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Silibinin inhibits endometrial carcinoma via blocking pathways of STAT3 activation and SREBP1-mediated lipid accumulation

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Title page

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Abstract

Aims: To seek new conservative treatments for young women with early-stage endometrial carcinoma (EC) who desire to retain fertility, we investigated the effects and the underlying mechanism of silibinin in EC, which exhibits promising anti-cancer and tumour-suppressing properties in many malignant tumours.
Main methods: Through relevant experiments such as MTT assay, cell colony

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formation assay and subcutaneous xenograft experiment, we showed that silibinin inhibited the proliferation of EC cells and tumours. Silibinin significantly induced cell cycle arrest and promoted apoptosis *in vitro. In vivo* TUNEL assay confirmed the apoptotic effect caused by silibinin. STAT3 is activated in the development of tumours. Silibinin notably inhibited the expression of STAT3 phosphorylation and regulated the expression of downstream genes involved in cell cycle and apoptosis at protein and mRNA levels in EC cells. Furthermore, silibinin decreased the expression of intranuclear SREBP1, which is a key regulator of lipid metabolism in the nucleus, and reduced the lipid accumulation in EC cells. Downregulation of the expression levels of SREBP1 and its downstream genes associated with lipid metabolism was also observed in silibinin-treated EC cells.

Key findings: The results revealed that a novel anticancer drug, silibinin, markedly suppressed cell proliferation, cell cycle progression, apoptosis inhibition and lipid accumulation by blocking STAT3 and SERBP1 signalling pathways in EC cells.

Significance: Silibinin has anti-tumour characteristics and inhibits abnormal lipid metabolism in EC. This compound is expected to contribute to the conservative and adjuvant treatment of EC and should therefore be investigated further.

Key words: silibinin, endometrial carcinoma, STAT3, SREBP1, proliferation, apoptosis, lipid metabolism

Introduction

Endometrial carcinoma (EC) is one of the most common malignant tumours in the female reproductive system. In recent years, the incidence and mortality of EC have increased, and the age of onset has become younger, seriously harming women's health[1, 2]. Despite routine surgery, radiotherapy and chemotherapy, conservative treatments of early-stage EC with the purpose to preserve fertility are limited. Current research on the molecular mechanism of EC is gradually deepening, and its targeted therapy has received extensive attention[3]. Epidemiological studies have shown that several malignant tumours are closely related to metabolic syndrome, which is associated with abnormal lipid metabolism and insulin resistance, and both of these are extremely important risk factors for the occurrence of EC[4,5]. Therefore, inhibition of abnormal lipid metabolism has become a focus of treatment for EC.

Silibinin (SB) is the major active constituent of silymarin, a polyphenolic flavonoid extracted from milk thistle seeds[6]. Recent studies have shown that silibinin plays an important role in the treatment of many types of tumours, such as lung cancer, prostatic cancer, colon cancer, breast cancer, bladder cancer and hepatocellular carcinoma, by affecting cancer cell growth,

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proliferation, apoptosis and angiogenesis through many signalling pathways[7,8]. However, the effect of silibinin on EC has not been reported. Its anti-tumour mechanism can provide new ideas for the conservative treatment of early-stage EC.

Studies have indicated that the biological effects of sterol regulatory element binding proteins (SREBPs) on tumour cells are mainly achieved through mediating lipid metabolism pathways. Among them, SREBP1 is the main regulator of fatty acid metabolism and plays an important role in tumour proliferation, migration and invasion, especially in hormone-sensitive breast, prostate and endometrial cancers[9-11]. Li et al found that the expression of SREBP1 in EC was not only higher than that in atypical hyperplasia and normal endometrium, but the expression was also gradually enhanced in highly differentiated to low differentiated EC. This suggested that overexpression and activation of SREBP1 may be involved in the progression of EC[11]. Nambiar et al suggested that SREBP1 may become a novel target of silibinin and play a critical role in silibinin inhibitory effects on prostate cancer[12]. Therefore, it is speculated that silibinin can become a new feasible anti-tumour and anti-metabolic agent by inhibiting SREBP1 in EC.

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that is activated in various tumours and regulates cell proliferation, differentiation and apoptosis. Retrospective studies have identified the expression of phospho-STAT3 (p-STAT3) as a poor prognostic marker in breast, colon, prostate and lung cancer. Agarwal et al first reported that silibinin inhibited the activation of STAT3 in the DU145 cell line of prostate cancer[13]. Since then, numerous preclinical data from cancer models and evidence of the ongoing clinical trials explored silibinin as a STAT3 inhibitor in cancer[14].

Considering that EC is closely related to metabolic syndrome, the present study aims to investigate whether STAT3 and SREBP1 serve as targets for silibinin, which may play a role in inhibiting cell proliferation, promoting apoptosis and controlling abnormal lipid metabolism in EC cells.

Materials and Methods

Cell lines and materials

The EC cell lines Ishikawa and RL-952 were obtained from the Key Laboratory of Gynecologic Oncology of Shandong Province (Qilu Hospital, Shandong University, Jinan, China). Ishikawa cells were cultured in RPMI-1640 medium supplemented with 10% FBS (foetal bovine serum). RL-952 cells were cultured in DMEM/F-12 medium supplemented with 10% FBS. Both cells were cultured at 37˚C in an incubator with 5% CO_2. Silibinin (S0417) (chemical name: 2,3-Dihydor-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl)benzodioxin) was purchased from Sigma-Aldrich. The stock solution (1,000,000 μM) was

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prepared with dimethyl sulfoxide (DMSO) and stored at -20˚C. The working concentration of silibinin was diluted in the respective medium, and the final concentration of DMSO was <0.1% (v/v).

The antibodies of SREBP-1 (sc-8984, Santa Cruz Biotechnology, USA, dilution 1:200), STAT3 (ab68153, Abcam, UK, dilution 1:1000), p-STAT3 (S727, ab32143, Abcam, UK, dilution 1:2000), Acly (ab40793, Abcam, UK, dilution 1:2000)，p-Acly (S455, ab46796, Abcam, UK, dilution 1:2000), SCD-1 (AF7550-SP, NOVUS, USA, 1:2000), Bcl-2 (ab32124, Abcam, UK, dilution 1:1000), Bax (ab182733, Abcam, UK, dilution 1:2000), Survivin (#2808, Cell Signaling Technology, USA, 1:1000), CyclinB1 (sc-166210, Santa Cruz Biotechnology, USA, dilution 1:200), CyclinD1 (ab134175, Abcam, UK, dilution 1:1000), and β-Actin (13E5) (#4970, Cell Signaling Technology, USA, dilution 1:1000) were purchased. Goat Anti-Rabbit IgG, HRP conjugate: LOT: 2488759, Merck Millipore, Germany, dilution 1:2000; Goat Anti-Mouse IgG, HRP conjugate: LOT: 2488791, Merck Millipore, Germany, dilution 1:2000) were used.

MTT assay

The Ishikawa and RL-952 cells in the logarithmic growth period were cultured in 96-well plates at a density of 5000 cells/100 μL/well. After an overnight incubation at 37˚C with 5% CO_2, the cells were exposed to silibinin at 0, 50, 100, 150, 200 and 250 μM for 24, 48 and 72 h. A total of 20 μl of MTT solution (0.5 mg/ml) (Sigma) was added to each well, and the cultures were further incubated at 37°C for 4 h. The media was then removed, and 100 μl DMSO was added to all wells to dissolve the formazan product. The MTT colorimetric value was obtained by measuring the absorbance at 490 nm of a microplate reader.

Colony formation assay

For the clonogenic assays, EC cells (Ishikawa and RL-952) in the logarithmic growth period were plated onto 6-well plates at a density of 400 cells/well in culture medium containing the untreated control groups and treatment groups treated with different concentrations of silibinin (25, 50, 75 μM). Each group was allocated to three plates and was grown for 12 days. The colonies formed on each plate were stained with crystal violet staining solution, and the number of colonies was counted quantitatively with an ImageJ software.

Xenograft tumour experiment

All animal handling and experimental conditions were in accordance with the guidelines developed by the Institutional Animal Care and Use Committee of Qilu Hospital of Shandong University. RL-952 cells were injected subcutaneously into BALB/c female nude mice for tumorigenesis experiments. After 14 days, 25 tumour-bearing nude mice with similar tumour sizes were

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selected and divided into five per group. Intragastric administration with solvent-treated control or different amounts of silibinin (dosage as 50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg) were performed in five groups mice every day, and the tumour sizes were recorded every other day. Three weeks after gavage, nude mice were sacrificed, and each tumour was weighed and photographed.

Cell cycle assay

After treatment with either control or different concentrations of silibinin (100, 150, 200 μM) for 48 h, Ishikawa and RL-952 cells were fixed, stained with a PI/RNase staining buffer (BD Bioscience Pharmingen, San Diego, CA, USA) and analysed with a FACS flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) on the basis of 2N and 4N DNA content. The data were analysed by ModFit LT 3.2 software (Verity Software House, USA).

Cell apoptosis assay

After treatment with either control or different concentrations of silibinin (100, 150, 200 μM) for 48 h, cell apoptosis assay was performedin Ishikawa and RL-952 cells with a FACS flow cytometer using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience Pharmingen, San Diego, CA, USA) according to manufacturer's instructions. The data were analysed by CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA).

***In vivo* TUNEL assay**

The paraffin-embedded sections (5 μm) of xenograft tumours from five groups mice administered with either solvent-treated control or different dosages of silibinin (50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg) were deparaffinized. The TUNEL assay was performed following the manufacturer’s instructions of a commercial apoptosis detection kit (Roche. *In Situ* Cell Death Detection Kit, Fluorescein, Cat. No.11684795910). Apoptotic cells exhibit a strong nuclear green fluorescence, and all cells of sections stained with DAPI exhibit a strong blue nuclear fluorescence. The slides were observed and photographed under a fluorescence microscope (Nikon Eclipse Ti-E). The number of TUNEL-positive cells of each section was counted by Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) and the ratio of apoptosis of each group was calculated for statistical analysis.

Western blot analysis

Control- or silibinin-treated cells (100, 150 and 200 μM for Ishikawa and RL-952 cells) for 48 h were lysed in a mixed buffer that contained RIPA, NaF and PMSF. Protein concentrations were analysed using a BCA protein assay kit (Tiangen Biotech Co., Ltd., Beijing, China). Protein was resolved by 10 or 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After incubation with the indicated primary antibodies overnight at 4℃

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and secondary antibodies for 2 h at room temperature, the protein bands were detected using ImageQuant LAS4000 (General Electric Company, Boston, MA, USA) and quantified with ImageJ software. β-Actin was detected as a loading control.

Quantitative real-time RT-PCR (qRT-PCR)

Control- or silibinin-treated cells (100, 150 and 200 μM for Ishikawa and RL-952 cells) for 48 h were subjected to RNA extraction by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. After examining the concentrations using the NanoPhotometer Pearl (Implen GmbH, Munich, Germany), reverse transcription was performed using 1 μg total RNA into cDNA by the SuperScript™ II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific). The primers for amplifying target genes (Table 1) were synthesized by BioSure Biotechnology (Shanghai, China). The qRT-PCR was carried out to identify the expression of each mRNA in the cells using Applied Biosystems 7900HT Fast Real-Time PCR system with SYBR Premix Ex Taq (Tli RNaseH Plus) (cat. no. RR420A; Takara Bio, Inc., Otsu, Japan) in a 10-μl reaction system, and β-Actin served as the loading control.

Cell Immunofluorescence staining

Both control RL-952 cells and RL-952 cells treated with 150 μM silibinin for 48 h were cultured on coverslips in 6-well plates, fixed with 4% paraformaldehyde for 15 minutes and washed with PBS. Triton X-100 (0.2%) was allowed to permeabilize at room temperature for 20 minutes. Normal goat serum was added dropwise and blocked for 30 minutes at room temperature. After absorbing the blocking solution with an absorbent paper, a sufficient amount of diluted SREBP1 antibody (1:100) was added to both the control and treatment cells and incubated overnight at 4°C. Next, a fluorescent secondary antibody was added to the cells and shielded from light in an incubator for 1 h. DAPI was added dropwise and incubated for 5 min. PBST washed away excess DAPI. The liquid on the coverslips was blotted with an absorbent paper, and the slides were sealed with an anti-fluorescent quencher, and then, the images were acquired (at 400× magnification) under a Nikon inverted fluorescence microscope. Mean density of SREBPI in the nucleus was calculated by Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) for statistical analysis.

Oil Red O staining

Ishikawa and RL-952 cells were seeded on coverslips at 100,000 cells/ well in a 6-well plate. The control groups and the treatment group with the intervention of 150 μM silibinin after 48 h were washed twice with distilled water and then were fixed in 10% buffered formalin for 15 minutes. After washing gently twice with distilled water, permeabilization buffer was added

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into the well plate for 30 min. After washing again twice gently with distilled water, the well plate was kept in a dark place and stained with ORO stain for 10-15 minutes. After staining, cells were rapidly washed with 75% alcohol for 1-3 seconds. After haematoxylin staining, the slides were covered with appropriate acacia. Images were captured at 400x magnification under Nikon Eclipse Ti-E microscope, and the ratio of red lipid droplets according to their distribution in the whole cell area was calculated to reflect lipid content by Image-pro plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical methods

All experiments were repeated three times. The data were expressed as mean±standard deviation (SD). Relative quantification of RNA expression was evaluated using the $2^{-\Delta\Delta Ct}$ method. All statistical analyses were performed using SPSS 20.0 and GraphPad software 7.0. T-test and one-way ANOVA were used for the statistical analysis of classification data. P values < 0.05 were considered as significant.

Results

Inhibition of EC cells proliferation by silibinin

First, we examined the effects of silibinin treatment on EC cell viability and proliferation by MTT assay and determined the 50% inhibitory concentration (IC50). We treated Ishikawa and RL-952 cells with different concentrations of silibinin for 24, 48 and 72 h and found that as the silibinin concentration increased, the viability of both cells decreased (Figure 1A and 1B) in a dose- and time-dependent manner. The IC50 values of Ishikawa and RL-952 cells were 162 μM and 136.7 μM after treatment with silibinin for 48 h, respectively. We then performed colony formation assay on silibinin-treated EC cells. After 12 days of cell culture with silibinin or vehicle, the results showed that the number of colonies of Ishikawa (Figure 1C and 1D) and RL-952 (Figure 1E and 1F) cells treated with higher concentrations of silibinin was significantly reduced. In summary, silibinin can significantly inhibit EC cell viability and proliferation.

In vivo inhibition of EC growth by silibinin

Next, we studied the effect of silibinin on EC *in vivo*. A total of 25 nude mice were implanted with equal numbers of RL-952 cells subcutaneously. On the 14th day of growth, 25 nude mice were divided into 5 groups. Five in each group were daily treated with different dosages of silibinin (50 mg/kg, 100 mg/kg,150 mg/kg, 200 mg/kg) by intragastric administration, except the solvent-treated control group. The size and volume of tumours from nude mice were recorded every other day thereafter. After 21 days of treatment with silibinin or vehicle, nude mice were sacrificed, and subcutaneous tumours of

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each group were removed for photography and weighed. It was found that tumour-bearing nude mice showed the trend of reduction in EC tumours after silibinin feeding (Figure 2A). The tumour weight after silibinin treatment showed that the high-dose silibinin groups (150 mg/kg, 200 mg/kg) had significant therapeutic effects to slow the growth of the EC tumours compared with the control group (Figure 2B). Our results suggest that silibinin can potentially inhibit the development of EC .

The blocking effect of silibinin on EC cell cycle progression

Silibinin affected EC cell cycle progression. We used different concentrations of silibinin (0, 100 μM, 150 μM, 200 μM) for treating Ishikawa and RL-952 cells. The proportion cells of each period in the cell cycle were measured after silibinin treatment for 48 h by flow cytometry. The data showed that the increased concentrations of silibinin significantly increased the proportion of cells in G0/G1 phase and decreased the proportion of cells in S phase in Ishikawa cells (Figure 3A and 3B). In contrast, in the RL-952 cells, the proportion of cells in G2/M phase was significantly increased and the proportion of cells in G0/G1 phase was decreased after silibinin treatment (Figure 3C and 3D). This implied that the G0/G1 phase was arrested in the Ishikawa cells, while the G2/M phase was prevented in RL-952 cells by silibinin treatment. Although cell cycle arrest occurred at different stages in the two cell lines, cell cycle progression was blocked in both cell lines.

Promotion of EC cell apoptosis by silibinin *In vitro and in vivo*

Cell cycle arrest is often accompanied by cell apoptosis. We examined the number of apoptotic cells in Ishikawa and RL-952 cells after 48 h silibinin treatment with the concentration gradient of 100 μM, 150 μM and 200 μM by flow cytometry. Compared with the solvent-treated control cells, the proportion of apoptotic cells was notably increased in Ishikawa (Figure 4A and 4B) and RL-952 (Figure 4C and 4D) cells after silibinin treatment. Therefore, silibinin has the effect of promoting apoptosis of EC cells *in vitro*.

TUNEL assay of the tumour tissue further confirmed the apoptotic effect. The percentage of TUNEL-positive cells was determined by counting the number of green fluorescent cells per field of tumour paraffin sections of 25 nude mice in 5 groups treated with solvent-treated control and gradually increasing dosages of silibinin (50 mg/kg,100 mg/kg,150 mg/kg and 200 mg/kg). The results showed that the percentage of apoptotic cells was notably increased after higher dosages of silibinin (150 mg/kg, 200 mg/kg) treatment compared with the control group (Figure 5A and 5B). This indicated that silibinin promoted EC cell apoptosis *in vivo.*

Inhibition of STAT3 activation by silibinin and the effects on apoptosis and cell cycle-related genes expression in the downstream signalling

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pathway in EC cells

To clarify the underlying mechanism of silibinin-induced cell cycle arrest and cell apoptosis, EC cell lines Ishikawa and RL-952 were treated with different concentrations of silibinin for 48 h, and the expression levels of protein and mRNA of STAT3-related downstream genes were detected by western blot and qRT-PCR. The results showed that the expression level of serine 727 phosphorylation of STAT3 was reduced with silibinin treatment in Ishikawa and RL-952 cells and suggested that silibinin inhibited the activity of STAT3 (Figure 6A and 6B). The protein expression levels of apoptosis-related genes Survivin and Bcl-2 were decreased by silibinin. In addition, the expression of cell cycle-related protein Cyclin D1 was reduced in silibinin-treated Ishikawa cells (Figure 6A), and the expression of Cyclin B1 was reduced in silibinin-treated RL-952 cells (Figure 6B). This suggested that silibinin induced cell cycle arrest at different stages in Ishikawa and RL-952 cells and confirmed that Cyclin D1 mainly regulates the progress of G0/G1 phase, while Cyclin B1 mainly regulates the G2/M phase according to some researchers [15-16]. On the other hand, the mRNA expression levels of STAT3, Caspase3, Bcl-2, Bax, Survivin, Ki67, Cyclin D1 and Cyclin B1 were detected by qRT-PCR. The results also determined that silibinin significantly inhibited the expression of these genes in silibinin-treated Ishikawa (Figure 6C) and RL-952 (Figure 6D) cells compared with those in the control cells. Thus, silibinin markedly affected EC cell cycle and apoptosis through STAT3-mediated signalling pathways.

Suppression of the expression of SREBP1 and downstream genes of lipid metabolism in EC cells by silibinin

Silibinin plays a tumour-suppressing role by regulating the key transcription factor SREBP1 associated with abnormal lipid metabolism. The protein levels of SREBP1 were significantly reduced after adding 150 μM silibinin to Ishikawa and RL-952 cells for 48 h (Figure 7A). Treatment of Ishikawa (Figure 7B) and RL-952 cells (Figure 7C) with different concentrations of silibinin reduced the expression of SREBP1 and its downstream genes, such as SCD-1 and phosphorylation of ACLY Ser455. Total ACLY was not affected at the protein level. Results of qRT-PCR also verified the inhibitory effect of silibinin on the mRNA levels of SREBP1 and its downstream genes including SCAP, FASN, ACLY, SCD-1 and HMGCR (Figure 7D and 7E). Next, an immunofluorescence staining technique was used to investigate the inhibitory effect of silibinin on nuclear SREBP1. We visually observed that the expression of SREBP1 in the nucleus of silibinin-treated RL-952 cells was significantly decreased compared with that in the untreated cells (Figure 7F). These results validated that silibinin restrained EC development by suppressing the levels of SREBP1 and its downstream genes.

Reduction of lipid accumulation by silibinin in EC cells

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High expression of SREBP1 promotes cellular lipid synthesis. After Ishikawa and RL-952 cells were treated with 150 μM silibinin for 48 h respectively, the distribution of lipids in the cells was detected by oil red O staining (Figure 8A and Figure 8C). Compared with the untreated cells, the results showed that silibinin significantly decreased the distribution area of lipid droplets in Ishikawa (Figure 8B) and RL-952 (Figure 8D) cells. This implied that silibinin reduced the accumulation of lipid in EC cells.

Discussion

In recent years, the incidence of EC has increased, and the onset age has become younger. According to pathological classification, the first pathogenetic type of EC is more common and mostly observed in patients with excessive oestrogen level, anovulation, obesity and abnormal lipid or glucose metabolism[17]. To preserve the reproductive function of young patients with well-differentiated and early stage of Type I EC, high-efficiency progesterone therapy is recommended as the first choice. However, more than 30% of patients are not sensitive to progesterone or produce progesterone resistance during the therapy[18]. Thus, new effective drugs for conservative and adjuvant treatment are needed to be explored.

STAT3 exists in the cytoplasm and is the focal point of multiple oncogenic signal transduction pathways. With the influence of various extracellular signals, STAT3 is abnormally activated and phosphorylated in a variety of tumours. Studies have suggested that the JAK2-STAT3 pathway is one of the most important signal transduction and transcription activation pathway in EC. By modifying the expression of target genes, regulating cell proliferation, differentiation and apoptosis, it is closely related to the occurrence, development, invasion, metastasis and prognosis of EC [19]. Liu et al reported that a higher concentration of STAT3 protein kinase inhibitor AG490 reduced the expression of JAK2, p-STAT3, and Bcl-2 protein and promoted apoptosis in EC cells[20].

Silibinin has the function of protecting and stabilizing hepatic cell membrane and is a traditional hepatoprotective drug that can modulate the abnormality of lipid metabolism and anti-oxidation[21]. Accumulating evidence has shown that silibinin inhibits cell growth, proliferation, angiogenesis, and cell migration and induces apoptosis to produce anti-cancer effects[22-24]. Agarwal et al reported for the first time that silibinin could inhibit STAT3 activation[13]. Studies on prostate cancer showed that silibinin inhibited cell proliferation and angiogenesis, induced apoptosis and cell cycle arrest by increasing ERK1/2 phosphorylation, decreased JNK1/2, MAPK, Akt expression, inhibited STAT3 phosphorylation (especially phosphorylation of serine 727), activated caspase-3 and downregulated the expression of CDK-cyclin[25]. Bosch-Barrera et al believed that p-STAT3 expression is considered as a poor prognostic marker in breast, colon, prostate and lung

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cancers[14, 26, 27]. In the present study, we selected two well-differentiated cell lines Ishikawa and RL-952 representing Type I EC for *in vitro* and *in vivo* experiments. We found that silibinin indeed showed inhibitory effects towards STAT3-mediated cell proliferation, apoptosis inhibition and cell-cycle progression by downregulating the expression of p-STAT3, Caspase3, Survivin, Bcl-2/Bax, Ki67, Cyclin D1, and Cyclin B1 in shikawa and RL-952 cells.

Abnormal lipid metabolism is an important feature of tumour cells. The de novo synthesis pathway of fatty acid promotes tumour cell proliferation, metastasis, and invasion. SREBPs is a nuclear transcription factor, which has a basic helix ring, a helix leucine zipper (bHLH-LZ) structure, including three subtypes SREBP-1a, SREBP-1c and SREBP-2. SREBPs are synthesized as precursor proteins in the endoplasmic reticulum (ER). SREBP cleavage activating protein (SCAP) activates SREBP in the nucleus[28]. SREBP1 matures are mainly involved in fatty acid and triacylglycerol metabolism, while SREBP2 matures are major regulators of cholesterol metabolism[29]. Overexpression of SREBP1 causes disorders of glycolipid metabolism, leading to excessive accumulation of fat in non-adipose tissue, resulting in metabolic diseases such as obesity, insulin resistance, type 2 diabetes and fatty liver[30]. SREBP1 is abnormally expressed in many malignant tumours with abnormal lipid metabolism, such as prostate, liver, pancreatic, ovarian, breast, colorectal cancer, EC and human glioma. Li et al explored that knockdown of SREBP1 expression in EC cell line AN3CA inhibited cell proliferation, invasion and promoted cell apoptosis, as well as significantly suppressed the formation of xenografts in nude mice[11]. Qiu et al detected the SREBF-1 gene polymorphism in endometrioid carcinoma, and found that SNP (rs2297508) associated with high susceptibility to EC was significantly different in its distribution in EC patients and normal controls[31]. Nambiar et al demonstrated that silibinin can inhibit the entry of SREBP1 into the nucleus by activating AMPK, thus significantly reducing abnormal lipid synthesis and inhibiting the development of prostate cancer[12]. Therefore, the therapeutic strategy of inhibiting the expression of SREBP1 at the gene level or utilizing drugs to intervene in the SREBP1-mediated lipid metabolism pathway is expected to become a new and effective method for anti-tumour therapy and anti-metabolic disease treatment[32-34].

Gao et al revealed that in EC cell lines Ishikawa and HEC-1A, the SREBP chemical inhibitor fatostatin can promote caspase-mediated apoptosis by blocking SREBP-regulated metabolic pathways. It mainly decreased the expression of SREBP1 which transcriptionally regulate the expression of three lipogenic genes FASN, ACLY and SCD-1, and thus reducing the levels of intracellular fatty acids in EC cells[35]. According to the current investigation, silibinin blocked the formation of the SCAP-SREBP complex and inhibited the activation of SREBP1 matures, the pivotal transcription factor for abnormal lipogenesis in the nucleus. Furthermore, silibinin markedly suppressed

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SREBP1 target genes FASN, ACLY and SCD-1, which were the three rate-limiting enzymes involved in long-chain fatty acid biosynthesis, and decreased the expression of cholesterogenic gene HMGCR, resulting in the reduction of lipid accumulation in EC cells. Similarly, this suggested that SREBP1 may play a crucial role in silibinin efficacy against EC cells. However, there is no research on the upstream regulatory pathways of silibinin targeting SREBP1 in EC.

In endocrine diseases, inhibition of STAT3 signalling suppressed the expression of SREBP1 and its downstream genes, thereby inhibiting lipid metabolism and insulin sensitivity[36]. In the mouse model of obesity, STAT3 was positively correlated with SREBP1 expression and promotes fat accumulation in mice[37]. Therefore, STAT3 and SREBP1 are closely related in diseases of abnormal lipid and glucose metabolism. In future studies, we would like to explore the regulation of SREBP1 by STAT3 and the potential mechanism by which silibinin regulates lipid metabolism possibly through STAT3/SREBP1 signaling pathway in EC cells. Our experimental design is to overexpress STAT3 with adenovirus treatment in EC cells and detect the expression of SREBP1 and the downstream genes. We assume that enhancement of STAT3 signalling in EC cells can suppress silibin-induced SREBP1 down-regulated expression and reduction of lipid accumulation. Accordingly, we assume silibinin inhibits SREBP1 by blocking STAT3 signalling, and STAT3 may be the upstream regulatory factor that controls the nuclear transcriptional activity of SREBP1 leading to lipid metabolic dysfunction.

Although silibinin is an “old drug” that has been clinically researched and applied for a long time, it is regarded as a new approach for the conservative and adjuvant treatment of Type I EC associated with abnormal lipid metabolism and insulin resistance, to achieve the purpose of inhibiting tumour growth and delaying disease progression. The inherent mechanism is still complex and has not been fully understood; thus, more in-depth research should be carried out in the future.

Conclusion

In this study, we discovered that silibinin can promote cell apoptosis, induce cell cycle arrest and inhibit the proliferation of EC cells in a concentration- and time-dependent manner by preventing the activation of STAT3. Moreover, silibinin can suppress the expression of SREBP1 and reduce lipid accumulation in EC cells. *In vivo e*xperiments confirmed that silibinin enhanced the apoptosis and inhibited the growth of transplanted tumours of EC in nude mice, which provided a credible basis for the application of silibinin in EC clinical trials. In conclusion, these basic findings validate that silibinin exhibits potential anti-cancer and anti-lipid accumulation effects on the therapy of EC. For the early stage of type I EC patients who wish to retain fertility, silibinin is anticipated to be a new, safe and effective option for

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conservative and adjuvant therapy.

Authors contribution to study

JJ conceived and designed the research. ZZS, QZ, SHG and XL performed the experiments. ZZS, QZ, WZL, PPJ analyzed the data. ZZS wrote the manuscript. WZL, ZML and PPJ revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Silibinin inhibited proliferation of EC cells. EC cell lines Ishikawa (A) and RL-952 (B) were treated with different concentrations of silibinin (SB) (0, 50 μM, 100 μM, 150 μM, 200 μM, 250 μM) and daily cell proliferation was measured by MTT assay in 24 h, 48 h, adn 72 h. Then Ishikawa (C) and RL-952 (E) cells were treated with different SB concentrations (0, 25 μM, 50 μM, 75 μM) and clone formation experiments were performed. (D) (F) The number of clones was counted. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 2. Silibinin inhibited the growth of EC tumour *in vivo*. The RL-952 cells were implanted subcutaneously in 25 nude mice, and nude mice divided into 5 per group were given intragastric administration with different dosages of silibinin (50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg) or vehicle. After 21 days, the mice were sacrificed and the tumours were removed. (A) In each group, two mice and their tumours were chosen randomly to be photographed and exhibited. (B) The weight of all tumours was recorded. All silibinin treatment groups were compared with the control group. *, p<0.05; **, p<0.01.

Figure 3. Silibinin blocked the progression of EC cell cycle. Ishikawa (A) and RL-952(C) cells were treated with different silibinin (SB) concentrations (0, 100 μM, 150 μM, 200 μM) for 48 h, cells were fixed and stained with PI, and the cell cycle was determined by flow cytometry. (B) (D) The proportions of cells in each phase of the cell cycle were counted. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 4. Silibinin promoted EC cell apoptosis. Ishikawa (A) and RL-952 (C) cells were treated with different concentrations of Silibinin (SB) (0, 100 μM, 150 μM, 200 μM) for 48 h, cell apoptotic rates were measured by flow cytometry. (B) (D) The proportions of early and late apoptotic cells were counted. *, p<0.05; **, p<0.01; ***, p<0.001.

Figure 5. Silibinin enhanced EC cell apoptosis *in vivo* by TUNEL assay. (A) The sections of xenograft tumours from 5 groups nude mice treated with different silibinin (SB) dosages (50 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg) or vehicle were subjected to DAPI (blue) and TUNEL (green) staining. The relative apoptotic cells (marked by white arrow photomicrographed at 400×) were determined by counting TUNEL-positive cells in three random fields (photomicrographed at 200×) for each sample. Negative control: antibody replaced by PBS. (B) The number of apoptotic cells divided by the total number of cells to calculate the rate of apoptosis. *, #, &, P<0.05; **, ##, &&, P<0.01; ***, ###, &&&, P<0.001. *, compared with the control group; #, compared with the 50 mg/kg SB treatment group; &, compared with 100 mg/kg SB treatment group.

Figure 6. Silibinin suppressed STAT3 activation and affected apoptosis and cell cycle-related gene expression in the downstream signalling pathway in EC cells. (A) (B) Ishikawa and RL-952 cells were treated with various concentrations of silibinin (SB) (0, 100 μM, 150 μM, 200 μM) for 48 h, and the protein expression levels of STAT3, p-STAT3, Survivin, Bcl-2, Bax, Cyclin D1, Cyclin B1 were detected by western blotting. (C) (D) Ishikawa and RL-952 cells were treated with SB (0, 100 μM, 150 μM, 200 μM) for 48 h, and the mRNA levels of STAT3, Caspase3, Bcl-2/Bax, Survivin, Ki67, CyclinD1, CyclinB1 were detected by qRT-PCR. β-Actin served as the loading control. *, p<0.05; **, p<0.01, ***, p<0.001.

Figure 7. Silibinin inhibited SREBP1 expression and down-regulated lipid

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metabolism gene expression in EC. (A) Ishikawa and RL-952 cells were treated with 150 μM Silibinin(SB) for 48 h and the protein expression of SREBP1 was detected by western blotting. (B) (C) Ishikawa and RL-952 cells were treated with various concentrations of SB (0, 100 μM, 150 μM, 200 μM) for 48 h and the protein expression of SREBP1, SCD1, ACLY and p-ACLY was detected by western blotting. (D) (E) Ishikawa and RL-952 cells were treated with SB (0, 100 μM, 150 μM, 200 μM) for 48 h, and the mRNA levels of SREBP1, SCAP, FASN, ACLY, SCD-1 and HMGCR were detected by qRT-PCR. β-Actin served as the loading control. (F) After RL-952 cells were treated with 150 μM SB for 48h, the intranuclear SREBP1 expression (in red) was detected by cell immunofluorescence staining (photomicrographs at 400×), and the fluorescence intensity was detected. DAPI (in blue) stains the nucleus. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Figure 8. Silibinin reduced lipid accumulation in EC cells. After treatment with solvent-vehicle and 150 μM silibinin (SB) for 48 h, Ishikawa (A) and RL-952 (C) cells were stained with oil red O solution, and the stained cells were photomicrographed (at 400×). (B) (D) Percentage of lipid droplets area is calculated. **, $p<0.01$; ***, $p<0.001$.

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Table 1. The primers sequences used for qRT‐PCR.F, forward; R, reverse.

Gene	Primer Sequence (5'-3')
SREBP1	F:CTGGTCTACCATAAGCTGCAC R:GACTGGTCTTCACTCTCAATG
SCAP	F:TATCTCGGGCCTTCTACAACCA R: ACACAACTCCTCCAAGCTCCTG
FASN	F:CGGTACGCGACGGCTGCCTG R:GCTGCTCCACGAACTCAAACACCG
HMGCR	F: GACTACCACAGAGGCTATGATTGAG R: CCCACCACCCACCGTTCC
SCD-1	F:CACTTGGGAGCCCTGTATGG R: AGCCGAGCTTTGTAAGAGCG
Acly	F: ACCTATGACTATGCCAAGACTATCC R: GATTGTGACTTCGTGCTCCTTC
STAT3	F:GAGCTGCACCTGATCACCTT R:TGGCAAGGAGTGGGTCTCTA
Survivin	F:AGGACCACCGCATCTCTACAT R:AAGTCTGGCTCGTTCTCAGTG
Bcl-2	F:TGGAGAGTGCTGAAGATTGATG R:GTCTACTTCCTCTGTGATGTTG
Bax	F:AGGTCTTTTTCCGAGTGGCA R:CCGGAGGAAGTCCAATGTCC
Ki67	F:TGCCTCCTAATACGCCTCTC R:CTTCAGGACAGGTGGAGTGTG
Caspase-3	F:ACTCCACAGCACCTGGTTATT R:TTCTGTTGCCACCTTTCGGT
Cyclin B1	F:TGGTGAATGGACTGTCAAGAAC R:CAGCTGTGGTAGAGTGCTGA
Cyclin D1	F:ATGCCAACCTCCTCAACGAC

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	R:TCTGTTCCTCGCAGACCTCC
β-Actin	F:CATGTACGTTGCTATCCAGGC R:CTCCTTAATGTCACGCACGAT

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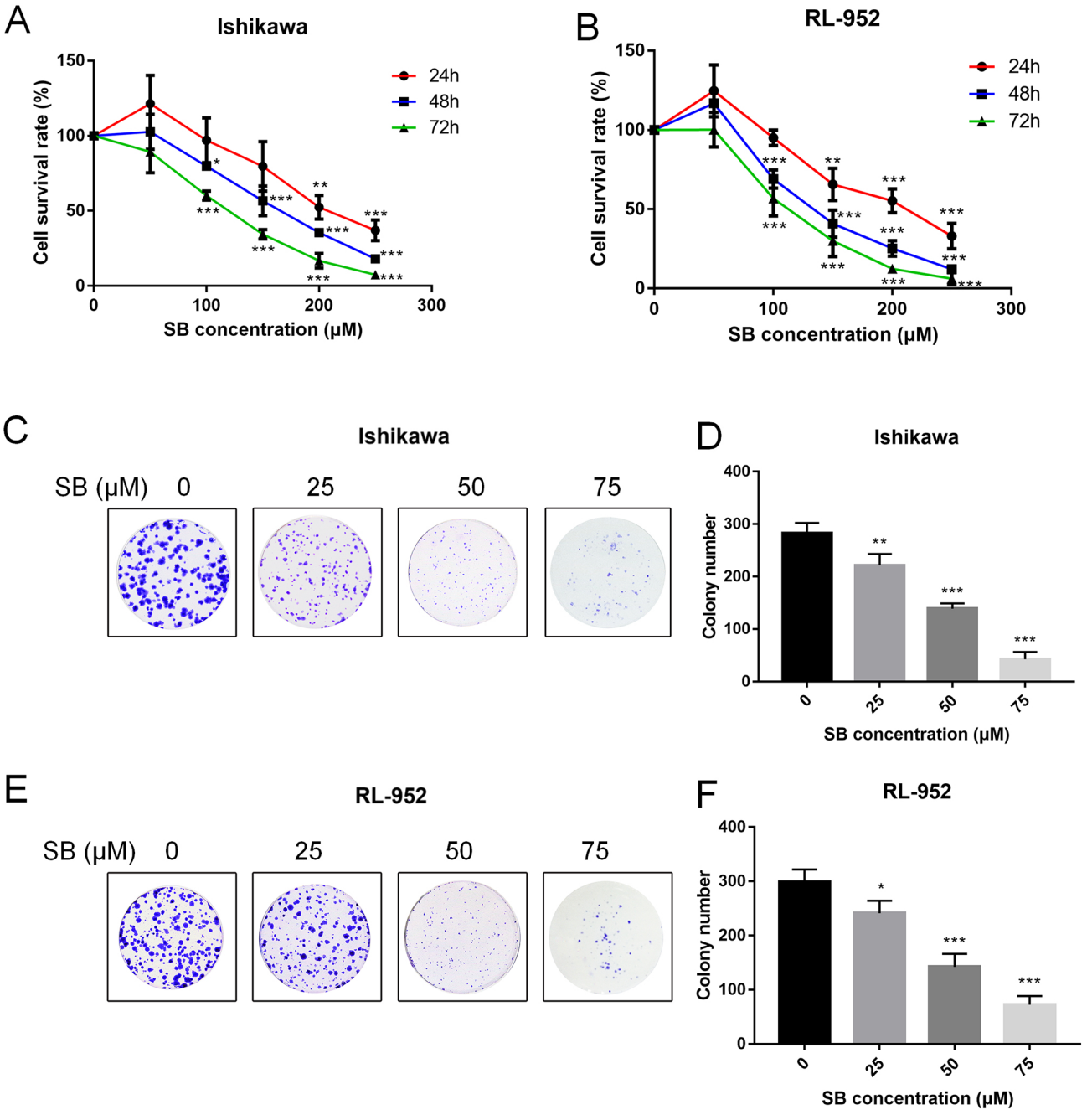

A
Ishikawa
Cell survival rate (%)
SB concentration (μM)
24h
48h
72h
B
RL-952
Cell survival rate (%)
SB concentration (μM)
24h
48h
72h
C
Ishikawa
SB (μM) 0 25 50 75
D
Ishikawa
Colony number
SB concentration (μM)
E
RL-952
SB (μM) 0 25 50 75
F
RL-952
Colony number
SB concentration (μM)

Figure 1

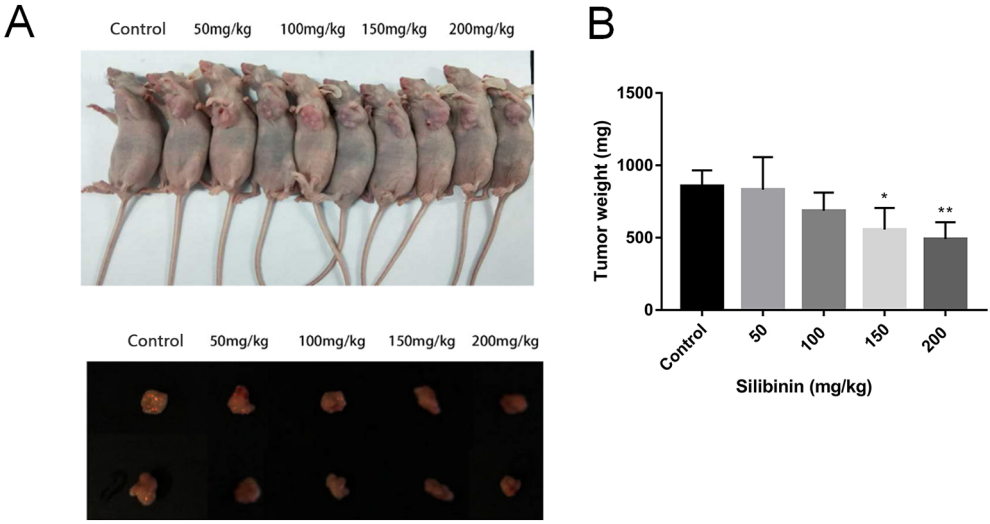

A
Control
50mg/kg
100mg/kg
150mg/kg
200mg/kg
Control
50mg/kg
100mg/kg
150mg/kg
200mg/kg
B
Tumor weight (mg)
1500
1000
500
0
*
**
Control
50
100
150
200
Silibinin (mg/kg)

Figure 2

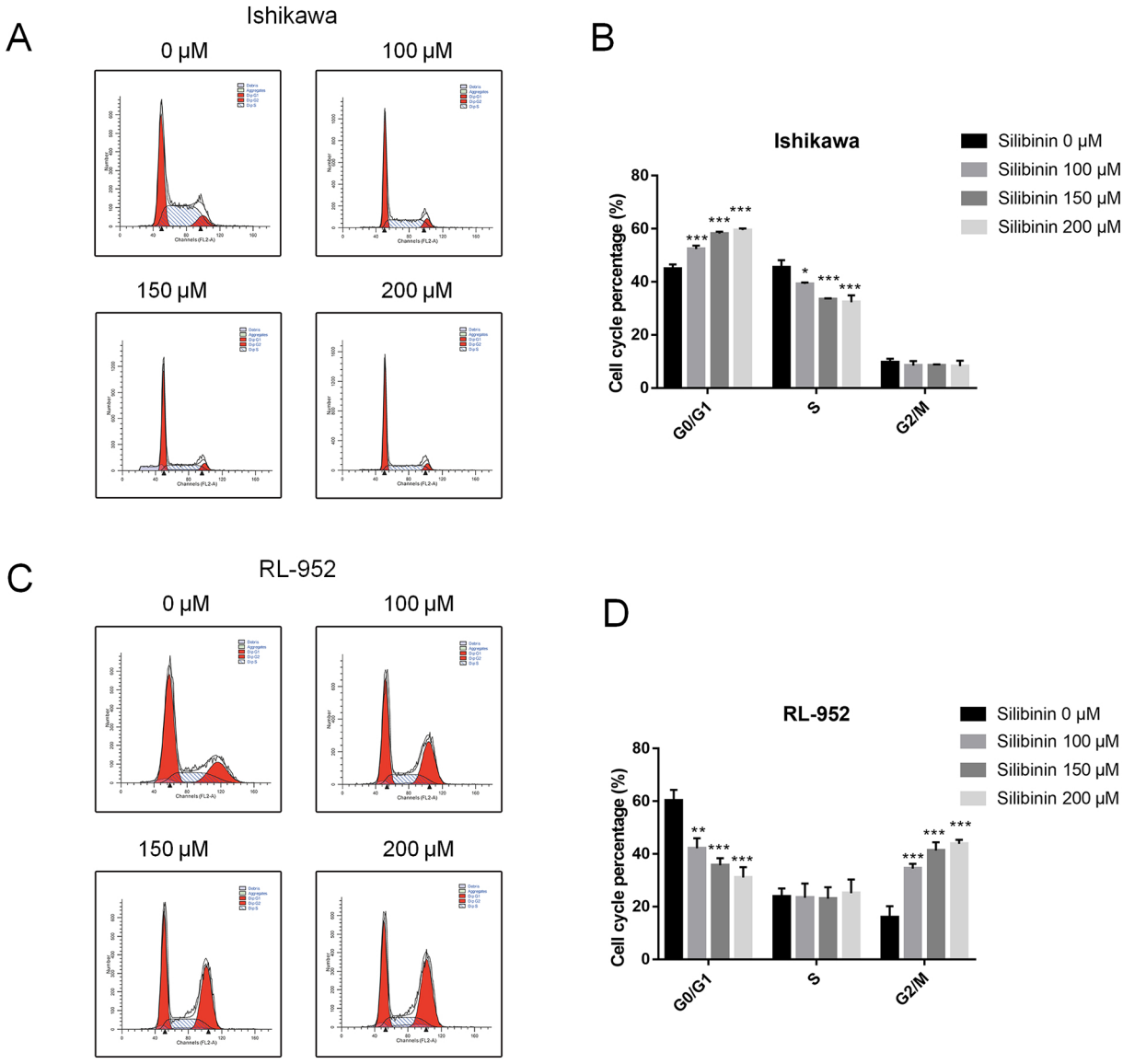

A
Ishikawa
0 μM
100 μM
150 μM
200 μM
B
Ishikawa
Silibinin 0 μM
Silibinin 100 μM
Silibinin 150 μM
Silibinin 200 μM
Cell cycle percentage (%)
G0/G1
S
G2/M
C
RL-952
0 μM
100 μM
150 μM
200 μM
D
RL-952
Silibinin 0 μM
Silibinin 100 μM
Silibinin 150 μM
Silibinin 200 μM
Cell cycle percentage (%)
G0/G1
S
G2/M

Figure 3

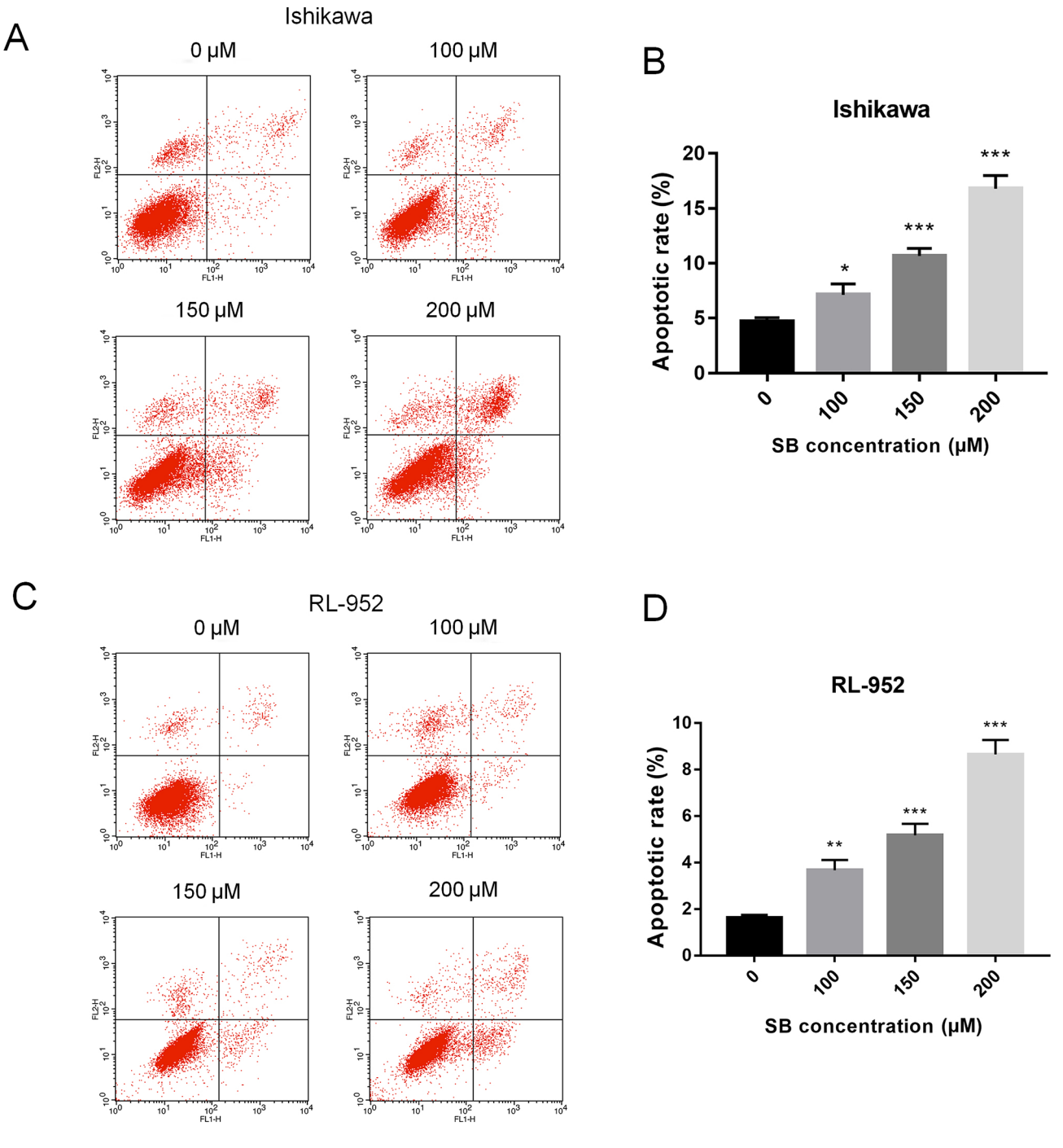
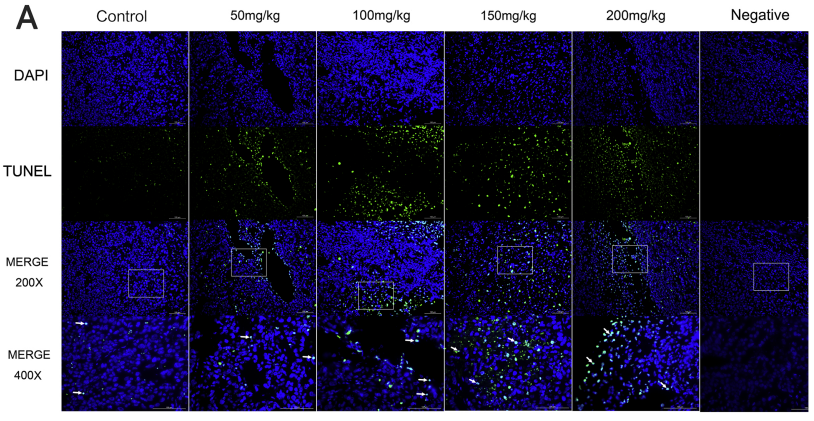

A
Ishikawa
0 μM
100 μM
150 μM
200 μM
FL2-H
FL1-H
B
Ishikawa
Apoptotic rate (%)
SB concentration (μM)
C
RL-952
0 μM
100 μM
150 μM
200 μM
D
RL-952
Apoptotic rate (%)
SB concentration (μM)

Figure 4


A
Control
50mg/kg
100mg/kg
150mg/kg
200mg/kg
Negative
DAPI
TUNEL
MERGE
200X
MERGE
400X

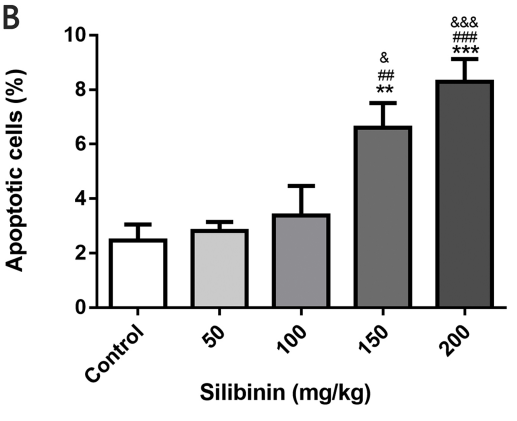

B
Apoptotic cells (%)
10
8
6
4
2
0
&
##
**
&&&
###
***
Control
50
100
150
200
Silibinin (mg/kg)

Figure 5

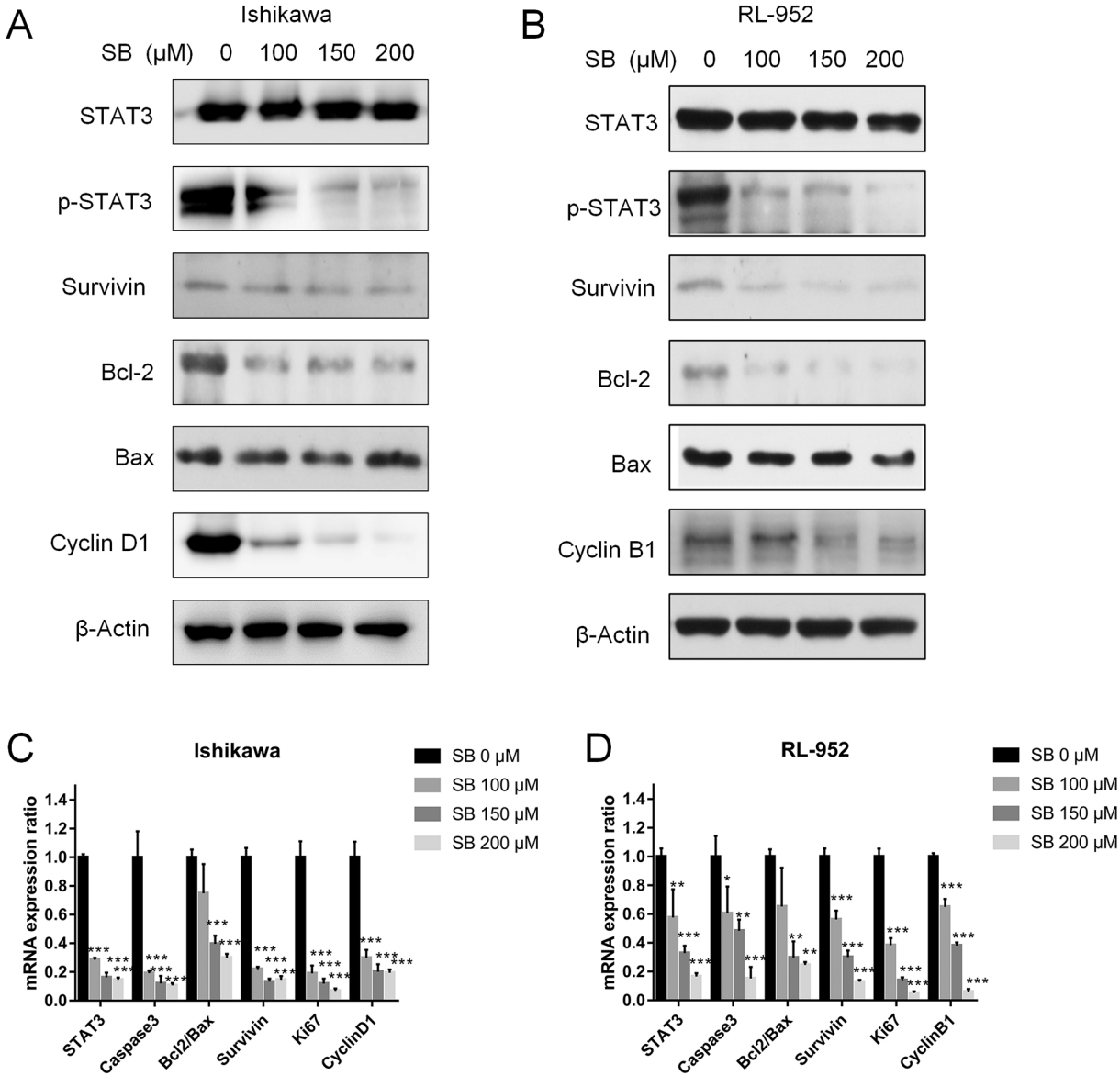

A
Ishikawa
SB (μM) 0 100 150 200
STAT3
p-STAT3
Survivin
Bcl-2
Bax
Cyclin D1
β-Actin
B
RL-952
SB (μM) 0 100 150 200
STAT3
p-STAT3
Survivin
Bcl-2
Bax
Cyclin B1
β-Actin
C
Ishikawa
SB 0 μM
SB 100 μM
SB 150 μM
SB 200 μM
mRNA expression ratio
STAT3
Caspase3
Bcl2/Bax
Survivin
Ki67
CyclinD1
D
RL-952
SB 0 μM
SB 100 μM
SB 150 μM
SB 200 μM
mRNA expression ratio
STAT3
Caspase3
Bcl2/Bax
Survivin
Ki67
CyclinB1

Figure 6

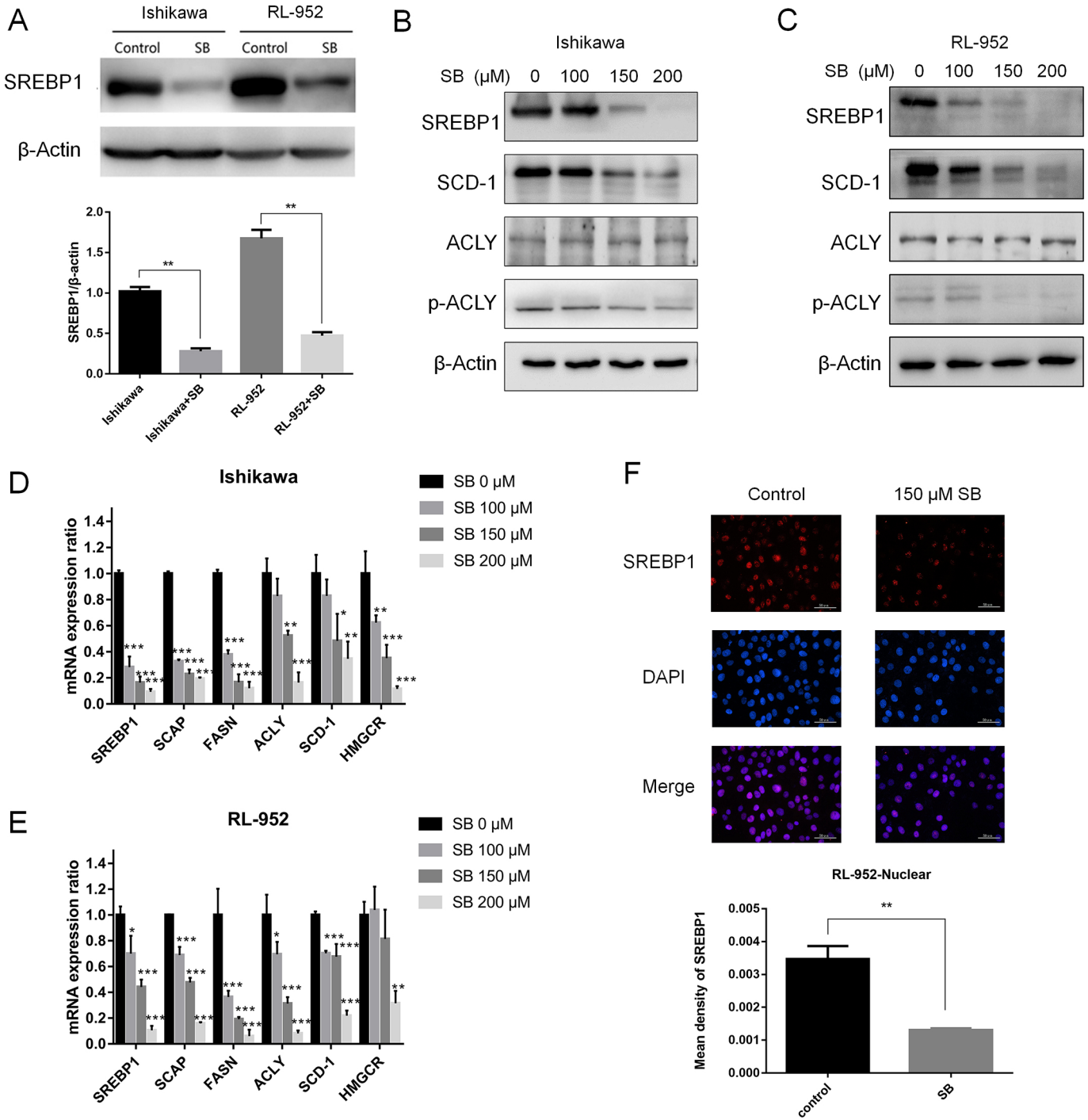

A
Ishikawa
RL-952
Control
SB
Control
SB
SREBP1
β-Actin
SREBP1/β-actin
Ishikawa
Ishikawa+SB
RL-952
RL-952+SB
B
Ishikawa
SB (μM) 0 100 150 200
SREBP1
SCD-1
ACLY
p-ACLY
β-Actin
C
RL-952
SB (μM) 0 100 150 200
SREBP1
SCD-1
ACLY
p-ACLY
β-Actin
D
Ishikawa
SB 0 μM
SB 100 μM
SB 150 μM
SB 200 μM
mRNA expression ratio
SREBP1
SCAP
FASN
ACLY
SCD-1
HMGCR
E
RL-952
SB 0 μM
SB 100 μM
SB 150 μM
SB 200 μM
mRNA expression ratio
SREBP1
SCAP
FASN
ACLY
SCD-1
HMGCR
F
Control
150 μM SB
SREBP1
DAPI
Merge
RL-952-Nuclear
Mean density of SREBP1
control
SB

Figure 7

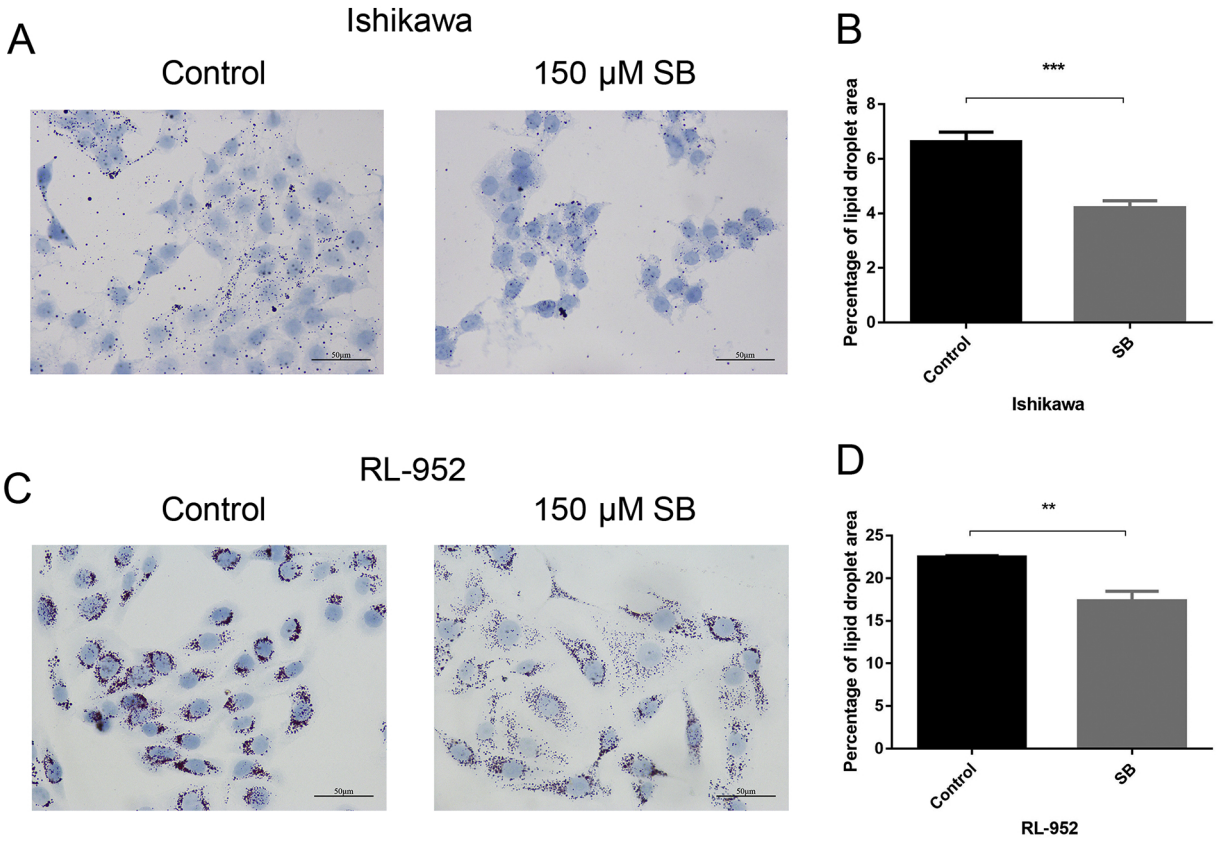

A
Ishikawa
Control
150 μM SB
50μm
50μm
B
***
Percentage of lipid droplet area
8
6
4
2
0
Control
SB
Ishikawa
C
RL-952
Control
150 μM SB
50μm
50μm
D
**
Percentage of lipid droplet area
25
20
15
10
5
0
Control
SB
RL-952

Figure 8