

## RESEARCH ARTICLE

**Resveratrol Inhibits Oesophageal Adenocarcinoma Cell Proliferation via AMP-activated Protein Kinase Signaling****Guang-Hua Fan<sup>1,2&</sup>, Zhong-Ming Wang<sup>1,3&</sup>, Xi Yang<sup>1&</sup>, Li-Ping Xu<sup>1</sup>, Qin Qin<sup>1</sup>, Chi Zhang<sup>1</sup>, Jian-Xin Ma<sup>3</sup>, Hong-Yan Cheng<sup>4</sup>, Xin-Chen Sun<sup>1\*</sup>****Abstract**

Resveratrol has been examined in several model systems for potential effects against cancer. Adenosine monophosphate-activated protein kinase (AMPK) is reported to suppress proliferation in most eukaryocyte cells. Whether resveratrol via AMPK inhibits proliferation of oesophageal adenocarcinoma cells (OAC) is unknown. The aim of this study was to determine the roles of AMPK in the protective effects of resveratrol in OAC proliferation and to elucidate the underlying mechanisms. Treatment of cultured OAC derived from human subjects or cell lines with resveratrol resulted in decreased cell proliferation. Further, inhibition of AMPK by pharmacological reagent or genetical approach abolished resveratrol-suppressed OAC proliferation, reduced the level of p27<sup>Kip1</sup>, a cyclin-dependent kinase inhibitor, and increased the levels of S-phase kinase-associated protein 2 (Skp2) of p27<sup>Kip1</sup>-E3 ubiquitin ligase and 26S proteasome activity reduced by resveratrol. Furthermore, gene silencing of p27<sup>Kip1</sup> reversed resveratrol-suppressed OAC proliferation. In conclusion, these findings indicate that resveratrol inhibits Skp2-mediated ubiquitylation and 26S proteasome-dependent degradation of p27<sup>Kip1</sup> via AMPK activation to suppress OAC proliferation.

**Keywords:** AMPK - resveratrol - oesophageal adenocarcinoma - p27<sup>Kip1</sup> - proliferation

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**Introduction**

Carcinoma of the oesophagus is a common, aggressive tumor. Several histological types are seen, almost all of which are epithelial in origin. The vast majority of these tumors will be either oesophagus squamous cell carcinoma (OSCC) or oesophagus adenocarcinoma (OAC). OAC forms in the lower part of the oesophagus. It occurs when cells inside the mucous glands that line the oesophagus multiply abnormally. The mucous glands produce a slimy substance to help food slide down the oesophagus more easily.

Resveratrol (3, 4', 5-trihydroxystilbene) is a phytoalexin, present in the skin of red grapes and other fruits (Baur et al., 2006). Resveratrol is reported to exert antitumor activities at various stages of tumor initiation, promotion and progression (Jang et al., 1997). This is corroborated by reports of chemo-preventive effect of resveratrol in various cancer cell lines like, HeLa, A549 and MCF-7 (Cal et al., 2003). Various mechanisms for anti-proliferative action of resveratrol have been proposed including increase in tumor suppressor proteins like, p53, phosphorylation of Rb protein and transcription factors like NF- $\kappa$ B and AP-1 (Liu et al., 2010). Resveratrol is also

reported to inhibit PI3K pathway and affects glycolysis to cause cell cycle arrest in B cell lymphomas (Faber et al., 2006). However, these findings do not explain the role of resveratrol in the growth of OAC cells.

The AMP-activated protein kinase (AMPK) is a heterotrimeric protein composed of  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits. The  $\alpha$  subunit imparts catalytic activity, while the  $\beta$  subunit contains a glycogen-binding domain (GBD) that also regulates the activity and the  $\gamma$  subunit forms the broad base of the protein and is required for AMP binding. AMPK is well-conserved among eukaryotic cells. Recently, it was reported that activation of AMPK mediates belinostat-induced apoptosis and growth inhibition in cultured pancreatic cancer cells and lung cancer cells (Wang et al., 2013; Storozhuk et al., 2013). Additionally, AMPK activation not only inhibits cancer cell growth but also non-cancer cells, such as vascular smooth muscle cells, endothelial cells, etc (Zhang et al., 2008; Song et al., 2011). Subcutaneous injection of AICAR, which is an AMPK activator, for 2 weeks suppresses neointimal formation after transluminal mechanical injury of the rat femoral artery (Nagata et al., 2004). In deed, resveratrol has been reported to stimulate AMPK in kidney collecting duct cells and neurons (Dasgupta et al., 2007; Weixel

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et al., 2013). Based on these reports, we hypothesized that the activation of AMPK mediates suppressive effect of resveratrol on OAC proliferation. Here, we provide evidence of a novel molecular mechanism in which resveratrol activates AMPK to inhibit OAC cell growth through a 26S-proteasome-Skp2-p27<sup>Kip1</sup> signaling pathway.

## Materials and Methods

### Materials

Resveratrol and AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) were purchased from Sigma (St. Louis, MO, USA). Resveratrol was dissolved in DMSO to make a 500 mM stock solution (0.1% v/v final concentration) and stored at -80 °C. AMPK $\alpha$ 1/2 siRNA, Skp2 siRNA and antibodies against p27<sup>Kip1</sup>, phospho-p27<sup>Kip1</sup> (Thr187) and Skp2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies against AMPK $\alpha$ , phospho-AMPK $\alpha$  (Thr172), GAPDH, and second antibodies were obtained from Cell Signaling Technology (Beverly, MA). The siRNA delivery agent Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). MG132 and compound C were obtained from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Other chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

### Cell culture

The human OAC cell lines OE33 and OE19 obtained from the European Collection of Cell Cultures (Wiltshire, UK) were seeded into 96-well plates. 48 hours after culturing, cells were serum starved for 24 h and then treated as appropriate with resveratrol or not.

### Cell Proliferation Assay

Cells were split into 96-well plates before the cell proliferation assay as described previously (Song et al., 2011). It was performed by using the CellTiter96 nonradioactive cell proliferation assay (Promega, Madison, WI) according to the manufacturer's directions. The absorbance at 570 nm was read by an enzyme-linked immunosorbent assay plate reader. Absorbance was normalized to initial readings to verify equal cell numbers at the start of the assay. Data are presented as the mean of four measurements per condition.

### Cellular DNA Synthesis

Cellular DNA synthesis was assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation as per manufacturer's instructions (Roche, Mannheim, Germany) (Song et al., 2011). Briefly, mouse VSMCs ( $1 \times 10^4$  cells/well) were seeded onto 96-well plates and incubated in culture medium overnight, followed by synchronization via serum starvation for 24 h. Cells were then incubated in mouse VSMC culture medium (with 10  $\mu$ M BrdU) for 16 h.

### Transfection of siRNA into cultured cells

OAC were transfected in 6-well plates according to a previously described protocol (Wang et al., 2008). Briefly, a 10  $\mu$ M stock solution of siRNA was prepared in 20 mM

KCl, 6.0 mM HEPES (pH 7.5), and 0.2 mM MgCl<sub>2</sub>. For each transfection, 100  $\mu$ l transfection media (Gibco) containing 4  $\mu$ l siRNA stock solution was incubated with 100  $\mu$ l transfection media containing 4  $\mu$ l transfection reagent (Lipofectamine 2000, Invitrogen) for 30 min at room temperature. The siRNA-lipid complex was then added to each well, which contained 1 ml transfection media. After incubation for 6 h at 37°C, the transfection media was replaced with normal growth media, and cells were cultured for an additional 48 h.

### Semi-quantitative Reverse Transcription Polymerase Chain Reaction

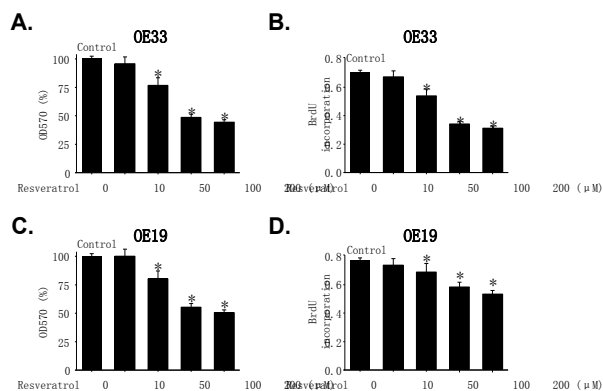
Cultured OAC were washed with cold PBS and total RNA was extracted in 1 ml of TRIZOL reagent (Invitrogen) per 100-mm dish. Total RNA (400 ng) from each sample was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions and as described previously (Wang et al., 2010). Prepared cDNA samples were amplified and analyzed by PCR using the following primers: p27<sup>Kip1</sup>, 5'-CGCTTTTGTTCGGTTTTGTT-3' (forward) and 5'-TTCGGAGCTGTTTACGTCTG-3' (reverse). Reactions were run for 30 cycles at conditions as follows: denaturation for 30 seconds at 94 °C, annealing for 30 seconds at 57 °C, and extension for 30 seconds at 72 °C. Constitutively expressed GAPDH mRNA was amplified as control.

### Western blot

Cells were homogenized on ice in cell-lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1  $\mu$ g/ml leupeptin, and 1 mM PMSF. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed using specific antibodies. Band intensity (area  $\times$  density) was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). Background intensity was subtracted from all calculated areas.

### 26S proteasome activity determination

The 26S proteasome function was measured, as described previously (Xu et al., 2007). Briefly, cells were washed with cold PBS and then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl<sub>2</sub>, 2 mM ATP). The cells were then pelleted by centrifugation. Homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 250 mM sucrose) was added, and cells were vortexed for 1 min. Cell debris was removed by centrifugation at 1000 x g for 5 min followed by 10,000 g for 20 min. Protein concentration was determined by the BCA (bicinchoninic acid) protocol (Pierce, Rockford, Ill). Protein (100  $\mu$ g) from each sample was diluted with buffer I to a final volume of 1 ml. The fluorogenic proteasome substrate Suc-LLVY-7-amido-4-methylcoumarin (chymotrypsin-like, Sigma, St. Louis, Mo) was added at a final concentration of 80  $\mu$ M in 1% DMSO. Cleavage activity was monitored continuously by detection of free 7-amido-4-methylcoumarin with a



**Figure 1. Resveratrol Inhibits OAC Proliferation.** (A) Cultured OAC of cell line OE33 were incubated with resveratrol (10, 50, 100, 200 μM) for 24 hours after starvation overnight. Cell proliferation assay was performed as per the manufacturer's protocol. N is 5 in each group. \* $P < 0.05$  VS control. (B) BrdU incorporation was measured in OAC of cell line OE33. N is 5 in each group. \* $P < 0.05$  VS control. (C) Cultured OAC of cell line OE19 were incubated with resveratrol (10, 50, 100, 200 μM) for 24 hours after starvation overnight. Cell proliferation assay was performed as per the manufacturer's protocol. N is 5 in each group. \* $P < 0.05$  VS control. (D) BrdU incorporation was measured in OAC of cell line OE19. N is 5 in each group. \* $P < 0.05$  VS control

fluorescence plate reader (Gemini, Molecular Devices, Sunnyvale, Calif) at 380/460 nm at 37 °C.

#### Statistical Analysis

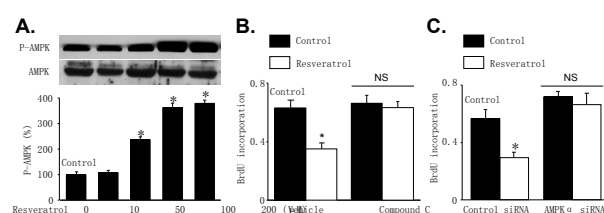
Results are expressed as the mean  $\pm$  SD. Statistical significance for comparisons between two groups was calculated using the two-tailed Student's t test. To assess comparisons between multiple groups, analysis of variance (ANOVA) followed by the Bonferroni procedure was performed using the Graph-Pad Prism 4 program (GraphPad Software, Inc, San Diego, CA). A  $P$  value of  $< 0.05$  was considered to be statistically significant.

## Results

### Resveratrol inhibits OAC proliferation

We first hypothesized resveratrol can suppress OAC proliferation. To test this notion, we determine the effect of resveratrol on OAC cell growth. Cultured OE33 cell line was quantified by OD570 assay. As shown in Figure 1A, resveratrol inhibited OE33 cell line growth in a dose-dependent manner. Following treatment of 50-200 μM resveratrol for 24 h, the growth speed of OE33 significantly began to reduce from 75% to 47% ( $P < 0.05$  VS 100% in control). Consistent with this, incorporation of the thymidine analog BrdU, which indicates DNA synthesis, was significantly decreased in 50-200 μM resveratrol-treated OAC, compared to control OAC (Figure 1B).

To further confirm the suppressive effect of resveratrol on OAC cell growth, we used another OAC cell line, which is OE19, to test this hypothesis. As expected in Figure 1C and Figure 1D, resveratrol effectively inhibited OAC OE19 cell line proliferation in a dose-dependent manner. These results demonstrated that resveratrol is an effective drug to inhibit OAC proliferation.



**Figure 2. Inhibition of AMPK $\alpha$  Abolished Resveratrol-suppressed OAC Proliferation.** (A) Phosphorylation of AMPK $\alpha$  Thr172 in OAC of cell line OE33 was determined by Western blot. This blot is from 3 independent experiments. \* $P < 0.05$  VS control. (B) Cultured OAC pretreated with compound C (10 μM) for 30 minutes were incubated with resveratrol (100 μM) for 24 hours. Cell proliferation assay was performed as per the manufacturer's protocol. N is 3 in each group. \* $P < 0.05$  VS control. NS indicates no significance. (C) Cultured OAC transfected with AMPK $\alpha$  siRNA for 48 hours were incubated with resveratrol (100 μM) for 24 hours. Cell proliferation assay was performed as per the manufacturer's protocol. N is 3 in each group. \* $P < 0.05$  VS control. NS indicates no significance

### Resveratrol increases AMPK Thr172 phosphorylation

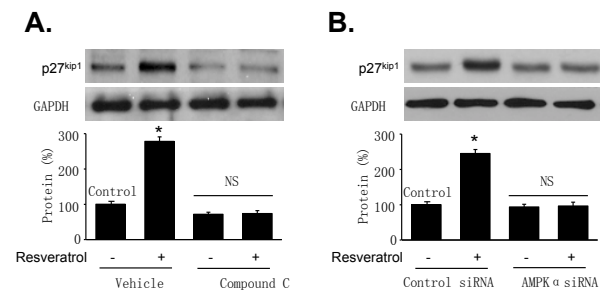
AMPK activation leads to cell cycle arrest in tumor cells, fibroblasts, and neural stem cells (Jiang et al., 2008; Zang et al., 2009). To investigate whether resveratrol via AMPK inhibits OAC proliferation, confluent OAC OE33 cell line was treated with varying concentrations of resveratrol for 24 hours. AMPK activation was indirectly assessed by western blot analysis of AMPK phosphorylation at Thr172, which is essential for AMPK activity. As shown in Figure 2A, the phosphorylation of AMPK was gradually increased beginning from 50 μM to 200 μM of resveratrol. Resveratrol treatment did not alter total levels of AMPK, suggesting that resveratrol-induced phosphorylation of AMPK was not due to altered expression of these proteins.

### Inhibition of AMPK abolished resveratrol-induced suppression on OAC proliferation

We next investigate the role of AMPK in resveratrol-suppressed OAC proliferation. Cell proliferation assays demonstrated that resveratrol remarkably attenuated cultured OAC proliferation in vehicle group, but not OAC pretreated with compound C, which is an AMPK inhibitor (Figure 2B). Consistent with this, resveratrol dramatically decreased incorporation of the thymidine analog BrdU, which indicates DNA synthesis, in OAC transfected with control siRNA but not in OAC transfected with AMPK $\alpha$  siRNA (Figure 2C). Taking all data together, it suggested that resveratrol suppressed OAC proliferation via AMPK activation.

### Resveratrol via AMPK activation increases p27<sup>Kip1</sup> levels in OAC

p27<sup>Kip1</sup> plays an important role in cell cycle (Boehm et al., 2004). Therefore, we investigated whether resveratrol via AMPK altered its expression. The level of p27<sup>Kip1</sup> protein was increased dramatically in resveratrol-treated OAC, compared with vehicle-treated OAC (Figure 3A). However, resveratrol did not increase p27<sup>Kip1</sup> protein in OAC incubated with AMPK inhibitor, compound C.



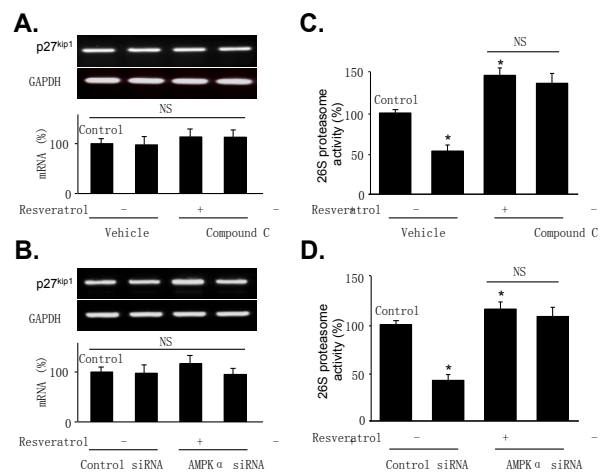
**Figure 3. Resveratrol via AMPK $\alpha$  Activation Increased Level of Total p27Kip1 Protein in OAC.** (A) Cultured OAC pretreated with compound C (10  $\mu$ M) for 30 minutes were incubated with resveratrol (100  $\mu$ M) for 24 hours. The total and phosphorylated p27Kip1 protein level was assessed by Western blot analysis. This blot is a representative blot from 3 independent experiments. \* $P$ <0.05 VS control. NS indicates no significance. (B) Cultured OAC transfected with AMPK $\alpha$  siRNA for 48 hours were incubated with resveratrol (100  $\mu$ M) for 24 hours. The total and phosphorylated p27Kip1 protein level was assessed by Western blot analysis. This blot is a representative blot from 3 independent experiments. \* $P$ <0.05 VS control. NS indicates no significance. (C) Cultured OAC pretreated with MG132 (0.5  $\mu$ M) for 30 min were incubated with or without compound C (10  $\mu$ M) for 24 hours. The total and phosphorylated p27Kip1 protein level was assessed by Western blot analysis. This blot is a representative blot from 3 independent experiments. \* $P$ <0.05 VS control. NS indicates no significance

To further validate the role of AMPK in p27<sup>Kip1</sup> protein expression, we performed siRNA knockdown to test the contribution of AMPK $\alpha$  in increasing p27<sup>Kip1</sup> expression in resveratrol-treated OAC. As depicted in Figure 3B, the level of p27<sup>Kip1</sup> protein was enhanced significantly by resveratrol in control siRNA-transfected OAC, but not in AMPK $\alpha$  siRNA-transfected OAC (Figure 3B). Collectively, these results demonstrate that AMPK plays a key role in resveratrol-increased p27<sup>Kip1</sup> protein expression in OAC.

In order to determine whether AMPK regulates p27<sup>Kip1</sup> stability through Thr 198 phosphorylation, we checked the phosphorylated p27<sup>Kip1</sup> ( $P$ -p27<sup>Kip1</sup>) levels. As showing in Figure 3A to 3C, the ration of  $P$ -p27<sup>Kip1</sup> to T-p27<sup>Kip1</sup> was not changed by AMPK activation or AMPK inhibition, indicating that AMPK regulates p27<sup>Kip1</sup> protein stability independent of phosphorylation. Also, the effects of compound C on p27<sup>Kip1</sup> stability were totally blocked by MG132 treatment (Figure 3C), suggesting AMPK inhibition through upregulation of 26S proteasome activity to breakdown p27<sup>Kip1</sup>.

#### Reduced p27<sup>Kip1</sup> protein level in AMPK-deficient OAC is independent on gene expression but on 26s proteasome-dependent degradation

Next, we examined how AMPK regulates p27<sup>Kip1</sup> protein expression in OAC. As shown in Figure 4A, RT-PCR analysis indicated that resveratrol did not alter p27<sup>Kip1</sup> mRNA expression in OAC treated with either vehicle or compound C. Further, resveratrol also did not increase p27<sup>Kip1</sup> mRNA level in OAC transected with control or AMPK $\alpha$  siRNA (Figure 4B). Taken together, these data suggest that resveratrol increases p27<sup>Kip1</sup> protein



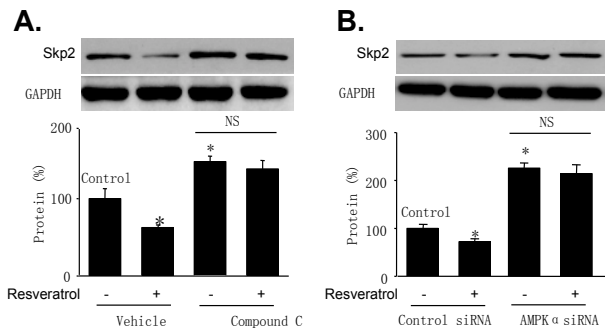
**Figure 4. Resveratrol-induced AMPK $\alpha$ -mediated p27Kip1 Elevation in OAC is 26S Proteasomes-Dependent, but not Gene Transcription Dependent.** (A) Cultured OAC pretreated with compound C (10  $\mu$ M) for 30 minutes were incubated with resveratrol (100  $\mu$ M) for 24 hours. The p27Kip1 mRNA level was assessed by RT-PCR analysis. N is 3 in each group. NS indicates no significance. (B) Cultured OAC transfected with AMPK $\alpha$  siRNA for 48 hours were incubated with resveratrol (100  $\mu$ M) for 24 hours. The p27Kip1 mRNA level was assessed by RT-PCR analysis. N is 3 in each group. NS indicates no significance. (C) The 26S proteasome activity were assessed by in situ fluorescence substrate in A. N is 3 in each group. \* $P$ <0.05 VS control. NS indicates no significance. (D) The 26S proteasome activity were assessed by in situ fluorescence substrate in B. N is 3 in each group. \* $P$ <0.05 VS control. NS indicates no significance

expression via a gene expression-independent pathway.

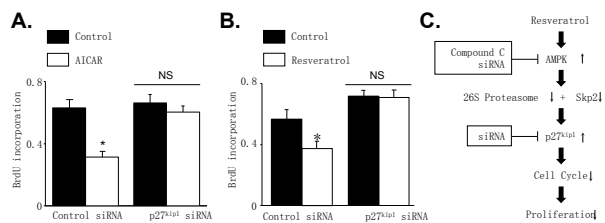
It has been reported that p27<sup>Kip1</sup> protein can be degraded by 26S proteasome in vascular smooth muscle cell (Song et al., 2011). Then we determined the 26S proteasome activity in OAC. As depicted in Figure 4C, 26S proteasome activity was decreased dramatically in resveratrol-treated OAC, compared with vehicle-treated OAC. However, resveratrol did not reduce 26S proteasome activity in OAC incubated with AMPK inhibitor, compound C. Similar to inhibition of AMPK by compound C, 26S proteasome activity was inhibited significantly by resveratrol in control siRNA-transfected OAC, but not in AMPK $\alpha$  siRNA-transfected OAC (Figure 4D). All data demonstrate that 26S proteasome pathway but not gene transcription is involved in resveratrol-increased p27<sup>Kip1</sup> protein level.

#### AMPK mediates resveratrol-reduced Skp2 protein expression in OAC

26S proteasome-dependent protein degradation depends on ubiquitin E3 ligase (Engel et al., 2013). Because S-phase kinase-associated protein 2 (Skp2), as an E3 ubiquitin ligase, is the main rate-limiting regulator of p27<sup>Kip1</sup> protein ubiquitylation and degradation in various cell types (Kleiger et al., 2009), we assessed Skp2 expression in OAC. As depicted in Figure 5A, the amount of Skp2 protein was decreased in OAC treated with resveratrol, compared with OAC treated with vehicle. Moreover, the levels of Skp2 protein were augmented in



**Figure 5. Resveratrol Reduced Skp2 Expression Via AMPK $\alpha$  Activation.** (A) Cultured OAC pretreated with compound C (10  $\mu$ M) for 30 minutes were incubated with resveratrol (100  $\mu$ M) for 24 hours. The Skp2 protein level was assessed by Western blot analysis. N is 3 in each group. \* $P$ <0.05 VS control. (B) Cultured OAC transfected with AMPK $\alpha$  siRNA for 48 hours were incubated with resveratrol (100  $\mu$ M) for 24 hours. The Skp2 protein level was assessed by Western blot analysis. N is 3 in each group. \* $P$ <0.05 VS control



**Figure 6. Decreased OAC Proliferation by AMPK Activation is p27<sup>Kip1</sup>-dependent.** (A and B) OAC were transfected with either control or p27<sup>Kip1</sup> siRNA for 48 hours and then left untreated or were treated with (A) AICAR (1 mM) or (B) resveratrol (100  $\mu$ M) for 24 hours. BrdU incorporation was used to measure OAC proliferation. N is 5 in each group. \* $P$ <0.05 VS control, NS indicates no significance. (C) Proposed molecular mechanism for suppressive effects of resveratrol on OAC proliferation

compound C-treated OAC. However, no changes were detected in the expression of Skp2 protein in compound C-treated OAC which is co-incubated with resveratrol. The role of AMPK in Skp2 protein regulation was further validated by siRNA knockdown to silence AMPK protein expression. The decrease of Skp2 protein level caused by resveratrol in OAC was bypassed by AMPK $\alpha$  siRNA but not control siRNA transfection to OAC (Figure 5B). Overall, these results indicate that Skp2 is downregulated by resveratrol via AMPK activation.

#### p27<sup>Kip1</sup> mediates resveratrol-reduced OAC proliferation

Finally we determined whether p27<sup>Kip1</sup> is required for resveratrol-reduced OAC proliferation. To determine the effect of p27<sup>Kip1</sup> inhibition on OAC proliferation, we transfected OAC with control or p27<sup>Kip1</sup> siRNA for 48 hours and then treated OAC with AICAR (1 mM, a known AMPK activator, used as a positive control) or resveratrol (100  $\mu$ M) for 24 hours. Cell proliferation was assayed by BrdU incorporation. As seen in Figure 6A and 6B, transfection with p27<sup>Kip1</sup>-specific siRNA but not control siRNA attenuated AICAR- or resveratrol-reduced OAC proliferation. These findings indicate that p27<sup>Kip1</sup> is responsible for the decreased proliferation in OAC if AMPK is activated.

## Discussion

Resveratrol is a parent compound of a family of molecules including glucosides and polymers (Cal et al., 2003), existing in cis and transconfigurations in narrow range of spermatophytes of which vines, peanuts and pines are the prime representatives (Kiselev et al., 2011; Nakata et al., 2012; Neves et al., 2012). Previous studies have reported that resveratrol exerted antitumor activities at various stages of tumor initiation, promotion and progression (Cimino et al., 2012; Stefanska et al., 2012; Whitlock et al., 2012), including in esophageal squamous cell carcinoma (Tang et al., 2013). However, the molecular mechanism remains unclear. In the present study, we have shown that AMPK $\alpha$  mediates resveratrol-suppressed OAC proliferation. The mechanism underlying this process is due to a novel pathway in which OAC proliferation is inhibited by resveratrol as a result of p27<sup>Kip1</sup> upregulation, which is controlled by downregulations of both Skp2 and 26S proteasome (Figure 6C). These findings indicate that AMPK is an important mediator for OAC cell growth and suggest that resveratrol therapy that modulates AMPK $\alpha$  signaling in OAC may be beneficial in treating oesophageal adenocarcinoma.

The most important finding of this study is to uncover the underlying mechanisms by which resveratrol activates AMPK, which downregulates ubiquitin E3 ligase Skp2 and 26S proteasome activity, and consequent p27<sup>Kip1</sup> upregulation, which contributes to inhibition of OAC cell growth. Although it has been reported (Liang et al., 2007) that LKB1-AMPK pathway regulates p27<sup>Kip1</sup> phosphorylation and thereby increases the p27 stability, our data indicated that AMPK activation can upregulate p27<sup>Kip1</sup> through 26s-proteasome. Several lines of evidence are consistent with the current hypothesis. First, inhibition of AMPK profoundly abolished resveratrol-suppressed ubiquitin-proteasome system. Second, p27<sup>Kip1</sup> protein but not mRNA level was significantly elevated in resveratrol-treated OAC, indicating resveratrol increases p27<sup>Kip1</sup> protein stability. Third, AMPK inhibition with Compound C or silence by siRNA transfection markedly limits p27<sup>Kip1</sup> protein upregulation in OAC. Fourth, p27<sup>Kip1</sup> siRNA transfection notably eliminated OAC proliferation induced by AICAR and resveratrol, which are AMPK activators. Taken together, these findings demonstrate that AMPK is a target of resveratrol, which potently modulates OAC activation and function through p27<sup>Kip1</sup> reduction. Recently, it has been shown that Skp2 promotes vascular smooth muscle cell proliferation and neointima formation in Skp2<sup>-/-</sup> mice (Wu et al., 2009), supporting our results and previous reports (Bishayee et al., 2009; Woodall et al., 2009) that Skp2 is a viable target of resveratrol for anticancer aimed at inhibiting OAC proliferation, including oesophagus squamous carcinoma and oesophagus adenocarcinoma.

In summary, we have shown that AMPK $\alpha$  activation is critical for resveratrol-reduced OAC proliferation. AMPK $\alpha$ -decreased OAC proliferation may emerge AMPK as an important therapeutic target in oesophageal adenocarcinoma. The identity of the downstream targets of AMPK and the manner in which they regulate AMPK-mediated OAC function remain to be further elucidated.

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