



中國醫藥大學  
癌症生物學研究所  
碩士學位論文

FOX2 於白藜蘆醇抑制肺癌細胞轉移之相關探討  
FOX2 is critical for resveratrol-mediated inhibition of  
metastasis in lung cancer

研究生：鄭羽真 (Yu-Jhen Cheng)

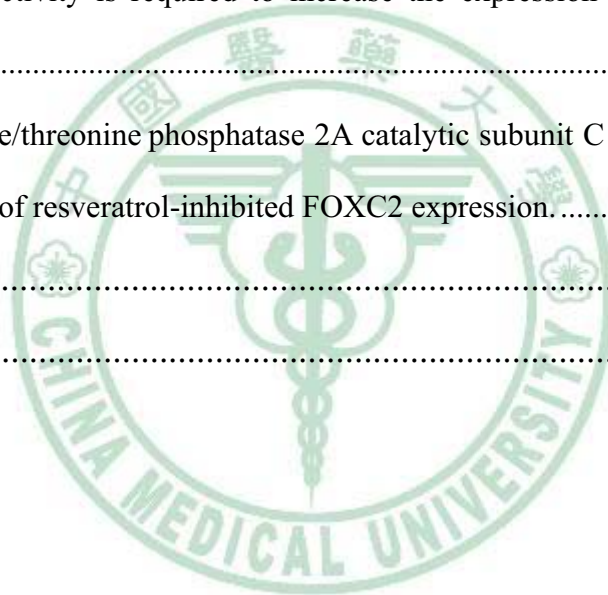
指導教授：蘇振良 (Jen-Liang Su, Ph.D.) 教授

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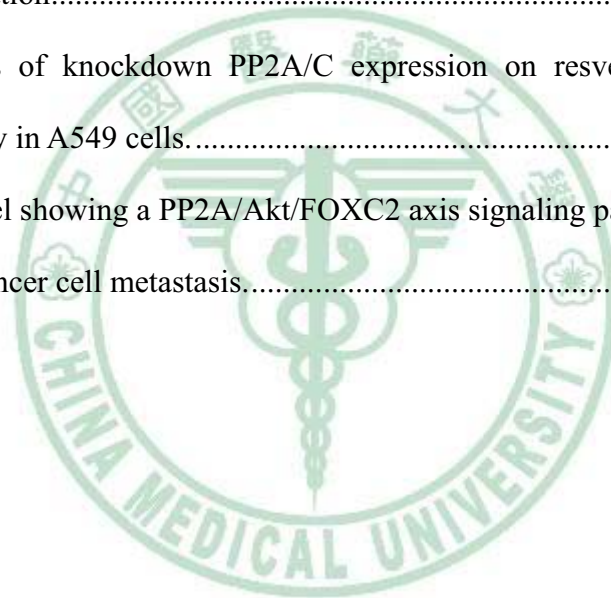
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## 中文摘要

白藜蘆醇(resveratrol)是一種天然的植物抗毒素(phytoalexin)，主要存在於葡萄、花生以及桑椹中。上皮-間質轉化(epithelial-mesenchymal transition, EMT)是胚胎發育過程中的生理現象，為上皮細胞型態(epithelium)轉變成間葉細胞(mesenchyme)型態的一種過程，是正常發育、傷口癒合以及惡性上皮腫瘤發生的基礎。目前的研究顯示上皮-間質轉化對多種腫瘤的發展進程具有重要的影響，上皮-間質轉化可促進腫瘤細胞的浸潤以及腫瘤的轉移，在人類以及動物實驗中發現上皮-間質轉化會導致預後不良。實驗結果顯示白藜蘆醇會降低上皮-間質轉化相關的轉錄因子 FOXC2 的表現，也發現白藜蘆醇能抑制不同肺癌細胞的移動及侵襲能力，改變上皮-間質轉化相關的蛋白質表現，例如增加 E-cadherin 的含量，降低 fibronectin、N-cadherin 和 vimentin 的表現。為了解白藜蘆醇抑制上皮-間質轉化的機制是否透過降低 FOXC2 的表現，因此在肺癌細胞中過度表現 FOXC2，當過度表現 FOXC2 時會破壞白藜蘆醇抑制上皮-間質轉化的現象，增加肺癌細胞轉移及侵襲能力，說明了白藜蘆醇是藉由降低 FOXC2 的表現來抑制上皮-間質轉化現象的發生。為進一步探討白藜蘆醇如何抑制 FOXC2 的表現，在肺癌細胞中加入 PI3K/Akt 特異性抑制劑以及 MAPK 抑制劑之後，結果顯示 PI3K/Akt 特異性抑制劑會抑制 FOXC2 的表現，表示藉由 Akt 訊息傳遞路徑會調控 FOXC2 的表現。已知絲氨酸/蘇氨酸去磷酸酶(protein serine/threonine phosphatase 2A, 簡稱 PP2A)能夠使 Akt 磷酸化的位點去磷酸化而使其失去活性。白藜蘆醇能活化絲氨酸/蘇氨酸去磷酸酶，加入絲氨酸/蘇氨酸去磷酸酶選擇型抑制劑 okadaic acid 或是以 shPP2A/C 抑制絲氨酸/蘇氨酸去磷酸酶的表現時會使得 FOXC2 的表現增加。這些結果顯示抗癌藥物白藜蘆醇能藉由活化絲氨酸/蘇氨酸去磷酸酶降低 PI3K/Akt-FOXC2 的表現，進而達到抑制肺癌細胞轉移的目的。

## Abstract

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene), a phytoalexin found in various plants such as grapes, has been shown to prevent cancer metastasis in murine models. Epithelial-mesenchymal transition (EMT) associates with pathological process such as fibrosis and cancer which leads to carcinoma progression and poor prognosis in various human and mouse model. Here we showed that EMT was inhibited by resveratrol through down-regulating the expression of FOXC2 (forkhead box C2), a critical transcription factor involved in EMT transition. Our data showed that treatment with resveratrol inhibited cell mobility and up-regulation of the expression of E-cadherin, an epithelial marker, we also found that treatment with resveratrol down-regulating several mesenchymal markers such as fibronectin, N-cadherin and vimentin in various lung cancer cells. Ectopic expression of FOXC2 in lung cancer cells recovered resveratrol-mediated suppression of cell mobility and EMT transition. To define the molecular mechanisms involved in resveratrol-regulated FOXC2 expression, PI3K/Akt and MAPK inhibitors were used. Treatment with PI3K/Akt inhibitor down-regulated FOXC2 expression and these data suggest that Akt signaling pathway may involved in the regulation of FOXC2 expression in lung cancer cells. Resveratrol also activated serine/threonine protein phosphatase 2A (PP2A), known to dephosphorylation of several protein kinase including Akt. Inhibition of PP2A by okadaic acid (OA), a phosphatase inhibitor, or knockdown the expression of PP2A by shPP2A/C increased the expression of FOXC2. These data indicated that the cancer-chemopreventive agent resveratrol induced PP2A/C activation and further inactivated the PI3K/Akt-FOXC2 pathway that resulted in decreasing metastatic ability of lung cancer cells.



## **1. Introduction**

### **1.1 Metastasis**

Metastasis is a disease that cancer cells spread from the primary neoplasm to distant organs. Metastasis is usually in the late stage of cancer and common cause death in cancer patients. Stephen Paget's in 1889 proposal that "seed and soil" hypothesis to explain metastasis which depends on cross-talk between selected cancer cells (the 'seed') and specific organ microenvironments (the 'soil') [1]. There is also a propensity for certain tumors to seed in particular organs. Metastasis is a multi-step process: the first step-"invasion"-some tumor cells invade into surrounding normal host tissue [2] and the second step-"intravasation"-tumor cells penetrate small vascular channels or lymphatics to entry into the circulation [3,4]. Only small part of tumor cells survival in the circulation, and then interacts with platelets, lymphocytes, and monocytes [5]. The surviving cancer cells complete the next step-"extravasation"-which arrest in the capillary beds of distant organs by adhering either to capillary endothelial cells or to subendothelial basement membrane. Finally, in the new host environment, tumor cells growth succeed from minute growths (micrometastasis) into malignant, secondary tumors [6,7].

### **1.2 Epithelial-Mesenchymal Transition**

Epithelial cells form a sheet or layers of cells that contact each other by tight junction, adherens junction, desmosome and gap junction to hold them tightly together and inhibit the movement of individual cell away from the epithelial layer [8]. Epithelial cells establish apical-basolateral polarity through association with a lamina layer at their basal surface, called the basement membrane [9]. In contrast to epithelial cells, mesenchymal cells interact with neighboring cells only focally contacts without forming organized cell layers, and exhibit a front-back end polarity [10]. In culture, epithelial cells grow as cluster and maintain complete cell-cell interaction with

neighbours, whereas mesenchymal cells look like spindle-shaped, fibroblast-like morphology and tend to be highly motile, but *in vivo* may not [11]. Epithelial-mesenchymal transition (EMT) was first recognized in series of elegant experiments in 1980s, the epithelial cells transformed to mesenchymal cells through a cellular program called EMT [12,13,14]. During EMT, cells would separate, lose the apical-basolateral polarity, and become more elongated, fibroblast-shaped, and change adhesions to facilitate cell movement and to invade extracellular matrix (ECM) [10,15]. EMT transition reduces cell-cell interaction via the transcriptional repression and delocalization of adherens junctions (cadherin), tight junctions (occluding and claudin), desmosomes (desmoplakin) and cytokeratin intermediate filaments (vimentin and fibronectin) [16,17,18,19]. Repression of the expression of E-cadherin [20], an important caretaker of the epithelial phenotype and induction of vimentin [21], a hallmark of mesenchymal type is characteristic of EMT. During embryogenesis, EMT plays an important role in development tissues, and required to gastrulation formation [22,23], neural crest development [24], heart-valve development [25,26] and secondary palate formation [27]. The reverse program epithelial cells become mesenchymal cells termed mesenchymal-epithelial transition (MET), occurs during embryogenesis and several pathological processes [15,28].

EMT transition also associates with pathological processes such fibrosis [29] and cancer [30] which leads to carcinoma progression and poor prognosis in various human and mouse tumors. Loss of E-cadherin, a tumor suppressor protein correlates with carcinoma progression and poor prognosis in various human and mouse model, such breast cancer and prostate cancer [31,32]. Mutation of E-cadherin leads protein loss of function and leads to about 50% of lobular breast carcinomas [33]. There are several pathways induce EMT transition, notably TGF- $\beta$  signaling which implicated as major induction signals [30,34], Wnt signal [35,36], Notch pathway [37,38],

Hedgehog signaling [39]. Hypoxia is one of physiological mechanisms that promote EMT program through up-regulation hypoxia-inducing factor-1 $\alpha$  (HIF1 $\alpha$ ), TWIST [40] and SNAIL [41]. Some transcription factors known to involved in EMT transition such as Snail[42,43], Slug [44], Twist [45], ZEB1/ $\delta$ EF [46], and ZEB2/SIP1 [47],E12/E47 [48], Goosecoid [49] and FOXC2 [50] associated with tumor invasion and metastasis. Twist is a basic helix-loop-helix (bHLH) transcription factor, plays a role in mesodermal differentiation and myogenesis [51]. Recently studies showed that increasing the expression of twist leads to cancer metastasis [45]. SNAI1 and Slug (SNAI2) are the members of SNAIL family which are conserved zinc-finger proteins and bind to the E-box elements of E-cadherin promoter to repress the expression of E-cadherin in epithelial cells [42,43,44].

### 1.3 Forkhead-Box C2

The FOXC2 (forkhead-box C2), previously termed *MFH1* (mesenchyme forkhead-1) or *FKHL14*, located on 16q24.1 is one of forkhead box family which have conserved DNA binding domain term forkhead box or winged helix domain [52]. The expression of *FOXC2* is mainly in mesoderm-derived tissues [53] and adipose tissue in adult [54]. *FOXC2* plays an important role in kidney embryonic development [55] and lymphatic sporting during vascular development [56]. *Foxc2* also plays a role in angiogenesis by regulating integrin  $\beta$ 3 expression [57]. The expression of FOXC2 leads to cancer cells migration and invasion *in vitro*, and *in vivo* studies show that expression of FOXC2 is associated with basal-like subtype breast cancer which is higher compared with HER2 overexpression tumor and luminal ER-positive subtype of tumor [50].

### 1.4 Resveratrol

Resveratrol (*trans*-3, 5, 4'-trihydroxystilbene) is a polyphenols which was first isolated from white hellebore (*Veratrum grandiflorum O. Loes*) in 1940, and since

then have been also found in various plants including grapes, peanuts, mulberries [58]. In 1963, the root in *Polygonum Caspidatum* used in traditional Chinese and Japanese medicine called Ko-jo-kon[59] found the existence of resveratrol. The sources of resveratrol was from red wine and red grape [60] which was synthesized in the leaf epidermis and in the grape skins [61]. Resveratrol can prevent or slow the progression of illnesses including cardiovascular [62], cancer [63] and ischemic injury [64,65] . There are studies shown that resveratrol could extend lifespan by silent information regulator 2 (Sir2)-dependent mechanisms in *Saccharomyces cerevisiae cerevisiae* [66], *Caenorhabditis elegans* [67] and *Dorsophila melanogaster* [68], and also extend lifespan of short-lived fish *Nothobranchius furzen* [69]. Resveratrol is an activator of SIRT1 which has the ability to prolong survival of calorie restriction mice treated with high-calorie diet [70].

The effects of resveratrol are dependent on the cell type, cellular condition, and concentrations and it can have opposing activities [61]. Because resveratrol has the ability to inhibit cancer formation at initiation, promotion and progression, it is shown to have chemoprevention ability [61]. Recently studies shows that resveratrol have chemoprevention functions in liver cancer [71,72], colon cancer [73,74], breast cancer [75], lung cancer [76,77], melanoma [78,79], head and neck squamous cell carcinoma [80,81] and ovarian and cervical carcinoma [82,83,84]. Resveratrol down-regulates the enzyme activity and transcriptional activity of cytochrome P-450 1A1 (CYP1A1) which is a carcinogen-activating enzyme, indicated that resveratrol can inhibit tumor progression at initiation stage [85] and resveratrol also can inhibit tumor formation by down-regulating the expression of cyclooxygenase 2 (COX2) [86]. Treatment with resveratrol could promote apoptosis through FasL pathway [87] , mitochondria pathway [88], p53 activation pathway [89,90,91,92] and Rb-E2F/DP pathway [93]. Resveratrol mediates cell-cycle arrest at difference stages in different cells

[89,94,95,96,97,98]. Some transcription factors are inhibited by resveratrol, including NF- $\kappa$ B [99,100,101,102], AP-1 [103], Egr-1 [104,105], and AP-2 $\alpha$  [106] and also down-regulate protein kinases such I $\kappa$ B $\alpha$  [100], JNK, MAPK, ERK1/2 [91,92,107,108], Akt [109,110], PKC [111], PKD [112] and CKII [113]. Pharmacokinetic studies shows that in human, resveratrol has rapid metabolism (~8 to 14 min) and converted to sulfate and glucuronide conjugate within ~30 min at liver and kidney [114]. Resveratrol inhibits vascular endothelial growth factor (VEGF)-induced angiogenesis in HUVECs [115] and also inhibits cancer cells invasion and migration in breast cancer [116]. Recently studies shown that resveratrol inhibits the invasion of tumor cells through repression of MMP-2 [116] or MMP-9 [117]. Resveratrol has the effect on prevention tumor growth and metastasis to lung in Lewis Lung Carcinoma-Bearing Mice [118]. Now, treatment with resveratrol in human colon cancer and colorectal cancer have several clinical trials at phase I stage [58].

Thus, we examined the signaling pathway involve in resveratrol-mediated cell metastasis in human lung cancer cells. These data showed that resveratrol induced the activation of serine/threonine protein phosphatase 2A, PP2A/C, in turn, down-regulation the signaling pathway PI3K/Akt-FOXC2. In this study, we found that inactivation of PP2A/C increase the expression of FOXC2 through up-regulation of Akt. These data also found the forkhead protein, FOXC2, was involved in resveratrol-mediated inhibition of metastasis in lung cancer and it may suggest to be a biomarker to early diagnosis in lung cancer. We provide evidence that resveratrol would be a critical therapeutic strategy for lung cancer.



## 2. Materials and Methods

**2.1 Reagents.** Resveratrol (>99% purify) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of resveratrol was made in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 5mM and stored at -80°C LY294002 (2-(4-Morpholino)-8-phenyl-4*H*-1-benzopyran-4-one), and okadaic acid (OA) was purchased from Sigma-Aldrich. UO126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) was purchased from Calbiochem (Nottingham, U.K.) Anti-E-cadherin, anti-fibronectin, and anti-N-cadherin were purchased from BD biosciences (San Jose, CA, USA). Anti-FOXC2 was from Abcam (Cambridge, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PP2A C subunit was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PP2A C subunit (52F8), anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-extracellular signal-regulated kinase (ERK) 1/2, and anti-ERK were all purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-vimentin was purchased from Neomarker (Fremont, CA, USA). Anti-twist, anti-slug, and anti-snail were from Santa Cruz. Anti- $\alpha$ -tubulin was from Sigma-Aldrich (St. Louis, MO, USA).

**2.2 Cell culture.** GP+E86 and PA317 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Invitrogen, Carlsbad, CA, USA). Lung cancer cell lines CL1-5, A549, H322, H1435, and 293T cells were supplemented with DME/F12 (1:1) (Hyclone Laboratories, Logan, Utah, USA). NCI-H520 cells were maintained in RPMI-1640 (Hyclone Laboratories, Logan, Utah, USA). The A549 overexpression FOXC2 cells and A549 and CL1-5 knockdown PP2A/C expression cells were cultured in DME/F12 containing 2 $\mu$ g/mL puromycin (Sigma-Aldrich). All of medium contained 10% fetal bovine serum and antibiotics (100IU/ml penicillin G and 100 $\mu$ g/ml streptomycin). Cell culture were maintained at 37°C in humidified 5% CO<sub>2</sub>



atmosphere. For treatment, the resveratrol was diluted in medium and added to cultures to give the desired final concentrations. Untreated cultures received the same amount of the carrier solvent DMSO.

**2.3 Western blotting analysis.** Resveratrol-treated cells were examined by Western blot analysis at the indicated time. The whole cell extracts were prepared by lysing cell in NETN lysis buffer (150mM NaCl, 20mM Tris-HCl pH8.0, 0.5% NP40 and 1mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich). After electrophoresis, the proteins were electrotransferred to PVDF membrane (Millipore Corporation, Bedford, MA, USA), blocked by 5% non-fat milk, and probed with the indicated antibodies at 4°C overnight. The blots were then washed, exposed to horseradish peroxidase-conjugated secondary antibodies (Abcam) 1:5000 dilution for 1hr at room temperature, and then detected by chemiluminescence reagent (Millipore).

**2.4 Migration and invasion assays.** For migration assay, cells pretreated by the indicated concentrations of resveratrol for 24h and suspended  $5 \times 10^4$  cells to layered in the upper compartment of a Transwell an 8- $\mu$ m-diameter polycarbonate filter (8 $\mu$ m pore size) which was from Corning Incorporated (Corning, NY, USA), and incubated at 37°C for 24h. For invasion assays,  $5 \times 10^4$  cells was suspend to the upper layer which coated with 30 $\mu$ L diluted matrigel (BD biosciences) and then treated the indicated concentrations of resveratrol for 48h. Each chamber was washed with PBS, cells were fixed by cold-methanol, stained by 0.05% crystal violet, removed noninvaded cells on the top of transwell with cotton swab, and then counted cell with three different fields under light microscope.

**2.5 Cell viability by MTS assay.** MTS assays were performed according to the manufacturer's instructions (CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA).  $5 \times 10^4$  cells were treated with resveratrol for the

indicated time in 24-well tissue culture plate with 1000 $\mu$ L of medium.

**2.6 Colony formation assay.** 500 cells were seeded in 6-well tissue culture plate and treated with resveratrol for 48h, replaced fresh medium and incubated for 4 days until colonies were visible. Cells were fixed with 3.7% formaldehy/PBS and were stained by 0.05% crystal violet. The total number of colonies formed on each well were counted.

**2.7 Reverse transcriptase–polymerase chain reaction (RT-PCR).** Total RNA was isolated using Trizol reagent (Invitrogen) and reverse transcribed into single-stranded cDNA with moloney murine leukemia virusreverse transcriptase (MMLV-RT; Invitrogen) as manufacturer’s instructions. The primer sequences for FOXC2 were: 5’-GCCTAAGGACCTGGTGAAGC-3’ (forward) and 5’-TTGACGAAGCACTCGTTGAG-3’ (reverse); for human  $\beta$ -actin were: 5’-GCTCGTCGTCGACAACGGCTC-3’ (forward) and 5’-CAAACATGATCTGGGTCATCTTCTC-3’ (reverse). The reaction mixture was first denatured at 94 $^{\circ}$ C for 5 min. For FOXC2, the PCR condition was 94 $^{\circ}$ C for 30 sec, 56 $^{\circ}$ C for 30 sec, and 72 $^{\circ}$ C for 30 sec for 35 cycles; for  $\beta$ -actin was 94 $^{\circ}$ C for 30 sec, 55 $^{\circ}$ C for 30 sec, and 72 $^{\circ}$ C for 30 sec for 22cycles followed by 72 $^{\circ}$ C for 10 min.

**2.8 Immunofluorescence staining.** Treatment with resveratrol for 48h, cells were fixed in 4% paraformaldehyde/PBS for 15min at room temperature and then permeabilized with 0.1% Triton X-100/PBS for 10min at room temperature. Nonspecific binding sites were block with 5% BSA/PBS and then the cells were incubated with in 1:100 polyclonal antibodies for E-cadherin (Cell Signaling, MA, USA) overnight at 4 $^{\circ}$ C and then at room temperature for 1h. After slide was rinsed, primary antibody was detection by Alex Fluoro 488-FITC (Molecular Probes, Eugene, OR, USA) for 1h at room temperature. The nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 10min at room temperature.

Images were captured with ZEISS fluorescence microscope using software program Axio Vision 4.7.2.

**2.9 Establish stable cell line by retrovirus infection.** The human FOXC2 cDNA was subcloned into the pBabe-Puro vector and was obtained from Addgene (Cambridge, MA, USA). Retroviral vector expressing FOXC2 gene or the control vector was transfected 4 $\mu$ g plasmid into an ecotropic packaging cell line by using lipofectamin<sup>TM</sup> LTX (Invitrogen), GP+E86 containing 4 $\times 10^5$  cells in 6-cm-diameter dish, and harvested overnight. Before infection to a second generation packaging cell line, PA317, the medium of GP+E86 containing polybrene (8 $\mu$ g/mL) were filtered by 0.22 $\mu$ m filter, and added 3mL medium to the 10-cm-diameter dish containing 5 $\times 10^5$  cells of PA317. After 2h, added the complete medium to 10mL in PA317 and harvested overnight. Packaging cells then were selected with 2 $\mu$ g/mL of puromycin until a confluent 10-cm-diameter dish of resistant cells was obtained. Amphotropic virus were collected from the medium of PA317 to infect cultured cells as described above, and then these cultured cells were selected with 2 $\mu$ g/mL puromycin. After selection, these cell lines dilute with 1:1000 to obtain single clone about four weeks.

**2.10 Construction of luciferase reporters.** The FOXC2 promoter (nucleotides -1990 to the Nru site +6) was amplified by PCR (sense: 5'-tgctcgagtgcccaaccagaccagcaac, and antisense: 3'-actaagcttctgcgtgcttccgagac) using FOXC2 BAC clone (Invitrogen) as template. The PCR product was inserted into the cutting site at XhoI and HindIII of pGEM T-easy vector (Promega). The fragment after confirming sequence was subsequently inserted into pGL3-basic reporter (Promega). For a series of deletion constructs was done by described above. For FOXC2-promoter from -1990 to -984, the primers are (sense: 5'-tgctcgagtgcccaaccagaccagcaac, and antisense: 3'-tgctcgagcctgccattccaatccagcg); for -984 to +6 using primers (sense: 5'-actaagcttctgcgtgcttccgagac, and antisense:

3'- actaagcttctgcgtgctgcttccgagac); from -215 to +6 using primers for (sense: 5'-tgctcgagatccgcccggtccgctgaag, and antisense: 3'- actaagcttctgcgtgctgcttccgagac). By restriction enzyme BlnI digested FOXC2-promoter from -984 to +6 to generate FOXC2-promoter from -984 to -460 and from -460 to +6.

**2.11 Transfection and reporter assay.** Transfection of plasmid DNA (pcDNA3.1 and myr-Akt) was performed using Lipofectamine™ LTX or Lipofectamine 2000 for reporter assay according to the manufacturer's instructions (Invitrogen). For luciferase reporter assays, firefly luciferase activities were normalized to total protein concentrations. After transfection, resveratrol were treated for 48hr. Luciferase assays were carried out using the Luciferase Assay System (Promega) according to manufacturer's protocol.

**2.12 The PP2A immunoprecipitation phosphatase activity.** A549 cells were treated with resveratrol at the indicated times and lysed by NETN lysis buffer containing aprotinin, PMSF. Equal amount of 500µg proteins were used to assay phosphatase activity according to manufacturer's instructions (Millipore). Briefly, adding 2 µg anti-PP2A C subunit to lysate, rotated at 4°C for overnight and then washed. Added phosphopeptide to lysate, incubated at 30°C for 10min, and then added malachite green detection solution to lysate incubation for 10 min at room temperature and detected activity for OD<sub>650</sub>.

**2.13 Construction and Production of shRNA in Lentiviral Vector.** shPP2A E1 (TRCN000002486) clone, shPP2A C1 (TRCN000002484) clone, pLKO.1-shLuc vector (shRNA against luciferase, act as a control), pMD.G plasmid and pCMVdeltaR8.91 plasmid were obtained form National RNAi Core Facility at the Genomics Research Center (Academia Sinica, Taipei, Taiwan). Recombinant lentiviruses were produced by co-transfecting 293T cells with the lentivirus expression plasmid, the lentivirus packaging vectors pCMVdeltaR8.91, and the

vesicular stomatitis virus G glycoprotein (VSVG) expression vector pMD.G using the Lipofectamine<sup>TM</sup> LTX according to manufacturer's instructions. The viruses were collected from the culture supernatants on 2 days post-transfection and filtered by 0.45 $\mu$ M filter. Cultured cells were incubated with lentivirus containing 8  $\mu$ g/mL polybrene for 24h, replaced medium and incubated for another 2 days. For stable clone, cells were then selected with 2 $\mu$ g/mL puromycin for 1 week.

#### **2.14 Statistical analysis.**

Results are expressed as mean  $\pm$  SD or SE, as indicated. One-tailed student's *t* test was used to compare the intergroup;  $p < 0.05$  was considered statistically significant.



### **3. Results**

#### **3.1 Resveratrol inhibits epithelial-mesenchymal transition in lung cancer cells.**

There were many studies shown epithelial-mesenchymal transition (EMT) plays an important role in regulating invasion and migration of cancer cells. To elucidate whether resveratrol inhibited cell mobility of lung cancer cells, CL1-5 and A549 lung carcinoma cells were treated with various concentrations of resveratrol, and cell mobility were determined by migration and invasion assay. Treatment with the indicated concentrations of resveratrol decreased the migration and invasion ability of lung cancer cells in a dose-dependent manner (Fig.1). During EMT transition, cancer cells lose the expression of E-cadherin which regulating cell-cell contact and acquired mesenchymal markers such as vimentin, fibronectin, and N-cadherin [21,31,119]. To examine that the effects of resveratrol in lung cancer cells would abolish EMT transition, lung cancer cells treated with 10 $\mu$ M resveratrol for 48h and then analyzed E-cadherin and mesenchymal markers expression by western blotting analysis. Resveratrol increased the expression of E-cadherin and decreased the expression of mesenchymal markers such as fibronectin, N-cadherin and vimentin in lung cancer cells (Fig.2). To confirm resveratrol-inhibited EMT with up-regulation of the expression of E-cadherin, immunofluorescence staining was preformed. The expression of E-cadherin was induced in resveratrol-treated A549, but not in control cells (Fig.3). The cell morphology of CL1-5 cells changed from spindle-shaped mesenchymal type to less-elongated epithelial cell morphology after treatment with resveratrol (Fig.4). The effect of resveratrol on cell viability of CL1-5 cells was down by MTS assay. Treatment with resveratrol for the indicated time had no effect on cell viability (Fig.5). These data indicated that treatment with resveratrol decreased cell mobility through down-regulation EMT and changed cell morphology from spindle-shaped to epithelial characteristics of lung cancer cells.



### **3.2 Resveratrol significantly inhibits EMT-inducing transcription factor, FOXC2, at transcriptional level in lung cancer cells.**

To elucidated how resveratrol-inhibited EMT, EMT-inducing transcription factors, FOXC2, twist, slug and snail were analysis by western blot analysis in lung cancer cell lines. Resveratrol significantly decreased the expression of FOXC2 in CL1-5 and A549 cells, but had less effect on twist and slug (Fig.6). Inhibition the expression of FOXC2 by resveratrol had the same result in other lung cancer cell lines (Fig.7). To examine that resveratrol-mediated inhibition of FOXC2 was through down-regulation of gene expression, RT-PCR was performed. Fig.7 revealed that resveratrol inhibited the expression of *FOXC2* was at transcriptional level in lung cancer cells. The inhibition ability of FOXC2 in response to resveratrol was for 48h and resveratrol enhanced E-cadherin expression was at time-dependent manner (Fig.8).

To determine the regulatory mechanism underlying the transcription of *FOXC2* gene by resveratrol, we analyzed the promoter region of human *FOXC2* gene. Within the ~1.9-kb fragment of upstream the start codon, there were several transcription factor binding sites. To identify the resveratrol-response elements, a series of 5' promoter deletion mutants of the *FOXC2* gene (F1-F6) were constructed. The F1, F3, F5 and F6 reporter constructs were down-regulated by resveratrol about 40-50% which indicating that resveratrol-response elements lie between -215 and +6 (F6) (Fig.9). These observations suggest that resveratrol-inhibition EMT were through down-regulated the expression of *FOXC2* in human lung cancer cells.

### **3.3 Ectopic expression of FOXC2 reverses resveratrol-mediated cell mobility and EMT.**

To investigate whether the expression of FOXC2 involved in resveratrol-mediated repression of cell mobility in lung cancer cells, FOXC2 was

ectopic expressed in A549 lung cancer cells. Overexpression of FOXC2 in A549 decreased the levels of E-cadherin and up-regulated mesenchymal markers, fibronectin, N-cadherin and vimentin expression (Fig.10). It was therefore of interest to determine whether resveratrol-inhibited EMT transition were exerted through the regulation of forkhead protein. Western blot and immunofluorescence staining experiments were performed. Stable expressed cells with FOXC2 increased the migration and invasion ability *in vitro* study, and rescued resveratrol-reduced cell mobility and resveratrol-inhibited the expression of EMT markers (Fig.11-13). Treatment with resveratrol had no effect on cell viability in A549 overexpression of FOXC2 cells (Fig.14-15). These results indicated that resveratrol-mediated repression of EMT transition through down-regulated the expression of FOXC2 in lung cancer cells.

#### **3.4 PI-3K/Akt activity is required to increase the expression of FOXC2 in A549 cells.**

Activation of FOXC2 is involved in phosphatidylinositol 3-kinase (PI3K) and ERK1/2 signaling pathways in adipocytes [120] and endothelial cells [121] but the role of FOXC2 in cell mobility and EMT in lung cancer cells is not clear. To address this issue, A549 cells were treated with PI-3K/Akt inhibitor, LY294002 and dual inhibitor of MEK1 and MEK2, U0126, and then FOXC2 expression were analyzed by western blot and by reporter assay. Fig.16 revealed that PI-3K/Akt inhibitor, LY294002, but not MEK inhibitor, U0126, reduced the expression of FOXC2 and FOXC2 promoter activities (F1). To confirm this observation, we used another approach to test the role of Akt in regulating FOXC2 expression by transfected constitutively activate myristoylated Akt, myr-Akt, in A549 cells with or without resveratrol. These results revealed that constitutive activation of Akt induced up-regulation FOXC2 expression, and rescued resveratrol-mediated inhibition of

FOXC2. Overexpression of Akt in cancer cells led to EMT induction and rescued resveratrol-mediated EMT markers expression (Fig.17). These data suggest the possible involvement of PI-3K/Akt, rather than MAPK signaling pathway, in the regulation of FOXC2 expression and EMT transition in lung cancer cells.

### **3.5 Protein serine/threonine phosphatase 2A catalytic subunit C (PP2A/C) acts as an up-regulator of resveratrol-inhibited FOXC2 expression.**

Many researchers have showed that PP2A, a serine/threonine phosphatase, interacts with Akt and thus down-regulates Akt activity by dephosphorylating Akt [122]. To examine that involvement of PP2A/C in resveratrol-mediated inhibition of PI-3K/Akt, A549 human lung cancer cells were treated with resveratrol with different concentrations or at different time and then analyzed the expression of PP2A/C and PP2A activity. Treatment with resveratrol increased the expression of PP2A/C and up-regulated the expression of PP2A/C in a time-dependent manner as well as PP2A activities (Fig.18). To determine the role of PP2A/C in regulating the expression of FOXC2 through dephosphorylated Akt, A549 cells were treated with resveratrol in the presence of okadaic acid (OA), a PP2A inhibitor. Data from western blot confirmed that decreased the expression of PP2A/C by PP2A inhibitor significantly increased FOXC2 and phospho-Akt expression and decreased resveratrol-induced PP2A activation (Fig.19). To further confirm this observation, we used another approach to test the role of PP2A/C by expression shLuc and shPP2A/C targeted knockdown control or PP2A/C by lentivirus infection, respectively. These results support those obtained above that down-regulation of PP2A/C increased the expression of FOXC2 and phospho-Akt by transient or stable infection (Fig.20). To ascertain the role of FOXC2 in resveratrol-mediated PP2A/C up-regulation, we knockdown PP2A/C and then treated with resveratrol. Down-regulated PP2A/C expression rescued resveratrol-inhibited FOXC2 and phosphorylated Akt expression and increased

resveratrol-inhibited cell mobility (Fig.21-22). These findings provided evidence that resveratrol-inhibited FOXC2 activation and cell mobility through down-regulation of Akt that inhibited by up-regulation the expression of PP2A/C.



#### 4. Discussions

Recent reports have shown that resveratrol is a potent cancer chemoprevention agent [63] that prevent, inhibits by either reducing angiogenesis [115], cancer cell metastasis [116,117] or inducing cancer cell apoptosis [87,88]. In this study, we verified that the regulation of the signaling pathway from protein serine/threonine phosphatase, PP2A/C, to subsequent Akt-mediated FOXC2 activation was critical for resveratrol-inhibited metastasis in human lung cancer cells.

The EMT program plays a role in cancer-related mortality: progression to distant metastatic disease and acquisition of therapeutic resistance and link to generation of cancer cells with stem cell-like properties [123]. To the best of our knowledge, there are currently no reports on EMT transition being involved in resveratrol-mediated inhibition of metastatic ability of cancer cells. In the present study, resveratrol inhibited the EMT transition and cell mobility in different lung cancer cells (Fig.1-3). We also showed that resveratrol significantly decreased FOXC2 expression but slightly on other EMT-related transcription factors, such as twist and slug (Fig.6-8). Overexpression of FOXC2 also significantly rescued resveratrol-inhibited EMT transition and cell mobility in lung cancer cells (Fig.11-13). In this study, resveratrol had the ability to repress EMT transition through down-regulation of FOXC2 expression. FOXC2 may be the potent downstream events that involved in resveratrol-inhibited cell metastasis in lung cancer cells.

FOXC1 and FOXC2 belong to the members of forkhead proteins. Overexpression of FOXC1 in MCF12A cells leads to complete EMT transition, as determined by cell morphology change from differentiated epithelial morphology to CD44<sup>+</sup> cell-like mesenchymal phenotype and decrease E-cadherin, increase N-cadherin expression, and increase cell motility and invasion ability [124]. Overexpression of FOXC2 in EpRas mouse mammary carcinoma cells leads to the



increased in cell metastatic ability to lung. Ectopic expression of Twist, Snail, Goosecoid or in response to EMT inducer, TGF- $\beta$ , in HMLE cells elevates FOXC2 expression and EMT transition [50]. Although the expression of FOXC2 may lead to cancer cells with higher metastatic potential, the mechanism involved in FOXC2 regulation remains unclear. Therefore, we suggest that resveratrol inhibits FOXC2 expression through PP2A-mediated Akt repression to decrease cancer mobility and overexpression of FOXC2 rescued resveratrol-inhibited cancer mobility. The results of our study showed that a signaling cascade of PP2A/Akt/FOXC2 in response to resveratrol-inhibited cancer metastasis.

The expression of *Foxc2* is up-regulated in the presence of insulin and TNF- $\alpha$  which through PI3K-Akt and ERK1/2-dependent pathway in 3T3-L1 adipocyte [120]. VEGF-activated PI3K and ERK pathways regulate the activity of Foxc1 and Foxc2 in Dll4 and Hey2 induction [121]. In this study, treatment with PI-3K/Akt inhibitor, LY294002, inhibited FOXC2 activation, but in the present of ERK1/2 inhibitor, U0126, did not decrease the inhibitor of FOXC2 analysis by western blot and reporter assay (Fig.16). Myr-Akt-transfected A549 cells induced the expression of FOXC2 and rescued resveratrol-inhibited FOXC2 expression (Fig.17). According to the above observation, we presume that resveratrol-mediated decreased FOXC2 expression is through PI-3K/Akt pathway, rather than ERK1/2 pathway in lung cancer cells. In this study, the questions of how Akt regulates the expression of FOXC2 need further investigation.

PP2A is a key protein serine/threonine phosphatase that is responsible for 30-50% of the total cellular serine/threonine dephosphorylation activity. PP2A is a holoenzyme contains an active core dimer composed by the catalytic subunit (PP2A/C) and a scaffold protein termed the A subunit (PP2A/A). The AC core dimer complex interact with regulatory B subunits (PP2A/B) to determine the substrate



specificity, subcellular localization and catalytic activity of the PP2A [125]. PP2A has been reported that regulates many signaling transduction processes, such as Akt [126,127] and MAPK [128]. PP2A has been shown to decrease Akt activity and dephosphorylate Akt at both Thr-308 and Ser-473 [126,127]. PP2A interacts with Akt [122] and PP2A inhibitors such as okadaic acid activate the expression of Akt means that PP2A plays an opposite role in regulation Akt [126,127]. Inhibition of PP2A activity by okadaic acid stimulates cell migration ability in Lewis lung carcinoma cells [129,130]. In this study, we showed that PP2A/C expression knockdown by shRNA induced cell metastatic ability and the expression of FOXC2 (Fig.20-22). Resveratrol-mediated activation of the expression of PP2A/C was critical for Akt dephosphorylation and FOXC2 down-regulation (Fig.19 and 21). Thus, we suggest that resveratrol-activated PP2A/C triggers dephosphorylation of downstream Akt to decrease the expression of FOXC2.

In conclusion, we present the first evidence demonstrating that the cancer-chemopreventive agent resveratrol induces a PP2A/C-dependent signaling pathway. We delineated the mechanism of resveratrol-inhibited metastasis. PP2A/C is activated after resveratrol treatment. The activated PP2A/C further inactivated the PI3K/Akt-FOXC2 pathway and resulted in the decreased metastatic ability of lung cancer cells (Fig.23).

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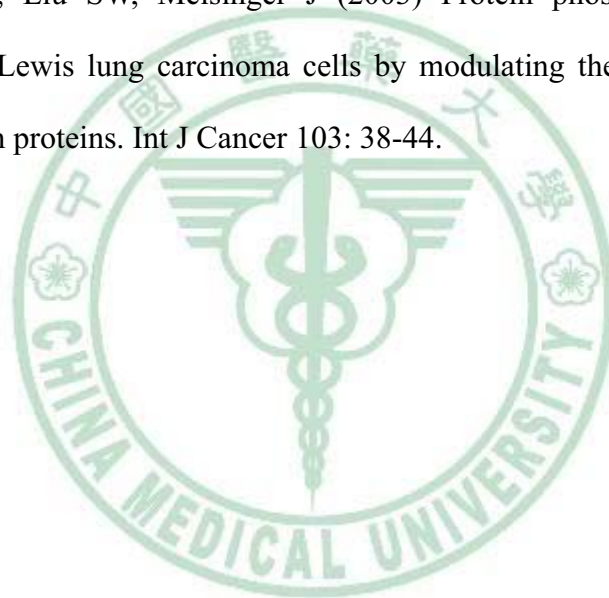


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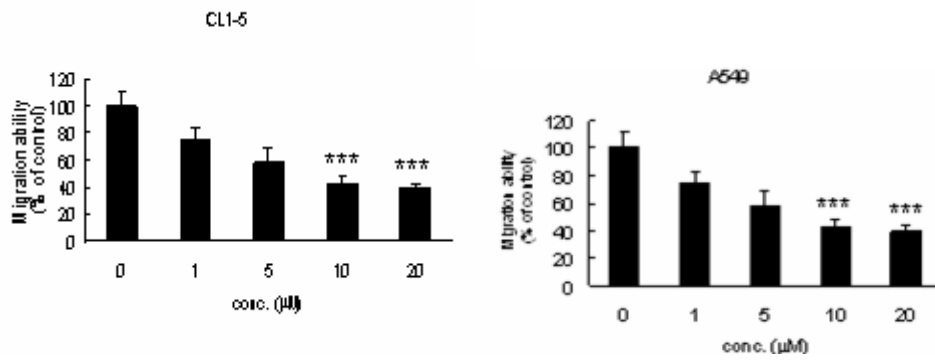
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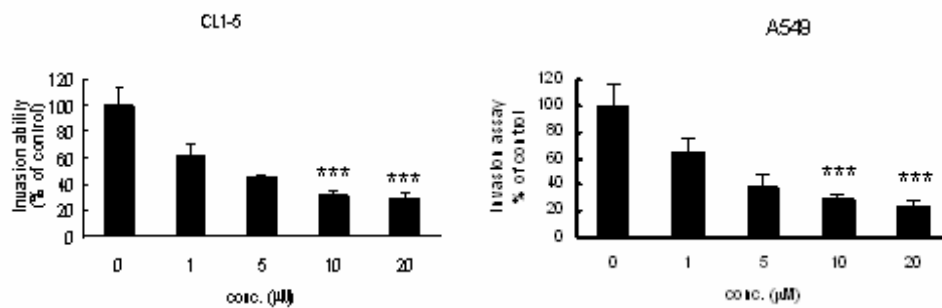
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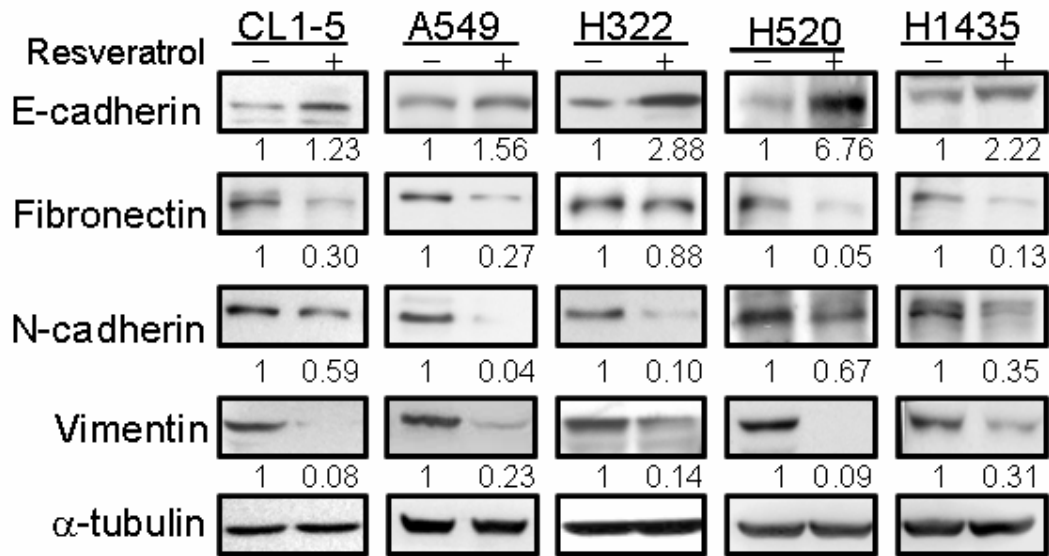
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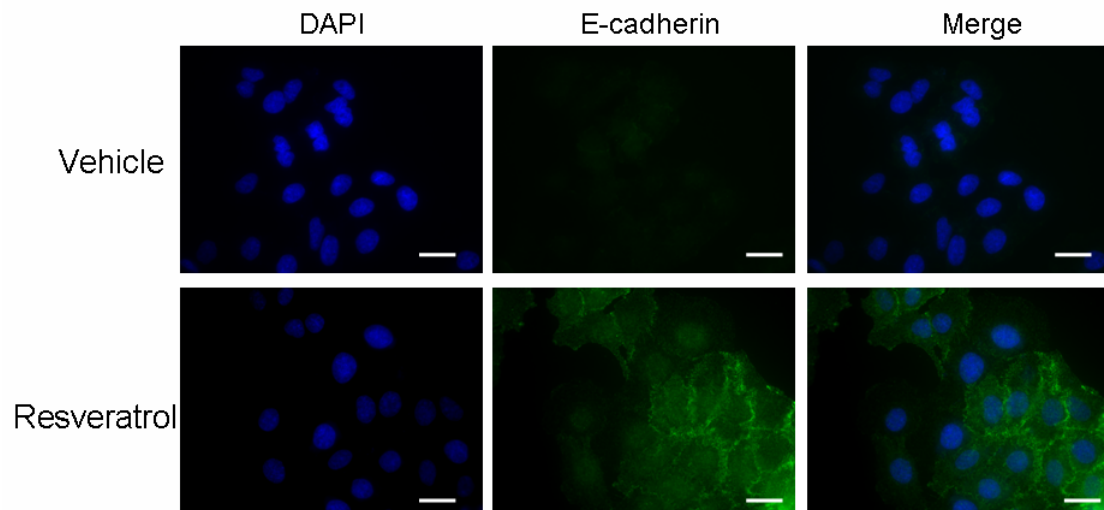


**Figure 1.** Resveratrol inhibited the cell metastatic and invasive ability in lung cancer cells. *A*, for migration assay, cells pretreated by the indicated concentrations of resveratrol for 24h and suspended cells to the upper layer of Transwell (8μm pore size) and harvested for 24h. *B*, for invasion assays, cancer cells was suspended to the upper layer of transwell coated with diluted matrigel and incubated for 48h. The indicated concentrations of resveratrol were added to both upper and bottom layers of transwell. Findings were representative of at least three separate experiments. *Bars* represent means  $\pm$  SD. *Asterisks* denote a significant difference compared with values for untreated control. (\*\*\*, p<0.001)

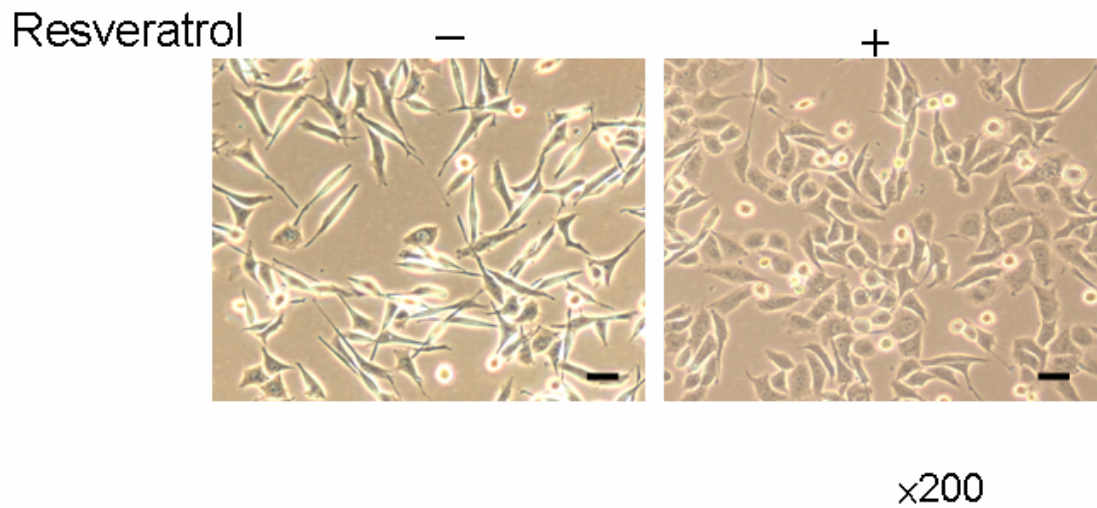


**Figure2.** Resveratrol repressed EMT transition in lung cancer cells. Western blot analyzed the expression of EMT markers with or without resveratrol (10 $\mu$ M) for 48h in different lung cancer cells as indicated. Numbers below lanes show folds of protein expression level. Findings were representative of at least three separate experiments.

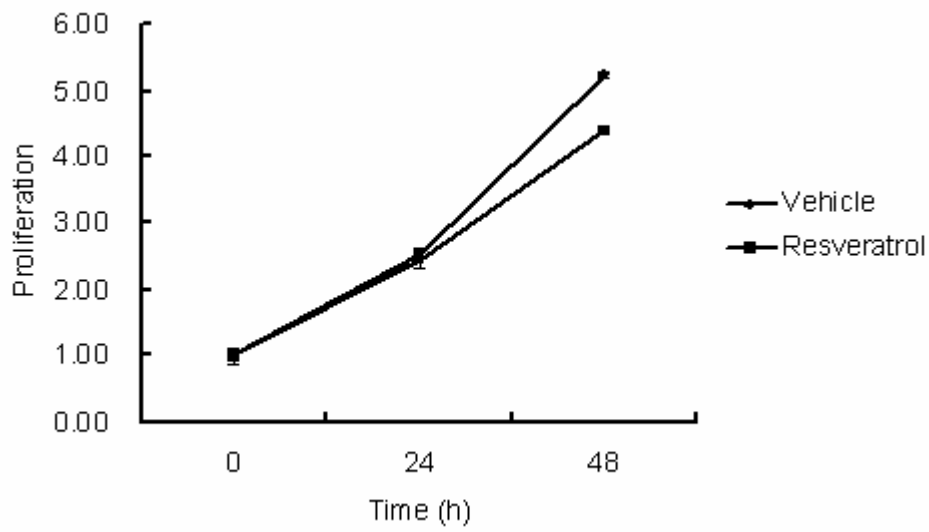




**Figure3.** Immunofluorescence staining for the effects of resveratrol on the expression of E-cadherin in lung cancer cells. Treatment with 10 $\mu$ M resveratrol A549 cells for 48h were fixed and then stained with anti-E-cadherin antibody (green). The nuclei were stained with DAPI (blue). Scale bars, 20 $\mu$ M. Findings were representative of three independent experiments.

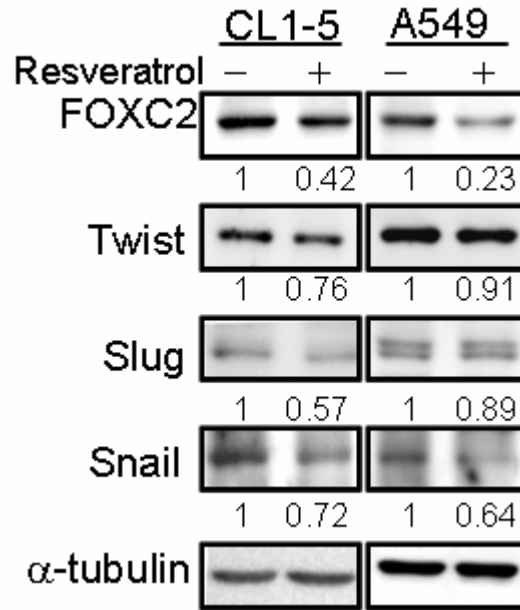


**Figure4.** Treatment with resveratrol changed cell morphology from mesenchymal-like to epithelial-like in CL1-5 cells. CL1-5 cells were cultured with 10 $\mu$ M resveratrol for 48h and then images were captured by phase-contrast microscopy. Scale bars, 100 $\mu$ M. Findings were representative of three separate experiments. Magnification:  $\times$ 200.



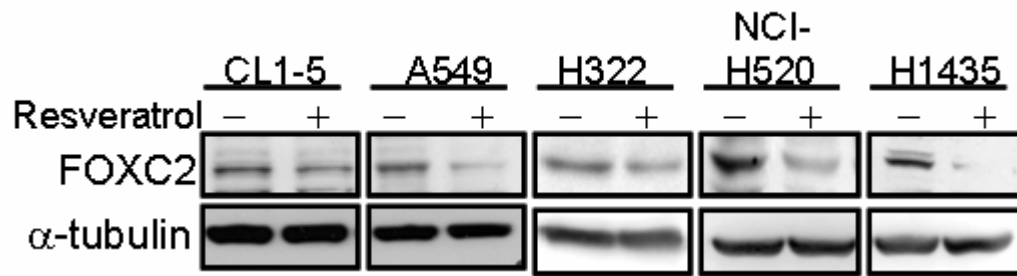
**Figure5.** Cell viability for treatment with resveratrol in CL1-5 cells. Treatment with 10 $\mu$ M resveratrol for 24h and 48h in CL1-5 cells. *Bars* represent means  $\pm$  SD.



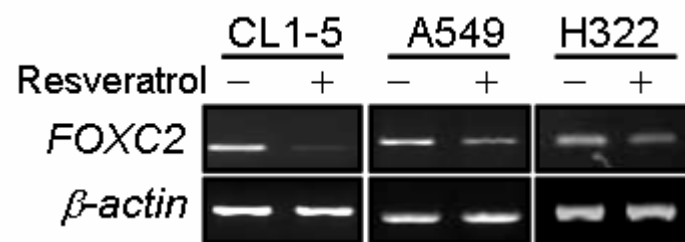


**Figure6.** Effect of resveratrol on EMT-inducing transcription factors in lung cancer cells. Western blotting analyzed the expression of twist, slug, snail and FOXC2 in response to resveratrol (10 $\mu$ M, 48h) in CL1-5 and A549 cells. Numbers below lanes show folds of protein expression level. Findings were reproduced on three separate occasions.

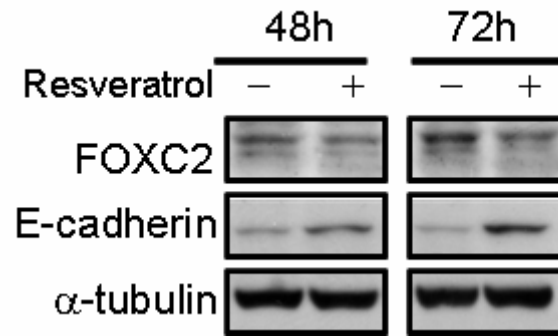
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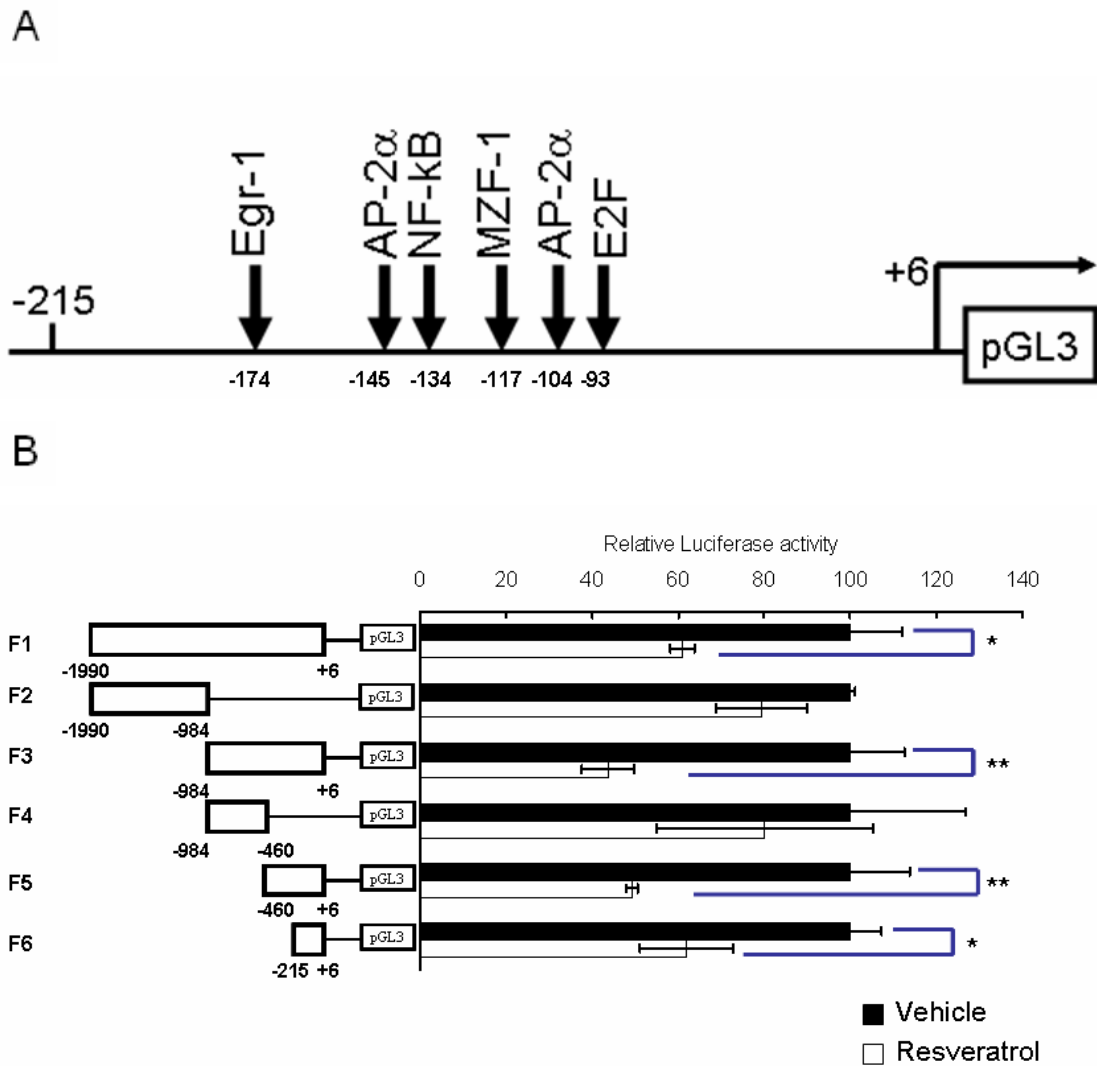
**Figure 7.** Resveratrol repressed the expression of FOXC2 in lung cancer cells. Western blotting (A) and RT-PCR (B) for analysis the effects of resveratrol (10 $\mu$ M, 48h) on the expression of FOXC2 in different lung cancer cells as indicated. Findings were reproduced on three separate experiments.



**Figure8.** Effects of resveratrol on the expression of FOXC2 and E-cadherin in A549 cells. A549 cells were treated with 10 $\mu$ M resveratrol for the indicated times, followed by measurements of FOXC2 and E-cadherin expression by western blot. Findings were representative of three separate experiments.

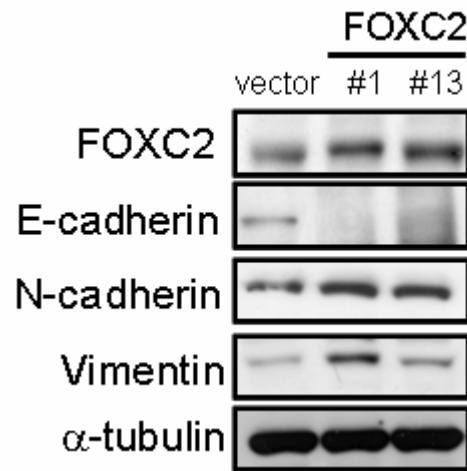




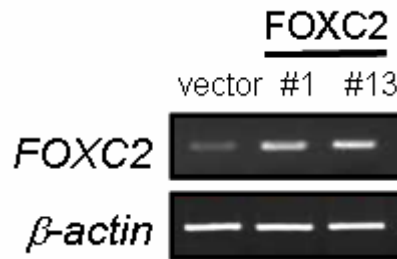


**Figure9.** Effect of resveratrol on *FOXC2* promoter activity in CL1-5 cells. *A*, schematic representation of human *FOXC2* promoter construct F6, Within the ~220-bp fragment, a potential Egr-1, AP-2 $\alpha$ , NF- $\kappa$ B, MZF-1 and E2F motifs exist. *B*, luciferase reporter assays using a series of 5' promoter deletion mutants of the *FOXC2* gene (F1-F6). The ~1.9-kb fragment upstream of the start codon contains the transcription factor-binding elements. CL1-5 cells were transfected with these reporter constructs, treated with or without resveratrol (20 $\mu$ M) for 48h and measured the luciferase activity. Bars represent means  $\pm$  SE from three independent experiments in triplicates. Asterisks denote a significant difference compared with values for untreated control (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

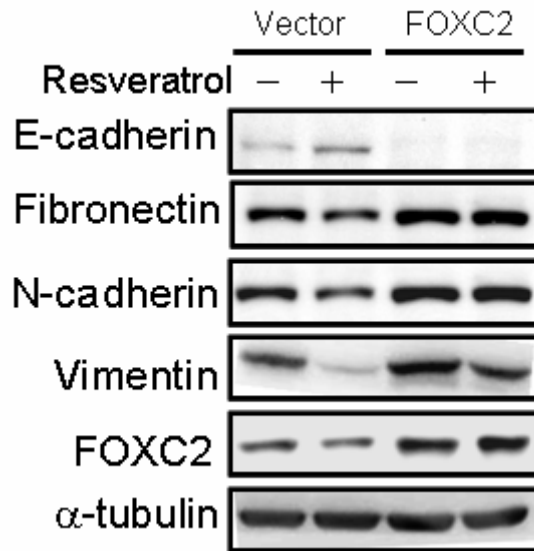
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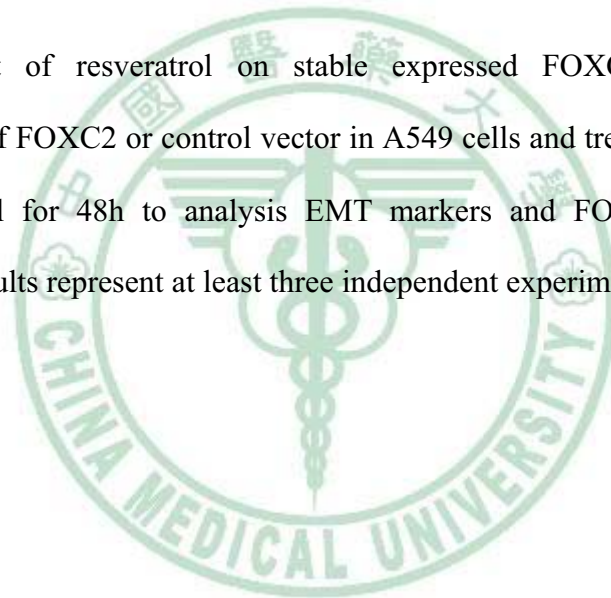
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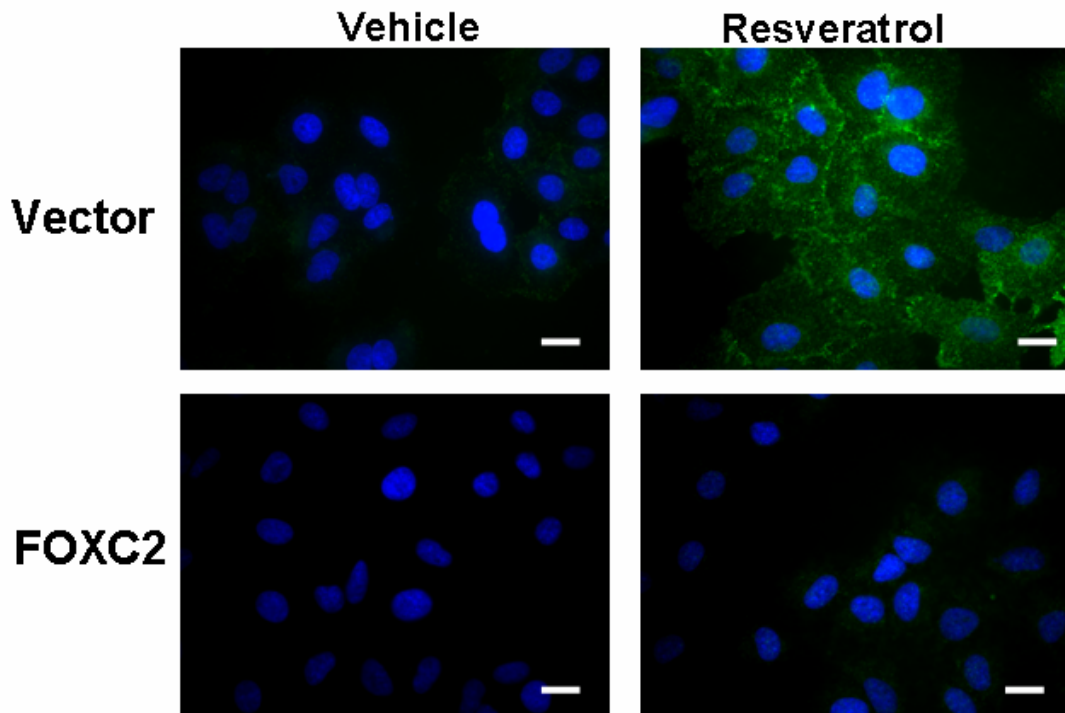


**Figure10.** Stable expression of FOXC2 in A549 cells. Overexpression of FOXC2 or control vector in A549 cells as described under “Materials and Methods,” followed by measurements of EMT markers and FOXC2 expression by western blot (A) and determined the expression of *FOXC2* by RT-PCR (B). Results were representative of three independent experiments.

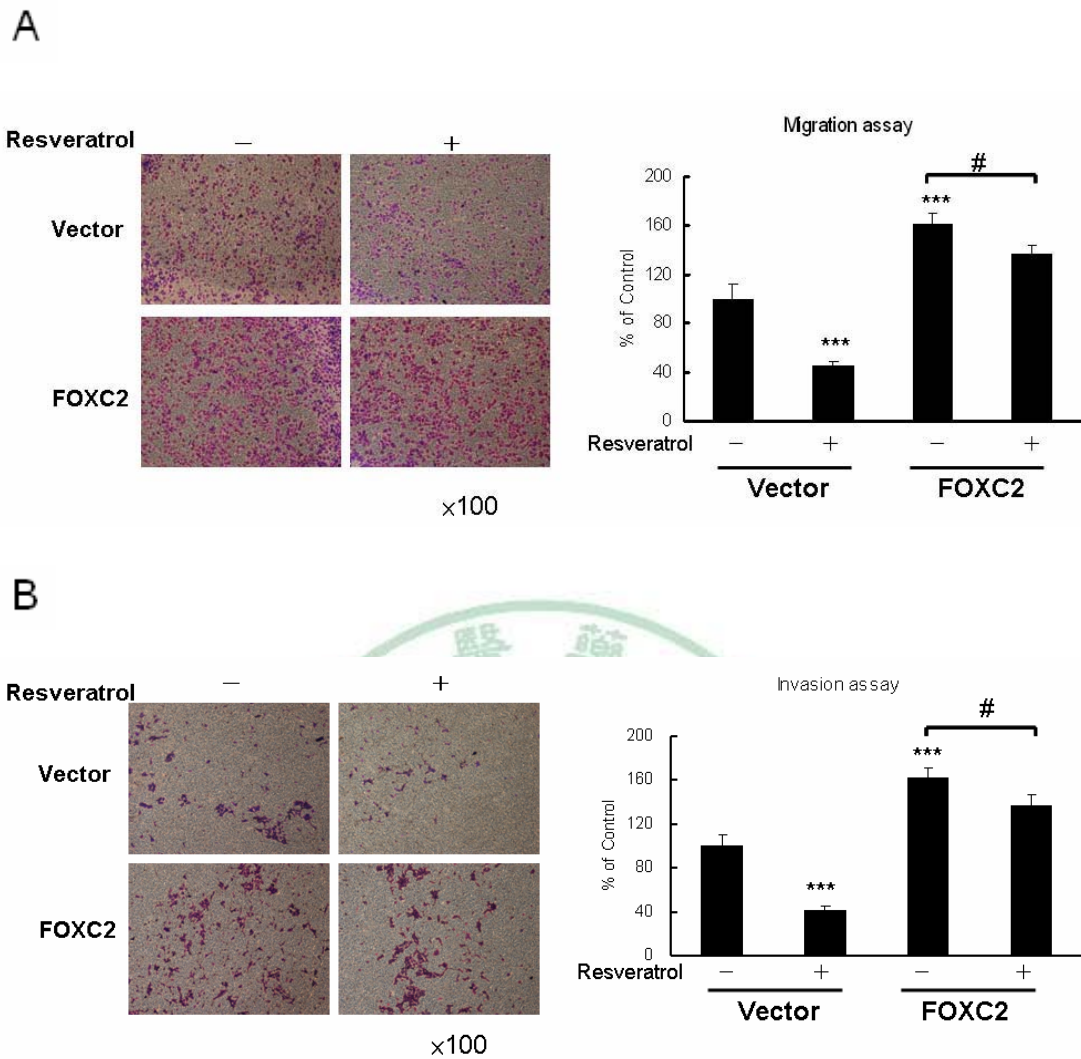


**Figure11.** Effect of resveratrol on stable expressed FOXC2 cells in A549. Overexpression of FOXC2 or control vector in A549 cells and treated with or without 10 $\mu$ M resveratrol for 48h to analysis EMT markers and FOXC2 expression by western blot. Results represent at least three independent experiments.

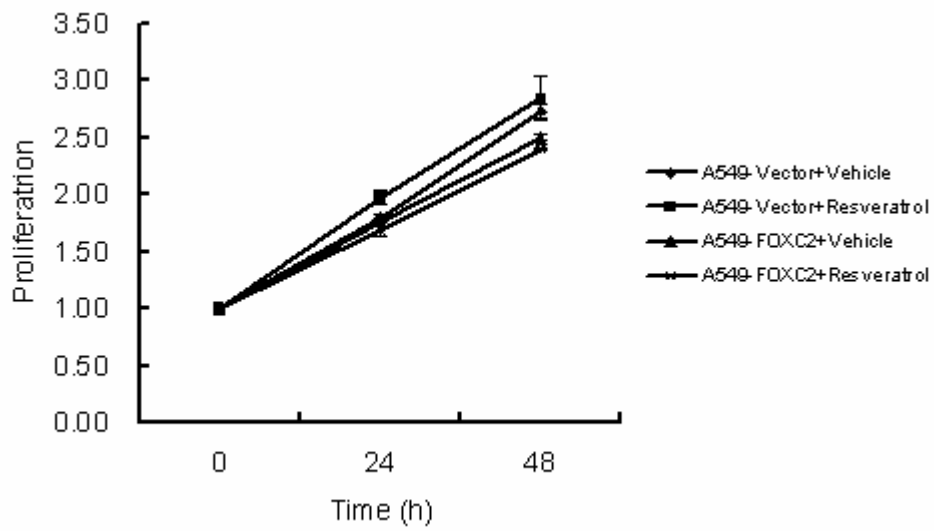




**Figure12.** Immunofluorescence staining for the effects of the expression of E-cadherin on resveratrol-treated A549-FOXC2 cells. A549-Vector and A549-FOXC2 cells cultured with or without resveratrol for 48h (10 $\mu$ M) and stained with anti-E-cadherin antibody (green). The nuclei were stained with DAPI (blue). Scale bars, 20 $\mu$ M. Results were representative of three separate experiments.



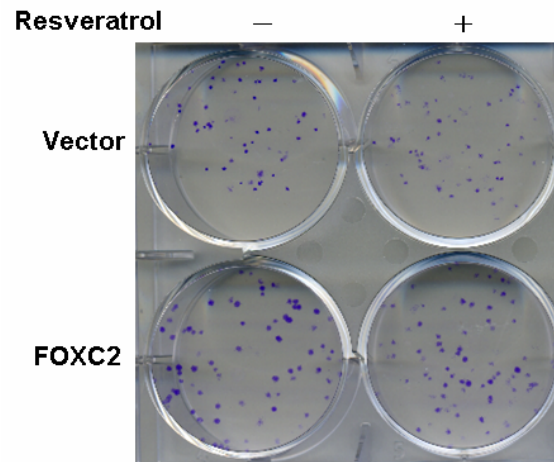
**Figure13.** Effects of resveratrol on cell metastatic and invasive ability in A549-FOXC2 cells. *A*, for migration assay, cells were treated with or without resveratrol (10 $\mu$ M) for 24h. *B*, for invasion assay cells were treated with resveratrol for 48h. Results represent findings of three independent experiments. *Bars* represent means  $\pm$  SD. *Asterisks* denote a significant difference compared with values for untreated control. *Hash* denote a significant difference compared with values for overexpression FOXC2-untreated group (\*\*\*,  $p < 0.001$ ; #,  $p < 0.05$ ).



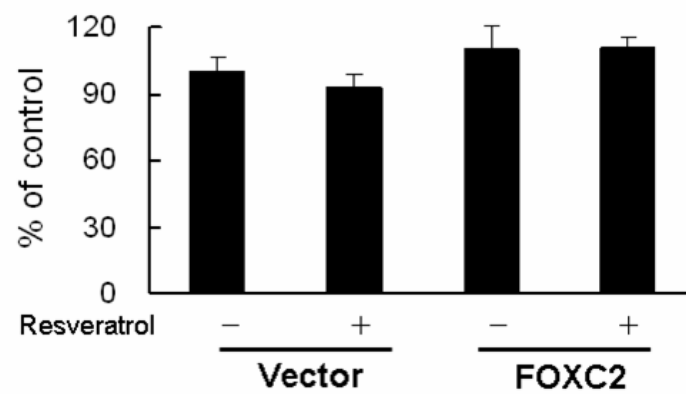
**Figure14.** Effects of resveratrol on cell viability in A549-FOXC2 cells. Cell viability for A549-Vector and A549-FOXC2 cells were cultured with 10 $\mu$ M resveratrol for the indicated time. *Bars* represent means  $\pm$  SD.



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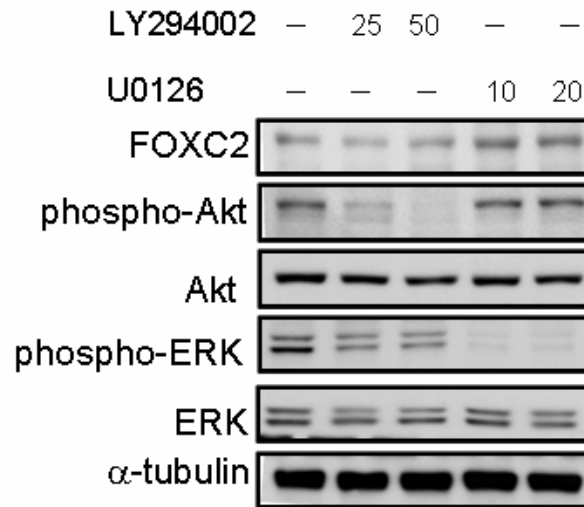


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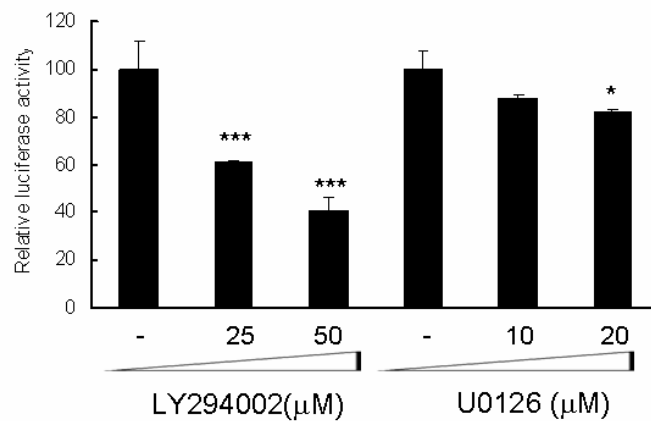


**Figure15.** Resveratrol inhibited colony formation in A549-FOXC2 cells. *A*, cells were treated with 10 $\mu$ M resveratrol for 2 days, replaced with fresh complete medium, and incubated for 4 days. Cell colonies were visualized by 0.05% crystal violet staining and photographed. *B*, quantitative analysis of colony numbers was shown. Results were representative of three independent experiments. *Bars* represent means  $\pm$  SD.

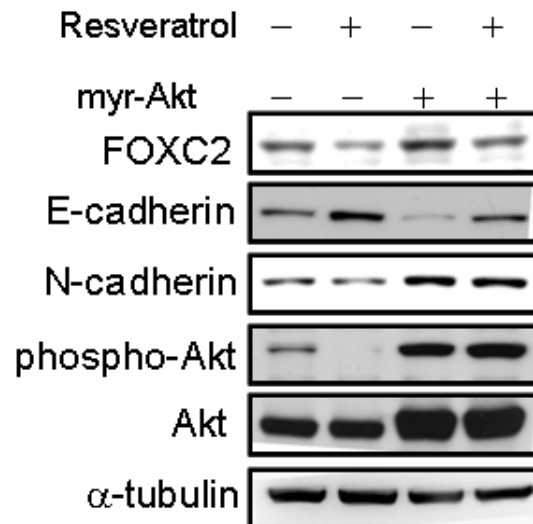
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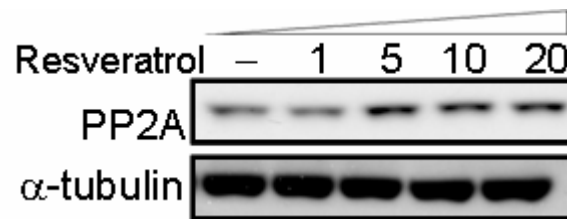


**Figure16.** Effects of PI-3K/Akt inhibitor, LY294002, or MAPK inhibitor, U0126, on the expression of FOXC2 in A549 cells. *A*, treated with 25μM and 50μM LY294002 and 10μM and 20μM U0126 for 24h in A549 cells and analyzed the expression of FOXC2 by western blot. *B*, A549 cells transfected with FOXC2-promoter reporter construct (F1). Cells were cultured with LY294002 and U0126 as indicated above for 24h and then luciferase activity were measured. Findings represent treatments performed in three separate experiments. *Bars* indicated means  $\pm$ SD. *Asterisks* denote a significant difference compared with values for untreated control. (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ).

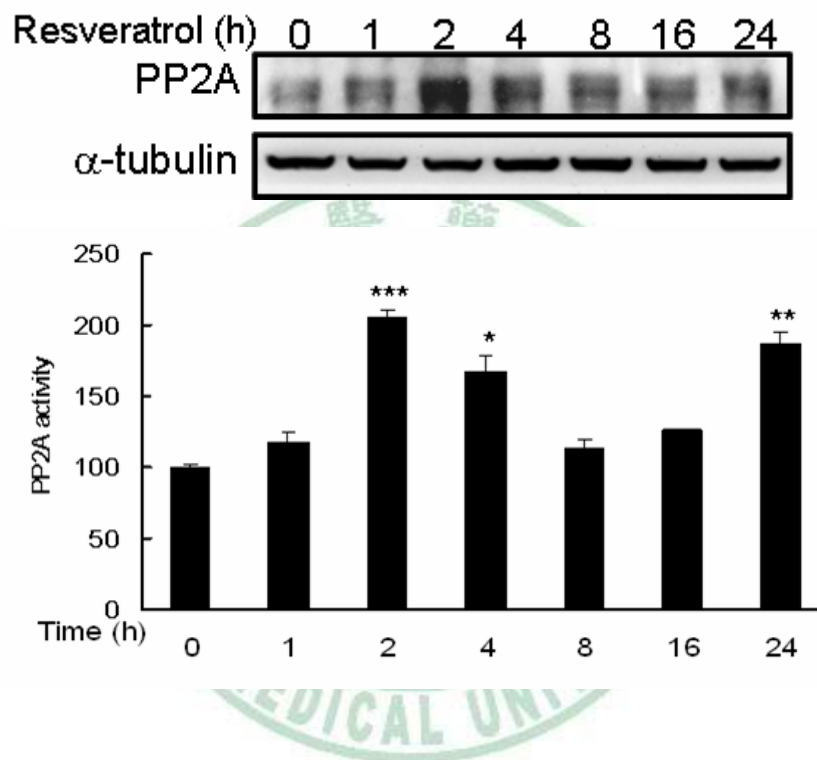


**Figure17.** Effects of overexpression Akt on resveratrol-inhibited FOXC2 expression. A549 cells were transfected with control vector or constitutively activated myristoylated Akt (myr-Akt) with or without resveratrol (10 $\mu$ M) for 48h and analysis the expression of the FOXC2, E-cadherin, N-cadherin and phosphorylated Akt by western blot. Findings represent performed in at least three independent experiments.

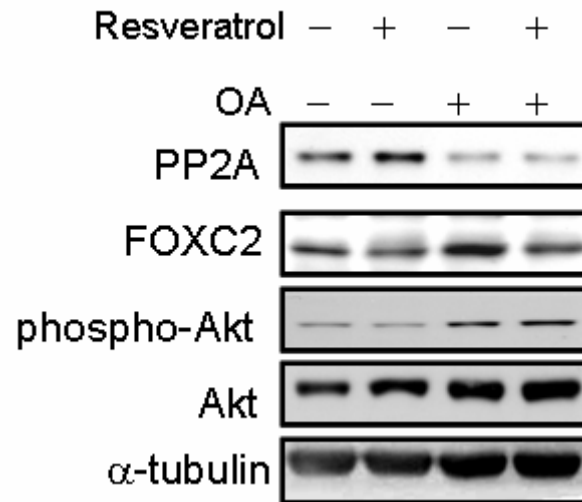
A



B

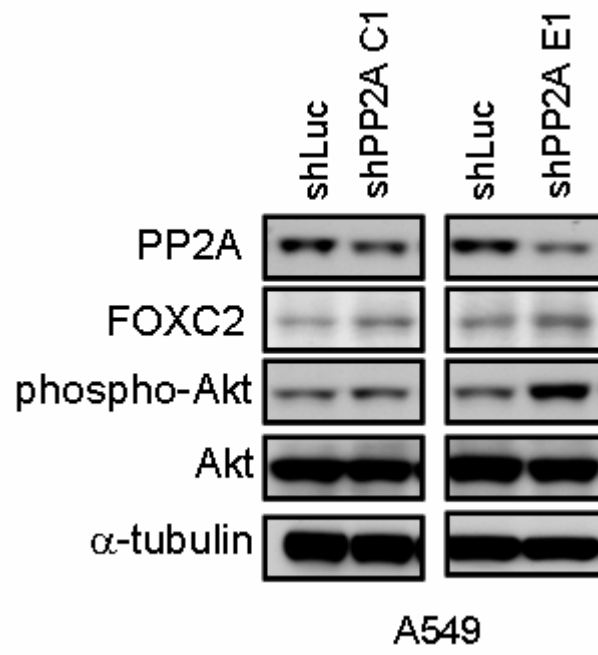


**Figure18.** Effects of resveratrol on PP2A/C protein expression and PP2A activity in A549 cells. *A*, A549 cells were treated with different concentrations of resveratrol for 2h, followed by measurements of PP2A/C expression by western blot. *B*, A549 cells were treated with resveratrol for the indicated times, and analyzed PP2A/C protein expression by western blot and the PP2A phosphatase activity was measurement according to manufacturer's instructions. Results were representative of three independent experiments. *Bars* indicated means  $\pm$ SD. *Asterisks* denote a significant difference compared with values for untreated control (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ).

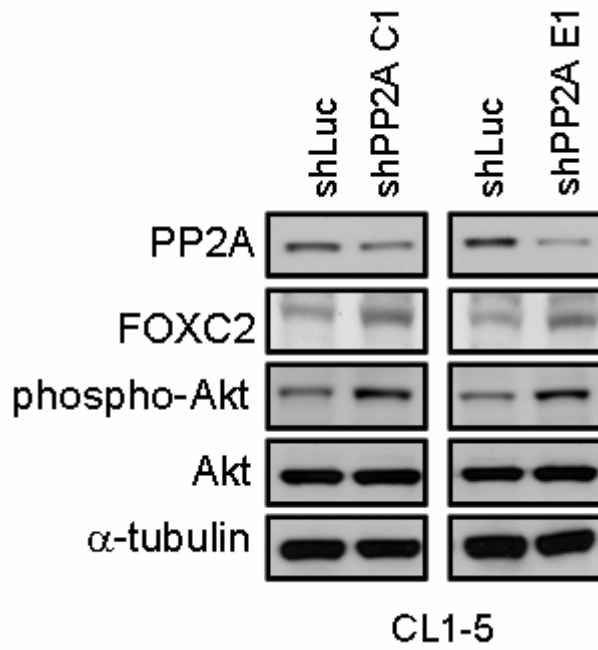


**Figure19.** Effects of okadaic acid (OA), a PP2A inhibitor, on resveratrol-inhibited FOXC2 expression in A549 cells. Control or OA-pretreated cells (150nM, 4h) were treated with or without resveratrol (5 $\mu$ M, 2h) for analysis PP2A/C, FOXC2 and phosphorylated Akt expression by western blot. Results were confirmed by three independent experiments.

A

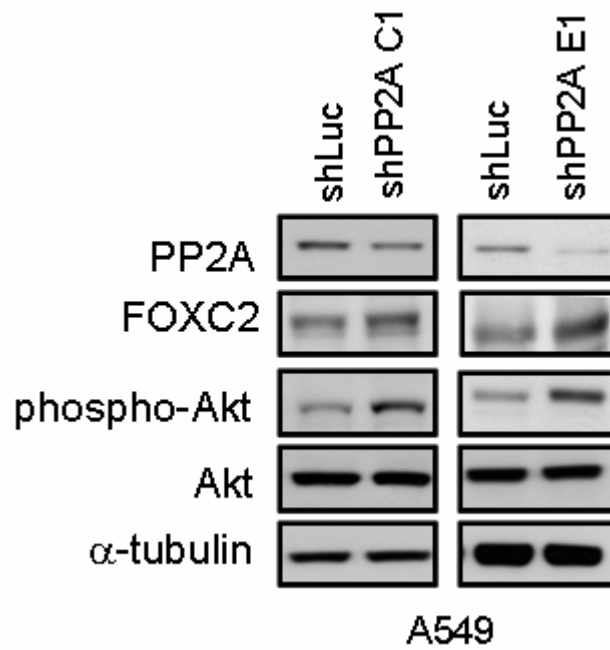


B

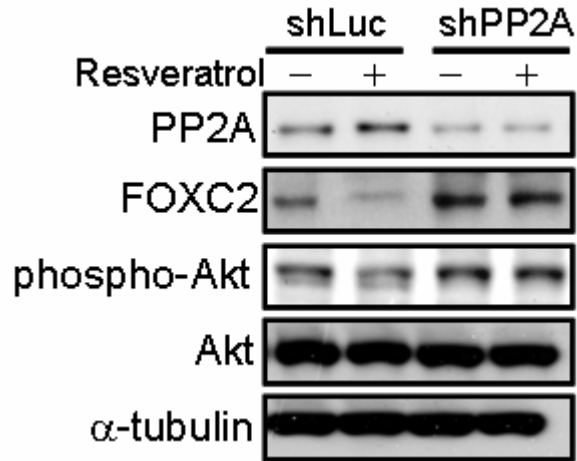




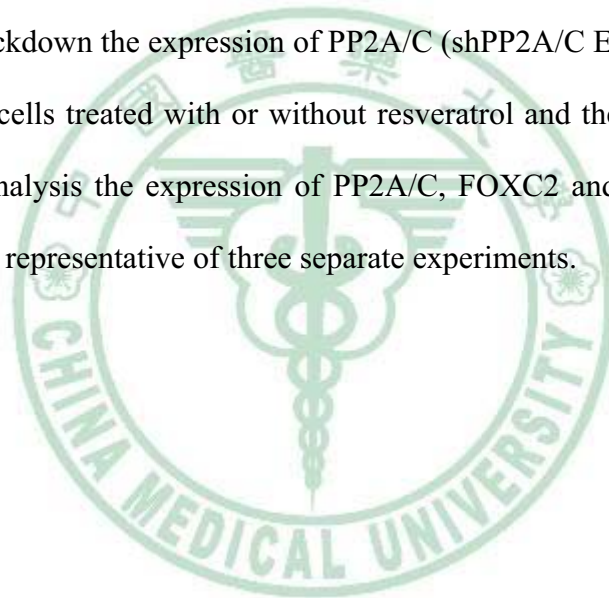
C

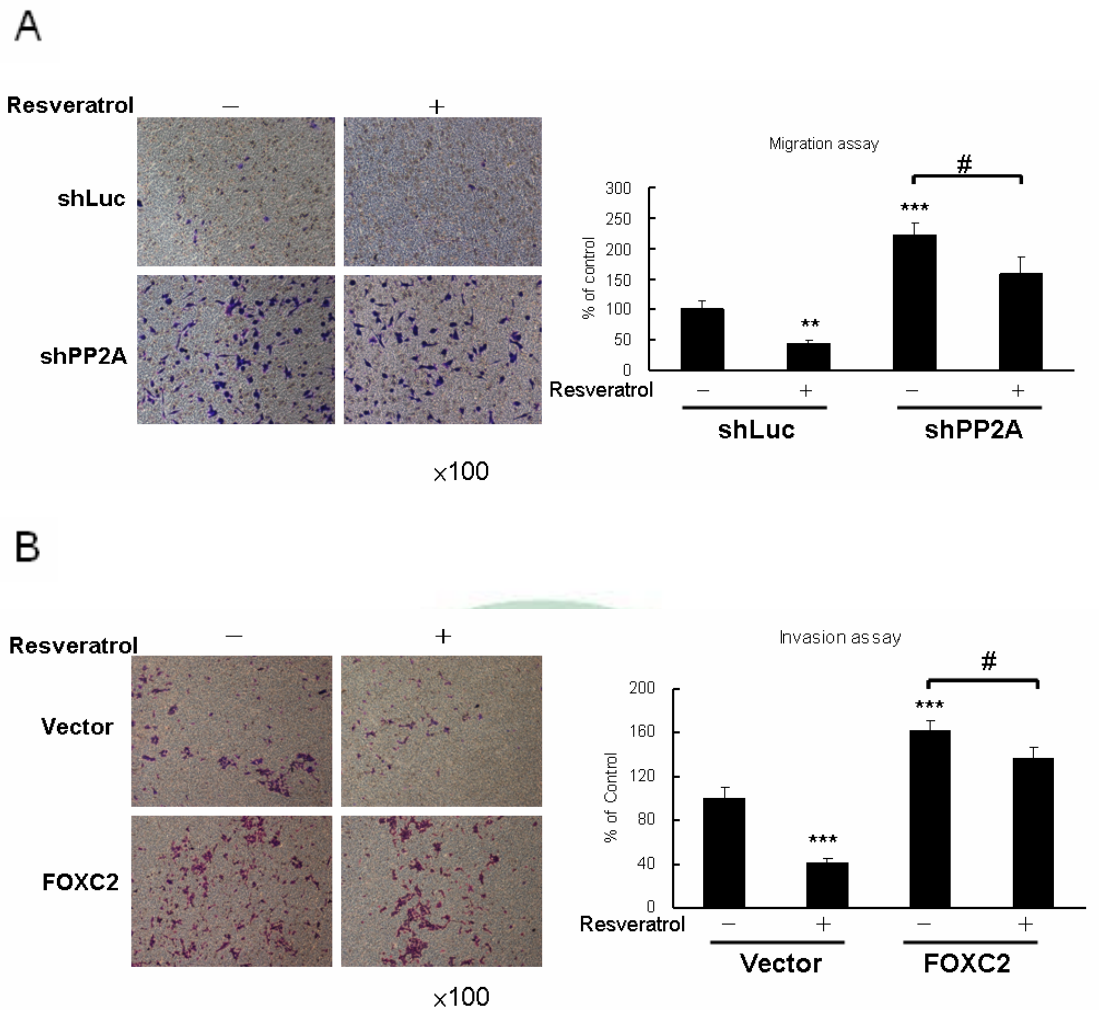


**Figure20.** Effects of PP2A/C on the expression of FOXC2 in lung cancer cells. Lung cancer cells were infected with shPP2A/C C1 and shPP2A/C E1 or control shRNA (shLuc) as described under “Materials and Methods” and were subjected to analysis the expression of FOXC2 and phosphorylated Akt by western blot. *A*, A549 cells were transient knockdown the expression of PP2A/C. *B* and *C*, lung cancer cells were stably knockdown PP2A/C expression after puromycin selection in CL1-5 and A549 cells, respectively. Data was representative of three separate experiments.

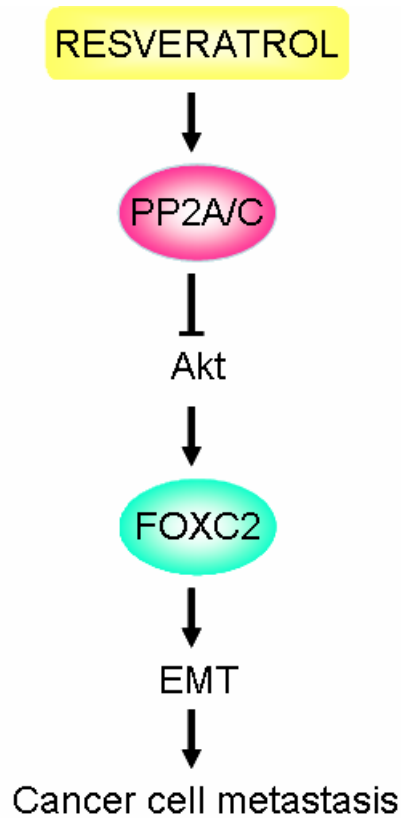


**Figure21.** Effects of knockdown PP2A/C expression on resveratrol-induced FOX C2 inactivation. Knockdown the expression of PP2A/C (shPP2A/C E1) or control shRNA (shLuc) in A549 cells treated with or without resveratrol and then were subjected to western blot to analysis the expression of PP2A/C, FOX C2 and phosphorylation of Akt. Results were representative of three separate experiments.





**Figure 22.** Effects of knockdown PP2A/C expression on resveratrol-inhibited cell mobility in A549 cells. *A*, for migration assay, cells were treated with or without resveratrol (10 $\mu$ M) for 24h. *B*, for invasion assay cells were treated with resveratrol for 48h. Findings were representative of three independent experiments. *Bars* indicated means  $\pm$ SD. *Asterisks* denote a significant difference compared with values for untreated control. *Hash* denote a significant difference compared with values for shPP2A untreat group (\*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; #,  $p < 0.05$ ).



**Figure23.** A model showing a PP2A/Akt/FOXC2 axis signaling pathway involved in lung cancer cell metastasis. Our data indicated that resveratrol treatment induces phosphatase activity of PP2A, which inhibits Akt and FOXC2 expression leading to inhibit cancer cell metastasis.