

# Resveratrol Suppresses Growth of Human Ovarian Cancer Cells in Culture and in a Murine Xenograft Model: Eukaryotic Elongation Factor 1A2 as a Potential Target

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## Abstract

The eukaryotic elongation factor 1A2 (eEF1A2) is known to retain oncogenic potential and is recognized as a novel target for cancer prevention and therapy. Resveratrol (*trans*-3,4',5-trihydroxystilbene), a phytoalexin present in grapes, has been reported to possess chemopreventive and chemotherapeutic activities. In the present study, we examined the growth-inhibitory effects of resveratrol in human ovarian cancer PA-1 cells, considering eEF1A2 as a potential molecular target. Pretreatment with resveratrol attenuated proliferation of serum-starved PA-1 cells stimulated with insulin or serum. Resveratrol also activated caspase-9, -7, and -3 and induced apoptosis in PA-1 cells in the presence of insulin or serum. Insulin or serum stimulation of PA-1 cells resulted in the marked induction of eEF1A2, which was suppressed by pretreatment with resveratrol. Moreover, resveratrol inhibited insulin- or serum-induced soft-agar colony formation in eEF1A2-transfected NIH3T3 cells. An antibody array directed to assess the phosphorylation of protein kinases revealed that treatment with insulin or serum induced the phosphorylation of Akt in PA-1 cells. Pharmacologic inhibition of Akt with LY294002 abrogated insulin- or serum-induced eEF1A2 expression and increased the caspase-3 activity. In another experiment, i.p. administration of resveratrol retarded the growth of PA-1 cell xenograft and the expression of eEF1A2 in athymic nude mice in association with decreased bromodeoxyuridine positivity, reduced expression of proliferating cell nuclear antigen, increased the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and caspase-3 staining, and diminished CD31 positivity. Taken together, eEF1A2 may be considered as a potential molecular target for the antiproliferative effects of resveratrol in PA-1 ovarian cancer cells. [Cancer Res 2009;69(18):7449–58]

## Introduction

Ovarian cancer is a "silent killer" that represents one of the leading causes of death from gynecologic malignancies (1, 2). In the

majority of patients, the early-stage malignancy is barely detected and symptoms are evident after spreading to the surfaces of the peritoneal cavity. The overall 5-year survival rate in patients with early-stage disease is >90%, while advanced-stage patients have a survival rate of 15% to 30% only (2). Conventional approaches, such as cytoreductive surgery and chemotherapy (3), have limited success in controlling ovarian cancer due to chemotherapy resistance and high recurrence rate (4, 5).

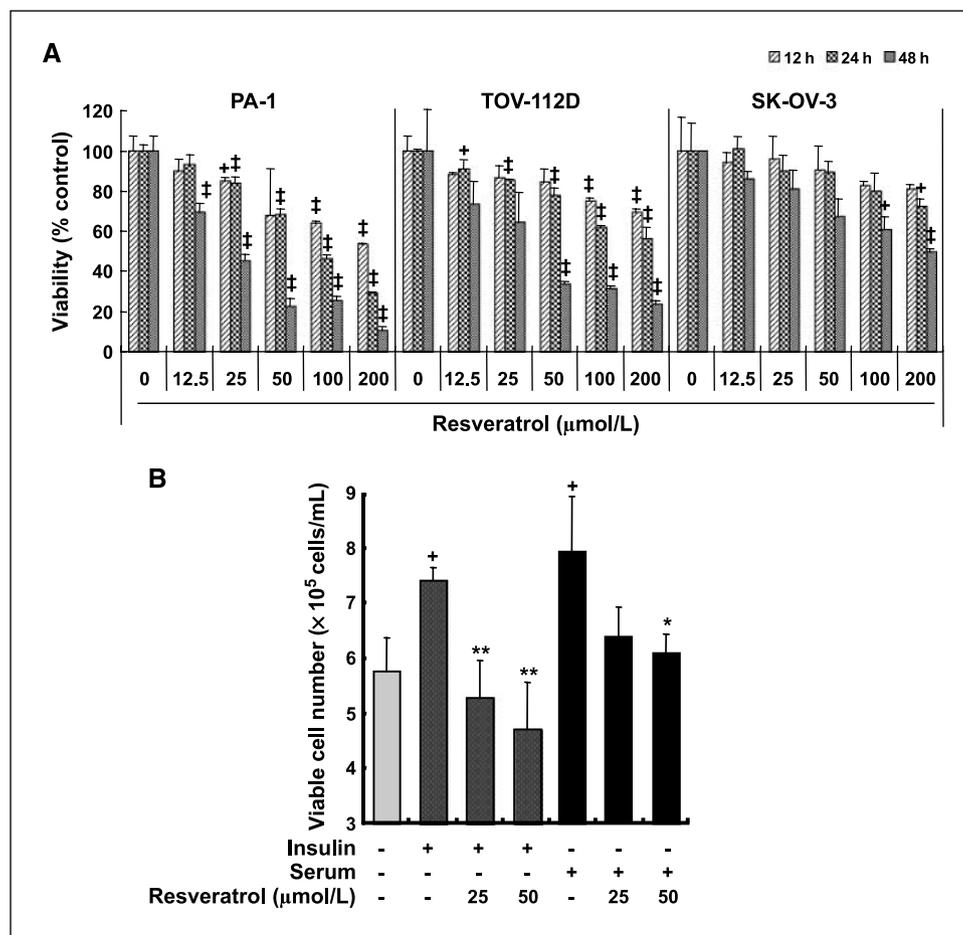
It has been well recognized that deregulated cell signaling pathways comprising a wide variety of proteins involved in cell proliferation, apoptosis, and angiogenesis largely account for the development and progression of cancer (6). The eukaryotic protein translation elongation factor appears to be a critical regulator of the protein repertoire of the cell. The eEF1A family of peptide elongation factor comprises two isoforms, eEF1A1 and eEF1A2, encoded by different chromosomes but sharing 98% homology (7). Whereas the eEF1A1 is widely expressed in almost all tissues, the eEF1A2 is normally expressed in brain, heart, and skeletal muscle (7–9). Recent studies have revealed that members of eEF1A family play a critical role in carcinogenesis (10, 11). The role of eEF1A2 in carcinogenesis has been addressed by Anand and colleagues (10), who have shown that eEF1A2 is overexpressed in 30% of ovarian tumors and established ovarian cancer cells. According to this study, the overexpression of eEF1A2 causes transformation of rodent fibroblasts (NIH3T3) cells and stimulates the growth of ovarian cancer (ES-2) cell xenograft in nude mice (10). Subsequent studies have revealed that eEF1A2 is overexpressed in primary breast tumors (12) and is positively correlated with the growth of hepatocellular carcinoma cells (13). Recently, eEF1A2 has been recognized as a biomarker for cancers of breast (12), lung (14), and prostate (15). Therefore, targeted inhibition of eEF1A2 may represent a pragmatic approach for the chemoprevention/chemotherapeutic intervention of ovarian cancer.

An adequate intake of fruits and vegetables is inversely correlated with the risk of ovarian cancer (16, 17). Resveratrol (*trans*-3,4',5-trihydroxystilbene), a phytoalexin present in grapes, has been extensively studied for its chemopreventive and chemotherapeutic effects in various cancer cells as well as in animal models (18). However, data on chemopreventive and/or chemotherapeutic potential of resveratrol in ovarian cancer are still limited. Resveratrol prevents angiogenesis (19), induces autophagocytosis (20), and alleviates chemotherapy resistance (21) in ovarian cancer cells. Here, we report that resveratrol inhibits insulin- or serum-induced growth of ovarian cancer PA-1 cells in culture and xenograft in nude mice by down-regulating the expression of eEF1A2 via the blockade of upstream Akt activation.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Effects of resveratrol on the growth of ovarian cancer cells. **A**, PA-1, TOV-112D, and SK-OV-3 cells were treated with resveratrol (12.5, 25, 50, 100, or 200 μmol/L) for 12, 24, and 48 h. The antiproliferative activity was determined by the MTT assay. +,  $P < 0.05$ ; †,  $P < 0.01$ . **B**, cell viability was determined by hemocytometer counts of trypan blue-excluding cells. Serum-starved PA-1 cells were stimulated with serum (1%) or insulin (10 μg/mL) plus serum. Number of viable cells was statistically analyzed by using Student's *t* test. \*,  $P < 0.05$ , serum versus resveratrol plus serum; \*\*,  $P < 0.01$ , insulin versus resveratrol plus insulin. All experiments were carried out in triplicate. +,  $P < 0.05$ , untreated versus insulin or serum.

## Materials and Methods

**Materials.** Resveratrol (purity >99%), insulin, and MTT were procured from Sigma-Aldrich. MEM, DMEM, RPMI 1640, and fetal bovine serum were purchased from Invitrogen (Life Technologies). Primary antibodies for caspase-3, -7, and -9, cleaved poly(ADP-ribose) polymerase, phospho-Akt, and Akt were obtained from Cell Signaling Technology. The eEF1A2 antibody was constructed by Takara Korea by changing the peptide sequence from mouse to human (22). Primary antibodies for proliferating cell nuclear antigen (PCNA) and CD31 were procured from Santa Cruz Biotechnology. Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Zymed Laboratory. LY294002 was purchased from Tocris.

**Cell culture and stable transfection.** PA-1, TOV-112D, and SK-OV-3 human ovarian cancer cells (American Type Culture Collection) were cultured in MEM supplemented with sodium pyruvate (1 mmol/L; for PA-1 and TOV-112D) and in RPMI 1640 enriched with 10% fetal bovine serum (for SK-OV-3), each containing 100 units penicillin and streptomycin. NIH3T3 cells were stably transfected with a blank vector or *eEF1A2* plasmid cloned into *EcoRI/XbaI* sites of pcDNA3.1-v5-HisA (Invitrogen) using Fugene-6 (Roche) and transfection was confirmed by using G418 (Roche). NIH3T3-vector and NIH3T3-eEF1A2 clones were grown in DMEM enriched with 10% bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 95% air/5% CO<sub>2</sub>.

**Cell proliferation assay.** Cells were cultured for 12, 24, and 48 h in the presence of varying concentrations of resveratrol dissolved in 0.1% DMSO. Cell viability was measured by MTT assay (23). For the trypan blue exclusion assay, PA-1 cells were kept starved for 48 h and then treated with different concentrations of resveratrol 1 h before addition of

serum or insulin. After 12 h, cells were harvested and counted after treatment with 0.4% trypan blue.

**Anchorage-independent growth assay.** PA-1 or NIH3T3 (vector-transfected or eEF1A2-transfected) cells (1 mL;  $2 \times 10^4$  cells/mL) suspended in 0.33% agarose solution were poured over hard-bottomed agar previously solidified in each 6-well plate. Cells were exposed to different concentrations of resveratrol twice a week for 3 weeks and incubated at 37°C in 95% air/5% CO<sub>2</sub>. Colonies (>100 μm) were scored and photographed after 3 weeks as described previously (24).

**Western blot analysis.** Serum-starved PA-1 cells were incubated with or without resveratrol or LY294002 for 1 h before treatment with insulin or serum for 12 h. Cells were lysed on ice for 30 min in cell lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na<sub>2</sub>EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] and centrifuged at  $20,817 \times g$  for 15 min. The supernatant was harvested as cellular protein extract. Protein samples were subjected to immunoblot analysis as described (24). Membranes were probed with antibodies against eEF1A2, actin, phospho-Akt (Ser<sup>473</sup>), and Akt and blots were visualized according to the procedure described previously (24).

**DNA fragmentation assay.** Serum-starved PA-1 cells were treated with resveratrol or LY294002 for 1 h followed by treatment with insulin (10 μg/mL) or serum (1%) for 12 h. Fragmented DNA in the cell lysates was extracted according to the procedure described earlier (25). The DNA fragments were separated by 1.8% agarose gel electrophoresis and visualized under UV light.

**Caspase-3 activity assay.** The caspase-3 activity was measured by using Ac-Asp-Glu-Val-Asp-*p*-nitroanilide (Biovision) as a substrate. Cells

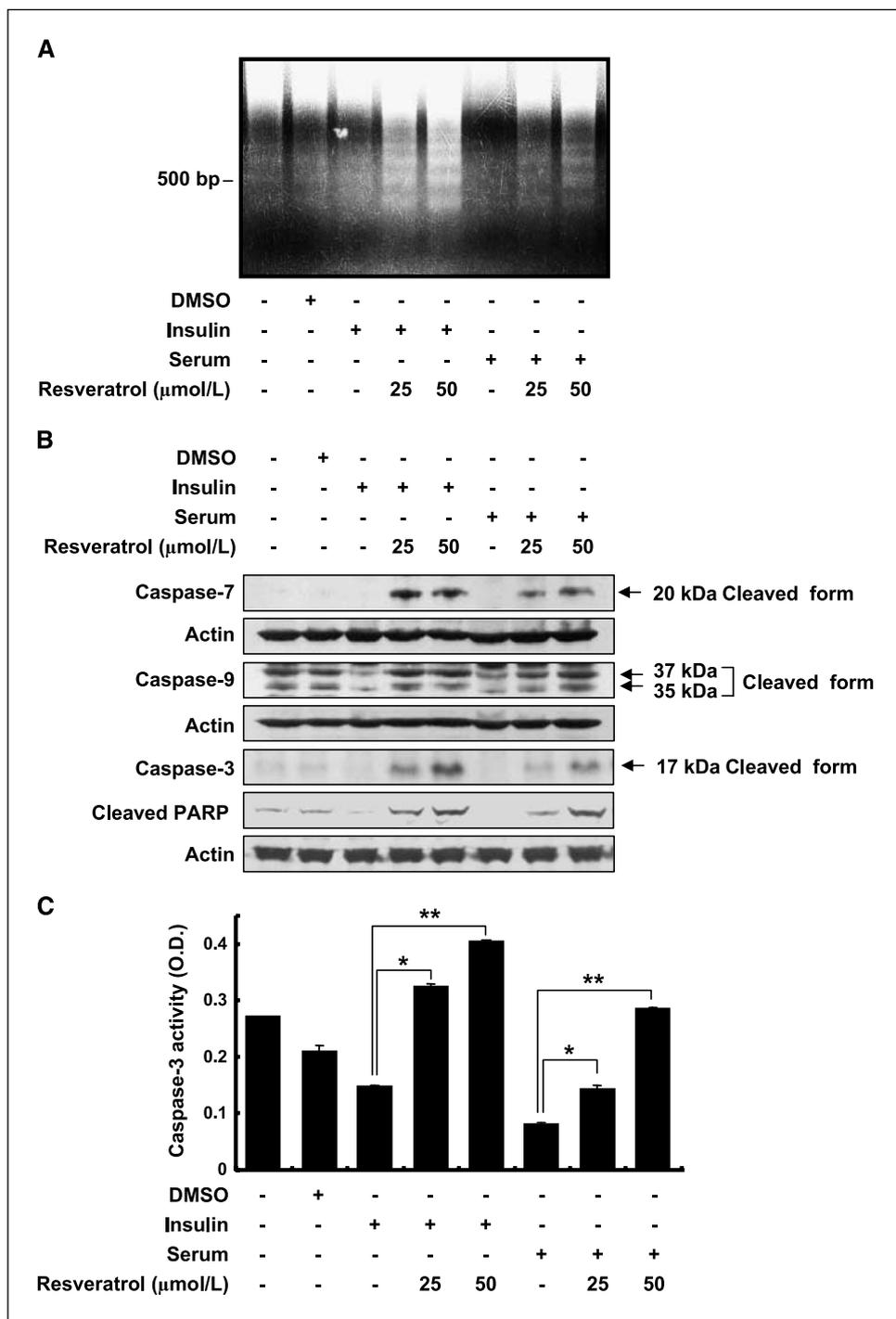
were incubated with insulin (10  $\mu\text{g}/\text{mL}$ ) or serum (1%) for 12 h in the presence or absence of resveratrol or LY294002. Whole-cell extracts (50  $\mu\text{L}$ ; adjusted to 80  $\mu\text{g}$  protein) were reacted with 50  $\mu\text{L}$  of 2 $\times$  reaction buffer containing DTT (10 mmol/L) and Ac-Asp-Glu-Val-Asp-*p*-nitroanilide (4 mmol/L) in a 96-well plate and incubated for 2 h at 37°C. Absorbance was measured at 405 nm.

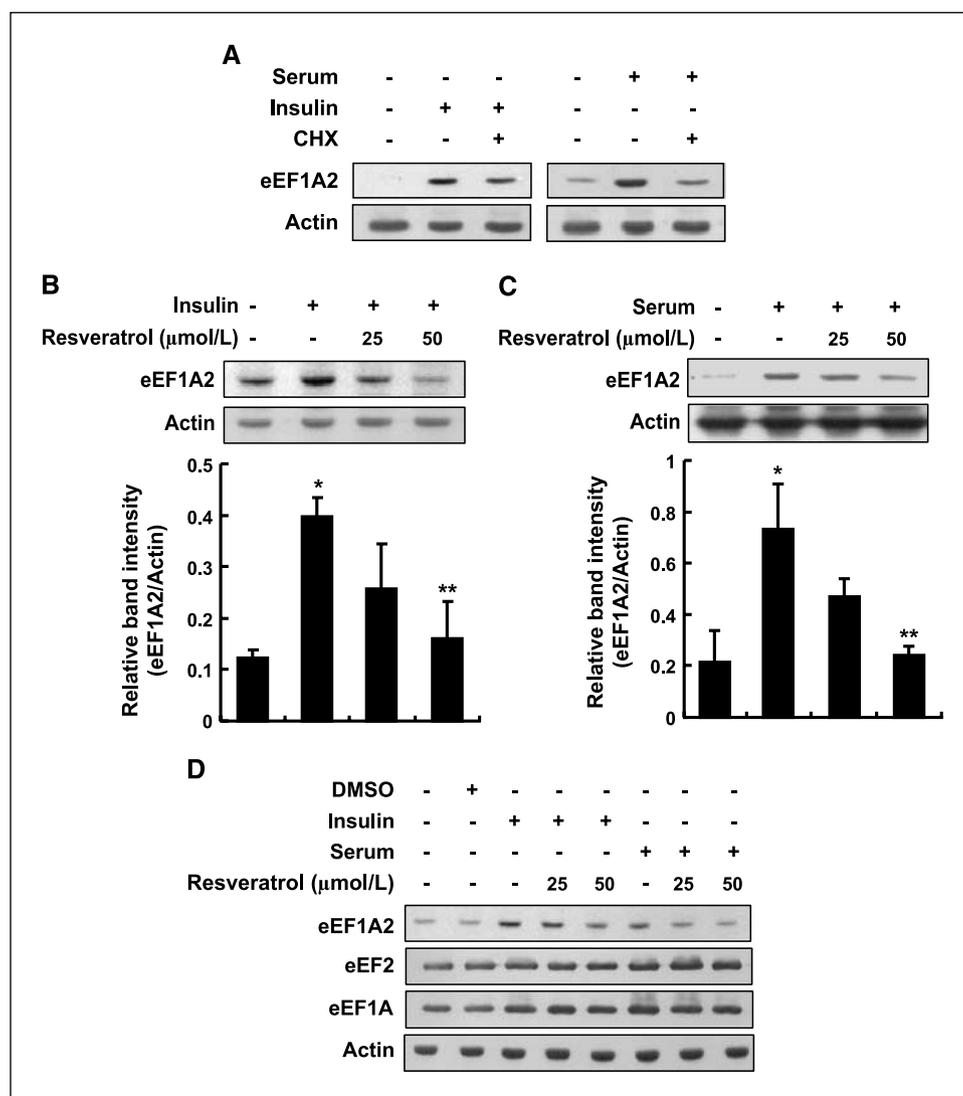
**Proteome profiler array.** Antibody array was done according to the manufacturer's protocol. Cell lysates were reacted with activated array membranes for overnight at 2°C to 8°C. Each array membrane was washed three times with 1 $\times$  wash buffer and incubated with the diluted antibody cocktail for 2 h at room temperature, washed three times with 1 $\times$  wash

buffer, and further incubated with streptavidin-horseradish peroxidase for 30 min at room temperature. Each array membrane was then washed three times with 1 $\times$  wash buffer and exposed with chemiluminescent reagents to X-ray film.

**Tumor growth in a xenograft model.** Female BALB/c (*nu/nu*) mice, 6 weeks old, were purchased from Charles River Laboratories (Japan) and were housed in a light/dark cycle of 12/12 h and fed with rodent chow and water *ad libitum*. All animal works were approved by the Seoul National University Ethics Research Board. PA-1 cells ( $1 \times 10^7$  in 200  $\mu\text{L}$  PBS) were injected s.c. on the right hind flank (26). Tumor volume (length  $\times$  width  $\times$  depth  $\times$  0.52) was measured three times a

**Figure 2.** Induction of apoptosis by resveratrol in PA-1 ovarian cancer cells. Serum-starved PA-1 cells were treated with resveratrol (25 or 50  $\mu\text{mol}/\text{L}$ ) for 1 h and then incubated in the presence of insulin (10  $\mu\text{g}/\text{mL}$ ) or serum (1%) for 12 h. **A**, DNA fragmentation assay was done as described in Materials and Methods. **B**, effects of resveratrol on the proteolytic cleavage of caspases and poly(ADP-ribose) polymerase (PARP). **C**, effect of resveratrol on caspase-3 activity. Each experiment was replicated for three times with three sets of samples each. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ .





**Figure 3.** Inhibition of insulin- or serum-induced eEF1A2 expression by resveratrol. **A**, effects of insulin or serum on the expression of eEF1A2 in PA-1 cells. Serum-starved cells were cultured in the presence or absence of cycloheximide (CHX) for 1 h and subsequently incubated with insulin or serum for additional 12 h. Whole-cell lysates were subjected to SDS-PAGE and immunoblotted with eEF1A2 antibody. Representative of three different experiments showing a similar trend. **B** and **C**, serum-starved PA-1 cells were pretreated with DMSO (lanes 1 and 2) or various concentrations of resveratrol for 1 h followed by stimulation with insulin (10 μg/mL) or fetal bovine serum (1%) for 12 h. Whole-cell extracts were separated by SDS-PAGE and immunoblotted with antibodies specific for eEF1A2 and actin. \*,  $P < 0.01$ , control versus insulin-treatment; \*\*,  $P < 0.05$ , insulin-treatment versus insulin plus resveratrol (**B**). \*,  $P < 0.05$ , control versus serum treatment; \*\*,  $P < 0.05$ , serum treatment versus serum plus resveratrol (**C**). **D**, effects of resveratrol on the expression of eEF1A2 and other peptide elongation factors in serum-starved PA-1 cells stimulated with insulin or serum.

week. After 10 days of implantation, two groups ( $n = 10$ ) were given resveratrol (dissolved in 5% ethanol and 25% polyethyleneglycol 400 in distilled water) i.p. at a daily dose of 50 or 100 mg/kg body weight for consecutive 4 weeks, whereas the other group received the vehicle only. Body weights were recorded everyday. Animals were given bromodeoxyuridine (BrdUrd; 10 mg/kg body weight, i.p.) 2 h before sacrifice. Xenograft tumors were weighed and frozen in liquid nitrogen or fixed in 10% formalin and embedded in paraffin. The BrdUrd-labeled cells in paraffin-embedded tissues were detected employing a monoclonal anti-BrdUrd antibody (Zymed Laboratory).

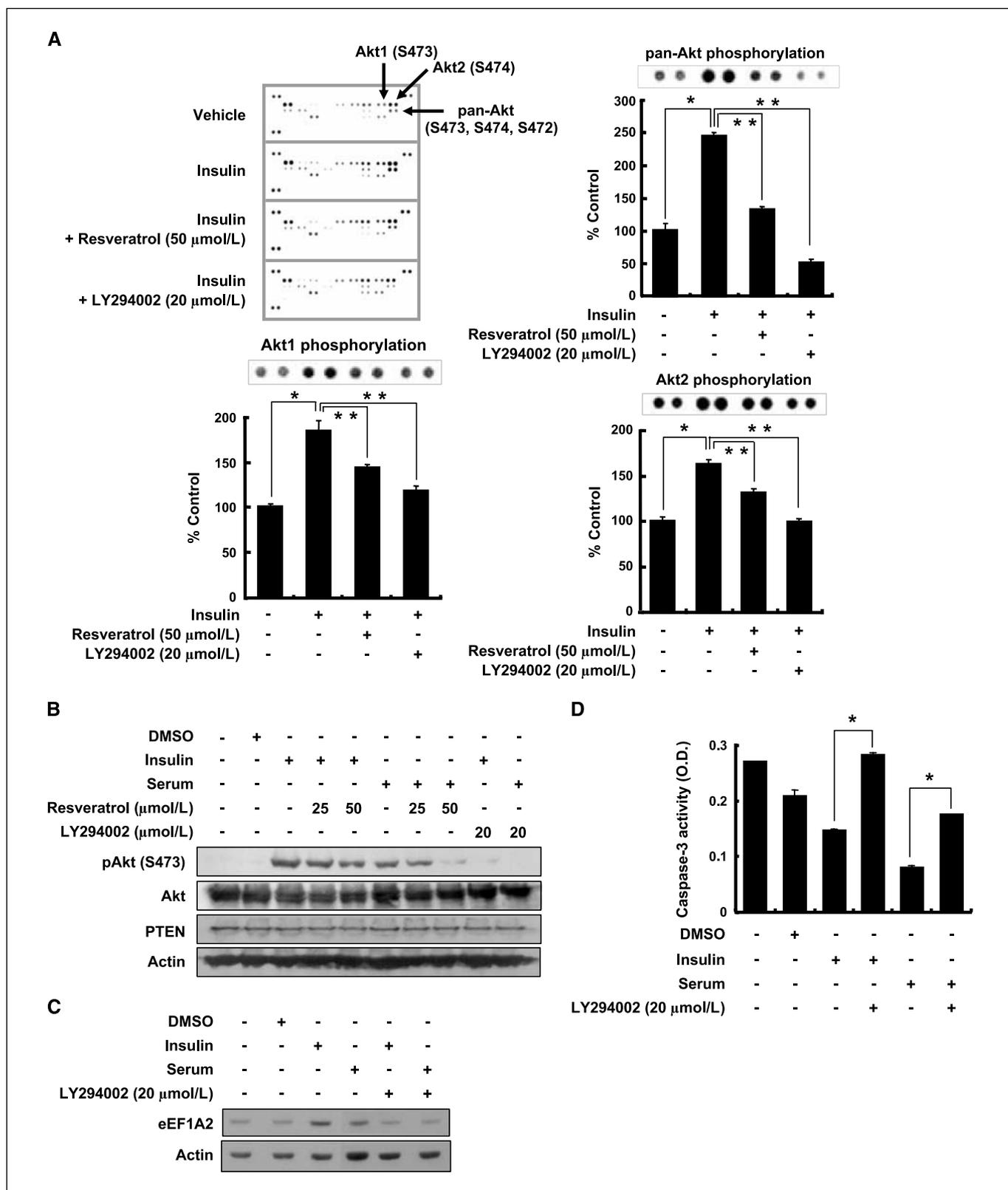
**Immunohistochemical analysis.** Slides using 4 μm sections of formalin-fixed and paraffin-embedded xenograft tumors from animals treated with or without resveratrol (50 or 100 mg/kg body weight) were prepared for immunohistochemical analysis as described previously (27). Slides were incubated separately with primary antibodies for eEF1A2 (1:200), PCNA (1:150), caspase-3 (1:50), or CD31 (1:50) and developed using anti-rabbit or anti-goat horseradish peroxidase Envision System (DAKO). Finally, counterstaining was done using Mayer's hematoxylin. Xenograft tissue sections were also subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (28).

**Statistical analyses.** Values were expressed as the mean  $\pm$  SE of at least three independent experiments. Statistical significance was determined by Student's  $t$  test and  $P < 0.05$  was considered to be statistically significant.

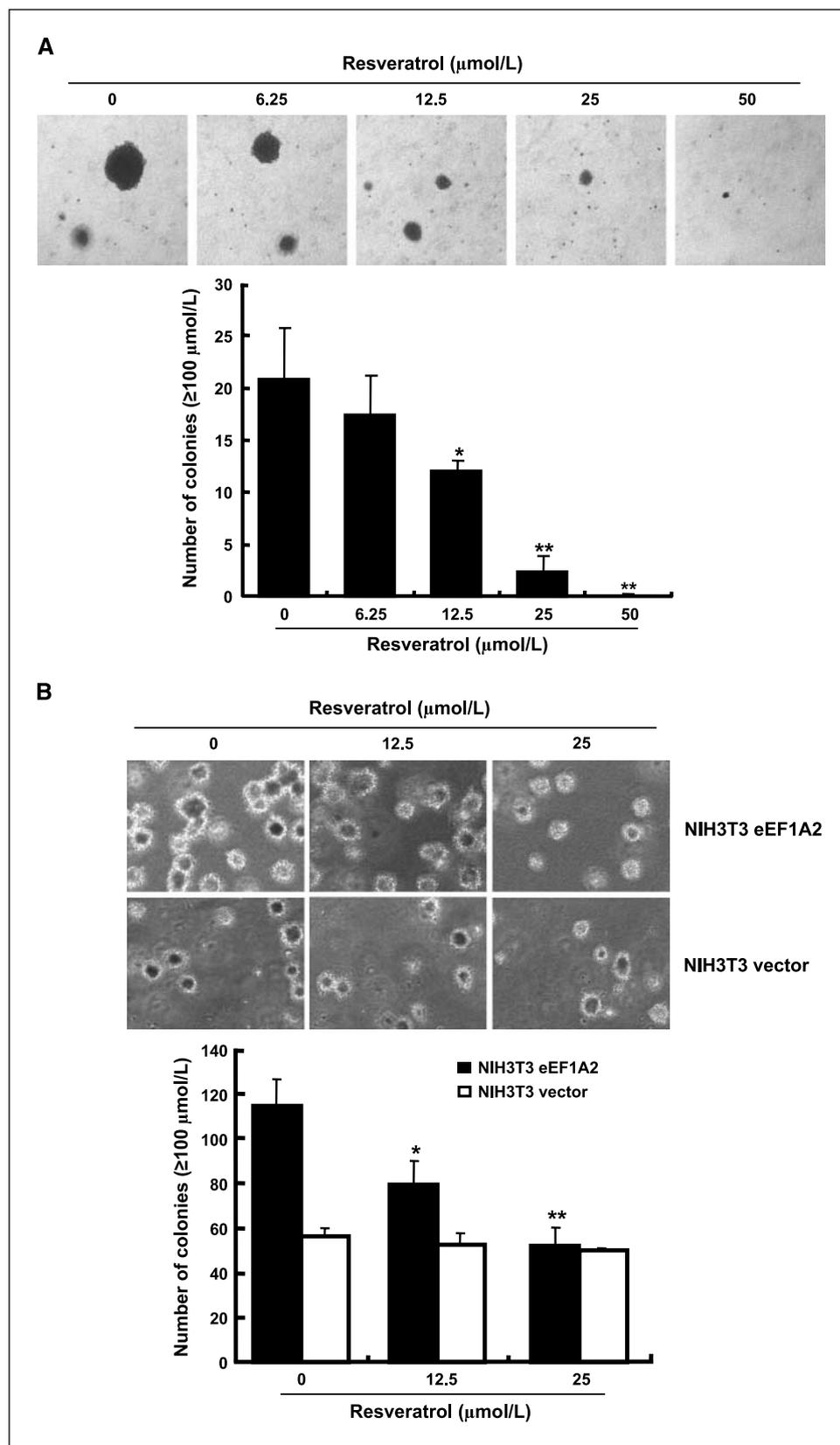
## Results

**Resveratrol inhibited serum- or insulin-induced growth of ovarian cancer PA-1 cells.** Treatment of human ovarian cancer (PA-1, TOV-112D, and SK-OV-3) cells with resveratrol (25, 50, 100, or 200 μmol/L) resulted in decreased cell viability in a concentration- and time-dependent fashion (Fig. 1A). Of these ovarian cancer cells, PA-1 cells were the most susceptible to growth inhibition by resveratrol. As shown in Fig. 1B, incubation of serum-starved PA-1 cells with insulin (10 μg/mL) or serum (1%) for 12 h increased cell viability, which was significantly diminished by pretreatment with resveratrol (25 or 50 μmol/L) in a concentration-dependent manner.

**Resveratrol induced apoptosis in PA-1 cells.** To clarify whether resveratrol-induced inhibition of PA-1 cell growth is partly attributed to its ability to induce apoptosis, we examined the effect of resveratrol on the activation of some representative caspases and the fragmentation of DNA, a biochemical hallmark of apoptosis. Pretreatment of PA-1 cells with resveratrol (25 or 50 μmol/L) caused fragmentation of DNA (Fig. 2A) as well as proteolytic activation of caspase-3, -7, and -9 and cleavage of poly(ADP-ribose) polymerase (Fig. 2B) in the presence of insulin



**Figure 4.** Effects of resveratrol on Akt phosphorylation and eEF1A2 expression in insulin- or serum-stimulated PA-1 cells. Cells were cultured in a serum-free medium and treated with resveratrol (25 or 50  $\mu\text{mol/L}$ ) or LY294002 (20  $\mu\text{mol/L}$ ) for 1 h followed by incubation in the presence of insulin or serum for 12 h. *A*, lysates from DMSO-, resveratrol-, or LY294002-treated cells were analyzed by antibody array using phospho-Akt antibody. The antibody array was composed of duplicate spots for each kinase on the single membrane. The experiment was done in triplicate and produced similar results. \*,  $P < 0.05$ , control versus insulin-treatment; \*\*,  $P < 0.05$ , insulin versus insulin plus resveratrol or insulin plus LY294002. *B*, effect of resveratrol on insulin-induced phosphorylation of Akt was confirmed by immunoblotting. *C*, effects of LY294002 on the expression of eEF1A2 in insulin- and serum-stimulated PA-1 cells. *D*, cell lysates from DMSO- or LY294002-treated cells were assayed for caspase-3 activity.



**Figure 5.** Effects of resveratrol on the anchorage-independent growth of PA-1 ovarian cancer cells and eEF1A2-transfected NIH3T3 cells. *A*, PA-1 cells were treated with resveratrol (6.25, 12.5, 25, or 50  $\mu\text{mol/L}$ ) twice a week for 3 wks in the soft-agar colony formation assay. *B*, NIH3T3-eEF1A2 or NIH3T3-vector cells were treated with resveratrol (12.5 or 25  $\mu\text{mol/L}$ ) as described in Materials and Methods. Colonies were counted using an inverted microscope (Nikon Diaphot 300, Japan). Colony counts from different treatment groups were subjected to statistical analysis. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , significantly different from vehicle control.

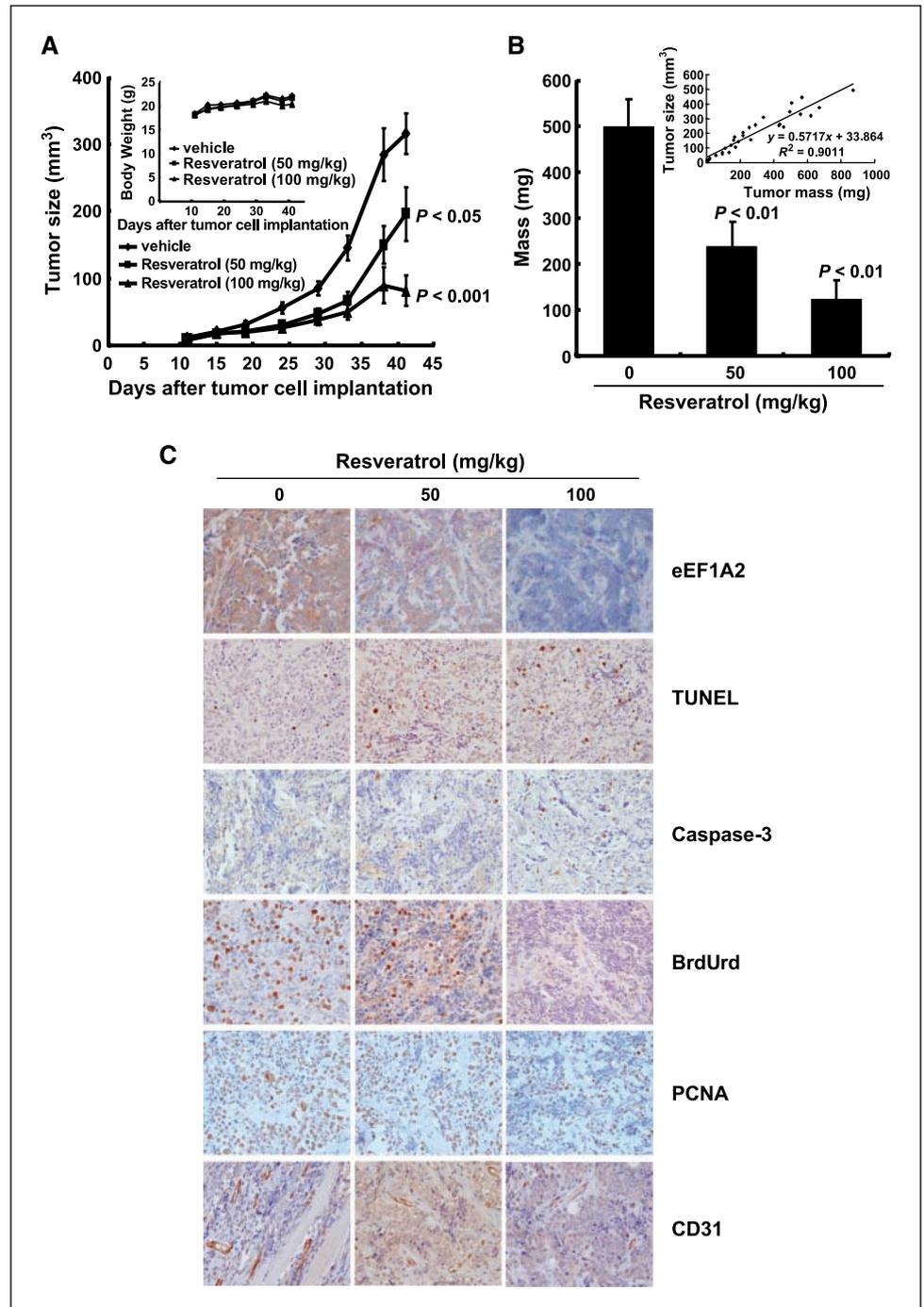
and serum. Under the same experimental conditions, resveratrol significantly increased the caspase-3 activity (Fig. 2C).

**Resveratrol down-regulated insulin- or serum-induced expression of eEF1A2 in PA-1 cells.** It has been reported that

eEF1A2 functions as an oncogene in various human malignancies (10, 12, 15) and protects against caspase-3-mediated apoptosis in differentiated myotubes (29). This prompted us to examine the role of eEF1A2 in insulin- or serum-induced proliferation of PA-1 cells

and its modulation by resveratrol. Incubation with insulin or serum induced the expression of eEF1A2 in serum-starved PA-1 cells, which was abolished by cycloheximide (Fig. 3A), suggesting that these growth stimuli can induce the *de novo* synthesis of eEF1A2. Pretreatment of PA-1 cells with resveratrol (50  $\mu$ mol/L) for 1 h significantly decreased insulin- or serum-induced expression of eEF1A2 (Fig. 3B and C), whereas treatment of cells with resveratrol alone decreased the basal expression of eEF1A2 (Supplementary Fig. S1). However, the insulin- or serum-induced expression of other peptide elongation factors, such as eEF1A1 and eEF2, remained unaltered by resveratrol pretreatment (Fig. 3D).

**Resveratrol inhibited eEF1A2 expression by blocking Akt in serum- or insulin-stimulated PA-1 cells.** To identify the upstream signaling kinases responsible for resveratrol-induced growth inhibition and apoptosis induction in insulin- or serum-stimulated PA-1 cells, we performed the proteome profiler antibody array analysis (Proteome Profiler; R&D Systems). This array can measure the relative levels of phosphorylation of nine representative mitogen-activated protein kinases, such as extracellular signal-regulated protein kinase 1/2, c-Jun NH<sub>2</sub>-terminal kinase 1-3, different p38 mitogen-activated protein kinase isoforms, and nine other serine/threonine kinases including Akt, glycogen synthase



**Figure 6.** Effects of resveratrol on the growth and proliferation of the ovarian tumor xenograft and eEF1A2 expression in athymic mice. *A*, effect of resveratrol on the volume of PA-1 cell xenograft tumor. *Inset*, comparison of body weight changes in mice treated with or without resveratrol. *B*, effect of resveratrol on the xenograft tumor mass. After 4 wks of treatment, xenograft tumors were collected by autopsy and weighed [vehicle control group 498.8  $\pm$  59.7 mg and resveratrol (50 mg/kg body weight)-treated group 237.9  $\pm$  53.1 mg;  $P < 0.01$ , compared with control; resveratrol (100 mg/kg body weight)-treated group 121.9  $\pm$  45.2 mg;  $P < 0.01$ , compared with control]. *Inset*, correlation between tumor mass and tumor volume. *C*, immunohistochemical analysis of eEF1A2, TUNEL, caspase-3, BrdUrd, PCNA, and CD31 expression in PA-1 cell xenograft tumors from vehicle- or resveratrol-treated groups. Effect of resveratrol on the proliferation of xenograft tumor as determined by BrdUrd staining. Treatment of animals and other experimental details are described in Materials and Methods. Magnification,  $\times 400$ .

kinase-3, and p70S6 kinase. We found that insulin induced phosphorylation of Akt1 (S473), Akt2 (S474), and pan (S473, S474, and S472), which was inhibited by resveratrol as well as the Akt inhibitor LY294002 (Fig. 4A). These results were further confirmed by Western blot analysis. PA-1 cells starved with serum-free medium for 48 h were treated with either resveratrol or LY294002 for 1 h before incubation with insulin or serum for additional 12 h. Intracellular levels of phospho-Akt were diminished by pretreatment with either resveratrol or LY294002 (Fig. 4B). However, the expression of PTEN, a negative regulator of phosphoinositide 3-kinase/Akt signaling, remained unchanged. To determine whether Akt could play a role in eEF1A2 up-regulation, the Akt inhibitor LY294002 was employed. As illustrated in Fig. 4C, pharmacologic inhibition of Akt abrogated eEF1A2 expression induced by insulin or serum. In addition, pretreatment of starved PA-1 cells with LY294002 restored the caspase-3 activity, which is prone to repression by insulin or serum supplementation (Fig. 4D), suggesting that Akt signaling is responsible, at least in part, for insulin- or serum-induced eEF1A2 expression and subsequent stimulation of proliferation in PA-1 cells.

**eEF1A2 as a molecular target for resveratrol-induced growth inhibition.** Resveratrol caused dose-dependent inhibition of anchorage-independent growth of PA-1 cells as revealed by the reduced number of colonies (Fig. 5A). To further investigate whether eEF1A2 is a plausible molecular target for antiproliferative activity of resveratrol, we examined the effect of resveratrol on anchorage-independent growth of NIH3T3 cells stably transfected with eEF1A2. As shown in Fig. 5B, there was a significant increase in the number of colonies in eEF1A2-overexpressing NIH3T3 cells (NIH3T3-eEF1A2) compared with that observed in the control (NIH3T3-vector) cells in the soft-agar colony formation assay. Treatment with resveratrol (12.5 or 25  $\mu\text{mol/L}$ ) dose-dependently attenuated the growth of NIH3T3-eEF1A2 cells compared with NIH3T3-vector control cells. Addition of insulin to the medium did not further increase the number of colonies in NIH3T3-eEF1A2 cells, but the average size of colonies was larger compared with that exhibited in the untreated cells (data not shown).

**Resveratrol attenuated the growth of PA-1 cell xenograft.** Because resveratrol suppressed the proliferation of PA-1 cells and diminished the expression of eEF1A2, we examined whether resveratrol could inhibit the growth of PA-1 cells transplanted to athymic mice and the expression of eEF1A2 *in vivo*. The average volume (Fig. 6A) and mass (Fig. 6B) of tumors were significantly reduced by resveratrol. The average tumor volume was reduced by treatment with resveratrol at a dose of 50 mg/kg body weight ( $195.5 \pm 124.8 \text{ mm}^3$ ;  $P < 0.05$ ) or 100 mg/kg body weight ( $81.7 \pm 70.5 \text{ mm}^3$ ;  $P < 0.001$ ) compared with the vehicle-treated animals ( $315 \pm 94 \text{ mm}^3$ ). There was a good correlation between the tumor volume and the tumor mass (Fig. 6B, *inset*). However, there were no significant differences in the body weight of animals between control and resveratrol-treated groups (Fig. 6A, *inset*). It was noticeable that the growth of PA-1 cell xenografts *in vivo* was associated with a marked increase in eEF1A2 expression (Fig. 6C), which was diminished by resveratrol. Immunohistochemical analysis revealed that resveratrol significantly inhibited the tumor cell proliferation as judged by decreased BrdUrd labeling and reduced PCNA expression. Compared with a vehicle control, PA-1 cell xenograft treated with resveratrol showed a marked increase in TUNEL positivity and caspase-3 staining (Fig. 6C). Moreover, resveratrol diminished the expression of CD31, a vascular endothelial cell marker (30), and microvessel density (Fig. 6C). Statistical

analysis of the TUNEL positivity, caspase-3, BrdUrd, and PCNA staining and that of microvessel density in PA-1 cell xenograft treated with or without resveratrol is presented in Supplementary Fig. S2.

## Discussion

Ovarian cancer is the leading cause of death from gynecologic malignancies (1). The early-stage ovarian cancer is asymptomatic, and ~75% of patients are diagnosed at an advanced stage (1). Despite substantial progress in the development of anticancer therapies, the 5-year survival rate of advanced-stage ovarian cancer patients still remains low (1, 31). An adequate intake of fruits and vegetables can halt or reverse the promotion and progression of certain cancers (6). Resveratrol, present in grapes and many other edible plants, has been extensively investigated as a promising candidate chemopreventive agent (18, 32). Resveratrol exerts its antitumorigenic effects by inhibiting the growth and inducing apoptosis in various cancer cells (18, 33). However, the molecular mechanisms underlying inhibitory effects of resveratrol on the growth of cancer cells remain elusive. In the present study, we report that resveratrol inhibits the growth of human ovarian cancer PA-1 cells through inhibition of cell proliferation and induction of apoptosis by targeting the protein elongation factor eEF1A2.

Insulin is one of the risk factors for ovarian cancer. High insulin levels have been suggested to cause obesity (34), which is associated with increased risk of ovarian cancer (35). Insulin, a growth stimulus for cancer cells, functions as a tumor promoter (34). It has been shown that insulin has high affinity for insulin-like growth factor-I receptor, which is overexpressed in ovarian carcinoma cells (36). A high incidence of ovarian cancer has been noted in insulin-treated type I diabetic patients (37). We therefore examined the antiproliferative effects of resveratrol in PA-1 cells on stimulation with insulin or serum. Incubation of serum-starved PA-1 cells with insulin and/or serum increased cell proliferation, which was significantly diminished by resveratrol pretreatment. The inhibitory effect of resveratrol on the growth of PA-1 cells was further confirmed by the significant suppression of anchorage-independent growth of these cells in a soft-agar colony formation assay. Tyagi and colleagues (38) also showed that resveratrol inhibited proliferation of PA-1 and SK-OV-3 cells in culture. Although resveratrol exerts antiproliferative effects by acting as a selective estrogen receptor modulator (39), the absence of estrogen receptor in PA-1 cells suggests that the antiproliferative effect of resveratrol in PA-1 cells is independent of estrogen receptor-mediated signaling.

Pretreatment of serum-starved PA-1 cells with resveratrol resulted in increased DNA fragmentation and caspase activation, even under growth stimulation by insulin or serum, corroborating the ability of resveratrol to induce apoptosis, which is in agreement with the induction of caspase-mediated apoptosis by the compound in A2780 ovarian cancer cells (20). In contrast, resveratrol did not alter cell viability in cisplatin-resistant A2780 (A2780/CP70) and OVCAR-3 ovarian cancer cells (19), suggesting that the compound exerts cell type-specific effects on the growth of ovarian cancer cells.

Although resveratrol has been widely investigated for its chemopreventive potential, a prime molecular target for its antiproliferative and apoptosis-inducing effects in ovarian cancer is yet to be identified. Within the myriad of cell signaling molecules implicated in cancer, resveratrol targets a series of protein

translation machinery (19). Resveratrol attenuated insulin-like growth factor-I-induced phosphorylation of different translation initiation factors in A2780/CP70 and OVCAR-3 cells (19). Besides translation initiation factors, peptide elongation factors have also been implicated in carcinogenesis (10, 40). Beyond its housekeeping role in protein synthesis, the peptide elongation factor eEF1A2 acts as an oncogene for cancers of the breast (12), liver (13), lung (14), pancreas (41), and ovary (10).

Because eEF1A2 functions as an inhibitor of caspase-mediated apoptosis as well as a putative oncogene for ovarian cancer (10, 29, 42), we examined the role of eEF1A2 in insulin- or serum-induced proliferation of ovarian cancer cells. Treatment of serum-starved PA-1 ovarian cancer cells with insulin or serum elevated the expression of eEF1A2, which was abrogated by pretreatment with cycloheximide, suggesting the *de novo* synthesis of eEF1A2 in response to growth stimuli. The significant decrease in insulin- or serum-induced expression of eEF1A2, but not of eEF1A1 and eEF2, by resveratrol suggests that eEF1A2 might be a prime target of resveratrol in retarding cell proliferation and inducing apoptosis in PA-1 cells. The significant increase in the number of colonies in eEF1A2-transfected NIH3T3 cells in comparison with vector-transfected NIH3T3 cells and its suppression by resveratrol further suggest that eEF1A2 is a potential molecular target for resveratrol-induced growth retardation.

Resveratrol is known to inhibit inappropriate activation of a panel of upstream kinases involved in cell proliferation and tumorigenesis (18, 32, 33). An antibody array directed to identify upstream cellular signaling molecules involved in the insulin-induced expression of eEF1A2 revealed an increase in the phosphorylation of Akt1 (at S473 residue), Akt2 (at S474 residue), and Akt pan (at S472, S473, and S474 residues), which were diminished by pretreatment with resveratrol or the Akt inhibitor LY294002. Consistent with our findings, resveratrol inhibited phosphorylation of Akt in A2780 cells (43) and insulin-like growth factor-I-stimulated A2780/CP80 and OVCAR-3 cells (19). Because Akt is a critical mediator of cell survival (44), the inhibition of insulin- or serum-induced phosphorylation of Akt in PA-1 cells by resveratrol suggests Akt as one of its key molecular targets.

Because eEF1A2 is phosphorylated on specific tyrosine residues by various tyrosine kinases (45), Akt may be an upstream regulator of eEF1A2. The suppression of insulin- or serum-induced expression of eEF1A2 in starved PA-1 cells preincubated with LY294002 suggests that Akt functions as an upstream regulator of eEF1A2. Because pretreatment of PA-1 cells with LY294002 restored caspase activity in the presence of insulin or serum, it can be inferred that resveratrol induced apoptosis in PA-1 cells by down-regulating eEF1A2 via suppression of Akt. In contrast, the expression of eEF1A2 causes activation of phosphoinositide 3-kinase/Akt, which in turn stimulates cell migration, invasion, and

filopodia formation in BT-549 cells (46). According to a recent study, eEF1A2 interacts with peroxiredoxin-I and inhibits apoptosis by down-regulating caspase-3 and -8 and up-regulating Akt in cultured cardiomyocytes and myotubes (47). Therefore, it would be worthwhile to examine if resveratrol induces apoptosis through modulation of peroxiredoxin-I and to clarify the presence of a positive feedback loop linking eEF1A2 expression and subsequent activation of phosphoinositide 3-kinase/Akt signaling.

The promoter region of eEF1A2 contains 12 E-boxes, 3 Egr family of proteins, 1 GATA motif, and 1 MEF2 binding site (48). However, insulin or serum stimulation of PA-1 cells failed to induce the DNA binding of Egr1 and MEF1 (data not shown). Analysis of promoter sequences of eEF1A2 revealed the presence of binding motifs for other transcription factors, such as activator protein-1, activator protein-2 $\alpha$ , and specificity protein-1. Incubation of PA-1 cells with insulin or serum also failed to induce the DNA binding of these transcription factors (data not shown). Further analysis of eEF1A2 promoter might help to identify transcription factors involved in insulin- or serum-induced transcriptional activation of eEF1A2.

Consistent with the inhibitory effects of resveratrol on the growth of other implanted cancer cells (49, 50), we show that the compound can also inhibit the growth of ovarian cancer cell xenografts. The decreased eEF1A2 expression in association with increased TUNEL positivity, caspase-3 activation, reduced BrdUrd labeling, and decreased PCNA expression in resveratrol-treated PA-1 cell xenograft is well correlated with the antiproliferative and apoptotic effects of the compound in the same animal tumor model. Moreover, the attenuation of CD31 expression and microvessel density in PA-1 cell-xenograft suggests the anti-angiogenic effect of resveratrol in ovarian cancer. In conclusion, the present study reveals that resveratrol significantly inhibits the growth of PA-1 human ovarian cancer cells in association with reduced eEF1A2 expression, suggesting this peptide elongation factor as a novel molecular target of resveratrol.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Resveratrol Suppresses Growth of Human Ovarian Cancer Cells in Culture and in a Murine Xenograft Model: Eukaryotic Elongation Factor 1A2 as a Potential Target

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