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# Resveratrol inhibits Erk1/2-mediated adhesion of cancer cells via activating PP2A/PTEN signaling network

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## Abstract

Resveratrol, a natural polyphenol compound, has been shown to possess anticancer activity. However, how resveratrol inhibits cancer cell adhesion has not been fully elucidated. Here, we show that resveratrol suppressed the basal or type I insulin-like growth factor (IGF-1)-stimulated adhesion of cancer cells (Rh1, Rh30, HT29 and HeLa cells) by inhibiting Erk1/2 pathway. Inhibition of Erk1/2 with U0126, knockdown of Erk1/2, or over-expression of dominant negative MKK1 strengthened resveratrol's inhibition of the basal or IGF-1-stimulated of Erk1/2 phosphorylation and cell adhesion, whereas ectopic expression of constitutively active MKK1 attenuated the inhibitory effects of resveratrol. Further research revealed that both PP2A and PTEN/Akt were implicated in resveratrol-inactivated Erk1/2-dependent cell adhesion. Inhibition of PP2A with okadaic acid or over-expression of dominant negative PP2A rendered resistance to resveratrol's suppression of the basal or IGF-1-stimulated phospho-Erk1/2 and cell adhesion, whereas expression of wild-type PP2A enhanced resveratrol's inhibitory effects. Over-expression of wild-type PTEN or dominant negative Akt, or inhibition of Akt with Akt inhibitor X strengthened resveratrol's inhibition of the basal or IGF-1-stimulated Erk1/2 phosphorylation and cell adhesion. Furthermore, inhibition of mTOR with rapamycin or silencing mTOR enhanced resveratrol's inhibitory effects on the basal and IGF-1-induced inhibition of PP2A/PTEN, activation of Akt/Erk1/2, and cell adhesion. The results indicate that resveratrol inhibits Erk1/2-mediated adhesion of cancer cells via activating PP2A/PTEN signaling network. Our data highlight that resveratrol has a great potential in the prevention of cancer cell adhesion.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

**Keywords**

Resveratrol; Cell adhesion; Erk1/2; PP2A; PTEN

## 1 INTRODUCTION

Resveratrol (3,4′,5-trihydroxystilbene; Res), a polyphenolic phytoalexin produced by grape and a few other plant species to protect them against fungal infections, possesses pharmacological activities such as cardioprotection, anti-aging, anti-inflammation and anti-carcinogenesis (Hasan and Bae, 2017; Park and Pezzuto, 2015). The anti-carcinogenic effect of resveratrol is attributed to its capability to inhibit proliferation, induce apoptosis and interfere with autophagy in cancer cells (Cucciolla et al., 2007; Shakibaei et al., 2009). Recently it has been reported that resveratrol exhibits anti-metastatic property in a spectrum of cancer cells (Bhattacharya et al., 2011; Salado et al., 2011; Yu et al., 2013). Adhesion of cancer cells to distant organs is a critical step during cancer metastatic process (Gupta and Massague, 2006; Steeg and Theodorescu, 2008). To date, several studies have shown that resveratrol reduced cell adherent ability in numerous malignancies (Bai et al., 2017; Mikula-Pietrasik et al., 2014; Park et al., 2009). However, how resveratrol inhibits cancer cell adhesion is not well understood.

Extracellular matrix (ECM), the non-cellular component present within all tissues and organs, is composed of water, proteins and polysaccharides (Frantz et al., 2010). ECM proteins, including collagen, elastin, fibronectin, vitronectin and laminin, mediate a wide variety of cellular interactions with ECM and play important roles in cell adhesion and migration (Frantz et al., 2010; Cescon et al., 2015; Pankov and Yamada, 2002; Yao, 2017). Cell adhesion to the ECM is mediated by ECM receptors (Humphries et al., 2006; Harburger and Calderwood, 2009; Leitinger and Hohenester, 2007). Integrins are the major receptors on the cell surface for adhesion to ECM (Hynes, 2002).

Cell adhesion is a multistep cellular process that is regulated by complex extracellular and intracellular signals (Homrich et al., 2015; Ridley et al., 2003). Increasing evidences have shown that extracellular signal-regulated kinase 1/2 (Erk1/2), an important member of mitogen-activated protein kinase (MAPK) family, is involved in cell adhesion regulation (Wang et al., 2013a; Zennadi et al., 2012). For example, shikonin inhibits proliferation, adhesion, migration and invasion by suppressing integrin β1 expression and the Erk1/2 pathway in A549 lung cancer cells (Wang et al., 2013a). Additionally, it has been reported that resveratrol regulates Erk1/2 activity in cancer cells (Kato et al., 2015; Kulkarni and Canto, 2015). For instance, resveratrol suppresses the Akt-GSK3β and Erk1/2 signaling pathways, resulting in reduction of nuclear cyclin D1 expression and cell cycle arrest in pancreatic cancer cells (Kato et al., 2015). Hence, we hypothesized that resveratrol may inhibit cancer cell adhesion by regulating Erk1/2 pathway.

Protein phosphatase 2A (PP2A) and phosphatase and tensin homologue on chromosome 10 (PTEN) are two tumor suppressor phosphatases, whose loss of function has been observed in many cancers (Kalev and Sablina, 2011; Perrotti and Neviani, 2013; Xu et al., 2014). Interestingly, recent studies have shown that both PP2A and PTEN negatively regulate

Erk1/2 pathway in several malignancies (Chetram and Hinton, 2012; Xie et al., 2015). Besides, PTEN negatively regulates Akt by its lipid phosphatase activity and Akt activates Erk1/2 through protein kinase C (PKC)/Raf signaling axis (Chetram and Hinton, 2012; Polak and Hall, 2009). Importantly, PP2A, PTEN and Akt function by regulating cell adhesion and migration (Haier and Nicolson, 2002; Kraus et al., 2002; Sontag and Sontag, 2006). In addition, studies have shown that resveratrol regulates PP2A, PTEN and Akt (Liu et al., 2015; Liu et al., 2014). Collectively, these findings suggest that resveratrol may affect Erk1/2 and cancer cell adhesion through mediating PP2A and PTEN/Akt signaling pathway.

Mechanistic/mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is a central controller of cell growth and metabolism (Laplante and Sabatini, 2012; Yang et al., 2013). It is dysregulated in many human cancers and contributes to cancer pathogenesis and therapy resistance (Guertin and Sabatini, 2007). mTOR lies downstream of type I insulin-like growth factor (IGF-1) receptor and functions at least as two complexes (mTORC1 and mTORC2) in mammalian cells (Laplante and Sabatini, 2012; Yang et al., 2013). mTORC1 phosphorylates p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1), while mTORC2 regulates phosphorylation or activity of Akt, glucocorticoid-inducible kinase 1 (SGK1), PKCα, focal adhesion proteins and small GTPases (Laplante and Sabatini, 2012; Yang et al., 2013). Recent studies have demonstrated that resveratrol inhibits mTOR signaling (Widlund et al., 2013). Interestingly, we recently found that both mTORC1 and mTORC2 are involved in the regulation of cell adhesion (Chen et al., 2015b). Taken together, we postulated that resveratrol might inhibit cancer cell adhesion via mediating PP2A/PTEN-Erk1/2 signaling network in an mTOR kinase activity-dependent manner.

Here we show that resveratrol attenuates IGF-1-induced adhesion of cancer cells in part by suppressing Erk1/2 pathway. Mechanistically, resveratrol blocks Erk1/2 pathway, not only by activating PP2A, but also via activating PTEN and inactivating Akt, thereby preventing IGF-1-induced adhesion in cancer cells. Our findings underline a potential beneficial role of resveratrol in the treatment of cancer, especially in the prevention of cancer cell adhesion.

## 2 MATERIALS AND METHODS

### 2.1 Materials

Resveratrol, collagen type IV (CN IV), fibronectin, laminin, U0126 and okadaic acid were purchased from Sigma (Saint Louis, MO, USA). Resveratrol was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution (100 mM), aliquoted and stored at −80˚C. IGF-1 was from PeproTech (Rocky Hill, NJ, USA), rehydrated in 0.1 M acetic acid to prepare a 10 μg/ml stock solution and stored at −80°C. RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM) and 0.05% trypsin-EDTA were obtained from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Akt inhibitor X was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA). CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay Kit was from Promega (Madison, WI, USA). Enhanced chemiluminescence solution was from Millipore (Billerica, MA, USA). Other chemicals were purchased from local commercial sources and were of analytical grade.

### 2.2 Cell line and culture

Human Ewing sarcoma (Rh1) and rhabdomyosarcoma (Rh30) cells were described previously (Chen et al., 2015b) and cultured in antibiotic-free RPMI 1640 medium supplemented with 10% FBS. Colon carcinoma (HT29) and cervical adenocarcinoma (HeLa) cells, obtained from American Type Culture Collection (Manassas, VA, USA), were grown in antibiotic-free DMEM supplemented with 10% FBS. The cells were maintained in a humid incubator (37°C, 5% $CO_2$).

### 2.3 Recombinant adenoviral constructs and infection of cells

The recombinant adenoviruses encoding FLAG-tagged constitutively active MKK1 (MKK1-R4F), dominant-negative MKK1 (MKK1-K97M), hemagglutinin (HA)-tagged dominant-negative PP2A catalytic subunit (dn-PP2A, L199P), FLAG-tagged wild-type rat PP2Acα (Ad-PP2A), wild-type human PTEN (Ad-PTEN), hemagglutinin (HA)-tagged dominant-negative Akt (dn-Akt, T308A/S473A) and the control vector expressing green fluorescent protein (GFP) alone (Ad-GFP) were described previously (Chen et al., 2009; Liu et al., 2010a). To construct recombinant adenoviruses expressing FLAG-tagged MKK1-R4F and MKK1-K97M, DNA fragments encoding the corresponding mutants were excised from pMCL-MKK1-R4F and pMCL-MKK1-K97M (Mansour et al., 1994) (gifts from Dr. Natalie Ahn, University of Colorado, Boulder, CO, USA), and then sub-cloned to FLAG-tagged pENTR11 shuttle vector. The recombinant adenovirus was generated using ViraPower™ Adenoviral Gateway™ Expression Kit (Invitrogen, Carlsbad, CA, USA) following the manufacture's instruction. For experiments, Rh30 and HeLa cells were grown in the growth medium and infected with the individual adenovirus for 24 h at 1 of multiplicity of infection (MOI = 1). Subsequently, cells were used for experiments. Ad-GFP served as a control. Expression of FLAG-tagged MKK1-R4F, MKK1-K97M and PP2A, as well as HA-tagged dn-PP2A and dn-Akt was determined by Western blot analysis with antibodies to FLAG and HA, respectively.

### 2.4 Lentiviral shRNA cloning, production, and infection

Lentiviral shRNAs to Erk1/2, mTOR and GFP (for control) were generated and used as described (Chen et al., 2008; Chen et al., 2015b). Rh30 and HeLa cells, when grown to about 70% confluence, were infected with above lentivirus-containing supernatant in the presence of 8 μg/ml polybrene for 24 h and then exposed to 2 μg/ml puromycin for 48 h. In 5 days, cells were used for experiments.

### 2.5 Cell adhesion assay

Cell adhesion was evaluated by using CN IV-, fibronectin- or laminin-coated assay, as described previously (Sawhney et al., 2004). Briefly, 48-well tissue culture plates were coated for 2 h at 37°C with CN IV (0.2 μg/ml), fibronectin (0.5 μg/ml) or laminin (0.5 μg/ml) respectively, followed by blocking with 3% bovine serum albumin for 3 h, and then rinsed once with PBS. Cells for determination of adhesion functions were changed to serum-free medium and grown for 24 h. After trypsinization, serum-starved cells were plated at $5 \times 10^4$ cells/well on CN IV, fibronectin or laminin-coated plates and incubated for 4 h in the absence or presence of resveratrol (0-100 μM) and/or IGF-1 (10 ng/ml), with 6 replicates of

each treatment. Non-adherent cells were removed by washing three times with serum-free medium. Afterwards, each well was added 20 μl of MTS reagent (one solution reagent) and incubated for 1 h. The relative number of attached cells was determined by measuring the optical density (OD) at 490 nm using a Victor X3 Light Plate Reader (PerkinElmer, Waltham, MA, USA).

### 2.6 Wound-healing assay

Cell motility was assessed by the wound-healing assay as described (Liu et al., 2006). Briefly, a monolayer of cells were grown in 6-well plates to 80% confluence and serum starved for 24 h, with 6 replicates of each treatment. Migration was initiated by removing a portion of the cell layer by scratching with a single-edge razor blade cut to ~27 mm in length. The scratch began at the diameter of the dish and extended over an area ~10 mm wide. The medium was changed to remove floating or damaged cells. Cells were pretreated with or without resveratrol (0–100 μM) for 4 h, followed by stimulation with or without IGF-1 (10 ng/ml) for 20 h. Cells migrated over the denuded area were observed and taken under a Leica inverted phase-contrast microscope equipped with Quick Imaging system (Leica DMi8, Wetzlar, Germany). The number of cells migrating per millimeter of scratch was counted.

### 2.7 Cell viability assay

Cell viability was evaluated using MTS assay, as described (Chen et al., 2014). Briefly, cells were seeded in a 96-well plate at a density of $1 \times 10^4$ cells/well in the growth medium and grown overnight at 37°C in a humidified incubator with 5% $CO_2$. After serum-starvation for 24 h, cells were treated with 0–100 μM resveratrol for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h, with 6 replicates of each treatment. Subsequently, cell viability, after incubation with MTS reagent (one solution reagent) (20 μl/well) for 3 h, was evaluated by measuring the optical density (OD) at 490 nm using a Victor X3 Light Plate Reader (PerkinElmer, Waltham, MA, USA).

### 2.8 Assay for live cell number

Live cell number was counted by using Trypan blue exclusion as described previously (Chen et al., 2014). In short, serum-starved cells, seeded in 6-well plates at a density of $2 \times 10^5$ cells/well, were treated with 0–100 μM resveratrol for 4 h and stimulated with/without IGF-1 (10 ng/ml) for 1 h, with 6 replicates of each treatment. Then, the floating and attached cells were collected and incubated with 0.4% trypan blue solution (Sigma, Saint Louis, MO, USA) for 3 min at room temperature. Afterwards, the viable (unstained) and nonviable (stained) cells in the trypan blue/cell mixture were counted separately under a microscope using a hemacytometer. Finally, the relative number of live cells was presented by calculating the ratio of the unstained cells / the total cells.

### 2.9 *In vitro* PP2A phosphatase assay

*In vitro* PP2A phosphatase activity was determined as described (Liu et al., 2010a), with some modifications. Briefly, after serum-starvation for 24 h, cells were incubated for 4 h in the absence or presence of resveratrol (0–100 μM) ± IGF-1 (10 ng/ml), with 6 replicates of

each treatment. Subsequently, the cells were lysed in 50 mM Tris-HCl buffer, pH 7.0, containing 1% Nonidet P-40, 2 mM EDTA, and protease inhibitor cocktail (Sigma, Saint Louis, MO, USA. 1:1000). PP2Ac in cell lysates was immunoprecipitated with antibodies to PP2Ac (Millipore, Temecula, CA, USA), and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the beads were washed three times with the above lysis buffer, followed by washing twice with the phosphatase assay buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM CaCl2). The phosphatase activity of immunoprecipitated PP2A was assayed with a Ser/Thr Phosphatase Assay kit 1 using p-nitrophenyl phosphate (pNPP) as the substrate (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. Finally, all data pooled from three different batches of experiments were statistically analyzed.

### 2.10 Western blot analysis

Western blotting was performed in three independent experiments, as described previously (Chen et al., 2014). Briefly, the indicated cells, after treatments, were washed with cold PBS, and then on ice, lysed in the radioimmunoprecipitation assay buffer. After that, lysates containing equivalent amounts of protein were separated on 6–12% sodium dodecyl sulfate - polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% nonfat dry milk to block nonspecific binding, and then with primary antibodies against phospho-Akt (p-Akt) (Thr308), p-Akt (Ser473), p-S6K1 (Thr389) and mTOR (Cell Signaling Technology, Danvers, MA, USA), PP5, p-Erk1/2 (Thr202/Tyr204), Erk2, demethylated-PP2A, Akt and S6K1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PP2Acα (BD Biosciences, San Jose, CA, USA), p-PP2A, p-PTEN (Thr366) and PTEN (Epitomics, Burlingame, CA, USA), PP2A-A subunit, PP2A-B subunit (Millipore, Bedford, MA, USA), MKK1, FLAG, HA and β-tubulin (Sigma, Santa Cruz, CA, USA) overnight at 4°C, respectively, followed by incubation with appropriate secondary antibodies including horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG (Pierce, Rockford, IL, USA) overnight at 4°C. Immunoreactive bands were visualized by using enhanced chemiluminescence solution (Millipore, Bedford, MA, USA). The blots for detected proteins were semi-quantified using NIH Image J software (National Institutes of Health, Bethesda, MD, USA).

### 2.11 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistically significant differences between treatment means were identified by using the Student's t-test for non-paired replicates. One-way or two-way ANOVA followed by Bonferroni's post-tests to compare replicate means was conducted to compare group variability and interaction. *p*-value of $<0.05$ was considered significant.

## 3 RESULTS

### 3.1 Resveratrol attenuates IGF-1-stimulated adhesion of cancer cells

Extracellular matrix (ECM) is tissue-specific due to unique composition and topology (Frantz et al., 2011). To determine the general cell adhesion function, representative ECM

substrates (CN IV, fibronectin and laminin) were selected as markers of cell adhesion assay according to our previous study (Chen et al., 2015b). To investigate the effect of resveratrol on cancer cell adhesion, human Ewing sarcoma (Rh1), rhabdomyosarcoma (Rh30), colon carcinoma (HT29) and cervical adenocarcinoma (HeLa) cells, after serum-starved for 24 h, were treated with 0–100 μM resveratrol for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. Adherent cells were determined using CN IV-, fibronectin- or laminin-coated cell adhesion assay. As shown in Figure 1a, exposure to resveratrol inhibited IGF-1-stimulated cancer cell adhesion in a concentration-dependent manner. In addition, we also determined the effect of resveratrol on cancer cell motility using the wound-healing assay, showing that treatment with resveratrol also inhibited IGF-1-stimulated cancer cell motility in a concentration-dependent manner (Figure S1). To exclude the possibility that resveratrol inhibits IGF-1-stimulated cancer cell adhesion by reducing cell viability or inducing cell death, we also examined the effect of resveratrol on cell viability and live cell number using MTS assay and trypan blue exclusion, respectively. As shown in Figure 1b, c, treatment with resveratrol (0–100 μM) for 4 h and then stimulation with/without IGF-1 (10 ng/ml) did not significantly influence cell viability or live cell number in all cell lines tested (Rh1, Rh30, HT29 and HeLa). The results indicate that resveratrol attenuates IGF-1-stimulated adhesion of cancer cells, which is not through reducing cell viability or live cell number.

### 3.2 Resveratrol intervenes in IGF-1-stimulated inhibition of PP2A and activation of Erk1/2 in cancer cells

Resveratrol may activate or inhibit Erk1/2, depending on the concentration of resveratrol used (Kato et al., 2015; Miloso et al., 1999). Besides, resveratrol can regulate PP2A activity (Liu et al., 2015). Importantly, both Erk1/2 and PP2A are involved in the control of cell adhesion (Sontag and Sontag, 2006; Wang et al., 2013a). To explore the molecular mechanism of resveratrol-inhibited cancer cell adhesion, we checked the effect of resveratrol on Erk1/2 and PP2A activity in cancer cells. As shown in Figure 2a, b, resveratrol inhibited IGF-1-stimulated phosphorylation of Erk1/2 in a concentration-dependent manner, suggesting a reduction of Erk1/2 activity in cancer cells. In addition, resveratrol did not alter cellular protein levels of PP2Ac, PP2A-A or PP2A-B, but reduced IGF-1-induced demethylated-PP2Ac (de-PP2A) and phospho-PP2Ac (p-PP2A) in a concentration-dependent manner, indicating that resveratrol activates PP2A. This is further supported by the finding that resveratrol attenuated IGF-1-induced decrease of PP2A activity, as determined by the *in vitro* Ser/Thr phosphatase assay (Figure 2c). To make sure that PP2A is actually the only protein specifically immunoprescipitated, we also conducted Western blot analysis for PP5. As expected, only PP2Ac, but not PP5, was seen in the immunoprecipitates with anti-PP2Ac antibody (Figure 2a), indicating that *in vitro* PP2A phosphatase assay is specific for PP2Ac.

### 3.3 Resveratrol suppresses IGF-1-stimulated cell adhesion through blocking Erk1/2 pathway in cancer cells

To unveil whether resveratrol inhibition of IGF-1-stimulated cancer cell adhesion is related to resveratrol blockage of Erk1/2 activation, Rh30 and HeLa cells was pre-incubated with/without U0126 (a selective inhibitor of MKK1/2, upstream of Erk1/2) alone, or in combination with resveratrol. We found that U0126 (5 μM) or resveratrol (100 μM) alone

obviously suppressed the basal and IGF-1-stimulated phosphorylation of Erk1/2 (Figure 3a, b). Furthermore, co-treatment with resveratrol/U0126 exhibited a stronger inhibitory effect on the basal and IGF-1-induced phospho-Erk1/2 (p-Erk1/2) in the cells (Figure 3a, b). Interestingly, the combination of resveratrol with U0126 also exhibited more potent inhibition of IGF-1-induced cell adhesion than resveratrol or U0126 alone (Figure 3c).

We also confirmed the above findings using RNA interference. As detected by Western blotting, lentiviral shRNA to Erk1/2, but not GFP, silenced the expression of Erk1/2 protein by ~90%, and silencing Erk1/2 blocked the basal and IGF-1-induced phosphorylation of Erk1/2 in Rh30 cells (Figure 3d, e) and HeLa cells (data not shown). Consistently, down-regulation of Erk1/2 reduced the basal and IGF-1-stimulated cell adhesion in Rh30 and HeLa cells (Figure 3f). Importantly, addition of resveratrol exhibited more inhibitory effects on IGF-1-induced cell adhesion in the cells infected with lentiviral shRNA to Erk1/2 than in the control cells infected with lentiviral shRNA to GFP (Figure 3f). In addition, we also employed recombinant adenoviruses Ad-MKK1-R4F and Ad-MKK1-K97M, encoding FLAG-tagged constitutively active and dominant negative MKK1, respectively. Infection with Ad-MKK1-R4F and Ad-MKK1-K97M, but not Ad-GFP (control virus), resulted in expression of high levels of FLAG-tagged MKK1 mutants in Rh30 cells (Figure 3g). Expression of MKK1-R4F led to robust phosphorylation of Erk1/2, whereas expression of MKK1-K97M downregulated phosphorylation of Erk1/2 in the cells (Figure 3g, h), indicating that the MKK1 mutants function in the cells as expected. Similar results were seen in HeLa cells infected by Ad-MKK1-R4F and Ad-MKK1-K97M (data not shown). Of note, expression of MKK1-R4F in Rh30 and HeLa cells significantly elevated the basal or IGF-1-stimulated cell adhesion, and conferred profound resistance to resveratrol's inhibitory effects (Figure 3i). In contrast, expression of MKK1-K97M in the cells dramatically reduced the basal or IGF-1-stimulated cell adhesion, and especially addition of resveratrol reinforced the events (Figure 3i). The results clearly indicate that resveratrol suppresses IGF-1-stimulated cell adhesion in part by blocking Erk1/2 pathway in cancer cells.

### 3.4 Resveratrol inhibits IGF-1-stimulated Erk1/2 activation and cell adhesion via PP2A-dependent manner in cancer cells

Since PP2A plays a crucial role in the regulation of Erk1/2 pathway (Xie et al., 2015), we next asked whether resveratrol's blockage of IGF-1-induced activation of Erk1/2 pathway and consequential cell adhesion was due to activation of PP2A. Rh1 and HeLa cells were pretreated with 100 nM okadaic acid (a relatively specific PP2A inhibitor) alone, or in combination with 100 μM resveratrol, followed by incubation with/without 10 ng/ml IGF-1. As shown in Figure 4a, b, IGF-1 markedly stimulated the expression of de-PP2Ac, p-PP2Ac and p-Erk1/2, which was inhibited by resveratrol. Okadaic acid profoundly increased the basal or IGF-1-stimulated de-PP2Ac, p-PP2Ac and p-Erk1/2, and reversed the inhibitory effect of resveratrol on IGF-1-stimulated events (Figure 4a, b). In line with this, resveratrol inhibited the basal or IGF-1 stimulated cell adhesion in Rh1 and HeLa cells, which was significantly attenuated by okadaic acid (Figure 4c). These results imply that resveratrol inhibits the basal or IGF-1-induced Erk1/2 phosphorylation and cell adhesion through upregulation of PP2A activity in cancer cells.

To gain more insights into the event that the upregulated PP2A activity is responsible for the inhibitory effects of resveratrol on the basal or IGF-1-induced Erk1/2 activation and cell adhesion, Rh30 and HeLa cells, infected with Ad-dominant negative (dn)-PP2A, Ad-PP2A and Ad-GFP (as control), respectively, were pretreated with/without U0126 (5 μM) for 1 h, and then treated with/without resveratrol (100 μM) for 4 h, followed by treatment with/without IGF-1 (10 ng/ml) for 1 h. A low basal level of p-Erk1/2 was observed in Ad-GFP-infected cells (control), whereas a higher basal level of p-Erk1/2 could be detected in Ad-dn-PP2A-infected Rh30 cells (Figure 4d, e) and HeLa cells (data not shown), suggesting the dn-PP2A was functioning in the cells. IGF-1 was able to stimulate the phosphorylation of Erk1/2 in Ad-GFP- and Ad-dn-PP2A-infected Rh30 (Figure 4d, e) and HeLa cells (data not shown). Of interest, expression of dn-PP2A markedly increased the basal cell adhesion, but conferred high resistance to resveratrol inhibition of the basal or IGF-1-stimulated cell adhesion (Figure 4f). However, in contrast, overexpression of wild-type PP2A powerfully suppressed the basal or IGF-1-induced p-Erk1/2 in the presence or absence of resveratrol in the cells (Figure 4g, h). Consistently, overexpression of wild-type PP2A notably inhibited the basal or IGF-1-induced cell adhesion in the presence or absence of resveratrol (Figure 4i). Taken together, our data strongly support the idea that resveratrol blocks the basal or IGF-1-induced Erk1/2 activation and cell adhesion via PP2A-dependent mechanism in cancer cells.

### 3.5 Resveratrol attenuates IGF-1-induced inactivation of PTEN and activation of Akt, leading to decreased Erk1/2 phosphorylation and cell adhesion in cancer cells

It is well-known that PTEN negatively regulates Akt pathway (Polak and Hall, 2009). In addition, Akt may activate Erk1/2 through PKC (Chetram and Hinton, 2012). Emerging studies have suggested that PTEN may also negatively regulate Erk1/2 pathway in several malignancies (Chetram and Hinton, 2012). Here, we hypothesized that resveratrol might block IGF-1-induced Erk1/2 activation and cell adhesion also partly by modulating PTEN/Akt signaling in cancer cells. As shown in Figure 5a, b, treatment with resveratrol attenuated IGF-1-induced phosphorylation of PTEN and Akt in Rh1, Rh30, HT29 and HeLa cells in a concentration-dependent manner. To identify the role and significance of PTEN/Akt pathway in resveratrol-inhibited IGF-1 stimulation of Erk1/2 activation and cell adhesion, Rh30 and HeLa cells, infected with recombinant adenovirus expressing wild-type human PTEN (Ad-PTEN) or Ad-GFP (as control), were pre-incubated with/without Akt inhibitor X (20 μM) for 1 h and then treated with/without resveratrol (100 μM) for 4 h, followed by incubation with/without IGF-1 (10 ng/ml) for 1 h. We observed that the infection with Ad-PTEN increased the expression of PTEN, compared to the infection with Ad-GFP in Rh30 cells (Figure 6a) and HeLa cells (data not shown). As expected, treatment with IGF-1 correspondingly increased phosphorylation of Akt and Erk1/2 in the cells infected with Ad-GFP (Figure 6a, b). Over-expression of PTEN blocked IGF-1-induced events in the cells (Figure 6a, b). Both resveratrol and Akt inhibitor X diminished IGF-1-induced p-Akt and p-Erk1/2 in the cells (Figure 6a, b). Furthermore, over-expression of PTEN was able to potentiate the inhibitory effects of resveratrol or Akt inhibitor X on IGF-1-induced phosphorylation of Akt and Erk1/2 in Rh30 cells (Figure 6a, b) and HeLa cells (data not shown). Moreover, we observed that over-expression of PTEN alone partially prevented IGF-1-induced cell adhesion in Rh30 and HeLa cells (Figure 6c). Addition of

resveratrol or Akt inhibitor X elicited more significant suppression of IGF-1-induced cell adhesion (Figure 6c). Collectively, the findings support the notion that resveratrol inhibits IGF-1-induced activation of Erk1/2 and consequential cell adhesion in cancer cells, by attenuating IGF-1-induced inactivation of PTEN and activation of Akt.

To further verify the role of Akt in resveratrol's blockage of IGF-1-induced Erk1/2 activation and cell adhesion in cancer cells, recombinant adenovirus expressing HA-tagged dominant negative Akt (Ad-dn-Akt) was utilized. As shown in Figure 6d, a high level of HA-tagged Akt mutant was seen in Rh30 cells infected with Ad-dn-Akt, but not in the cells infected with Ad-GFP (control virus). Similar results were observed in Ad-dn-Akt-infected HeLa cells (data not shown). Over-expression of dn-Akt remarkably suppressed IGF-1-triggered phosphorylation of Akt and Erk1/2 in the cells (Figure 6d, e). Resveratrol, but not U0126, powerfully attenuated IGF-1-increased Akt phosphorylation (Figure 6d, e). However, both resveratrol and U0126 obviously inhibited IGF-1-induced Erk1/2 phosphorylation in Rh30 cells (Figure 6d, e) and HeLa cells (data not shown). Interestingly, over-expression of dn-Akt was able to strengthen the inhibitory effects of resveratrol on IGF-1-induced phosphorylation of Akt or Erk1/2 in the cells (Figure 6d, e). Consistently, over-expression of dn-Akt also potently reinforced the inhibitory effect of resveratrol on IGF-1-induced cell adhesion in Rh30 and HeLa cells (Figure 6f). These data indicate that resveratrol blocks IGF-1-induced Erk1/2 activation and cell adhesion by suppressing IGF-1-induced activation of Akt in cancer cells.

### 3.6 Inhibition of mTOR potentiates resveratrol's activation of PP2A/PTEN, leading to more inhibition of Akt/Erk1/2 and cell adhesion in IGF-1-stimulated cancer cells

Increasing findings have shown that resveratrol can inhibit mTOR (Widlund et al., 2013). Importantly, we have demonstrated that mTOR is involved in the regulation of cell adhesion (Chen et al., 2015b). To determine whether inhibition of mTOR co-operates with resveratrol in activating PP2A and PTEN, leading to more inhibition of Erk1/2 and cancer cell adhesion, Rh30 and HeLa cells were pretreated with/without the mTORC1 inhibitor rapamycin (100 ng/ml) for 1 h, and then treated with/without resveratrol (100 μM) for 4 h, followed by incubation with/without IGF-1 (10 ng/ml) for 1 h. As shown in Figure 7a, b, rapamycin or resveratrol alone obviously suppressed the basal and IGF-1-stimulated de-PP2Ac, p-PP2Ac, p-PTEN and p-Erk1/2 in the cells. In line with previous observations (Chen et al., 2015b), rapamycin inhibited p-S6K1 and induced p-Akt. However, rapamycin potentiated the inhibitory effects of resveratrol on IGF-1-stimulated de-PP2Ac, p-PP2Ac, p-PTEN and p-Erk1/2. Consistently, the combination of resveratrol with rapamycin also exhibited more potent inhibition of IGF-1-triggered cell adhesion than resveratrol or rapamycin alone (Figure 7c). These results imply that inhibition of mTOR enhances the activating effect of resveratrol on PP2A and PTEN, resulting in more inhibition of Erk1/2 and cell adhesion, in response to IGF-1.

To confirm the above findings, RNA interference was used. As demonstrated in Figure 7d, e, lentiviral shRNA to mTOR, but not GFP, silenced the expression of mTOR protein by ~90% in Rh30 and HeLa cells, as detected by Western blotting. Down-regulation of mTOR significantly decreased the mTOR kinase activity, since the basal or IGF-1-induced

phosphorylation of S6K1 (Thr389), routinely used as an indicator of mTOR kinase activity, was almost not detectable by Western blotting (Figure 7f, g). Of note, down-regulation of mTOR blocked IGF-1-induced de-PP2Ac, p-PP2Ac, p-PTEN, p-Akt, and p-Erk1/2 in the cells even without treatment with resveratrol (Figure 7f, g). Furthermore, as expected, down-regulation of mTOR obviously suppressed IGF-1-induced cell adhesion, and potentiated the inhibitory effect of resveratrol (Figure 7h). Taken together, our data underscore the concept that inhibition of mTOR is co-operative with resveratrol in activating PP2A/PTEN, leading to more inhibition of Akt/Erk1/2 and cancer cell adhesion.

## 4 DISCUSSION

Resveratrol executes its anticancer activity in part through its anti-metastatic action in cancer cells (Chen et al., 2012; Kim et al., 2016; Lee et al., 2012). Cancer cell migration and attachment to specific distant organs are key events during cancer metastasis (Leber and Efferth, 2009; Woodhouse et al., 1997). Recently it has been shown that resveratrol inhibits cell adhesion in numerous malignancies (Wang et al., 2013b; Wu et al., 2008). Here, we report that resveratrol inhibited IGF-1-induced cell adhesion in a panel of cancer cell lines (Rh1, Rh30, HT29 and HeLa). Mechanistically, resveratrol inhibited IGF-1-induced cancer cell adhesion by activating PP2A/PTEN network, leading to inhibition of Erk1/2.

Here, we chose the most widely expressed ECM proteins such as CN IV, fibronectin and laminin to investigate the effect of resveratrol on cancer cell adhesion. The results showed that resveratrol inhibited cell adhesion in Rh1, Rh30, HT29 and HeLa cells. Since cell adhesion is an essential step in cancer metastatic process (Gupta and Massague, 2006; Leber and Efferth, 2009; Steeg and Theodorescu, 2008), inhibition of cancer cell adhesion may contribute to prevention of cancer metastasis. To verify the link between cancer cell adhesion and cancer metastasis, we further investigated the effect of resveratrol on cancer cell motility using the wound-healing assay according to our previous research (Liu et al., 2006). The results indicated that resveratrol indeed inhibited IGF-1-stimulated cell motility in Rh1, Rh30, HT29 and HeLa cells. Taken together, these data suggest that resveratrol has a great potential in the prevention of cancer metastasis by inhibiting cancer cell adhesion.

It is well known that multiple signaling molecules mediate cancer cell adhesion (Canel et al., 2013; Mitra and Schlaepfer, 2006). Erk1/2, a member of MAPK family involved in cell behavior control, has a well-established role as a regulator of cancer cell adhesion (Fincham et al., 2000; Liao et al., 2009). Similarly, PP2A, a negative regulator of Erk1/2, also regulates cell adhesion (Sontag and Sontag, 2006). Resveratrol can inhibit Erk1/2 at high concentrations (Roy et al., 2011). Recently, we have noticed that resveratrol activates PP2A activity (Liu et al., 2015). This led us to ask whether resveratrol inhibits IGF-1-induced cancer cell adhesion by targeting PP2A-Erk1/2 pathway. In this study, we found that resveratrol suppressed IGF-1-induced phosphorylation of Erk1/2 in Rh1, Rh30, HT29 and HeLa cells in a concentration-dependent manner (Figure 2a, b). Of note, resveratrol did not alter cellular protein expression of the catalytic subunit of PP2A (PP2Ac) and two regulatory subunits PP2A-A and PP2A-B, but remarkably attenuated IGF-1 induced expression of de-PP2Ac and p-PP2Ac (Tyr307) in the cells (Figure 2a, b). The *in vitro* PP2A phosphatase assay further confirmed that resveratrol reversed IGF-1-induced reduction of PP2A activity

(Figure 2c). The results suggest that resveratrol inhibits IGF-1-induced cancer cell adhesion, probably by activating PP2A, leading to inhibition of Erk1/2.

First, to corroborate that resveratrol inhibits the basal and IGF-1-stimulated cell adhesion through inhibiting Erk1/2 phosphorylation in cancer cells, pharmacological and genetic approaches were employed. We noticed that the combination of resveratrol with U0126 exhibited a more potent inhibitory effect on IGF-1-induced activation of Erk1/2 and cell adhesion than resveratrol or U0126 alone (Figure 3a-c). Furthermore, silencing Erk1/2 or expression of dominant negative MKK1 (MKK1-K97M) reinforced resveratrol's inhibition of IGF-1-stimulated Erk1/2 activation and cell adhesion, whereas expression of constitutively active MKK1 (MKK1-R4F) reversed resveratrol's inhibitory effect on IGF-1-induced Erk1/2 phosphorylation and cell adhesion (Figure 3d-i). Collectively, our findings indicate that resveratrol prevents IGF-1-induced cell adhesion, at least in part, by blocking activation of Erk1/2 pathway in cancer cells.

Next, to determine whether resveratrol inhibits the basal and IGF-1-stimulated cell adhesion through activating PP2A, resulting in inhibition of Erk1/2, pharmacological/genetic inhibition or rescue experiments for PP2A were conducted. We found that over-expression of wild-type PP2Ac potentiated resveratrol's suppression of IGF-1-induced phosphorylation of Erk1/2 and cell adhesion, whereas inhibition of PP2A by okadaic acid, or expression of dominant negative PP2A resulted in the robust phosphorylation of Erk1/2 and conferred high resistance to resveratrol inhibition of IGF-1-induced cell adhesion (Figure 4). These results support a model in which resveratrol blocks IGF-1-induced cancer cell adhesion by suppressing IGF-1 activation of Erk1/2, which is partly via activation of PP2A.

PTEN is a well-known phosphatase negatively regulating Akt pathway (Polak and Hall, 2009); Akt can activate Erk1/2 through PKC (Chetram and Hinton, 2012). Emerging studies have suggested that PTEN also negatively regulates Erk1/2 pathway in several malignancies (Chetram and Hinton, 2012). Both PTEN and Akt are involved in the regulation of cell adhesion (Haier and Nicolson, 2002; Kraus et al., 2002). In this study, treatment with resveratrol (0–100 μM) for 4 h potently blocked IGF-1-induced p-PTEN and p-Akt in Rh1, Rh30, HT29, and HeLa cells (Figure 5a, b). Putting all data together, we thus postulated that IGF-1 inactivation of PTEN and concurrent activation of Akt may result in activation of Erk1/2, which may be blocked by resveratrol. Here, for the first time, we present evidence that resveratrol inhibited IGF-1-induced cancer cell adhesion indeed by blocking IGF-1-induced inactivation of PTEN and activation of Akt, resulting in inhibition of Erk1/2. This is strongly supported by the findings that ectopic expression of wild-type PTEN or dominant negative Akt, or inhibition of Akt with Akt inhibitor X enhanced resveratrol's inhibitory effects on IGF-1-induced p-Erk1/2 and cell adhesion in Rh30 and/or HeLa cells (Figure 6a-f). Our data underscore that resveratrol has an ability to suppress IGF-1-induced inactivation of PTEN and activation of Akt, thereby attenuating IGF-1-induced Erk1/2 activation and adhesion in cancer cells.

In addition, PI3K is a kinase that converses phosphatidylinositol (3,4)-bis-phosphate ($PIP_2$) lipids to phosphatidylinositol (3,4,5)-tris-phosphate ($PIP_3$) (Hemmings et al., 2012). Many studies have documented that Akt is a major downstream effector of PI3K (Hemmings et al.,

2012). The PI3K/Akt signaling pathway plays an important role in regulating various cellular processes including cell adhesion and migration (Faes and Dormond, 2015). Emerging evidence has demonstrated that PI3K and Akt can act independently of each other (Mahajan and Mahajan, 2012; Bruhn et al., 2013). Definitely, more studies are needed to uncover the role of PI3K in the regulation of resveratrol-inhibited Akt and cancer cell adhesion.

Recently, we have demonstrated that both mTORC1 and mTORC2 are involved in the regulation of cell adhesion (Chen et al., 2015b). This prompted us to study whether inhibition of mTOR can potentiate the inhibitory effect of resveratrol on IGF-1-induced inhibition of PP2A/PTEN and activation of Akt/Erk1/2, as well as cell adhesion. We found that treatment with rapamycin alone inhibited p-S6K1 but induced p-Akt (Figure 7a, b), which is line with our previous observations (Chen et al., 2015b). It has been proposed that rapamycin inhibits S6K1, which may activate Akt through a S6K1-IRS negative feedback mechanism (Cornu et al., 2013; Lamming et al., 2013). Rapamycin treatment alone inhibited the basal and IGF-1-induced cancer cell adhesion (Figure 7c), which is also consistent with our recent finding (Chen et al., 2015b). Rapamycin blocked IGF-1-induced inactivation of PP2A and PTEN, thus potentiating the inhibitory effects on IGF-1-induced p-Erk1/2 and cell adhesion (Figure 7a-c). This is further supported by the findings that silencing mTOR more potently enhanced the inhibitory effects of resveratrol on IGF-1-induced inhibition of PP2A/PTEN and activation of Akt/Erk1/2 activation as well as cell adhesion (Figure 7d-h). These results clearly indicate that inhibition of mTOR potentiates the inhibitory effect of resveratrol on IGF-1-induced cell adhesion.

Dysregulation of cell adhesion molecules (CAMs), including integrins, cadherins, immunoglobulin-like CAMs, selectins and CD44s, is a characteristic in human cancers (Schmidmaier and Baumann, 2008). Focal adhesion proteins (FAK, paxillin and $p130^{Cas}$) and small GTPases (RhoA, Cdc42 and Rac1) have been recognized as key players in the regulation of cell motility and adhesion (Havel et al., 2015; Liu et al., 2008; Liu et al., 2010b). It has been reported that Erk1/2 pathway regulates integrins and cadherins (Chen et al., 2015a; Tashiro et al., 2016; Ying et al., 2017). Our group previously has found that IGF-I stimulates the phosphorylation of focal adhesion proteins and the activity of small GTPases, which is substantially attenuated by rapamycin (Liu et al., 2008; Liu et al., 2010b). Whether the suppression of Erk1/2 and Akt pathways leads to inhibition of cancer cell adhesion by altering the expression of these CAMs, focal adhesion proteins and small GTPases remains to be determined. Moreover, basement membrane (BM), a unique ECM, is not just a physical barrier, and it also contributes to cell adhesion, migration, proliferation, and survival (LeBleu et al., 2007). Laminins are major components of BM (Yao, 2017). Laminin-binding integrins are associated with both inhibitory and stimulatory roles in cancer (Ramovs et al., 2017). IGF-1 stimulated cell adhesion relies essentially on β1 integrins, which are privileged receptors for laminin molecules available to invading cancer cells in basal laminae (Takada et al., 2017). It has been shown that resveratrol affects ovarian cancer cell adhesion to human peritoneal mesothelial cells by decreasing cellular α5β1 integrin level (Mikula-Pietrasik et al., 2014). These data suggest that resveratrol inhibition of IGF-1-stimulated cancer cell adhesion seems through inhibiting laminin-β1 integrin interaction. Further research should be conducted to address this issue.

In addition, reactive oxygen species (ROS) has been found to regulate PP2A and PTEN/Akt activity (Nakahata and Morishita, 2014; Wang et al., 2016). Resveratrol, as a natural antioxidant, inhibits hypoxia- and hyperglycemia-driven ROS-induced invasion and migration of pancreatic cancer cells through suppression of Hedgehog and Erk/p38 MAPK signaling pathway, respectively (Cao et al., 2016; Li et al., 2016). Undoubtedly, more studies are required to address whether resveratrol suppresses ROS level, which results in activation of PP2A and PTEN, thereby leading to inhibition of Erk1/2-mediated adhesion in cancer cells.

In summary, here we have identified that resveratrol attenuates IGF-1-induced cancer cell adhesion by blocking Erk1/2 pathway. Mechanistically, resveratrol blocks Erk1/2 pathway, not only by activating PP2A, but also via activating PTEN and inactivating Akt, thereby preventing adhesion in cancer cells (Figure 8). Inhibition of mTOR enhanced the inhibitory effect of resveratrol on the events (Figure 8). Our findings suggest that resveratrol has a great potential in the inhibition of cancer cell adhesion, which may contribute to its prevention of cancer metastasis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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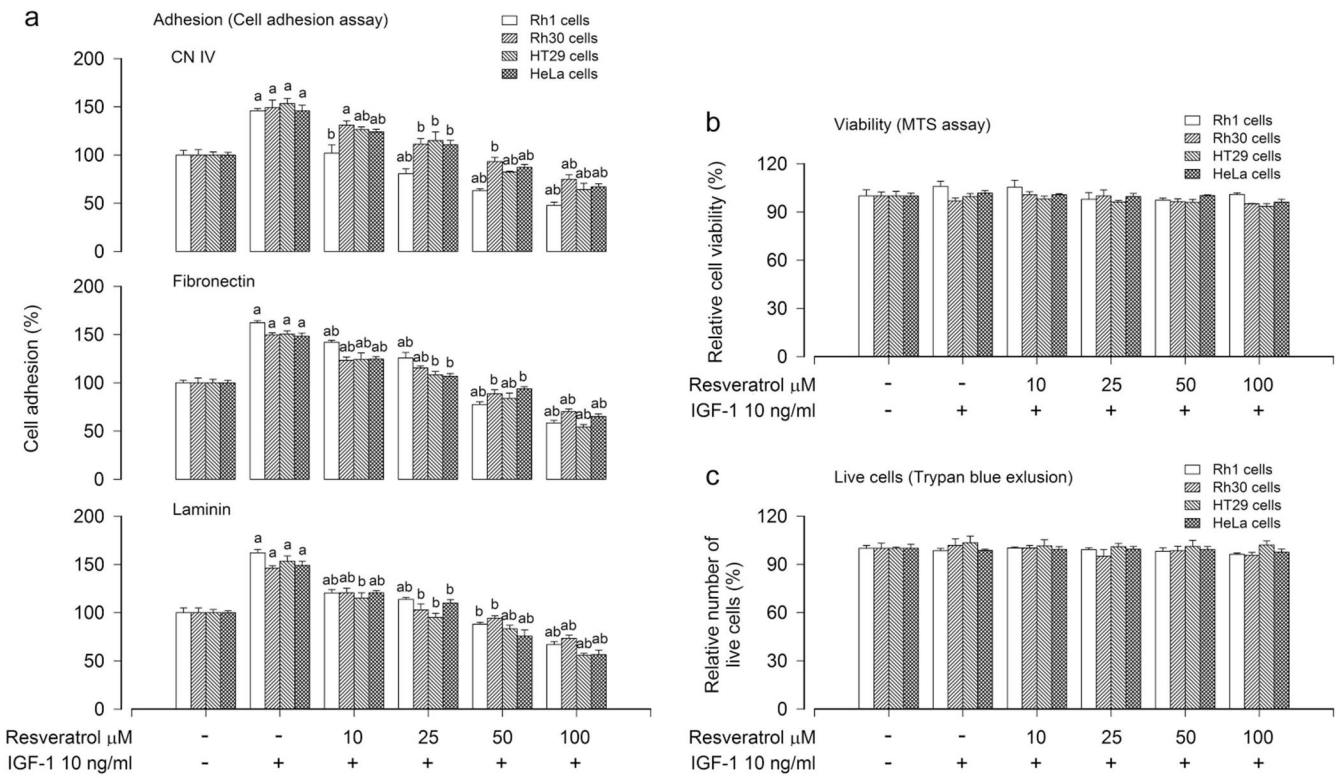


**FIGURE 1.**

Inhibitory effect of administered resveratrol in Rh1, Rh30, HT29 and HeLa cells on IGF-1-stimulated cell adhesion. Serum-starved Rh1, Rh30, HT29 and HeLa cells were treated with resveratrol (0–100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a) Adherent cells were evaluated using CN IV-, fibronectin- or laminin-coated cell adhesion assay. (b) Cell viability was determined by the MTS assay. (c) Live cells were detected by counting viable cells using trypan blue exclusion. Results are presented as mean ± SEM ($n = 6$). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group.

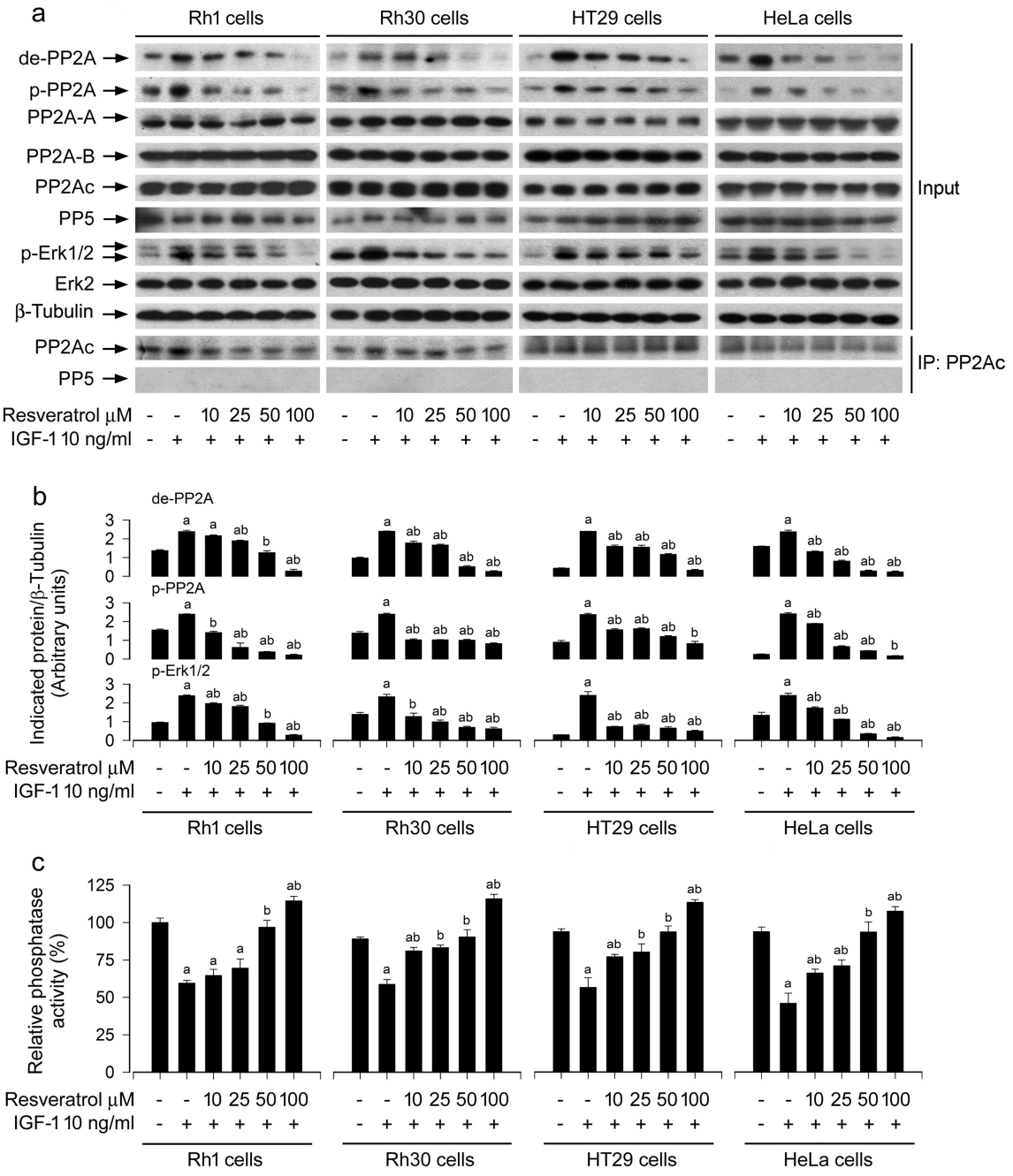


**FIGURE 2.**

Block effects of administered resveratrol in Rh1, Rh30, HT29 and HeLa cells on IGF-1-induced inhibition of PP2A and activation of Erk1/2. Serum-starved Rh1, Rh30, HT29 and HeLa cells were treated with resveratrol (0–100 μM) for 4 h and then stimulated with/without IGF-1 (10 ng/ml) for 1 h. (a) Total cell lysates were subjected to Western blotting using indicated antibodies. Immunoprecipitation (IP) was performed by incubation of cell lysates (500 μg) with antibodies to PP2Ac, followed by immunoblotting with antibodies to PP2Ac and PP5, respectively. The blots were probed for β-tubulin as a loading control.

Similar results were observed in at least three independent experiments. (b) The blots for de-PP2A, p-PP2A, and p-Erk1/2 were semi-quantified using NIH image J. (c) PP2A in cell lysates was immunoprecipitated with antibodies to PP2Ac plus protein A/G agarose beads, followed by *in vitro* phosphatase assay using Ser/Thr Phosphatase Assay Kit. Results are presented as mean ± SEM ($n = 3–6$). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group.

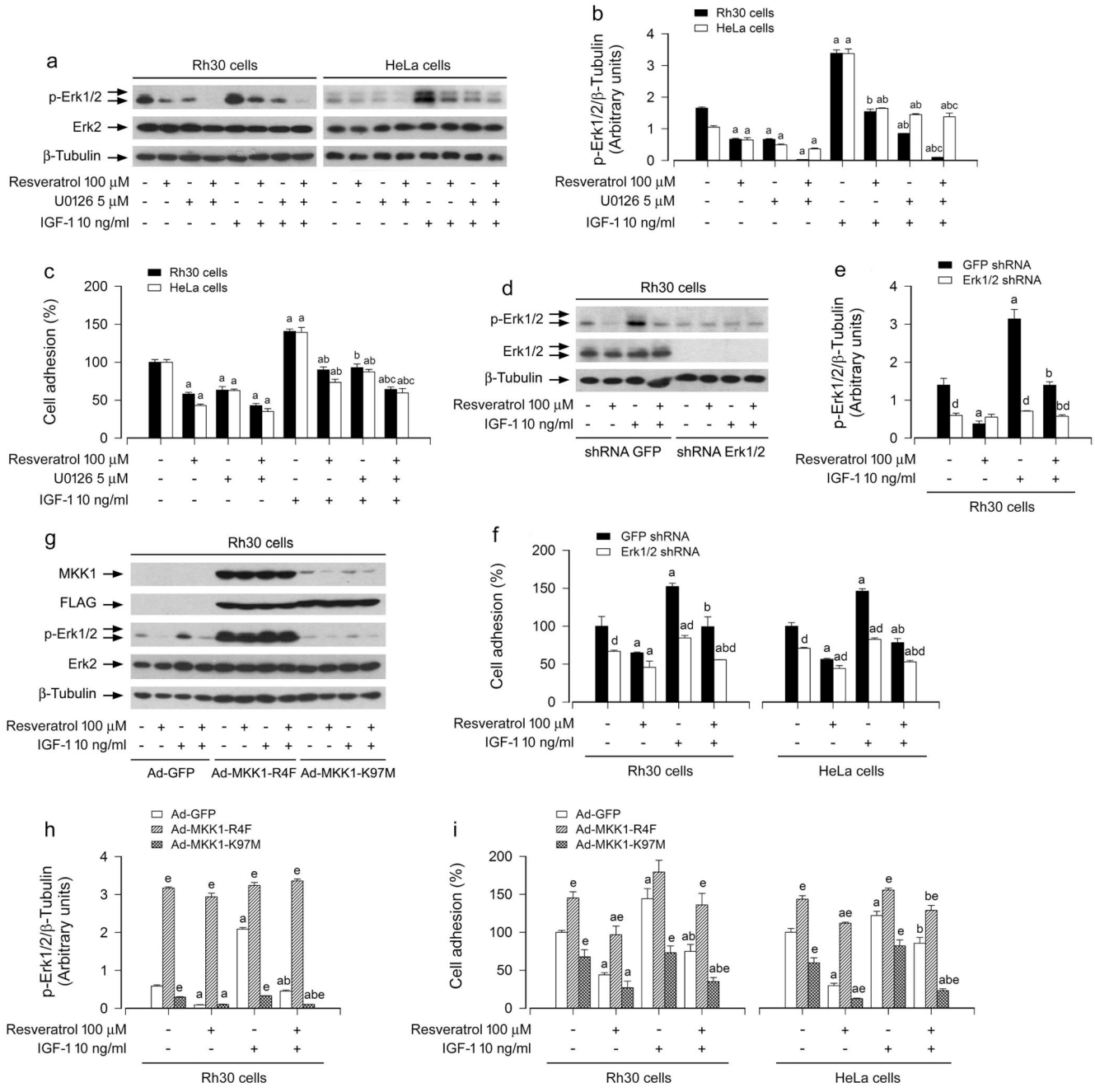


**FIGURE 3.**

Effects of inhibition of Erk1/2 by U0126, down-regulation of Erk1/2, or ectopic expression of MKK1-R4F or MKK1-K97M in Rh30 and HeLa cells on resveratrol's inhibition of IGF-1-induced Erk1/2 activation and cell adhesion. Serum-starved Rh30 and HeLa cells, pre-incubated with/without U0126 (5 μM) for 1 h, or serum-starved Rh30 and/or HeLa cells, infected with lentiviral shRNA to Erk1/2 or GFP (as control), or infected with Ad-MKK1-R4F, Ad-MKK1-K97M or Ad-GFP (as control), respectively, were treated with/without resveratrol (100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a, d, g) Total cell lysates were subjected to Western blotting using indicated antibodies. The

blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (b, e, h) The blots for p-Erk1/2 were semi-quantified using NIH image J. (c, f, i) Adherent cells were determined using CN IV-coated cell adhesion assay. Results are presented as mean ± SEM ($n$ = 3–6). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group; [c] $p < 0.05$, difference with IGF-1/resveratrol group or IGF-1/U0126 group; [d] $p < 0.05$, Erk1/2 shRNA group vs. GFP shRNA group; [e] $p < 0.05$, Ad-MKK1-R4F group or Ad-MKK1-K97M group vs. Ad-GFP group.

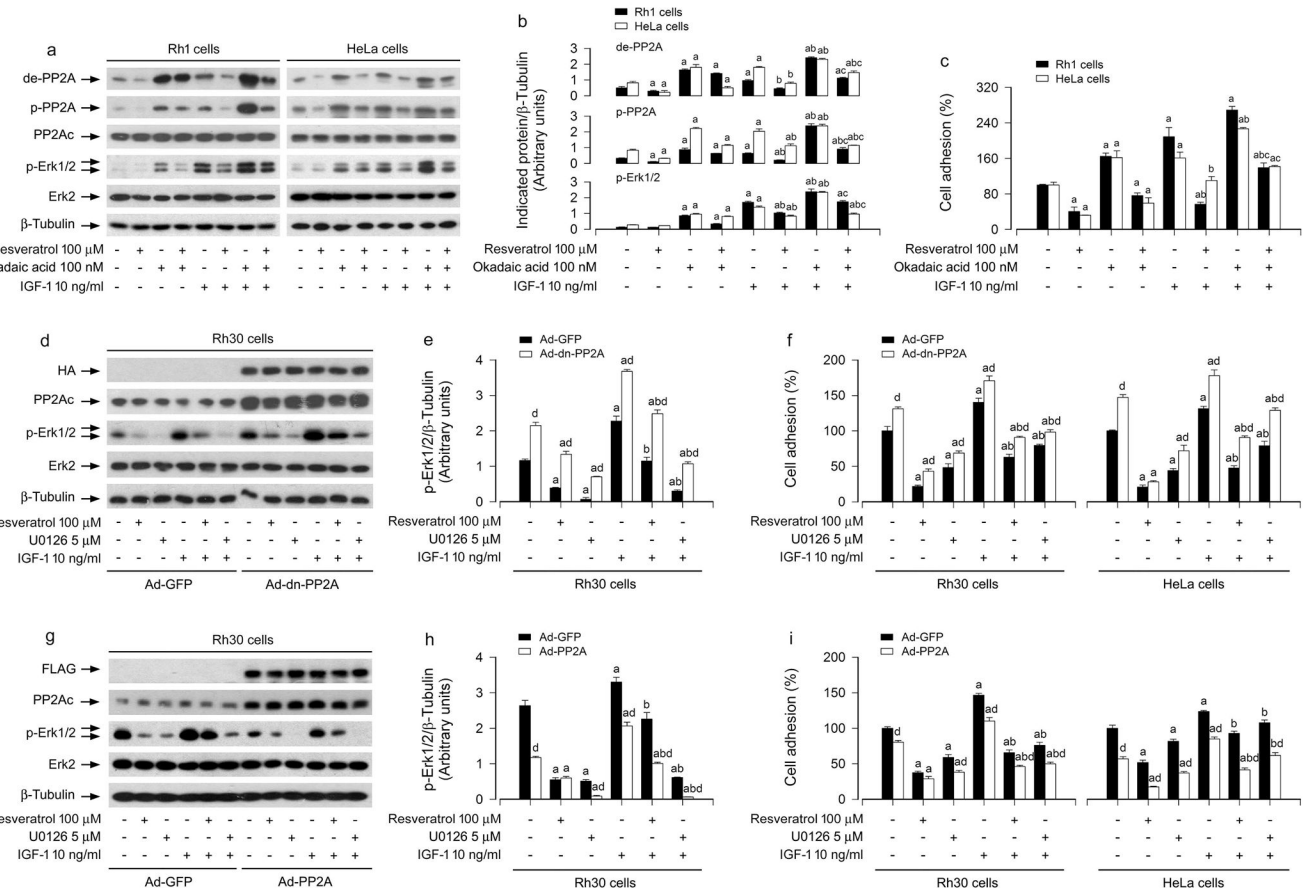


**FIGURE 4.**

Effects of inhibition of PP2A by okadaic acid or ectopic expression of dominant negative or wild-type PP2A in Rh1, Rh30 and/or HeLa cells on resveratrol's inhibition of IGF-1-induced Erk1/2 activation and cell adhesion. Resveratrol inhibits IGF-1-stimulated Erk1/2 activation and cell adhesion via activating PP2A. Serum-starved Rh1 and HeLa cells, pre-treated with/without okadaic acid (100 nM) for 1 h, or serum-starved Rh30 and/or HeLa cells, infected with Ad-GFP (as control), Ad-dn-PP2A and Ad-PP2A, respectively, and then pre-incubated with/without U0126 (5 μM) for 1 h, were treated with/without resveratrol (100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a, d, g) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (b, e, h) The blots for de-PP2A, p-PP2A, and p-Erk1/2 were semi-quantified using NIH image J. (c, f, i) Adherent cells were determined using CN IV-coated cell adhesion assay. Results are presented as mean ± SEM ($n = 3–6$). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group; [c] $p < 0.05$, difference with IGF-1/resveratrol group or IGF-1/okadaic acid group; [d] $p < 0.05$, Ad-dn-PP2A group or Ad-PP2A group vs. Ad-GFP group.

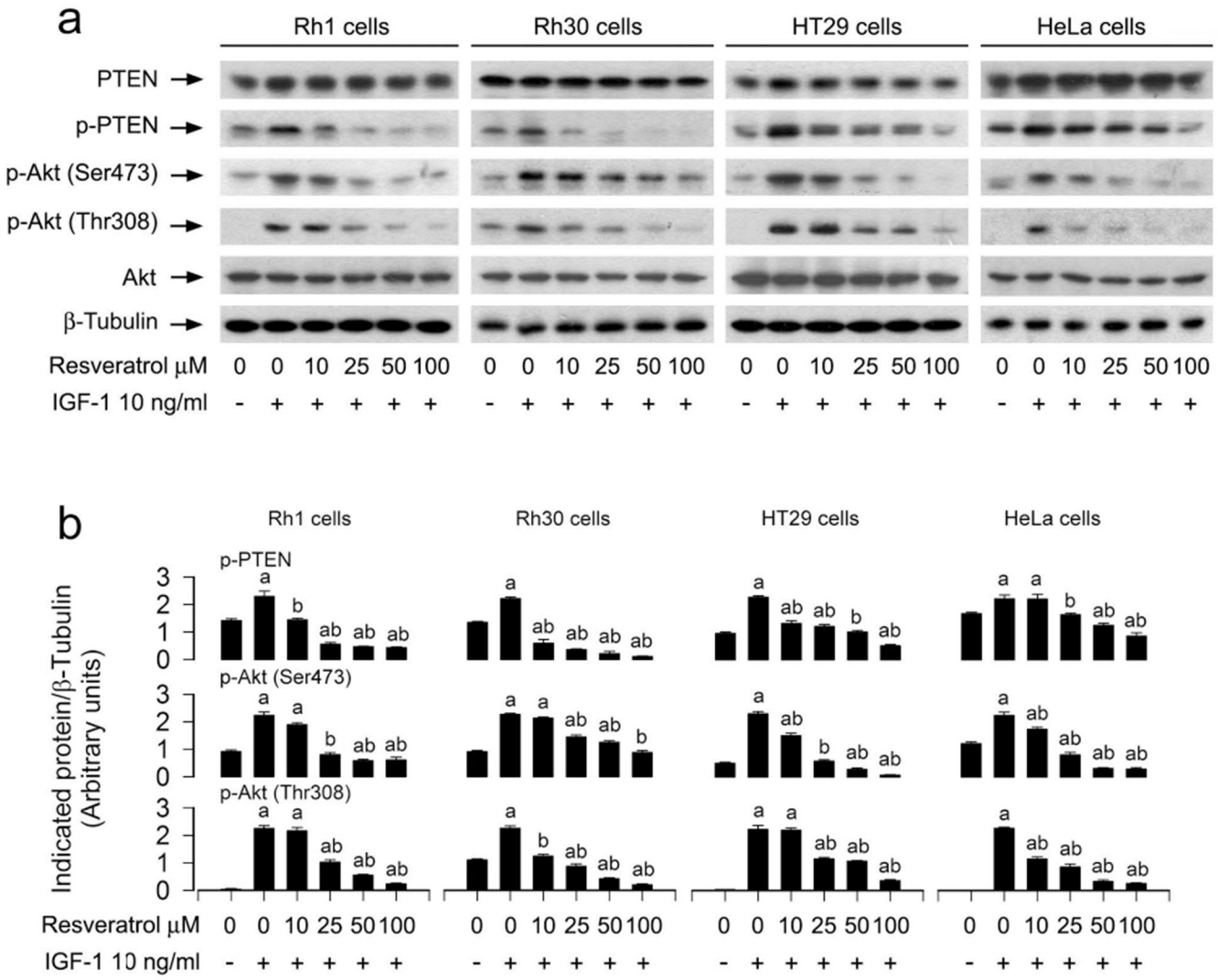


**FIGURE 5.**

Preventive effects of administered resveratrol in Rh1, Rh30, HT29 and HeLa cells on IGF-1-induced inhibition of PTEN and activation of Akt. Serum-starved Rh1, Rh30, HT29 and HeLa cells were treated with resveratrol (0–100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (b) The blots for p-PTEN, p-Akt (Ser473), and p-Akt (Thr308) were semi-quantified using NIH image J. Results are presented as mean ± SEM ($n = 3$). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group.

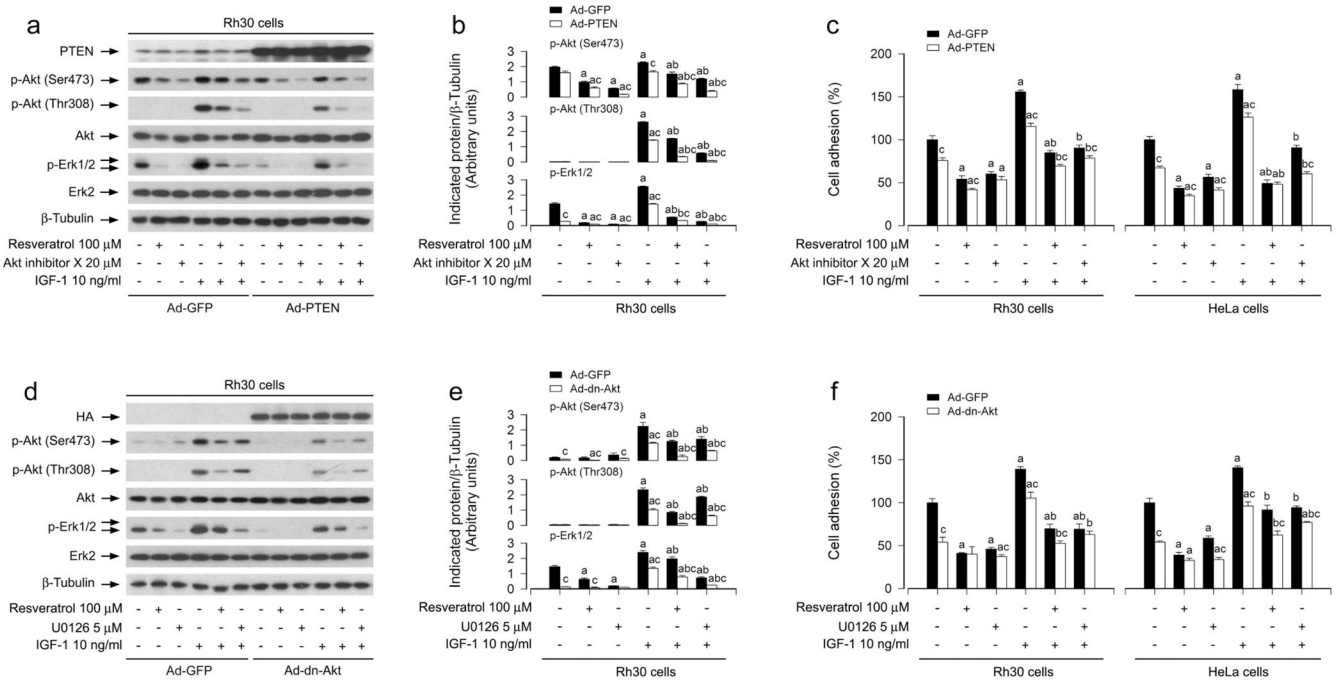


**FIGURE 6.**

Effects of ectopic expression of wild-type PTEN or dominant negative Akt in Rh30 and HeLa cells on resveratrol's suppression of IGF-1-stimulated Akt/Erk1/2 activation and cell adhesion. Serum-starved Rh30 and/or HeLa cells, infected with Ad-GFP (as control), Ad-PTEN or Ad-dn-Akt, respectively, and pretreated with/without Akt inhibitor X (20 μM) or U0126 (5 μM) for 1 h, were treated with/without resveratrol (100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a, d) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (b, e) The blots for p-Akt (Ser473), p-Akt (Thr308), and p-Erk1/2 were semi-quantified using NIH image J. (c, f) Adherent cells were determined using CN IV-coated cell adhesion assay. Results are presented as mean ± SEM ($n$ = 3–6). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group; [c] $p < 0.05$, Ad-PTEN group or Ad-dn-Akt group vs. Ad-GFP group.

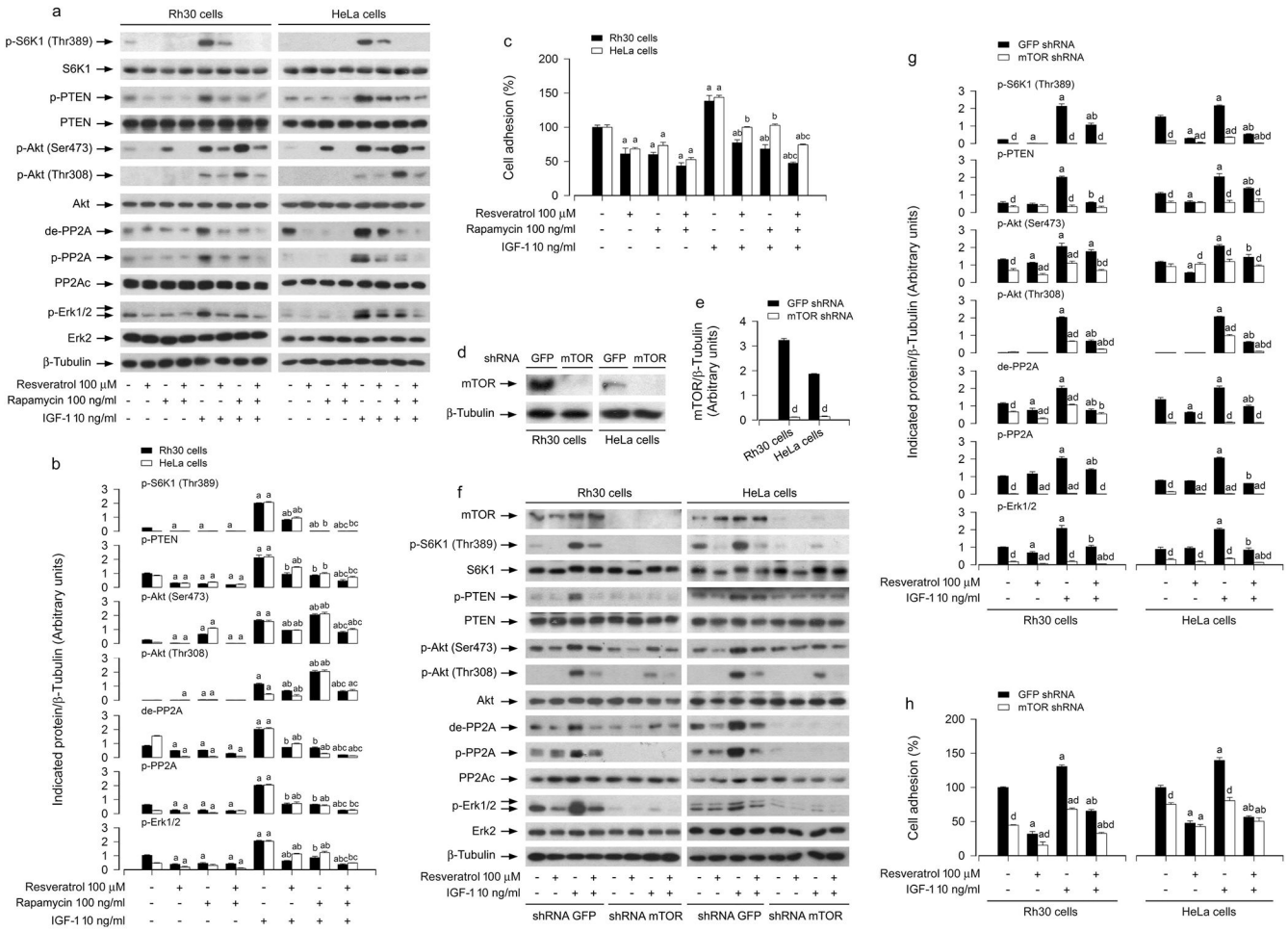


**FIGURE 7.**

Effects of inhibition of mTOR by rapamycin or down-regulation of mTOR in Rh30 and HeLa cells on resveratrol's prevention from IGF-1-induced PP2A/PTEN inhibition, Akt/Erk1/2 activation and cell adhesion. Serum-starved Rh30 and HeLa cells, pre-incubated with/without rapamycin (100 ng/ml) for 1 h, or infected with lentiviral shRNA to mTOR or GFP (as control), respectively, were treated with/without resveratrol (100 μM) for 4 h, followed by stimulation with/without IGF-1 (10 ng/ml) for 1 h. (a, d, f) Total cell lysates were subjected to Western blotting using indicated antibodies. The blots were probed for β-tubulin as a loading control. Similar results were observed in at least three independent experiments. (b, e, g) The blots for p-S6K1 p-PTEN, p-Akt (Ser473), p-Akt (Thr308), de-PP2A, p-PP2A, and p-Erk1/2 were semi-quantified using NIH image J. (c, h) Adherent cells were determined using CN IV-coated cell adhesion assay. Results are presented as mean ± SEM ($n = 3–6$). [a] $p < 0.05$, difference with control group; [b] $p < 0.05$, difference with IGF-1 group; [c] $p < 0.05$, difference with IGF-1/resveratrol group or IGF-1/rapamycin group; [d] $p < 0.05$, mTOR shRNA group vs. GFP shRNA group.

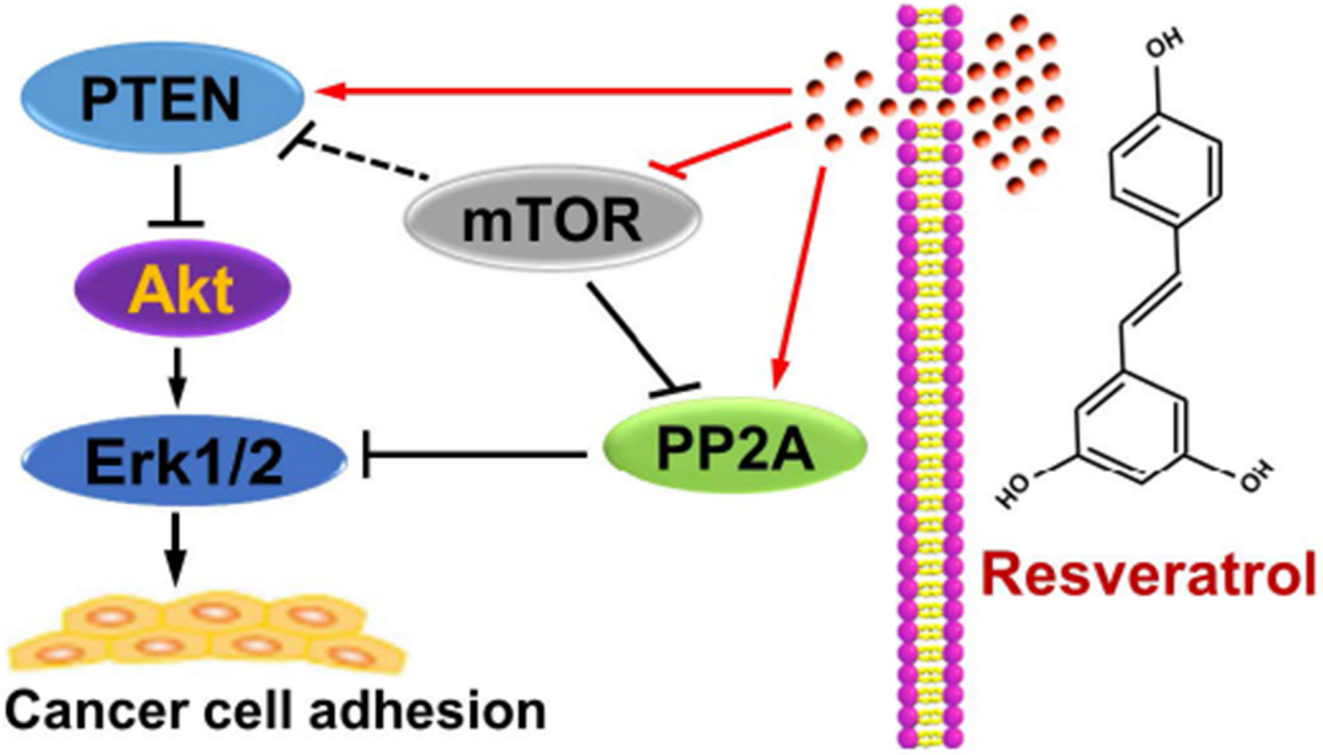


**FIGURE 8.**

Schematic model of the preventive effect of resveratrol on cancer cell adhesion. Resveratrol prevents cancer cells from Erk1/2-mediated adhesion, not only by activating PP2A, but also via activating PTEN and inactivating Akt. Inhibition of mTOR potentiates the inhibitory effect of resveratrol on the events.