

Survivin suppression through STAT3/ β -catenin is essential for resveratrol-induced melanoma apoptosis

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Abstract. Although many chemotherapies have been developed for melanomas, successful therapy would be aided by the identification of intrinsic mechanisms that are crucial for melanoma survival. Here, we used resveratrol, a phytoalexin, as an anti-melanoma reagent. Applying resveratrol to various human and murine melanoma cell lines, we show that survivin is essential for melanoma survival *in vitro* and *in vivo* and is targeted by resveratrol. Furthermore, we identify the downregulation of survivin transcription by resveratrol through the suppression of β -catenin and STAT3. In addition, overexpression of survivin protects melanoma cells from resveratrol-induced apoptosis. Collectively, these studies establish that targeting survivin could provide an opportunity to treat melanoma patients.

Introduction

Although melanoma only comprises <10% of all forms of skin cancer; it is responsible for the majority of skin cancer-related deaths (1-4). In 2013, it was estimated that 76,690 new cases of melanoma would be diagnosed in the United States, and that 9,480 people would die of this disease (5). When diagnosed in the early stage and when limited to the skin, melanoma is curable; however, in the case of metastatic melanoma, the prognosis is very poor with a 5-year survival rate of <20%. Recently, vemurafenib, a BRAF V600E inhibitor, was found to be an attractive drug for melanoma patients and to produce clinical responses in the majority of melanoma patients (6); however, the responses are variable and all patients eventually relapse. The available medicines for melanoma patients are

therefore not significantly effective (7), and a new strategy is required.

Resveratrol, a phytoalexin originating from plants such as grapes, is an attractive compound for cancer treatment and has a beneficial effect on various diseases, including cardiovascular disease (8), diabetes (9) and cancer (10). Subsequent preclinical and clinical studies reported that resveratrol has both anti-proliferative and anti-metastatic activity in leukemia, breast cancer, and colon cancer (11). Even though resveratrol is known to induce apoptosis in melanoma cells through the suppression of anti-apoptotic proteins such as BCL-2, BCL-xL, survivin (12-19), the responsible molecules and the detailed mechanism have not been fully elucidated.

In the present investigation we showed that resveratrol induces apoptosis in all human and murine melanoma specimens used, even though they were genetically and phenotypically different. Furthermore, we determined for the first time that the transcriptional suppression of survivin is essential for resveratrol-induced apoptosis. We also identified the inhibition of both tumor growth and survivin expression *in vivo* by resveratrol. Collectively, these results strongly suggest that targeting survivin by reagents such as resveratrol could provide an opportunity to treat genetically varied melanoma patients.

Materials and methods

Reagents and plasmid preparation. Resveratrol (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in DMSO to make a 100-mM stock solution. pcDNA3.1-polyP was a kind gift from Dr D.E. Fisher (Massachusetts General Hospital, Boston, MA, USA). Survivin cDNA was subcloned into the *Bam*HI-*Xho*I site of pcDNA3.1 after RT-PCR from cDNA of normal human melanocytes.

Cell culture. UACC257, SK-MEL-28, SK-MEL-2, UACC62, M14, MeWo, SK-MEL-5 cell lines were kind gifts for Dr D.E. Fisher and B16-BL6 cell lines were from Dr I.J. Fidler (MD Anderson Cancer Center, Houston, TX, USA). UACC257, UACC62, M14, MeWo cells were cultured in RPMI-1640. SK-MEL-28, SK-MEL-2, and SK-MEL-5 were cultured in

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Dulbecco's modified Eagle's medium (DMEM), and B16-BL6 cells were cultured in Eagle's minimal essential medium (EMEM). All of the media were supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

For the establishment of stable survivin-expressing cells, SK-MEL-28 cells were transfected with pcDNA3.1-polyP as a vector control, or pcDNA3.1-HA/survivin vector and selected with 500 μ g/ml G418 over a period of 6 weeks.

WST-1 assay. Cell viability was quantified using the cell proliferation reagent WST-1 (Dojindo, Kumamoto, Japan). Melanoma cells were seeded and then incubated for 24 h. Resveratrol-containing medium was added to the well at the indicated concentrations. After the indicated incubation time, WST-1 solution was added and absorbance was measured at 450 nm using a microplate reader. Cells viability was determined as percent viability compared with the control.

Western blot analysis. Whole cell lysates were prepared as described previously (20). Primary antibodies used were specific to caspase-3, poly (ADP-ribose) polymerase (PARP), BCL-2, BCL-xL, XIAP, Survivin, BCL2A1, MCL-1, STAT3, p-STAT3, β -catenin, p53 (Cell Signaling Technology, Beverly, MA, USA), specific to hemagglutinin (HA) (Roche, Indianapolis, IN, USA) and specific to β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). All antibodies were used at x2,000 dilution.

Annexin V and dead cell assay. Apoptotic cell number was determined using the MUSE Annexin V and Dead Cell kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, after cells were treated with resveratrol, all cells were collected and diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) as a dilution buffer to a concentration of 5x10⁵ cells/ml. The cell suspension (100 ml) was then added to 100 μ l MUSE Annexin V and dead cell reagent (2X dilution), incubated for 20 min at room temperature, and analyzed using the MUSE Cell Analyzer.

Real-time PCR. Expression of *survivin* mRNA was quantitatively determined by real-time PCR on an ABI PRISM 7300 sequence detection system (Life Technologies Corp., Carlsbad, CA, USA). Total RNAs were prepared using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany). Expression level of *survivin* mRNA was normalized to the β -actin gene. The primers used were: 5'-TGC CTG GCA GCC CTT TC-3' (sense) and 5'-CCT CCA AGA AGG GCC AGT TC-3' (antisense) for *survivin* mRNA and 5'-GCA CAG AGC CTC GCC TT-3' (sense) and 5'-GTT GTC GAC GAC GAG CG-3' (antisense) for β -actin mRNA.

Chromatin immunoprecipitation assay (ChIP). ChIP assays were performed as described previously (21). The antibody used was anti-polymerase II serine 2 phosphorylation (Abcam, Cambridge, MA, USA). The primers were: 5'-GCA GTT CTG GTA ACG GTG ATA G-3' (sense) and 5'-GGG CAG AGA AGG GCA TTA TT-3' (antisense) for the *survivin* gene region,

5'-GAC ATT GAT GGA GAC GGT AAG G-3' (sense) and 5'-ATA GCC AGG ACT AGA CAG AAG T-3' (antisense) for the *THBS1* gene region, and 5'-CAT CCT CAC CCT GAA GTA CCC-3' (sense) and 5'-TAG AAG GTG TGG TGC CAG ATT-3' (antisense) for the β -actin gene region.

Animal model. C57BL/6 mice (6-week-old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The study was conducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals and approved by the ethics committee of the University of Toyama (permit no. A2012INM-6). Tumor inoculation was performed under isoflurane anesthesia, and all efforts were made to minimize suffering. The B16-BL6 cells were inoculated s.c (2.5x10⁵ cells/100 μ l 50% Matrigel in PBS/mice) into the flank of anesthetized mice. Mice in each group received resveratrol in 0.5% carboxymethyl cellulose solution (100 mg/kg/day) or vehicle by oral administration every day. The tumor volume was assessed every two days starting from day 9. The primary tumor was measured using a caliper square along the longer (a) and shorter (b) axis, and tumor volume was calculated by the following formula: tumor volume (mm³) = ab²/2.

Statistical analysis. The results are expressed as the mean \pm standard deviation. Statistical significance (p<0.05) was evaluated by either Student's two-tailed t-test or one-way ANOVA followed by the Bonferroni post-hoc test, comparing the results to the control.

Results

Resveratrol induces apoptosis in melanoma cells. To examine the cytotoxicity of resveratrol against melanoma, seven human melanoma cell lines with various genetic backgrounds (Table I) were treated with resveratrol. As summarized in Table I, all the human melanoma cell lines tested showed a similar IC₅₀ range (120.4-257.0 μ M) even with different genetic backgrounds; therefore, there seemed to be no specific genetic requirement for the efficacy of resveratrol in melanoma cell lines. Because of their different backgrounds, further experiments were performed using UACC257, SK-MEL-28, and SK-MEL-2 cells. The cytotoxicity of resveratrol against human melanoma cell lines was also observed in a dose-dependent manner and a similar result was confirmed in murine B16-BL6 cells (Fig. 1A). To determine whether the cytotoxicity is mediated through canonical apoptotic pathways, we next assessed the biomarkers for apoptotic cell death. As shown in Fig. 1B and C, resveratrol induced the cleavage of caspase-3 and PARP in both human and murine melanoma cell lines and subsequently induced Annexin-V-positive apoptotic cells in a dose-dependent manner. Collectively, these results indicate that resveratrol showed cytotoxicity in human and murine melanoma cells through the induction of canonical apoptotic pathways regardless of their genetic background.

Resveratrol suppresses survivin expression in melanoma cells. In order to determine whether apoptosis by resveratrol could be mediated via the suppression of anti-apoptotic

Table I. Oncogenic mutation and IC₅₀ of resveratrol in human melanoma cells.

Melanoma cells	BRAF	NRAS	p53	PTEN	IC ₅₀ (μM)
UACC257	V600E	wt	wt	wt	143.4
SK-MEL-28	V600E	wt	L145R	wt	257.0
SK-MEL-2	wt	Q61R	G245S	wt	159.5
M14	V600E	wt	G266E	wt	232.8
MeWo	wt	wt	Q317X/E258K	wt	120.4
SK-MEL-5	V600E	wt	wt	wt	158.8
UACC62	V600E	wt	wt	p245fs5	218.8

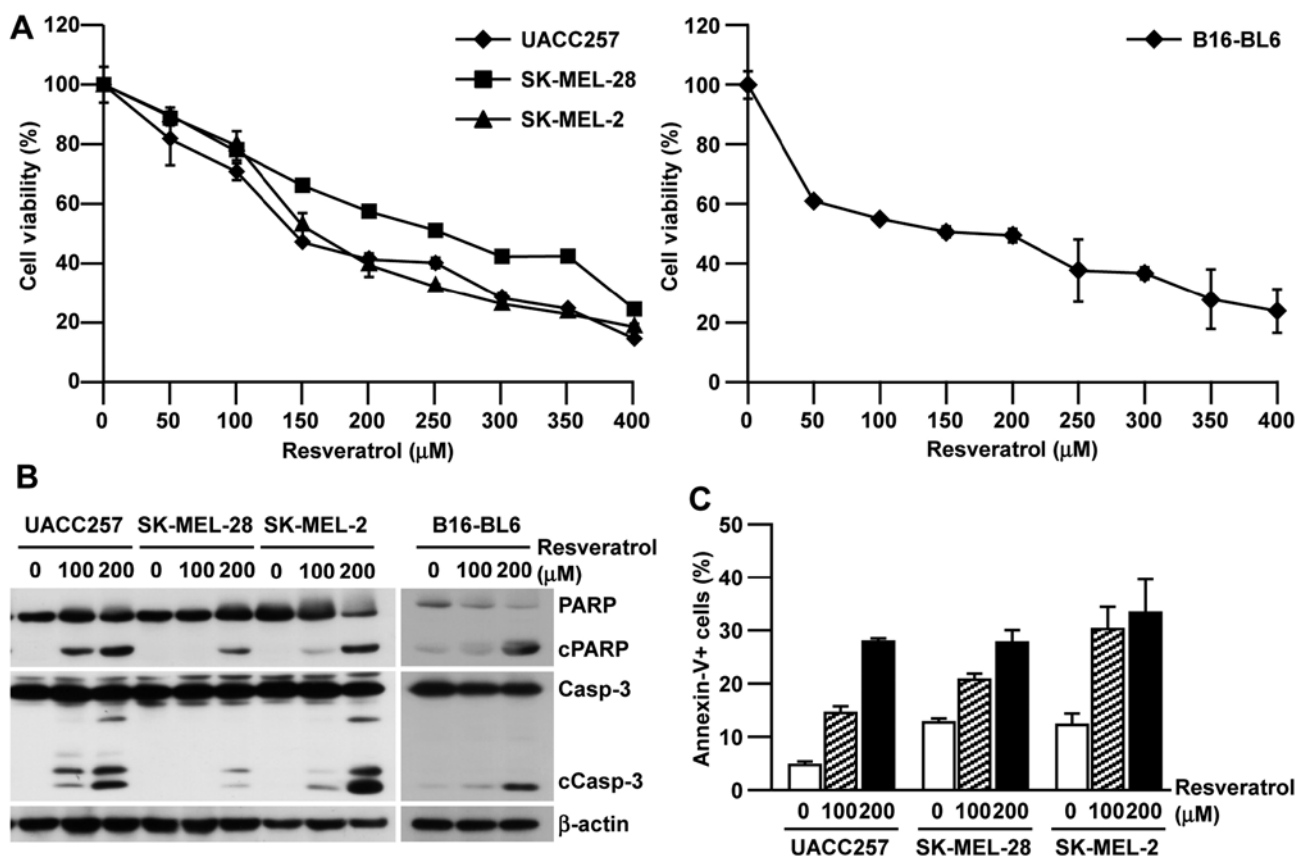


Figure 1. Resveratrol induces apoptosis in melanoma cells. (A) Human melanoma cells (upper panel) or murine melanoma cells (lower panel) were treated with the indicated concentrations of resveratrol for 24 h, and cell viability was determined by the WST-1 assay. Data are shown as the mean \pm SD of three independent experiments. (B) Protein expressions of apoptotic markers were determined with antibodies against caspase-3 and PARP. Other conditions were similar to (A). (C) Total apoptotic cells were determined by Annexin V and dead cell assays. Data are shown as the mean \pm SD of three independent experiments. Other conditions were similar to (A). ** $p < 0.01$ vs. untreated cells by two-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test.

proteins, the expression levels of BCL-2, BCL-xL, XIAP, survivin, BCL2A1, and MCL-1 were determined after treatment with resveratrol (Fig. 2). While suppression of BCL-2, XIAP, and BCL-xL was observed only in SK-MEL-2 cells, not in SK-MEL-28 and UACC257 cells, the expressions of survivin, BCL2A1 and MCL-1 were strongly suppressed in all three human melanoma cell lines. Since only survivin was consistently downregulated in murine B16-BL6 melanoma cells as well as human melanoma cell lines, we decided to further focus on the role of survivin in the anticancer efficacy

of resveratrol in melanoma cells as a conserved mechanism in both humans and mice.

Requirement of survivin suppression for resveratrol-induced apoptosis. To investigate the requirement of survivin for resveratrol-induced apoptosis in melanoma, we established hemagglutinin (HA)-tagged survivin-overexpressing cells (SK-MEL-28/Survivin) and control cells (SK-MEL-28/Vector, Fig. 3A) to test the pro-apoptotic effect of resveratrol. As shown in Fig. 3B, the induction of Annexin-V-positive apoptotic cells

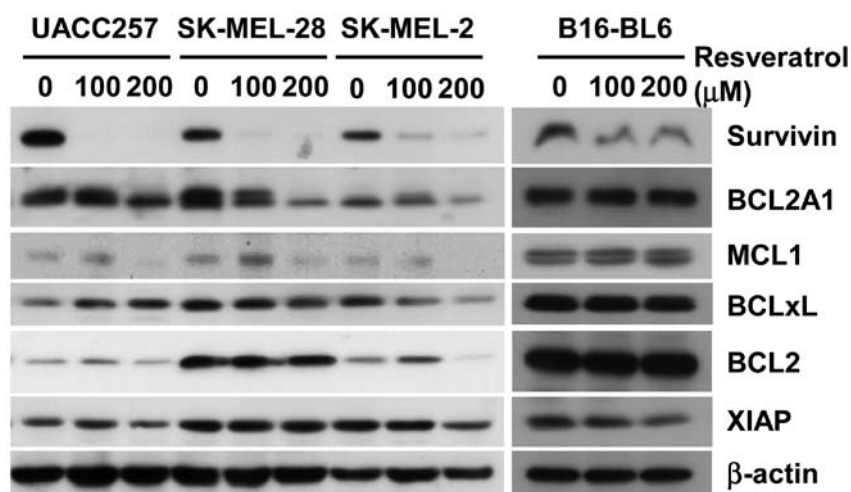


Figure 2. Resveratrol suppresses survivin expression in melanoma cells. Human melanoma cells (left panel) or murine melanoma cells (right panel) were treated with the indicated concentrations of resveratrol for 24 h. Anti-apoptotic proteins were examined with antibodies against survivin, BCL2A1, MCL1, BCLxL, BCL2 and XIAP.

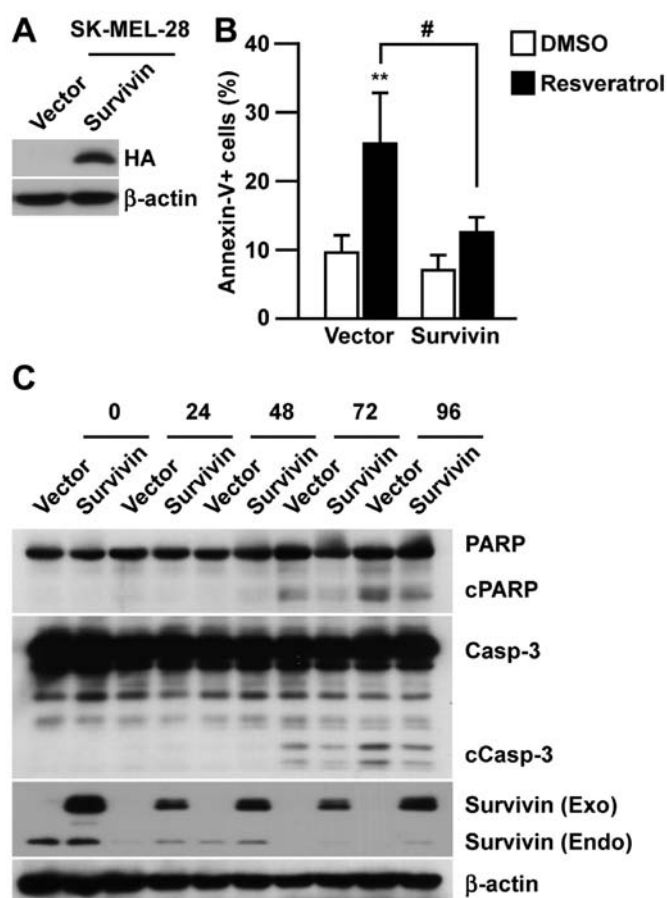


Figure 3. Requirement of survivin suppression for resveratrol-induced apoptosis. (A) Exogenous survivin expression was determined using an antibody against HA in HA/survivin-overexpressing cells (SK-MEL-28/Survivin, shown as Survivin) and in vector control cells (SK-MEL-28/Vector, shown as Vector). (B) SK-MEL-28/Vector or SK-MEL-28/Survivin was treated with 100 μ M resveratrol for 24 h and total apoptotic cells were determined by Annexin V and dead cell assays. ** $p < 0.01$ vs. DMSO-treated cells, and # $p < 0.05$ vs. SK-MEL-28/Vector with 100 μ M resveratrol by two-way ANOVA followed by the Bonferroni post-hoc test. (C) SK-MEL-28/Vector or SK-MEL-28/Survivin was treated with 100 μ M resveratrol for the indicated time. Protein expressions were determined with antibodies against PARP, caspase-3 and survivin.

was significantly suppressed by overexpressing survivin in SK-MEL-28. Consistent with the reduction in Annexin-V-positive cells, the overexpression of survivin in SK-MEL-28 reduced the cleavage of PARP and caspase-3 upon resveratrol treatment (Fig. 3C). This indicated the direct involvement of survivin suppression in resveratrol-induced apoptosis.

Transcriptional regulation of survivin by resveratrol in melanoma cells. In order to determine the molecular mechanism by which resveratrol inhibits survivin expression, we next examined the effect of resveratrol on *survivin* mRNA expression using real-time RT-PCR and the transcriptional levels of survivin by chromatin immunoprecipitation assay. As shown in Fig. 4A, the expression of *survivin* mRNA was decreased in all three human melanoma cell lines after resveratrol treatment. Consistently, the occupancy of pol-II S2 in the *survivin* gene region specifically decreased after resveratrol treatment compared with the β -actin region or *THBS1* region, in which *THBS1* expression is known to be increased by resveratrol treatment (Fig. 4B) (22). To further investigate which transcription factor is responsible for the suppression of *survivin* transcription, we next determined the expression of STAT3, β -catenin, p65 and p53, which are known to be involved in *survivin* transcription (23). Interestingly, resveratrol strongly inhibited β -catenin expression and STAT3 phosphorylation in all three melanoma cell lines, but did not show a significant effect on p53 expression (Fig. 4C). Suppression of p65 phosphorylation was only seen in SK-MEL-2 cells, not in SK-MEL-28 or UACC257 cells. Collectively, these results indicate that resveratrol inhibits survivin expression at the transcriptional level through the regulation of β -catenin and STAT3 pathways.

In vivo antitumor effect of resveratrol in B16-BL6 melanoma model. Finally, we tested the efficacy of resveratrol against highly malignant B16-BL6 melanoma in syngenic immunocompetent C57BL/6 mice. Consistent with our *in vitro* data, we observed that the administration of resveratrol significantly inhibited B16-BL6 melanoma growth compared to the vehicle

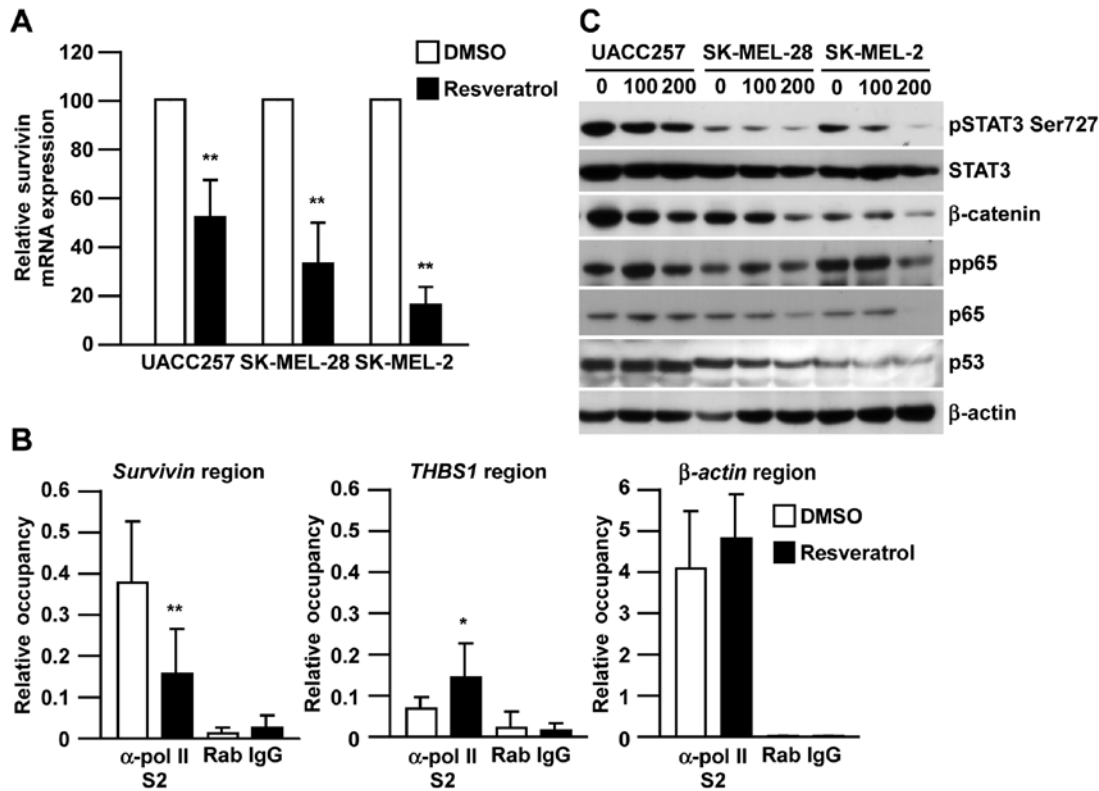


Figure 4. Resveratrol inhibits transcription of survivin in melanoma cells via STAT3 and β -catenin. (A) Human melanoma cells were treated with 200 μ M resveratrol for 24 h. *Survivin* mRNA was quantified by real-time RT-PCR. Data are shown as the mean \pm SD of three independent experiments. ** $p < 0.01$ vs. each cell with DMSO by two-way ANOVA followed by the Bonferroni post-hoc test. (B) SK-MEL-28 cells were treated with 200 μ M resveratrol for 24 h. Chromatin immunoprecipitation assay was performed by using the antibody against the polymerase II phosphorylated serine 2 site (α -pol II S2). Immunoprecipitated DNA was quantified by real-time PCR using primers specific to the *survivin*, *THBS1* and β -actin genes. Results are normalized to 1% input of genomic DNA. Data are shown as the mean \pm SD of three independent experiments. ** $p < 0.01$ vs. α -pol II S2-precipitated DNA with DMSO by two-way ANOVA followed by the Bonferroni post-hoc test. (C) Protein expressions of transcription factors related to survivin expression were examined with antibodies against STAT3, p-STAT3^{ser727}, β -catenin, p65, p-p65 and p53. Other conditions were the same as in Fig. 2.

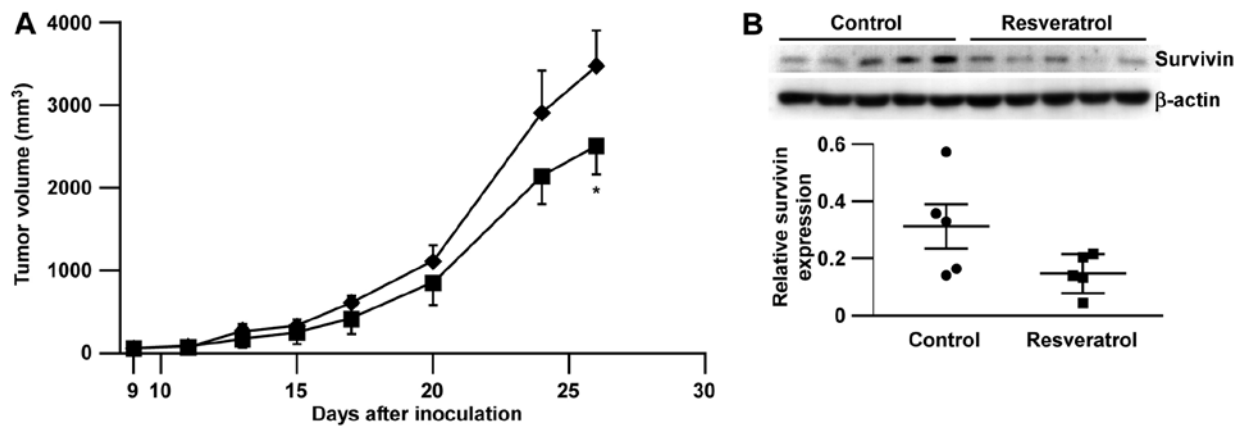


Figure 5. Resveratrol inhibits B16-BL6 melanoma tumor growth in C57BL/6 mice. (A) B16-BL6 cells were s.c. inoculated into mice. Mice received resveratrol in 0.5% carboxymethyl cellulose solution (100 mg/kg/day) or vehicle by oral administration every day. Tumor volume was measured at the indicated times. Data are shown as the mean \pm SEM (n=5). * $p < 0.05$ vs. control group at day 26 by two-way ANOVA followed by the Bonferroni post-hoc test. (B) At 14 days after inoculation, tumors were excised and the proteins were extracted from each tumor. Protein expressions were examined with antibodies against survivin. Other conditions were the same as in (A). Lower panel shows the intensities of survivin normalized to β -actin. Data are represented as the mean \pm SEM (n=5).

control without any body weight loss (Fig. 5A and data not shown) and further, the reduction of survivin expression in tumors after consecutive treatment with resveratrol (14 days, Fig. 5B).

Discussion

In this study, we showed that resveratrol induces apoptosis through survivin in various melanoma cell lines *in vitro*

without any genetic requirement and further demonstrated the efficacy of resveratrol against melanoma tumor growth in a mouse model. The transcriptional suppression of *survivin* by resveratrol was mediated through either the β -catenin or STAT3 pathway.

Although we concluded that survivin was critically involved in resveratrol-induced apoptosis, a substantial level of apoptosis (Fig. 3C) and *in vitro* growth inhibition (Fig. 5A) were observed in SK-MEL-28/Survivin cells treated with resveratrol. These results may imply some additional mechanisms of resveratrol in melanoma growth inhibition other than the transcriptional suppression of survivin. Indeed, there are several reports regarding the downstream targets of β -catenin or STAT3 other than survivin that might be related to resveratrol-induced apoptosis. In our results, BCL2A1, MCL-1, and BCL-xL were not consistently regulated by resveratrol in some melanoma cells (Fig. 2). In addition, resveratrol-induced apoptosis was not rescued by overexpression of BCL2A1 in melanoma cells (data not shown), suggesting that BCL2A1, MCL-1 and BCL-xL are unlikely to be responsible for resveratrol-induced apoptosis. The mTOR pathway is also known as an important target of resveratrol in certain cancers (24,25) and is a potential repressor of survivin translation (26). Consistent with previous reports (24,25), we observed the suppression of p70 S6K phosphorylation by resveratrol, one of the downstream molecules in the mTOR pathway in some melanoma cell lines (data not shown); therefore, inhibition of the mTOR pathway might be an alternative mechanism of resveratrol-induced apoptosis in melanoma.

Considering that survivin is known as a therapeutic target for various cancer types such as leukemia, prostate, breast, bladder, colon and melanoma (27-31) and that resveratrol reduced cell viability in all melanoma cell lines regardless of their genetic background in BRAF/NRAS/p53/PTEN (Fig. 1 and Table I), the utility of resveratrol in cancer treatment is feasible. Importantly, survivin expression was higher in melanoma than in nevus and normal skin when we re-analyzed the published microarray data set (32) (data not shown) and survivin expression was also very limited in normal adult tissues (33). In addition, resveratrol could suppress UV damage in normal human keratinocytes, resulting in adverse effects of the prevention of photocarcinogenesis (34). Considering that we did not observe any weight loss in mice with resveratrol *in vivo* (data not shown), survivin inhibitors including resveratrol could be attractive reagents for cancer therapy regardless of the cancer type. Of note, resveratrol even reduced the cell viability of SK-MEL-5 cell line, which is known to have high β -catenin activity (35), suggesting that resveratrol might be effective against various target cancer cells by overcoming the high transcriptional activity of β -catenin or STAT3.

Collectively, these results strongly suggest that targeting survivin with reagents such as resveratrol could provide a new opportunity for melanoma therapy, regardless of the genetic background.

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