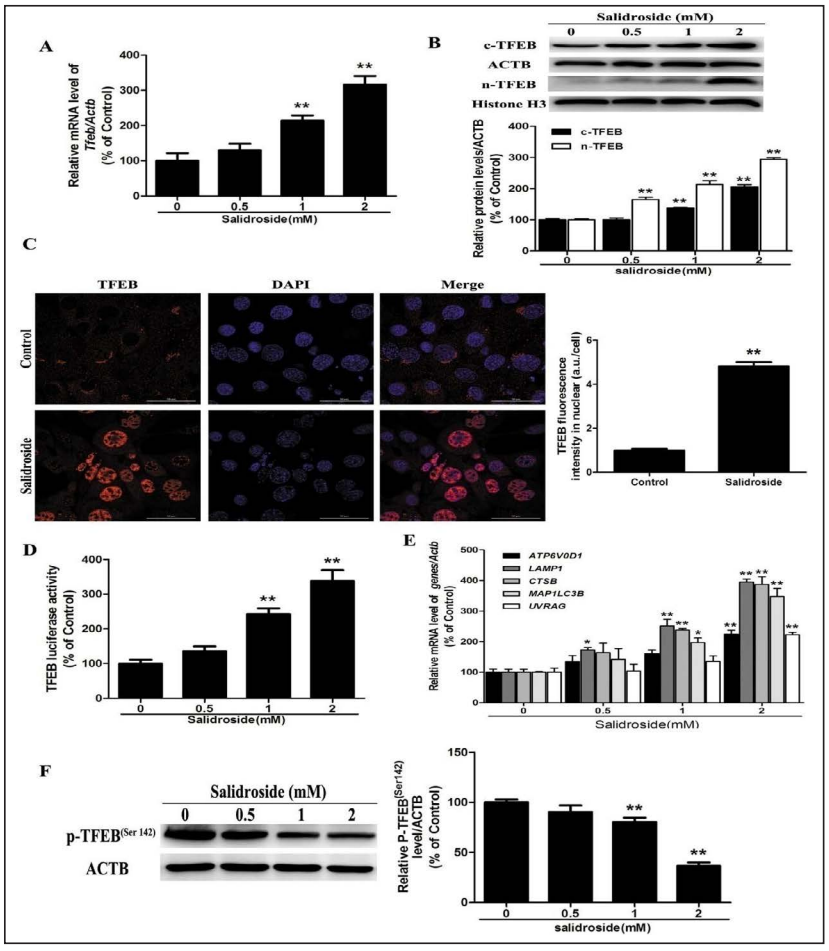

Fig. 4. Salidroside increases TFEB activity in SW1353 cells. (A) SW1353 cells were treated with different concentrations of salidroside for 24 h, and the mRNA level of TFEB was then analyzed by RT-PCR; (B) SW1353 cells were treated with different concentrations of salidroside for 24 h, and the levels of cytoplasmic and nuclear TFEB were then analyzed by western blotting, as shown in left panel; GAPDH and Histone H3 were the internal standards for protein loading, respectively. (C) Immunofluorescence of SW1353 cells incubated with anti-TFEB antibody and DAPI after salidroside (2 mM) treatment for 24 h;(D)SW1353 cells were transfected with TFEB-luciferase expression vector and incubated with different concentrations of salidroside. The luciferase activity was then measured.(E) The level of TFEB$^{(Ser142)}$ protein was analyzed by western blotting; GAPDH was the internal standard for protein loading. (F)The mRNA levels of TFEB target genes were measured using RT-PCR. The values are presented as the means ± SEM. *P<0.05, **P<0.01versus the control group (n=6).

Inhibition of salidroside-induced autophagy sensitizes SW1353 cells to salidroside -induced apoptotic cell death

Increasing evidence indicates that autophagy can prolong the survival of cancer cells and enhance their resistance to apoptosis [27, 28]. Because the manipulation of autophagy may improve the efficacy of anticancer therapeutics, we were eager to assess the effect of salidroside-elicited autophagy on the survival of SW1353 cells. Beclin1 is an essential protein for autophagy activation, and beclin1 deficiency can significantly interrupt autophagy [29]. Therefore, autophagy was inhibited in SW1353 cells by transfecting them with Beclin1-siRNA to determine the precise role of autophagy in salidroside-exposed SW1353 cells. SW1353 cells transfected with Beclin-1 siRNA showed a reduced LC3-II accumulation after salidroside treatment compared with a scrambled siRNA-transfected control cells, indicating the involvement of Beclin-1 in salidroside-mediated autophagy in SW1353 cells(Fig. 3A). To test the potential cytoprotective role of autophagy in our system, we inhibited autophagy in SW1353 cells using bafilomycin A1 (Baf A1), which is an inhibitor of lysosomal V-ATPase and results in the accumulation of autophagosomes due to a defect in the fusion between autophagosomes and lysosomes [26]. We observed that the presence of 10 nM Baf A1 significantly increased the level of LC3II and cleaved PARP in SW1353 cells treated with 2 mM salidroside (Fig. 3B). In agreement with the data derived from pharmacological inhibitors, the knockdown of Beclin-1 by siRNA enhanced the level of cleaved PARP, as assessed using

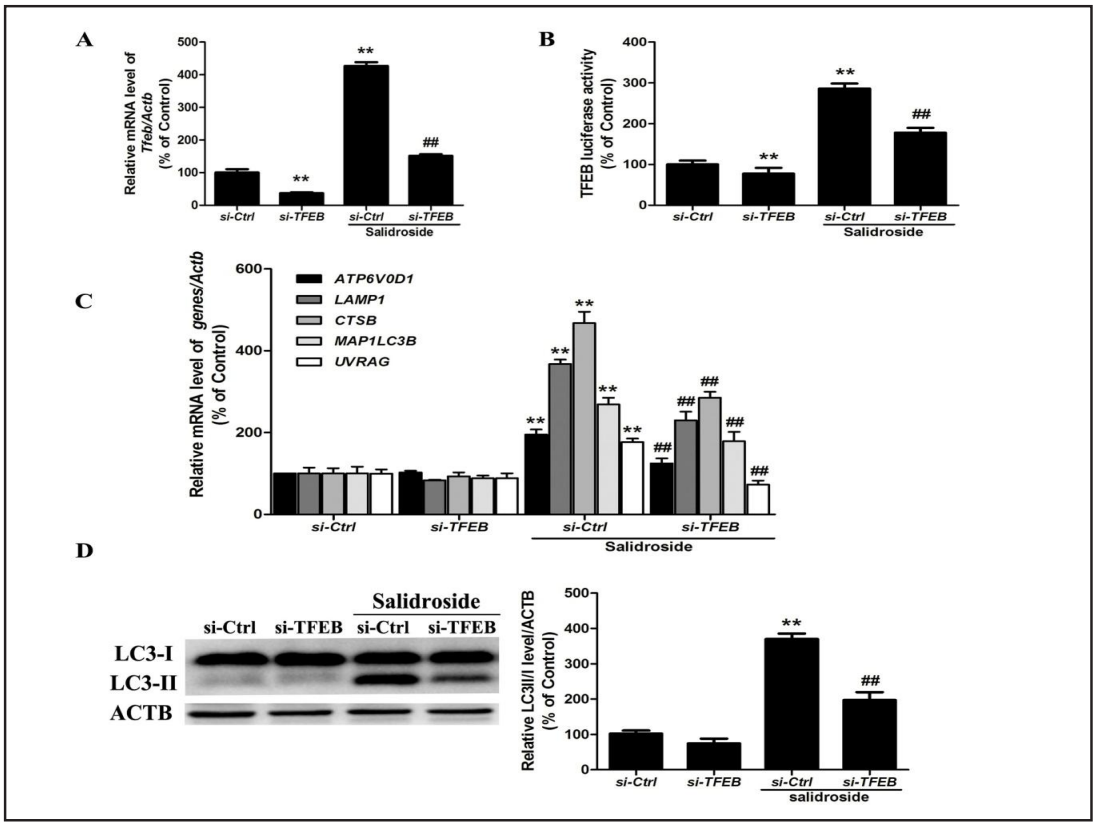

Fig. 5. TFEB mediates salidroside-induced autophagy. (A) The mRNA levels of autophagy-related genes were determined in TFEB siRNA-transfected SW1353 cells using RT-PCR. (B)SW1353 cells were treated with siRNA against TFEB or control siRNA and then incubated with 2mM salidroside for another 24 h before measuring the Luciferase activity. (C)The mRNA levels of TFEB target genes were measured using RT-PCR. (D) The levels of LC3II/LC3I were measured using western blotting. The values are presented as the means ± SEM. **P<0.01versus the control group, ##P< 0.01 versus the si-Ctrl+ salidroside (2 mM) group (n=6).

western blotting analysis (Fig. 3C). Moreover, salidroside markedly increased the caspase-3 activity when autophagy flux was inhibited (Fig. 3D and 3E). These results show that inhibiting of salidroside-induced autophagy sensitized SW1353 cells to salidroside-induced apoptotic cell death.

Salidroside induces TFEB nuclear translocation in SW1353 cells

Transcription factor EB (TFEB), which is a member of the MiT/TFE family, is a major regulator of lysosomal biogenesis [30]. Many target genes of TFEB participate in substrate degradation and lysosome-associated processes, such as autophagy and endocytosis [31], and recent studies have suggested a correlation between autophagy and the TFEB pathway in human cancer [19]. Based on these results, we investigated the involvement of TFEB in the effect of salidroside on SW1353 cells. As shown in Fig. 4A, TFEB mRNA expression increased significantly after exposure to different concentrations of salidroside for 24 h. The activation of TFEB was measured based on the detection of its translocation into cell nuclei from its initial location in the cytoplasm where it exists in an inactive form [32]. Western blotting for TFEB protein in the nuclear and cytosolic fractions of SW1353 cells indicated that salidroside treatment increased the level of nuclear TFEB (Fig. 4B), and immunofluorescence staining produced similar results (Fig. 4C). Moreover, the entry of TFEB into the nucleus was accompanied by an upregulation of "TFEB-responsive genes"[33]. As expected, salidroside significantly increased the TFEB luciferase activity and the mRNA abundance of 5 tested genes, including ATP6V0D1, LAMP1, CTSB, MAP1LC3B and UVRAG(Fig. 4D and 4E). To

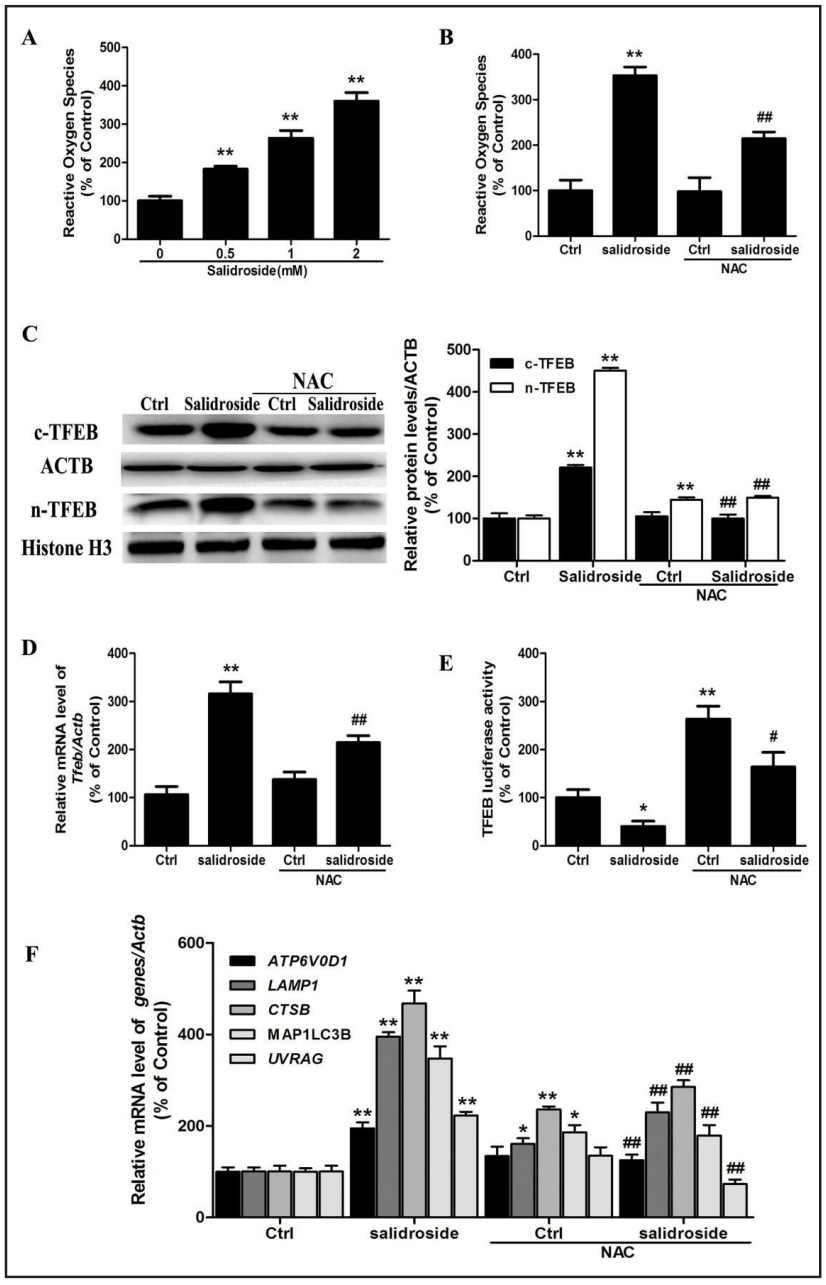

Fig. 6. ROS is an upstream signaling molecule that activates the TFEB-dependent autophagy pathway. (A) SW1353 cells were treated with salidroside (0. 5, 1 or 2 mM) for 24 h and then examined for the intracellular accumulation of ROS using DCFH-DA; ROS levels were measured using anInfinite™ M200 Microplate reader; SW1353 cells were exposed to NAC (5mM) for 2 h, followed by treatment with salidroside(2 mM) for 24 h. (B) The ROS levels were measured with an Infinite™ M200 Microplate reader; (C) The expression levels of nuclear and cytoplasmic TFEB were measured. (D) The mRNA level of TFEB was detected by RT-PCR. (E) The TFEB Luciferase activity was then measured. (F)The mRNA levels of TFEB target genes were measured using RT-PCR. The values are presented as the means ± SEM. **P<0.01, versus the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 versus the salidroside (2 mM) group. (n=6)

investigate the mechanism underlying salidroside-elicited TFEB nuclear translocation, we assessed the phosphorylation status of TFEB. Because Ser142 dephosphorylation is the primary, and sufficient event leading to TFEB nuclear translocation, we assessed the level of TFEB phosphorylation at Ser142. Salidroside treatment significantly decreased TFEB phosphorylation at Ser142 (Fig. 4F). Collectively, these data show that salidroside induced TFEB$^{(Ser142)}$ dephosphorylation, subsequently activated TFEB nuclear translocation, and increased TFEB reporter activity, which contributed to the expression of autophagy-related genes and lysosomal biogenesis.

TFEB mediates salidroside-induced autophagy in SW1353 cells

To further assess the ability of TFEB to salidroside-induced autophagy, TFEB was inhibited with TFEB-specific siRNA prior to salidroside in SW1353 cells. As shown in Fig. 5A and 5B, TFEB-siRNA abrogated salidroside-induced the TFEB expression and luciferase

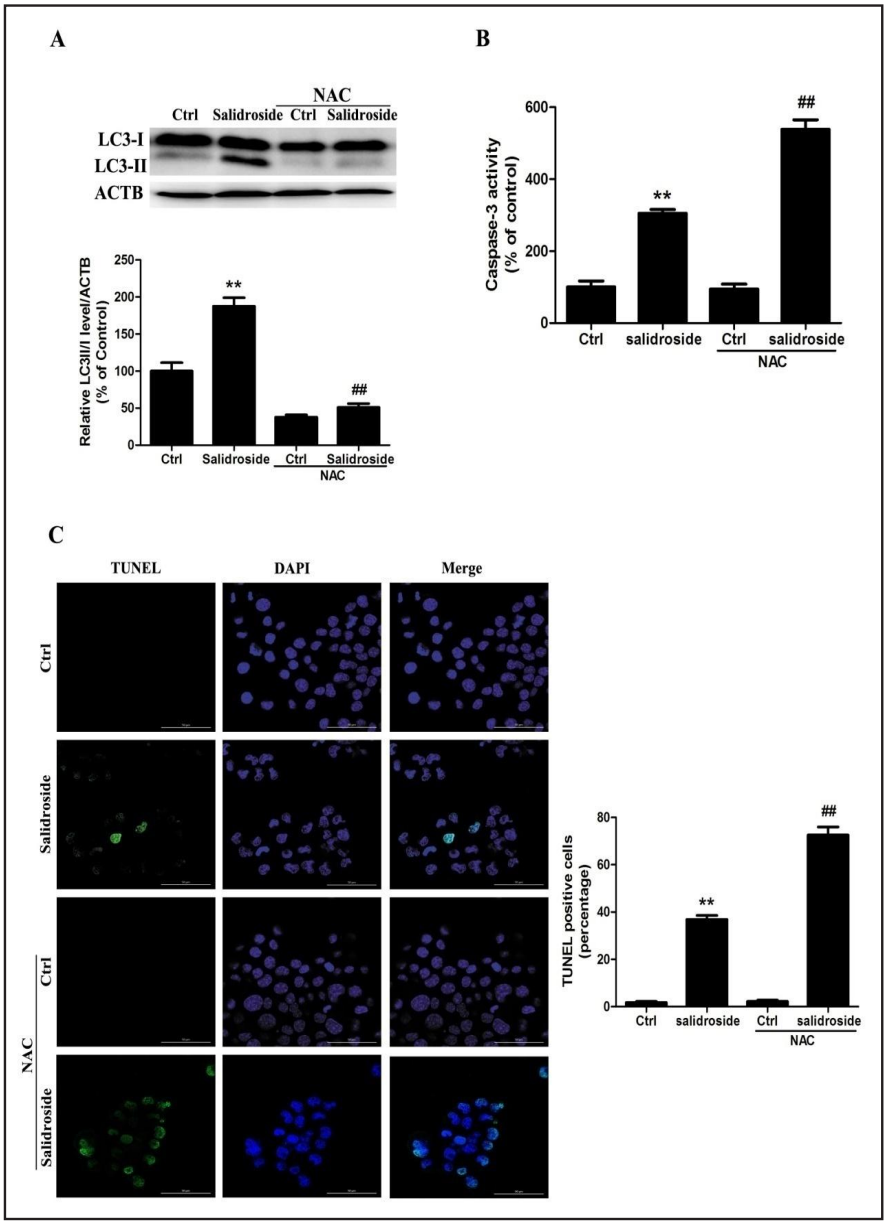

Fig. 7. NAC increased salidroside-induced apoptosis after the inhibition of autophagy. SW1353 cells were treated with salidroside (2 mM) for 24 h after pre-incubation with or without NAC (5 mM) for 2 h. (A) The levels of LC3II/LC3I were measured using western blotting. (B) The caspase-3 activity was measured. (C) apoptotic cells stained with TUNEL. The values are presented as the means ± SEM. **P<0.01, versus the control group; ## P<0.01 versus the salidroside (2 mM) group (n=6).

activity. Notably, the inhibition of TFEB activity decreased salidroside-induced lysosomal biogenesis and autophagy-related genes expression (Fig. 5C). Additionally, the protein levels of LC3 II/LC3 I were also suppressed (Fig. 5D). These results suggested that salidroside induced autophagy by activating TFEB pathway in SW1353 cells.

ROS is an upstream signaling molecule that activates the TFEB-dependent autophagy pathway

Accumulating evidence indicates that intracellular ROS can induce apoptosis and autophagy in cancer cells [34-37]. Thus, we examined the role of ROS generation in salidroside induced apoptosis and autophagy in SW1353 cells. Salidroside significantly induced ROS generation in a dose-dependent manner (Fig. 6A) and the ROS scavenger N-acetyl-L-cysteine (NAC) significantly decreased intracellular ROS generation (Fig. 6B). In addition, pretreatment with NAC significantly inhibited the activation of TFEB in salidroside-treated cells, indicating that ROS is an upstream signaling molecule that activates the TFEB pathway (Fig. 6D-E). NAC also significantly suppressed the protein expression of LC3-II, suggesting

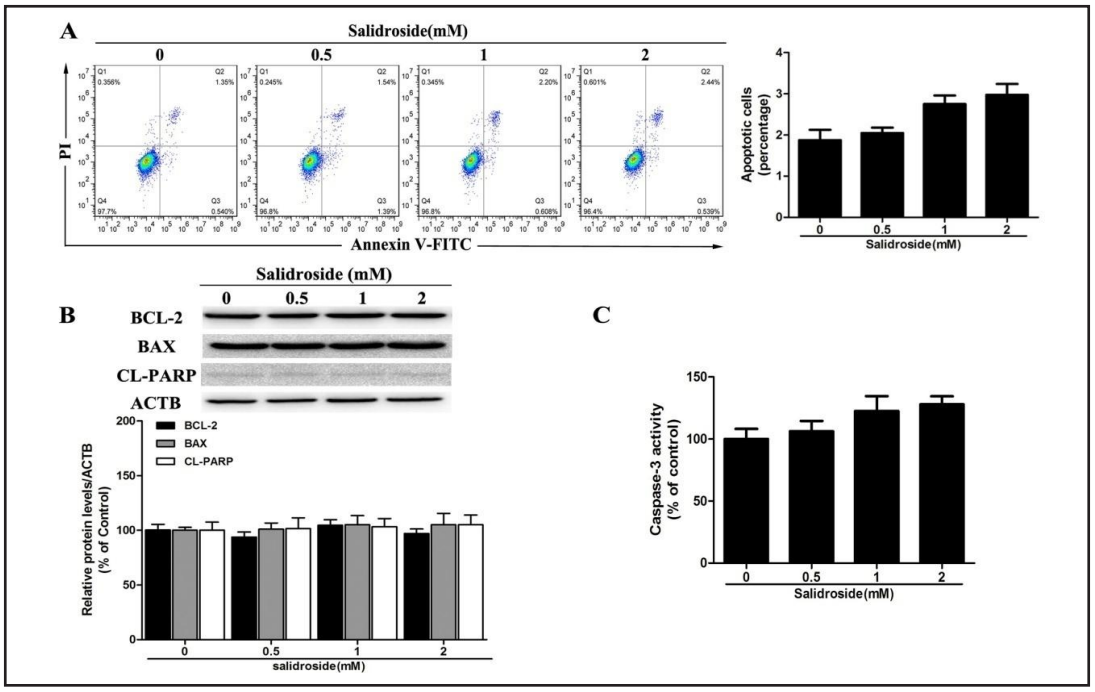

Fig. 8. Salidroside does not induce apoptosis in OBs. (A) OBs were treated with 0.5 mM, 1 mM, and 2 mM of salidroside for 24 h and stained with Annexin V/PI, then analyzed by flow cytometry. The percentages of Annexin V-positive cells were presented in bar charts; (B) OBs were treated with different concentration of salidroside for 24 h then western blot analysis was performed to assess the expression level of Cleaved-PARP (CL-PARP), BCL-2 and BAX. (B) caspase-3 activity of OBs after 24 h of salidroside treatment at the indicated concentrations. The percentages of caspase-3 activity were presented in bar charts; (n=3)

that autophagy was inhibited by NAC administration (Fig. 7A). Moreover, the incubation of cells with NAC for 2 h prior to treatment with salidroside increased Caspase-3 activity and the proportion of apoptotic cells (Fig. 7B and 7C). Taken together, these results showed that ROS is involved in salidroside-induced autophagy in SW1353 cells, and salidroside-promoted TFEB signaling partly depends on ROS generation.

Discussion

Chondrosarcoma is a common malignant histological tumor; worldwide, the incidence of chondrosarcoma is second only to that of primary tumors of bone, and male-to-female ratio of the incidence of this disease is 1.5:1[38]. Recently, the combination of curative surgery with chemotherapy has improved the five-year of patients with chondrosarcoma, but many of these patients either do not respond to chemotherapy or develop drug resistance to current chemotherapy regimens [39]. Therefore, the discovery of effective anticancer activities with few side effect chemotherapeutic agents is necessary and urgent.

Salidroside is a phenylpropanoid glycoside isolated from a popular traditional Chinese medicinal plant, Rhodiola rosea L, and has been proven to inhibit cell proliferation, arrest the cell cycle, and promote the apoptosis of human cancer cells [40]. Salidroside also plays an antitumor role by inhibiting tumor metastasis and reducing angiogenesis [10]. In our study, cleaved PARP, the ratio of Bax to Bcl-2 protein and caspase-3 activity increased in SW1353 cells after treatment with salidroside, which indicated that salidroside is a potential treatment for chondrosarcoma. Importantly, apoptosis was not detected in primary rat osteoblasts (OBs) after salidroside treatment, which confirmed the selective cytotoxic action of salidroside against human chondrosarcoma cells (Fig. 8).

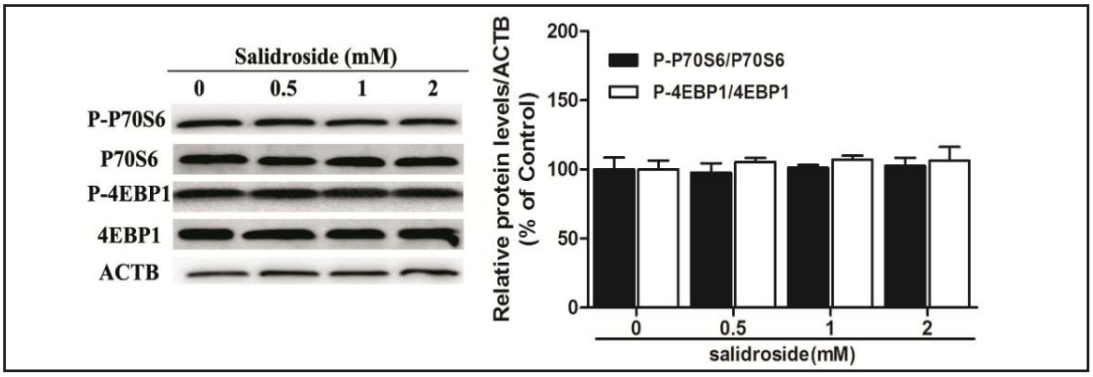

Fig. 9. Salidroside has no effect on mTORC1 pathway in SW1353 cells. SW1353 cells were treated with different concentration of salidroside for 24 h then western blot analysis was performed to assess the expression level of p-p70S6K and p-4EBP1.(n=4)

Autophagy is a lysosome dependent mechanism by which dysfunctional or damaged intracellular organelles are broken down and recycled through the lysosomes. As a putative adaptive catabolic process, autophagy plays an important role in many human cancers, and the inhibition of autophagy in these conditions can lead to increased chondrosarcoma cell death. Reumann et al. reported that the inhibition of autophagy increased 2-Methoxyestradiol-induced cytotoxicity in SW1353 chondrosarcoma cells [41], and Xing et al. found that the knockdown of HOTAIR inhibited autophagy, which favored chondrosarcoma cell death [42]. Moreover, Gao et al. observed that autophagy protected chondrosarcoma cells from siBMPR2-induced apoptotic cell death [43]. Consistent with these findings, our experiments showed that the pharmacological or genetic inhibition of autophagy leads to increased salidroside-induced apoptosis, indicating that salidroside-mediated autophagy is a pro-survival mechanism rather than a pro-death mechanism. We also examined the potential mechanisms underlying salidroside-induced autophagy. TFEB has been shown to regulate autophagy in various human cancers and has been well demonstrated to play key roles in several biological activities, including autophagy, apoptosis, inflammation, survival and metabolic disorders [44]. The activation of TFEB was measured based on the detection of its translocation into cell nuclei from its initial location in the cytoplasm where it exists in an inactive form. The nuclear translocation of TFEB stimulates autophagy in various cell types by inducing lysosomal biogenesis and increasing the expression of numerous autophagy-related genes [45]. In our research, salidroside treatment activated TFEB nuclear translocation and increased TFEB reporter activity, which contributed to lysosomal biogenesis and the expression of autophagy-related genes, including genes encoding several subunits of v-ATPase (ATP6V0D1), lysosomal transmembrane proteins (LAMP1), lysosomal hydrolases (CTSB), and proteins that play an essential role in the formation of autophagosomes (MAP1LC3B) as well as their degradation (UVRAG) [46]. Moreover, the siRNA-mediated down-regulation of TFEB suppressed salidroside-induced autophagy indicating that the TFEB signaling pathway is involved in salidroside-induced autophagy in the human chondrosarcoma cells.

However, the mechanism that underlies the salidroside-mediated enhancement of TFEB activity and nuclear translocation remains elusive. Recent evidence has suggested that mTORC1 regulates the intracellular localization of TFEB. In certain circumstances, TFEB is recruited to the lysosomal surface, where it undergoes mTORC1-dependent phosphorylation. Specifically, mTORC1 phosphorylates TFEB at several residues, but Ser142 is particularly relevant because the phosphorylation of this residue creates a binding site for the cytosolic chaperone 14-3-3. Interaction with 14-3-3 results in sequestration of the TFEB in the cytosol. Conversely, inactivation of the mTORC1, together with dephosphorylation of Ser142, prevents binding to 14-3-3, which results in the rapid accumulation of TFE3 in

the nucleus [45, 47]. However, the phosphorylation of p-70S6K and p-4EBP1, which are mTORC1 substrates, did not significantly change after salidroside treatment, suggesting that mTORC1 signaling is not involved in salidroside-induced TFEB-dependent autophagy (Fig. 9)[48]. Other studies have linked the accumulation of ROS to TFEB activation in the invasion and migration of cancer [49]. We found that salidroside induced ROS generation in a dose-dependent manner, an effect that was abolished by pretreating cells with NAC. Furthermore, the incubation of human chondrosarcoma cells with NAC for 2 h prior to treatment with salidroside revealed that the inhibition of ROS generation abrogated the effect of salidroside on the TFEB pathway. Based on these results, we suggest that ROS are important cellular mediator that triggers the TFEB-dependent pathway after the administration of salidroside in chondrosarcoma cells.

Collectively, we report that salidroside induces marked apoptosis in human chondrosarcoma cells and rapidly activates an autophagic process. A link between salidroside-induced autophagy and ROS production was also observed. Moreover, the pharmacological or genetic inhibition of TFEB-dependent autophagy sensitized chondrosarcoma cells to salidroside-induced apoptosis. These findings indicate that autophagy plays a cytoprotective role in chondrosarcoma cells treated with salidroside, and the inhibition of autophagy may improve the therapeutic efficacy of salidroside treatment for chondrosarcoma.

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Disclosure Statement

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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