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ARTICLE

Resveratrol Modulates Tumor Cell Proliferation and Protein Translation via SIRT1-Dependent AMPK Activation

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Resveratrol functions as an agonist for estrogen receptor (ER)-mediated transcription. However, other researchers have reported that resveratrol decreases proliferation of breast cancer cells that are either ER-positive or ER-negative, which suggests that the interaction of resveratrol with the ER may not fully explain its inhibitory effect on proliferation. Similar to those effects associated with caloric restriction (CR), resveratrol has multiple beneficial activities, such as increased life span and delay in the onset of diseases associated with aging. One key enzyme thought to be activated during CR is the AMP-activated kinase (AMPK), a sensor of cellular energy levels. The suppression of nonessential energy expenditure by activated AMPK along with the CR mimetic and antiproliferative properties of resveratrol has led us to hypothesize that resveratrol activity might have an important role in the activation of AMPK. Here, we show that resveratrol activated AMPK in both ER-positive and ER-negative breast cancer cells. Once activated, AMPK inhibited 4E-BP1 signaling and mRNA translation via mammalian target of rapamycin (mTOR). Moreover, we also found that AMPK activity mediated by resveratrol in cancer cells was due to inducing the expression of Sirtuin type 1 (SIRT1) via elevation in the cellular NAD^+/NADH in ER-positive cells. To our knowledge, we demonstrate here for the first time that resveratrol induces the expression of SIRT1 protein in human cancer cells. These observations raise the possibility that SIRT1 functions as a novel upstream regulator for AMPK signaling and may additionally modulate tumor cell proliferation. Targeting SIRT1/AMPK signaling by resveratrol may have potential therapeutic implications for cancer and age-related diseases.

KEYWORDS: Resveratrol; caloric restriction; AMPK; SIRT1; LKB1

INTRODUCTION

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is produced by several plants, including peanuts, mulberries, grapes, and root extracts of the weed of *Polygonium cuspidatum* (the traditional Chinese and Japanese medicine). Resveratrol has been reported to have functions as a phytoestrogen exerting both estrogenic and antiestrogenic effects based on its ability to bind to estrogen receptor (ER) (*1*) for its activation. Many researchers have demonstrated that resveratrol suppresses cell viability and proliferation in ER-positive and ER-negative cells (*1*, *2*), although the mechanism by which resveratrol inhibits cell proliferation is not clearly understood. Since then, several studies have shown that resveratrol can prevent or slow the progression of a wide range of illnesses, including cancer (*3*), cardiovascular disease (*4*), diabetes (*5*, *6*), ischemic injuries (*7*), and age-related diseases (*8*).

Caloric restriction (CR) is a 20−40% lowering of caloric intake, known to extend lifespan in a wide spectrum of species (*9*). It has been suggested that CR extends longevity and reduces age-related pathologies by reducing the levels of DNA damage and mutations that accumulate with age (*10*). An age-related accumulation of mutation has been associated with the increased incidence of most cancers. Many of the activities of resveratrol are similar to the beneficial effects offered by CR, including slowed aging and delaying the onset of chronic disease (*11*, *12*).

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One key enzyme thought to be activated during CR is AMP-activated kinase (AMPK). AMPK is a heterotrimeric serine/threonine protein kinase that is composed of a catalytic α-subunit and regulatory β- and γ-subunits. AMPK activity is regulated allosterically by AMP and through phosphorylation at Thr172 in the activation loop of the α-subunit (*13*−*15*). AMPK acts to maintain the intracellular AMP/ATP ratio, repress pathways that consume energy, and promote ATP-producing catabolic pathways by phosphorylation of downstream targets (*16*). The AMPK pathway is linked to tumor growth and proliferation through regulation of the mammalian target of rapamycin (mTOR) pathway. There is currently a high level of interest in understanding signaling through the mammalian target of rapamycin. Rapamycin is an anticancer drug, which inhibits the growth of a broad spectrum of cancers via mTOR and can cooperate with other agents to induce apoptosis. The best-understood roles of mTOR in mammalian cells are related to the control of mRNA translation by the eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) (*17*−*19*). In its hyperphosphorylation form via mTOR, 4E-BP1 ultimately initiates translation of certain mRNAs, including those needed for cell cycle progression and those involved in cell cycle regulation (*20*).

There have been occasional reports of antiproliferative activity of resveratrol in various experimental models (*21*). In a wide variety of species, resveratrol is a potential target for CR mimetic, exerting via silent information regulator 2 (Sir2) enzymes, collectively called sirtuins, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase (*22*). SIRT1 is a human orthologue of Sir2, which regulates important aspects on mitochondria function. Activities of mitochondria have been linked to aging and diseases associated with aging. Hence, SIRT1 might provide a key link between mitochondria dysfunction and aging along with age-related diseases including cancer, diabetes, and neurodegenerative disorders (*23*). Importantly, SIRT1 facilitates the formation of heterochromatin, the more tightly packed form of chromatin associated with histone hypoacetylation, in which gene repression is achieved. In addition, it is known that SIRT1 is increased in response to CR and that the enzymatic activity of SIRT1 is required for resistance to apoptosis induced by CR (*24*).

The mechanism by which resveratrol inhibits ER-positive and ER-negative cell proliferation is not clearly understood. Recent observations have found that resveratrol has multiple beneficial activities similar to those associated with CR, and these might be related to uncharacterized direct actions of resveratrol on cancer cells. However, the underlying aspects of mechanism have not been fully explored. In the current study, we demonstrate that resveratrol modulates translation and proliferation of ER-positive and ER-negative cells through AMPK activation that is dependent on SIRT1.

MATERIALS AND METHODS

Chemicals. Cell culture materials were obtained from Invitrogen (Burlington, ON, Canada). Antibodies to phospho-4E-BP1 (Thr37/46), phospho-AMPKα (Thr172), AMPKα, and phospho-mTOR (Ser2448) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to SIRT1 and LKB1 were purchased from Upstate Biotechnology Inc. (Charlottesville, VA). Anti-β-actin antibody, horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and enhanced chemiluminescence (ECL) reagents were purchased from Sigma-Aldrich (St. Louis, MO). Resveratrol, nicotinamide, metformin (1,1-dimethylbiguanide), and MTT (3-(4,5 dimethylthiaxol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma-Aldrich. Compound C and AICAR (5-aminoimidazole-4-carboxamide-1-β-ribofuranoside) were purchased form Toronto Reserch Chemicals (Toronto, ON, Canada). The RNAi constructs of AMPKα were made by the National RNAi Core Facility (Taipei, Taiwan).

Cell Culture. All cell lines were purchased from the American Type Culture Collection (Manassas, VA). MCF-7, MDA-MB-231, A549, SKOV3.ip1, SAS, and HeLa cell lines were cultured in DMEM. Cells of BT-474 and PC-3 were cultured in DMEM/F12 and RPMI-1640, respectively. All media were supplemented with 10% fetal bovine serum (FBS) and 100 units/mL gentamicin at 37 °C. Cells were incubated in 75 cm^2 flasks at 37 °C with 5% CO_2 and were passaged by 0.25% trypsin−EDTA when they reached 80% confluence.

Cell Viability. Cells were treated with various doses of resveratrol for 48 or 72 h before cell proliferation was examined by MTT assay according to the manufacturer's instructions (Promega, Madison, WI). The absorbance was measured at 590 nm.

Western Blotting. Cells were treated with various agents as indicated in figure legends. After treatment, Western blotting was done as described previously (*25*). Expression levels of mTOR, AMPK, 4E-BP1, SIRT1, LKB1, β-actin were detected by using specific antibodies in combination with enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL).

Short Hairpin RNA. RNAi reagents were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica, supported by the National Research Program for Genomic Medicine Grants of NSC (NSC 97-3112-B-001-016). Short hairpin RNAs (shRNAs) were designed to target specific sequences of human AMPK (clone ID, TRCN000000861; target sequence, 5′-GTT GCC TAC CAT CTC ATA ATA-3′). One day before transfection, cells were seeded at the density of 30−40% without antibiotics. Twenty nanomolar AMPK shRNAs were transfected into cells by lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were incubated for an additional 24 h before addition of resveratrol as previously described. The cell lysates were analyzed by Western blot as previously described.

^{35}S-Methionine Metabolic Labeling. Cells seeded in 6-well plates were incubated with increasing doses of resveratrol for 24 h in DMEM supplemented with 10% FBS. After 24 h, cells were washed once with PBS and incubated with resveratrol in methionine-free DMEM without FBS for 2 h. The medium was then replaced with methionine-free DMEM containing ^{35}S-protein labeling mix (20 μCi/mL). After 4 h, the cells were washed with cold PBS and lysed in lysis buffer, and radioactivity incorporated into the TCA precipitable material was measured.

Determination of NAD^+/NADH Ratio. Cells were treated with different concentrations of resveratrol for 1 h and lysed with NAD^+/NADH extraction buffer by alternating freezing and thawing twice. Samples were vortexed and then centrifuged for 5 min. Supernatants were transferred to new tubes. Nicotinamide nucleotides were assayed by using BioVision's NAD^+/NADH Quantification Kit (Mountain View, CA).

In Vivo Studies. Female BALB/c nude mice (18−20 g; 6−8 weeks of age) were purchased from the National Animal Center and maintained in pressurized ventilated cages according to institutional regulations. MDA-MB-231 cells (5×10^6) were inoculated subcutaneously (sc) into the right flanks of the mice. After 7 days, 18 tumor-bearing mice were randomly divided into three groups. The first group received only vehicle. The second and third groups were intraperitoneally (ip) given resveratrol every 3 days at 50 and 100 mg/kg, respectively. Mice were weighed, and tumors were measured using calipers every 3 days. Tumor volumes were determined by measuring the length (l) and the width (w), and the volumes were calculated as $V = lw^2/2$. On the final day of the treatment, mice were sacrificed; tumors were excised, weighed, and sectioned, and the tumor sections were embedded in OCT compound and frozen at −70 °C.

Immunohistochemical Staining of Frozen Tissue Sections. Sections frozen in OCT were fixed in acetone and chloroform. After overnight incubation with rabbit polyclonal anti-Sir2 antibody (1:100 dilution; Upstate Biotechnology Inc.), the slides were washed again and then incubated with biotinylated secondary antibodies before subsequent incubation with avidin−biotin−horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Antibody detection was done with 3,3′-diaminobenzidine, and the tissue sections were counterstained with Mayer's hematoxylin, washed, mounted with Universal Mount, and dried on a 56 °C hot plate. The prepared slides were examined by light microscopy.

Statistics. All values are expressed as mean ± SD. Each value is the mean of at least three separate experiments in each group. Student's t test

was used for statistical comparison. Asterisks indicate that the values are significantly different from control (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

RESULTS

Resveratrol Up-regulates AMPK Activity in ER-Positive and ER-Negative Breast Cancer Cells. As previous work demonstrated that the metabolic actions of resveratrol require AMPK (*26*), we tested whether resveratrol could increase the phosphorylation in ER-positive and -negative breast cancer cells. We examined the phosphorylation status of AMPK in MDA-MB-231 and MCF-7 cells. Western blot analysis indicated that resveratrol stimulates AMPK phosphorylation in a dose-dependent manner (**Figure 1A**). AMPK activation is associated with decreased phosphorylation of mTOR and 4E-BP1 (**Figure 1A**). A similar antiproliferative effect was found when cells were treated with AICAR, an AMP analogue, and metformin, a biguanide commonly used in the treatment of type 2 diabetes mellitus (**Figure 1B**). To determine if resveratrol suppresses protein synthesis through inhibition of mTOR pathway by activating AMPK, we added compound C, an AMPK inhibitor, in the absence or presence of resveratrol. The AMPK activity was suppressed by compound C in the presence of resveratrol, and the mTOR activity was recovered (**Figure 1B**). In addition, we also treated cells with AMPK shRNA to silence the expression of AMPK. As shown in **Figure 1C**, AMPK shRNA reduced the stimulatory effect of resveratrol. This was correlated with reduced activity of mTOR, as detected by Western blot analysis. We hypothesized that resveratrol, by up-regulating AMPK activity, would inhibit mTOR activation and downstream signaling events. Consistent with this prediction, resveratrol inhibited the level of phospho-4E-BP1, whereas AMPK shRNA had the opposite effect.

Reseveratrol Decreases General Translation. In view of the effect of resveratrol on mTOR and 4E-BP1 activation, we hypothesized that protein translation would be decreased in the presence of resveratrol in MDA-MB-231 and MCF-7 cells. As shown in **Figure 1D**, treatment of cells with resveratrol indeed led to a reduction of general translation with a maximal inhibition of 80%, which is consistent with our hypothesis.

Resveratrol-Stimulated AMPK Activation Is Dependent on SIRT1. SIRT1 participates in many crucial functions, including protection from stress, aging, and cell cycle regulation (*27*). Because a number of biological effects of resveratrol and other polyphenols have been known to depend on SIRT1 function (*28*), we explored whether AMPK activation by resveratrol depended on SIRT1. First, we found that SIRT1 was up-expressed in MDA-MB-231 and MCF-7 cells determined by using Western blot analysis (**Figure 2A**). Next, we stimulated cells with 40 μM resveratrol for 24 h in the presence or absence of inhibitor of SIRT1 (50 mM nicotinamide). We observed that nicotinamide inhibited the resveratrol-induced SIRT1 expression in MDA-MB-231 and MCF-7 cells (**Figure 2B**). The SIRT1 inhibitors attenuated the robust activation of AMPK by resveratrol for 72 h as judged by the increased phosphorylation of AMPK and reduced phosphorylation of its downstream target mTOR and 4E-BP1 (**Figure 2C**). These results suggested that the effect of resveratrol on AMPK is dependent on SIRT1 activity.

Resveratrol Is Capable of Increasing Cytosolic NAD^+/NADH Redox Potentials. A unique feature of Sir2 histone deacetylases is their utilization of NAD^+ as a cosubstrate, which has led to the suggestion that Sir2 activity reflects the cellular energy state (*29*). We consequently asked whether resveratrol affects glycolysis in human cancer cells that can be monitored by measurement of the NAD^+/NADH ratio. As shown in **Figure 2D**, resveratrol increases the ratio of NAD^+/NADH in cells of MCF-7, not in those of MDA-MB-231. We concluded from these studies that resveratrol effectively changes the redox status of human cancer cells, although with cell type specificity.

Resveratrol-Induced SIRT1 Expression Increases AMPK Activity Independent of LKB1 in Cancer Cells. Two kinases, serine/threonine kinase 11 (STK11), also known as LKB1, and Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ), have been identified as upstream activators of AMPK (*30*, *31*). To examine the effect of resveratrol on proliferation more generally, cell viability assays were carried out on several cell lines. Three breast cancer cells, including MCF-7, MDA-MB-231, and BT-474, were used. Other cancer cells with different origin, including A549 (lung cancer), PC-3 (prostate cancer), SKOV3.ip1 (ovarian cancer), SAS (oral cancer), and HeLa (cervical cancer), were also used. As shown in **Figure 3A**, growth of both HeLa cells and A549 cells, which have no functional LKB1 allele, was also inhibited by resveratrol. Next, we stimulated cells with 40 μM resveratrol for 24 h and confirmed by Western blot analysis that SIRT1 is still expressed in cancer cells without functional LKB1 (**Figure 3B**). These results suggested that SIRT1 but not LKB1 is required for resveratrol-stimulated AMPK activation in cancer cells.

Growth Inhibition of MDA-MB-231 Cells by Resveratrol in Vivo. To investigate the in vivo antitumor activity of resveratrol, we carried out xenografts with MDA-MB-231 cells in nude mice. Eighteen female nude mice were individually sc injected with MDA-MB-231 cells. One week after inoculation, the mice were divided into three groups (six mice per group) and treated with vehicle alone or resveratrol at 50 or 100 mg/kg. Animals monitored for vital signs and weight changes for the duration of the experiment did not lose weight. After 4 weeks, the animals were killed, and no pathologic signs were seen. As shown in **Figure 4A**, ip administration of resveratrol induced a dose-dependent inhibition of MDA-MB-231 tumor growth, with the resveratrol (100 mg/kg) treated group (175.66 mm^3) being more significant ($P < 0.001$) compared to the vehicle-treated one (931.84 mm^3). These results showed that resveratrol significantly inhibited MDA-MB-231 tumor growth in a mouse xenograft model.

Immunohistochemical Analysis of Tumor Sections Confirms Resveratrol-Dependent Up-regulation of SIRT1. To determine whether SIRT1 up-regulation by resveratrol observed in vitro can be found in vivo, we did immunohistochemical staining assays on tumors from the xenograft mice. On the final day of the MDA-MB-231 antitumor experiment, tumor sections were stained separately with SIRT1 to determine if the level of protein in the tumors is altered. Representative immunohistochemical photographs of SIRT1 are shown in **Figure 4B**. Compared with the control group, SIRT1 was induced by resveratrol. These findings indicate that resveratrol can, through the up-regulation of SIRT1 expression, modulate tumor cell proliferation both in vitro and in vivo.

DISCUSSION

Most studies of the effects of resveratrol on cell signaling networks have been carried out in the context of cancer research. It has been noted that resveratrol mimics CR-induced metabolic changes in response to AMPK activation (*6*, *11*). We focus on the effect of AMPK activation on cellular proliferation and explore the possibility that AMPK might be a therapeutic target for cancer.

There is currently a high level of interest in signaling through mTOR. The role of mTOR in tumor acts as a sensor for energy, growth factors, and nutrients, all of which are required for protein translation (*32*). The mTOR pathway is aberrantly activated in

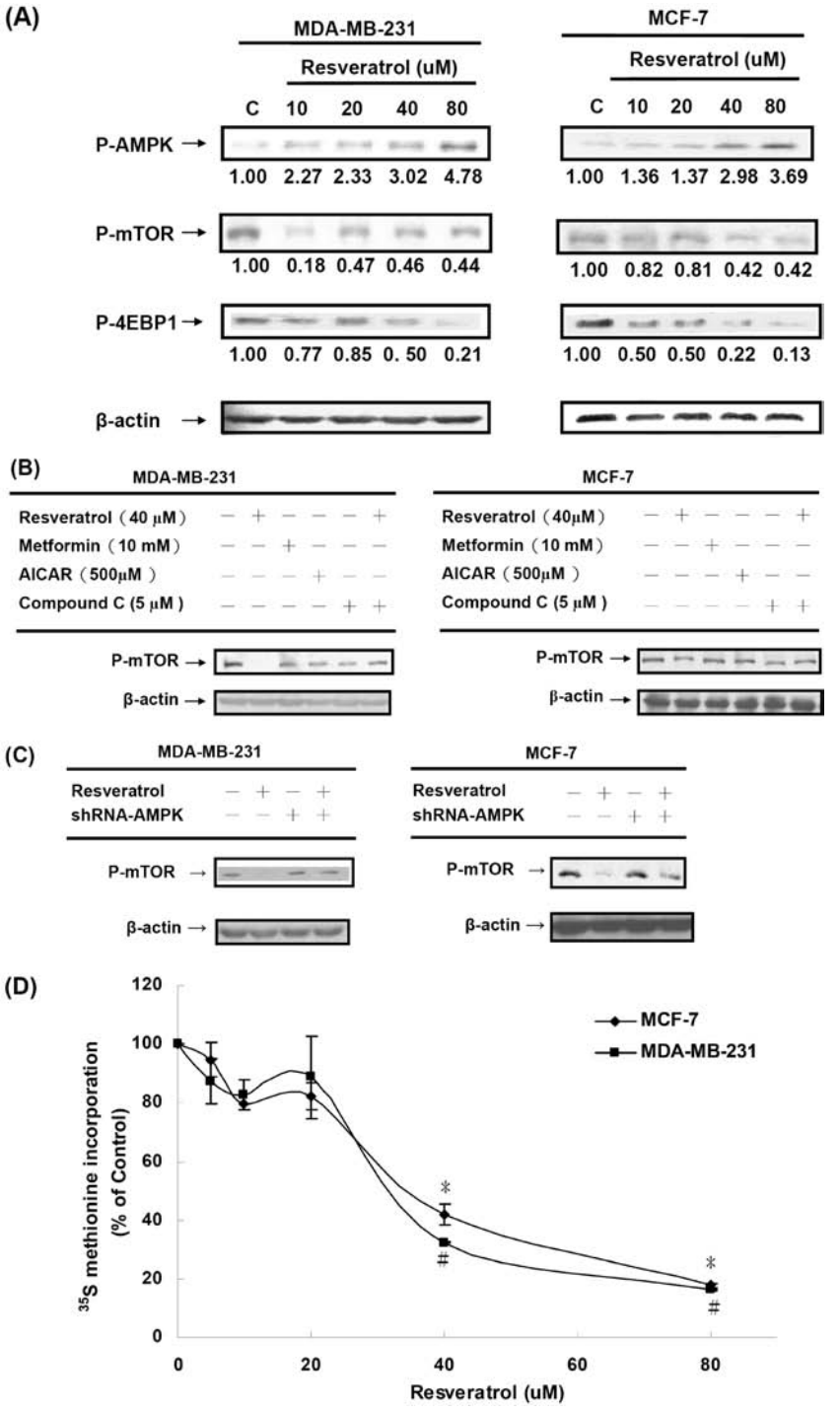

Figure 1. Resveratrol activates AMPK and decreases general mRNA translation in both ER- positive and -negative breast cancer cells. (**A**) MDA-MB-231 and MCF-7 cells were treated with various concentrations of resveratrol for 72 h, after which cell extracts were analyzed for levels of phosphorylated AMPK (Thr 172), mTOR (Ser 2448), 4E-BP1 (Thr 37/46), and β-actin by Western blotting. (**B**) MDA-MB-231 and MCF-7 were stimulated for 48 h with 40 μM resveratrol only, or 10 mM metformin, 500 μM AICAR, or 5 μM compound C in the absence or presence of resveratrol. After harvesting, cells were lysed and prepared for Western blot analysis using antibodies against phospho-mTOR (Ser2448) and β-actin. Metformin and AICAR were used as AMPK activators, and compound C was used as an AMPK inhibitor. (**C**) MDA-MB-231 and MCF-7 cells were transfected with or without 50 nmol/L AMPKα1-shRNA using Oligofectamine. After 24 h of transfection, cells were treated with resveratrol for 48 h. After harvesting, cells were lysed and prepared for Western blot analysis using antibodies against phospho-mTOR (Ser2448). β-Actin was used as a loading control. (**D**) MDA-MB-231 and MCF-7 cells were incubated with the indicated doses of resveratrol for 24 h before the addition of ^{35}S-methionine-protein labeling mix (20 μCi/mL) for 4 h. Cells were harvested after labeling, and radioactivity incorporated into the TCA precipitable material was measured. Protein synthesis levels are displayed as percentage of that exhibited in the absence of resveratrol. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression normalized to β-actin.

many human cancers (*33*, *34*). Thus, approaches to block the pathway are being actively pursued in many laboratories and pharmaceutical companies. In view of the mTOR being regulated by multiple factors, there are a number of target proteins for

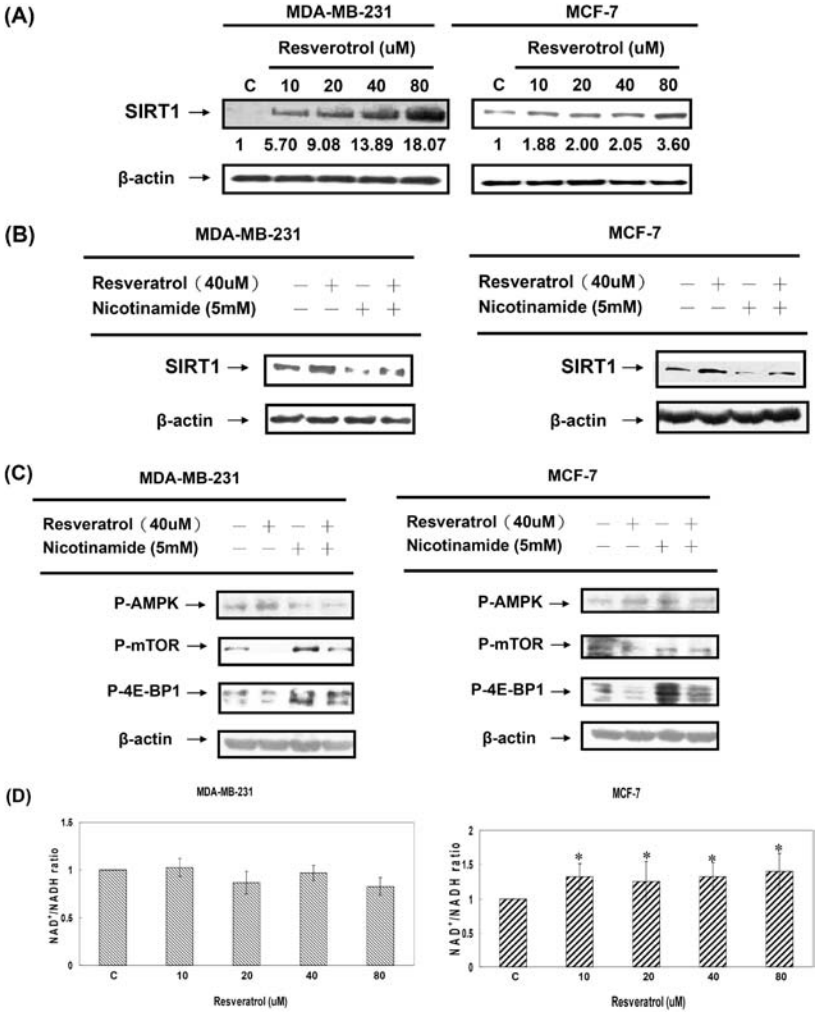

Figure 2. SIRT1 stimulation by resveratrol. (**A**) MDA-MB-231 and MCF-7 cells were treated with various concentrations of resveratrol for 24 h, after which cell extracts were analyzed for levels of SIRT1 by Western blotting. (**B**) MDA-MB-231 and MCF-7 cells were pretreated with nicotinamide (50 mM) for 2 h, then in the presence or absence of the resveratrol (40 μM) for 24 h, and cell extracts were analyzed for level of SIRT1 by Western blot analysis. β-Actin was used as a loading control. Nicotinamide was used as an inhibitor of SIRT1. (**C**) MDA-MB-231 and MCF-7 cells were pretreated with nitotinamide (5 mM) for 2 h, then in the presence or absence of the resveratrol (40 μM) for 72 h. The expression of phospho-AMPK, phospho-mTOR, and phospho-4E-BP1 was determined by Western blot analysis. β-Actin was used as a loading control. (**D**) MDA-MB-231 and MCF-7 cells were treated with different concentrations of resveratrol for 1 h, and cells were lysed using the extraction buffer by freeze/thaw. Samples were centrifuged for 5 min, and the supernatants were transferred to new tubes. The NAD^+/NADH ratios were assayed by using BioVision's NAD^+/NADH Quantification Kit. Western blot data presented are representative of those obtained in at least three separate experiments. The values below the figures represent change in protein expression normalized to β-actin.

which intervention would be predicted to lower mTOR activity and have an impact on cancer cells. Activation of AMPK results in a decrease mTOR signaling and in turn inhibition of protein synthesis. The data presented here show that the inhibition of protein translation via the AMPK−mTOR pathway by resveratrol works for both ER-positive and ER-negative cancer cells.

The ER, expressed in about 60% of breast cancers, is an important predictive and prognostic marker in human breast cancer. It has become evident that estrogen/ER signaling exhibits pleiotropic effects through nongenomic interactions with growth factor signaling pathways. In particular, long-term estrogen-deprived breast tumor cells exhibit increased Akt/mTOR activation (*35*). Moreover, the pathway has been strongly implicated in resistance to antiestrogen therapeutics (*36*). Therefore, the combination of resveratrol with endocrine therapies supports the potential for the effective therapy of endocrine-dependent breast cancers.

Many studies have shown that both resveratrol and CR increase SIRT1 activity (*22*, *24*). The activation of SIRT1 by resveratrol is an allosteric regulation. It is an attractive hypothesis that resveratrol might use the same pathways activated by CR in mammals. Moreover, our results clearly show that treatment with resveratrol significantly increases the protein expression of SIRT1. The mechanism underlying the expression control of SIRT1 is poorly understood. To the best of our knowledge, we demonstrate here for the first time that resveratrol induces the expression of SIRT1 protein.

SIRT1 is a conserved NAD^+-dependent deacetylase that regulates lifespan in accord with nutritional provision. AMPK is activated by alterations in the intracellular AMP/ATP ratio. Both AMPK and SIRT1 signaling pathways are energy sensing. We hypothesize that resveratrol may affect these two singling pathways. Our data show that a significant dose-dependent effect of resveratrol for the NAD^+/NADH ratio can be found in MCF-7 cells, but not in MDA-MB-231 cells, although treatment of resveratrol stimulates AMPK activity in both cells. According to this model, CR or resveratrol treatment increases NAD^+, which activates the SIRT1 function that leads to the deacetylation of

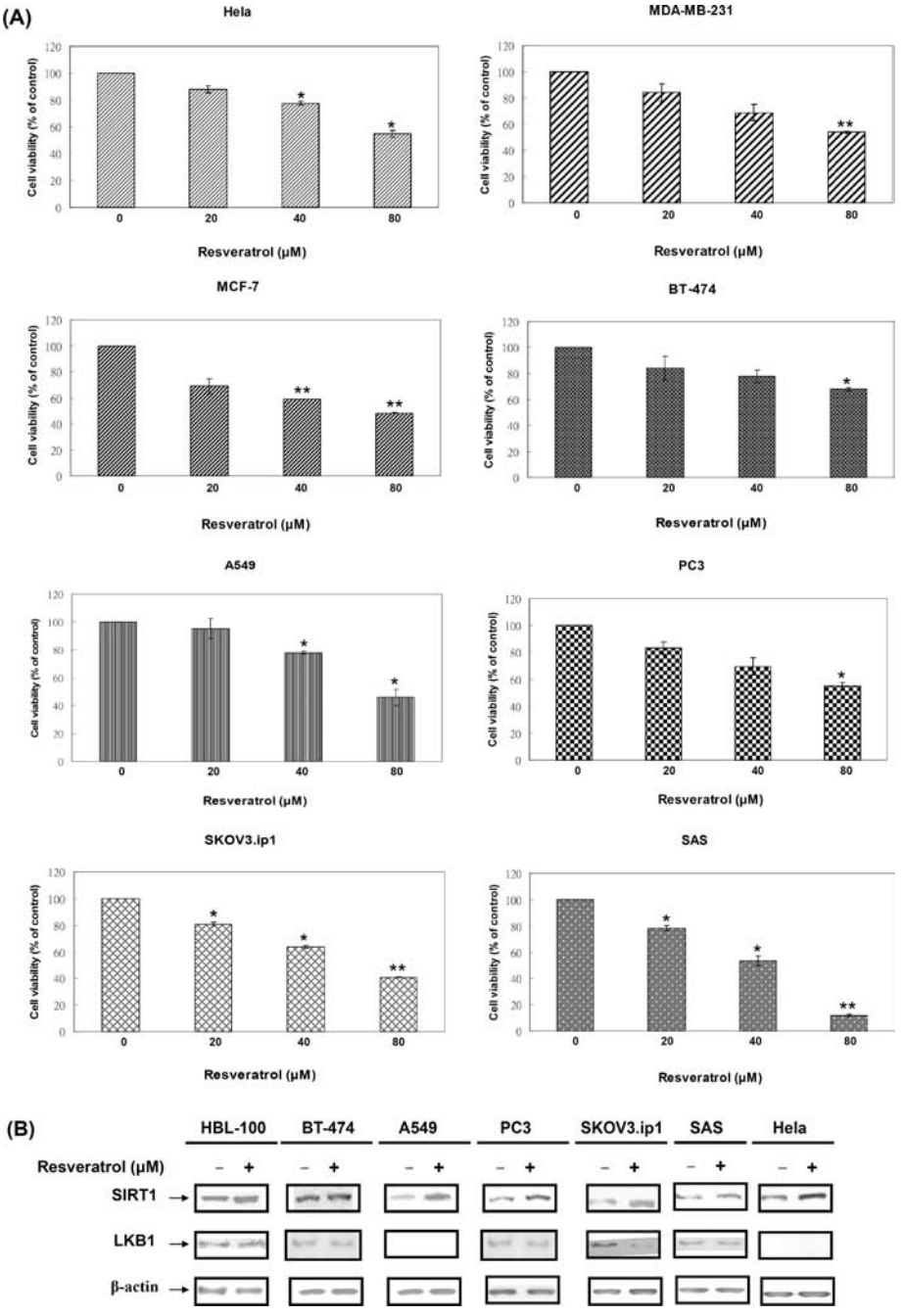

Figure 3. Resveratrol activates SIRT1 through an LKB1-independent pathway. (**A**) Cells were seeded into 24-well plates in the presence of 10% FBS for 24 h before treatment with various concentrations of resveratrol at 37 °C for 72 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption value from cells without treatment of resveratrol as 100%. This experiment was repeated three times. Bars represent the SD. (**B**) Various cell lines were treated with 80 μM resveratrol for 24 h, and extracts were analyzed for levels of SIRT1 and LKB1 by Western blotting. Western blot data shown are representative of those obtained in at least three separate experiments.

critical protein targets. However, this idea does not consider the compartmentalization of nicotinamide adenine dinucleotides, in which much of the NAD^+ and NADH is bound to protein, which is thereby incapable of participating SIRT1 regulation, or the vast excess of free NAD^+ over NADH.

SIRT1 is an antiaging protein that has therapeutic potential for a range of diseases involved in aging, including metabolic disorders, neurodegenerative disorders, cancer, and cardiovascular disease. The function of SIRT1 has received much attention from investigators mainly due to its crucial roles in regulating a variety of molecular and cellular processes including antiapoptosis (*37*), neuronal protection (*38*), calorie restriction (*6*), glucose metabolism (*39*), fat storage (*40*), insulin secretion (*41*), and cellular senescence (*37*). Interestingly, the endogenous expression of SIRT1 in cancer cells is higher than that in normal human cells, suggesting the possibility that SIRT1 is involved with tumorigenesis. However, the present study has shown that SIRT1 overexpression can block hormonal activation of AR. Overexpression of SIRT1 has been shown to inhibit the in vitro growth and proliferation of prostate cancer cells that express the AR (*42*), suggesting that resveratrol and other SIRT1 activators might be good chemopreventive and chemotherapeutic agents for prostate cancer. Moreover, SIRT1 has also been shown to deacetylate NF-κB (*43*−*45*). A direct interaction between endogenous SIRT1 and

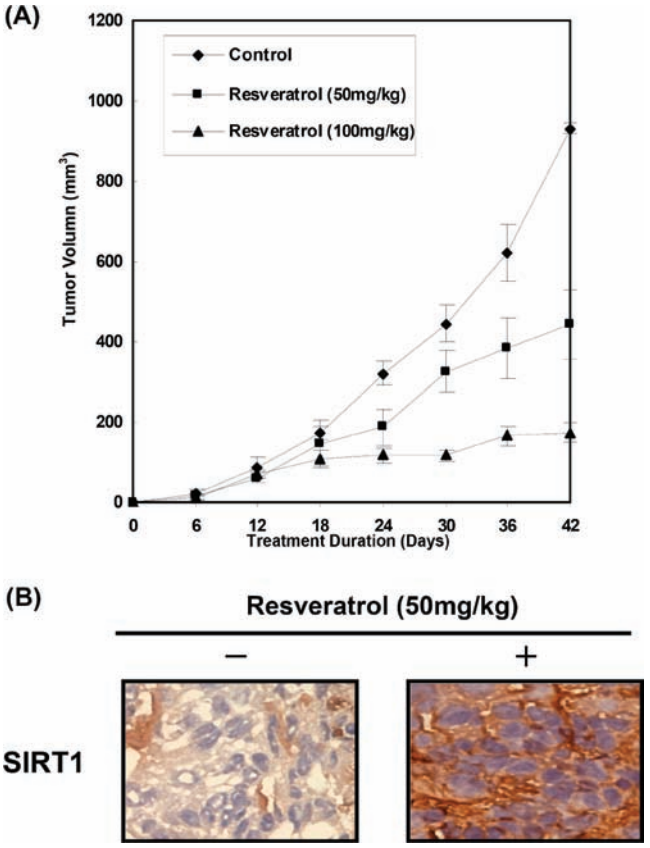

Figure 4. Effect of resveratrol on MDA-MB-231 tumor growth in a mouse xenograft model. (**A**) Female nude mice received sc MDA-MB-231 transfectants to induce tumor xenografts. Seven days later, mice were divided into three groups. The first group was a control group treated with vehicle. The second and third groups were given ip resveratrol (50 mg/kg) and resveratrol (100 mg/kg), respectively, every 3 days for a total of 42 days. Tumor volume was measured and calculated as described under Materials and Methods. Points, mean tumor volume; bars, SD. (**B**) Immunohistochemical staining of tumor sections removed from xenograft mice. The micrographs with 200 times magnification of the original ones show protein expression of SIRT1.

NF-κB promotes not only the deacetylation of NF-kB on lysine 310 but also that of HATs on target promoters and local histone proteins to actively repress target gene expression (*46*). As chronic inflammation and activation of NF-κB may contribute to several types of cancers, treatment with resveratrol may be a potential chemopreventive or chemotherapeutic agent. Resveratrol inhibits NF-κB-dependent transcription, but it is not known if this is directly and solely through activation of SIRT1 or is also dependent on other regulators of NF-κB.

Several research groups have reported LKB1 being the upstream kinase of AMPK (*47*). LKB1 is mutated in patients with Peutz−Jeghers syndrome and in sporadic lung tumors. The role of LKB1 in AMPK activation has been demonstrated in some LKB1-deficient cancer cells such as HeLa and MDA-MB-231. Data presented here show that treatment of MDA-MB-231 (LKB1−) and MCF-7 (LKB1+) cells with resveratrol leads to an increase in the phosphorylation of Thr172 on AMPK. Our finding reveals that AMPK activation by resveratrol is independent of the tumor suppressor LKB1 (**Figure 3B**).

In summary, the AMPK signaling pathway that regulates mTOR is dysregulated in a large number of human cancers, which leads to an increase in mTOR activity, resulting in enhanced mRNA translation and increased cellular proliferation.

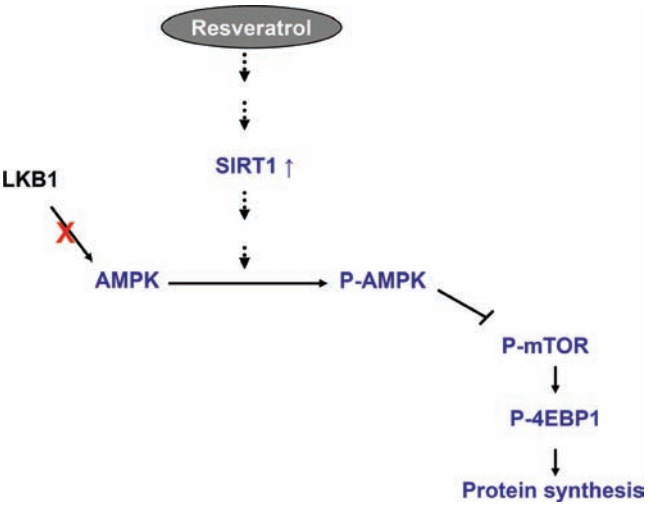

Figure 5. Schematic representation of the molecular mechanism of resveratrol modulating tumor cell proliferation.

We propose a mechanism through which resveratrol could inhibit protein translation by activating AMPK-mTOR pathway, a process that is independent of the LKB1. Our data show that resveratrol-induced AMPK activation decreases tumor cell proliferation, which depends on the expression of SIRT1 (**Figure 5**). The direct action of resveratrol as an activator of the SIRT1/AMPK tumor suppressor pathway in epithelial cells reported here suggests the possibility of broader clinical relevance.

ABBREVIATIONS USED

AMPK, AMP-activated protein kinase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; AICAR, 5-aminoimidazole-4-carboxamide-1-β-ribofuranoside; CaMKK, Ca^{2+}/calmodulin-dependent kinase kinase; CR, calorie restriction; DMEM, Dulbecco's Modified Eagle Medium; eIF-4E, eukaryotic translation initiation factor 4E; ER, estrogen receptor; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein-1; FBS, fetal bovine serum; LKB1, serine/threonine kinase 11; mTOR, mammalian target of rapamycin; mM, millimolar; NAD, nicotinamide adenine dinucleotide; PBS, phosphate buffer saline; rpm, revolutions per minute; RNAi, RNA interference; Sir2, silent information regulator 2; SIRT1, sirtuin type 1; siRNA, small interfering RNA; Tris, tris-(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; μM, micromolar.

LITERATURE CITED

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