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## Benzo(a)pyrene-Caused Increased G<sub>1</sub>-S Transition Requires the Activation of c-Jun through p53-Dependent PI-3K/Akt/ERK Pathway in Human Embryo Lung Fibroblasts

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### Keywords

B(a)P; Signaling Pathway; Cell cycle; c-Jun; Akt; p53

### Introduction

Benzo(a)pyrene (B(a)P) is a typical compound of polycyclic aromatic hydrocarbons present in industrial pollution, auto exhaust, tobacco smoke and coal tar (Boström et al, 2002). B(a)P have been proven to be a complete carcinogen in many animal models (reviewed in Rubbin, 2001). A generally accepted concept is that B(a)P-induced genetic mutation is a crucial initiating step during carcinogenesis (Hornberg et al, 1996; Jeffy et al, 2002). Nevertheless, other evidence indicates that cell cycle is regulated by oncogenes and tumor suppressor genes (Schreiber et al. 1999), and cell cycle alterations occur in the response of cells to various carcinogens (Khan and Dipple, 2000; Sherr, 1996), suggesting that cell cycle control is necessary for carcinogenic activity of B(a)P.

Progression through the cell cycle is controlled by a set of proteins, including the different cyclins, cyclin-dependent kinases (cdks) and their inhibitors (Bakiri, 2000). The regulation of mammalian cell proliferation by mitogen and stress occurs largely during the G<sub>1</sub> phase of the cell cycle (Schreiber et al, 1999). B(a)P has been documented to accelerate cell cycle progression from G<sub>1</sub> phase to S phase and induce cell proliferation in human embryo lung fibroblasts (Du et al, 2006; Jia et al, 2006; Gao et al, 2006a). However, the molecular mechanisms involved in the cell cycle alternations upon B(a)P exposure are still largely unknown. Thus, the identification of signaling molecule and related pathways involved in B(a)P-induced cell cycle alterations, not only contributes to our knowledge of signaling

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transduction processes, but also is essential for understanding the carcinogenic effect of B(a)P.

Previous studies have shown that transcription factor c-Jun plays a role in the regulation of normal cell cycle progression (reviewed in Weiss and Bohmann, 2004; Wisdom et al, 1999). There are growing evidence that c-Jun is implicated in chemicals-induced tumor and malignant transformation (Eferl et al, 2003; Huang et al, 1996, 1998). Although much is known about c-Jun involvement in cell cycle control, the precise mechanisms of its activation and contribution in B(a)P-induced cell cycle alternations remain unclear. Our recent results have shown that B(a)P stimulated the transactivation of activator factor 1 (AP-1) (Gao et al, 2006b), suggesting that c-Jun, acting as a primary member of AP-1, might play a role in the regulation of cell cycle upon B(a)P exposure. In view of above data, we sought to extend these earlier observations in the present study and, therefore, investigated the potential role of c-Jun in B(a)P-induced alternations of cell cycle as well as the likely interaction of several signaling pathways on the regulation of c-Jun activation.

## Materials and Methods

### Reagents and plasmids

B(a)P was purchased from Sigma Chemical Co (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) at 2 mmol/L stock concentration. RPMI1640 medium was obtained from Gibco Co (Gibco BRL, NY, USA). The p53-specific inhibitor pifithrin- $\alpha$  was obtained from Calbiochem (San Diego, CA, USA). Transfectam reagent and the luciferase assay substrate were bought from Promega (Madison, WI, USA). The phospho-specific Rb antibody at Ser780 was bought from Cell Signaling Biotechnology (Beverly, MA, USA). The other antibodies used in the studies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CMV-neo vector plasmid, c-Jun dominant negative mutant plasmid (TAM67) and cyclin D1-luciferase reporter plasmid were described in previous studies (Ding et al, 2006; Huang et al, 1999; Ouyang et al, 2004).

### Cell culture

Human embryo lung fibroblasts (HELFL) were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. HELFL-sip53 cells, HELFL-AP-1/vector cells, HELFL-AP-1-DN-Akt cells and HELFL-AP-1-DN- $\Delta$ p85 cells were described previously (Gao et al, 2007; Wang et al, 2005). HELFL cells and their derivations were cultured in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, 50 mg/ml gentamycin sulfate at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were detached with trypsin and transferred to 75-cm<sup>2</sup> culture flasks two to three times per week.

### Stable transfection

HELFL cells were cultured in a six-well plate until they reached 60-70% confluence. Fifteen microlitre of Transfectam reagent mixed with 15  $\mu$ g of plasmids (1  $\mu$ g of CMV-neo vector, 2  $\mu$ g of cyclin D1-luciferase reporter plasmid, and 12  $\mu$ g of TAM67 or vector control) were used to transfect each well in the absence of serum. Transfected cells were selected in the presence of 400  $\mu$ g/ml G418 for 2 weeks. The expression of TAM67 in the transfectant cells was detected by Western blot with an antibody that recognizes the COOH terminus of c-Jun. The stable transfectants were identified by measuring both the basal level of luciferase activity and the inhibition of c-Jun phosphorylation.

### Growth curves

HELF-cyclin D1-TAM67 cells, HELF-cyclin D1/vector cells and parent HELF cells were seeded in 6-well plates ( $1.5 \times 10^4$ /well). Cell viability was measured at daily intervals by counting the number of trypan blue-excluding intact cells. Cell number was determined daily in triplicate for 6 days and mean values were plotted into growth curves.

### Cell proliferation assay (MTT)

Cells were seeded in 96-well plates ( $1 \times 10^4$ /well) at 37°C overnight, and then exposed to B(a)P at various concentrations of 0.5-16  $\mu\text{mol/L}$ . Following treatment for 24 h and 48 h, the culture medium was removed and replaced with a medium containing 0.5 mg of MTT dissolved in PBS (pH 7.2). After 4 h, the formed crystals were dissolved with 200  $\mu\text{l}$  DMSO. The intensity of the color in each well was measured at a wavelength of 490 nm using a Dynex Technologies Microplate Reader.

### Cell cycle analysis

Cells were cultured in 75-cm<sup>2</sup> culture flasks to 80–85% confluence, and then serum-starved with 0.5% FBS RPMI-1640. After 24 h, cells were untreated or treated with B(a)P for 24 and 48 h. The cells were harvested and fixed with ice cold 70% ethanol overnight at 4°C. The fixed cells were incubated with 1mg/ml RNase A for 30 min at 37°C, and then stained with 1 mg/ml propidium iodide (PI) for 40 min at 4°C without light. The DNA content was determined by flow cytometry (Beckman Coulter, San Diego, CA) and EXPO 32 software.

### Reporter gene assay

HELF-cyclin D1 cells ( $2 \times 10^5$ ) were seeded in each well of a 6-well plate. After being serum-starved for 24 h, the cells were exposed to B(a)P at the concentrations and time periods as indicated in the figure legends. The cultures were extracted with lysis buffer and luciferase activity was measured using luciferase assay reagent with a luminometer (TD-20/20, Turner Designs Instrument). The results are expressed as cyclin D1 induction (relative cyclin D1 induction).

### Western blot

Whole-cell extracts were prepared by lysing the cells directly in SDS sample buffer. After sonication, protein samples (10-20  $\mu\text{l}$ ) were separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membrane. Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline, pH 7.6, 0.1% (w/v) Tween-20 (TBST) for 1 h at room temperature (RT) and incubated with primary antibodies (1:1000) overnight at 4°C. After several washes with TBST, blots were incubated with the peroxidase-conjugated secondary antibody (1:2000) for 1 h at RT and developed with an enhanced chemical luminescence (ECL) detection system according to manufacture's instructions.

### Indirect immunofluorescence

Cells were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed with PBS and permeabilized with 0.1% Triton X-100 in PBS on ice. The fixed cells were blocked with 5% goat serum for 1 h, and then incubated with primary antibodies in 5% goat serum at 4°C overnight. Antibodies included a monoclonal antibody against phosphorylated c-Jun at position Ser63 or a monoclonal antibody against cyclin D1. Bound antibodies were detected using FITC- or TRITC-conjugated secondary antibodies for 1 h at room temperature. After several washes, samples were examined using an OLYMPUS fluorescence microscope.

## Statistical Analysis

The significance of the difference between the treated and untreated groups was determined with the Student's *t* test. The results are expressed as mean±SD. The differences were considered significant at *P* < 0.05.

## Results

### B(a)P-induced c-Jun phosphorylation in HELF cells

In response to various toxic stimuli, c-jun mRNA expression is rapidly induced several fold above the basal level in a wide variety of tissues and/or cell types (reviewed in Reddy and Mossman, 2002). In general, the mRNA levels of c-jun peak within 15–30 min of stimulation and return to basal level within 1–2 h, whereas c-Jun protein expression remains elevated above basal level for 2–24 h, depending upon the stimuli (Hazzalin et al, 2002). Consistent with this observation, under our experiment condition, B(a)P exposure markedly induced the increases in the phosphorylation of c-Jun at Ser63 and Ser73, but had no effect on total c-Jun expression as compared to those in cells of DMSO control (Fig.1A). The phosphorylation levels of c-Jun maximally occurred at 12 h and decreased at 24 h after exposure, paralleled the kinetics of AP-1 transactivation reported previously (Gao et al, 2006b).

### B(a)P-induced c-Jun phosphorylation through PI-3K/Akt pathway

c-Jun, as a downstream transcription factor, is modulated by a variety of signaling pathways (reviewed in Weiss and Bohmann, 2004). We recently found that PI-3K/Akt/p70<sup>S6K</sup> were implicated in AP-1 transactivation induced by B(a)P (Gao et al., 2007), but their role in B(a)P-induced c-Jun activation has not been identified. To elucidate the role of PI-3K/Akt pathway in B(a)P-induced c-Jun phosphorylation, we investigated whether PI-3K and Akt were required for c-Jun activation caused by B(a)P exposure. Consequently, HELF-AP-1-Δp85 and HELF-AP-1-DN-Akt, which are well characterized transfectants in our prior study (Gao et al, 2007), were employed for this investigation. After B(a)P stimulation for 6 h, immunofluorescence assay was performed to compare c-Jun phosphorylation in transfected cells with that in control cells. As shown in Fig. 2A, vector control cells exhibited marked nuclear staining with phospho-specific c-Jun (Ser63) after B(a)P treatment (panel b), indicative of an increase in c-Jun phosphorylation. This effect was not seen in both HELF-AP-1-Δp85 cells (panel d) and HELF-AP-1-DN-Akt cells (panel f), indicating that disruption of either PI-3K or Akt could inhibit B(a)P induced c-Jun phosphorylation. Western blot analysis also showed that overexpression of dominant-negative mutant PI-3K potently blocked Akt activation (data not shown) and c-Jun phosphorylations in response to B(a)P (Fig. 2B). In contrast, B(a)P-induced c-Jun phosphorylation was suppressed partly in cells transfected with dominant negative mutant Akt (Fig. 2B). In addition, B(a)P treatment had little or no effect on c-Jun expression in any of cell lines tested. These observations reveal that B(a)P is able to induce c-Jun phosphorylation through PI-3K/Akt-dependent pathway.

### PI-3K/Akt pathway modulated B(a)P-induced c-Jun phosphorylation via the activation of ERK but not JNK

The conventional position of the c-Jun protein in the signaling transduction cascades is close to mitogen activated protein kinase (MAPK) family (Kennedy and Davis, 2003). We hypothesized that PI-3K/Akt might mediate B(a)P-induced c-Jun phosphorylation through MAPK pathway based on our recent observation that two subgroups of the MAPK family, extracellular signaling regulated kinase (ERK) and c-Jun NH<sub>2</sub>-terminal kinase (JNK), were involved in B(a)P-induced c-Jun activation (Jiao et al, 2007). To test it, the effect of B(a)P

on the phosphorylation levels of ERK and JNK were analyzed in the cells transfected with dominant-negative mutants of PI-3K ( $\Delta p85$ ) or Akt. Figure 3 showed that exposure of HELF cells to B(a)P led to an increase in the phosphorylations of ERK and JNK. Overexpression of dominant negative mutant PI-3K specially inhibited B(a)P-induced phosphorylations of ERK but not JNK (Fig. 3). Identical effects were observed in HELF-AP-1-DN-Akt cells (Fig. 3). These results clearly indicate that PI-3K/Akt pathway regulates B(a)P-induced c-Jun activation specially via ERK, but not JNK.

### **p53 mediated B(a)P-induced c-Jun phosphorylation through PI-3K/Akt/ERK pathway**

Previous studies have indicated that p53 protein functions as a down-regulator in AP-1 activity (Wang et al, 2005; Wu, 2004). Other studies have identified that p53 can suppress cell transformation caused by oncogene activation (Lin and Lowe, 2001). Together, these observations suggest that p53 might provide an inhibitory signaling for B(a)P-induced c-Jun activation. To test such a hypothesis, HELF cells was pretreated with pifithrin- $\alpha$ , a p53 inhibitor, for 30 min and then exposed to B(a)P. Pretreatment of cells with pifithrin- $\alpha$  led to dramatic inhibition of B(a)P-induced p53 overexpression, whereas it markedly increased B(a)P-induced c-Jun phosphorylation (Fig. 4A). Because it has been reported recently that pifithrin- $\alpha$  is also able to inhibit the activity of cytochrome P450 1B1 (Sparfel et al, 2006), p53 siRNA was further used to address the causality between loss of p53 function and elevation of c-Jun activation. The results indicated that p53 siRNA can inhibit the expression of p53, which subsequently leads to the increase in phosphorylation of c-Jun induced by B(a)P (Fig. 4C). More importantly, knockdown of p53 expression in HELF cells by either its chemical inhibitor or specific siRNA caused the elevation of B(a)P-induced the phosphorylation of ERK (Fig. 4A and 4C) and Akt (Fig. 4B and 4D). Based on the above data, we anticipate that basal level of p53 expression might inhibit B(a)P-induced c-Jun activation by regulating the activity of Akt and ERK.

### **B(a)P-induced cell proliferation was inhibited in HELF by overexpression of TAM67**

To test potential role of c-Jun phosphorylation in B(a)P-induced cell proliferation, we established a stably transfectant expressing dominant negative mutant of c-Jun (TAM67) in HELF (HELF-cyclin D1-TAM67). As shown in Fig.5A, Western blot showed a 29 kDa TAM67 protein expression as compared with vector control transfectant (Fig. 5A). TAM67 transfectant displayed growth rate identical to that for control cells (Fig. 5B). We further showed that overexpression of TAM67 prominently repressed phosphorylation of c-Jun at Ser63 and Ser73 as compared with that of control cells (Fig. 5C). To determine the effect of TAM67 on B(a)P-induced cell proliferation, HELF cells was treated with B(a)P at various concentrations of 0.5-16  $\mu\text{mol/L}$  for 24 h and 48 h, and then MTT assay was performed. As shown in Fig. 6 that overexpression of TAM67 significantly hampered B(a)P-induced cell proliferation by >50% at 24 h after treatment, whereas it only showed a slight inhibition of B(a)P-induced cell proliferation at 48 h after B(a)P exposure. These results clearly indicate that c-Jun activation is only involved in the regulation of cell proliferation upon B(a)P exposure at early phase rather than later phase.

### **B(a)P-induced cell cycle alternations was reversed in HELF-cyclin D1-TAM67 cells**

Numerous published reports have shown that c-Jun is required for progression through the G<sub>1</sub> phase of the cell cycle in fibroblasts (Schreiber et al, 1999; Wisdom, et al, 1999). In view of these, together with recent finding that B(a)P-induced cell proliferation is accompanied by increased G<sub>1</sub>/S transition (Gao et al, 2006a; Jia et al, 2006), it was of interest to investigate whether TAM67 had a suppressive function on alternations of the cell cycle caused by B(a)P. Thus, cell cycle distribution was determined in HELF-cyclin D1/vector cells and HELF-cyclin D1-TAM67 cells after a 24- and 48- h incubation of 2  $\mu\text{mol/L}$  B(a)P. Figure 7 showed exposure of cells to B(a)P evoked a dramatic alteration in cell cycle

distribution with a decrease in the fraction of cells in G<sub>1</sub> phase and a corresponding increase in the fraction of cells in S phase, the percentages in S phase were 30.4% and 41.5% after B(a)P treatment for 24 h and 48 h, respectively (panel b and d). In contrast, TAM67 overexpression dramatically reduced those alternations (panel f and h), which were consistent with cell proliferation assay. These data strongly indicate that c-Jun activation is essential for cell cycle alternations elicited by B(a)P.

### **B(a)P up-regulation of cyclin D1 pathway was blocked by overexpression of TAM67**

Cyclin D1 serves as a key sensor and integrator of extracellular signals of cells in early to mid-G<sub>1</sub> phase, mediating its function through binding both the CDKs and other signaling molecules to modulate the genes that are involved in regulation of cell proliferation and differentiation (Neumeister et al, 2003; Wang et al, 2004). We have shown previously that B(a)P exposure induces cyclin D1 expression in human embryo lung fibroblasts (Du et al, 2006). Consistently, under the same condition, exposure of HELF-cyclin D1/vector cells to B(a)P obviously induced cyclin D1 transcription in a dose- (Fig. 8A) and time-dependent manners (Fig. 8B). c-Jun has been linked directly to transcriptional regulation of cyclin D1 in many experimental models (Hennigan and Stambrook, 2001; Wisdom et al, 1999). To determine whether c-Jun regulates cyclin D1 induction by B(a)P, reporter gene assay was performed to examine the induction of cyclin D1 transcription after B(a)P stimulation for 12 h. As indicated in Fig. 8C, B(a)P-induced cyclin D1 transcription was dramatically blocked in TAM67-transfected cells as compared to vector control transfectant. Western blot analysis also showed that overexpression of TAM67 significantly inhibited the cyclin D1 protein expression by B(a)P (Fig. 8D). Identical result was obtained from the immunofluorescence assay (Fig. 8E). Together, these findings mentioned above suggest that c-Jun activation is required for the induction of cyclin D1 by B(a)P.

Cyclin D1 is the regulatory subunit of the cyclin D1-CDK4 complex that phosphorylates and inactivates the cell-cycle inhibiting function of the retinoblastoma protein (Rb) (Andrysiak et al, 2007). Rb is thought to silence specific genes that are active in the S phase of the cell cycle through repression of E2F transcriptional activity (Stacey, 2003). Cyclin D1, pRb and E2F comprise a pivotal signaling pathway in mediating cell cycle from G<sub>1</sub> to S. To further evaluate the role of c-Jun in the control of cell cycle regulators, phosphorylation of Rb and expression of E2F1 were analyzed by Western blot after B(a)P treatment. Figure 8D showed that B(a)P led to obvious increases in E2F1 expression and Rb phosphorylation at Ser780, and suppression of c-Jun phosphorylation by using TAM67 potentially impaired B(a)P-induced overexpression of E2F1 and hyperphosphorylation of Rb. Taken together, the aforementioned observations provide compelling evidence that c-Jun participates in the modulation of G<sub>1</sub> regulators - cyclin D1, pRb and E2F1, and in turn, mediates cell cycle alterations induced by B(a)P.

## **Discussion**

Cell cycle regulation has been increasingly recognized as one of the important mechanisms required for the carcinogenic effect of B(a)P during the past several years, but the molecular basis of this is still obscure. Here, we focused on the role and related activating pathways of c-Jun in B(a)P-induced cell cycle alternations. Our current findings suggest that c-Jun is an important player for mediating B(a)P-induced cell cycle alternations through p53-dependent PI-3K/Akt/ERK signaling pathway. These data provide the evidence that activation of c-Jun is involved in B(a)P-induced cell cycle alternations, and reinforce the concept that signaling mechanisms for c-Jun activation are cell-type and inducer specific.

c-Jun, acting as immediate-early genes and early response proto-oncogenes, is activated by a broad range of physiological and pathological stimuli, including cytokines, growth factors,

stress signals and infections, as well as toxic stimuli (Hoffer et al, 1996; Jochen et al, 2004). Aberrant expression and/or activation of c-Jun are often associated with many diseases, especially cancer (Jochum et al, 2001; Zhou and Thompson, 1996). In this study, exposure of HELF cells to B(a)P evoked c-Jun phosphorylation at Ser63 and Ser73, which is known to extend half-life of the protein and improve its transactivation potential (Weiss and Bohmann, 2004). Consistently, treatment of cells to benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE), ultimate metabolite of B(a)P, also led to marked increases in c-Jun phosphorylation (data not shown). Similarly, exposure of mouse epidermal Cl41 cells to BPDE induces significant increases in phosphorylation and expression of c-Jun (Li et al, 2004; Ding et al, 2006; Huang, et al, 2006). Additionally, it has been reported that aryl hydrocarbon receptor (AhR) could increase the mRNA and protein levels of c-Jun, stimulating AP-1 expression (Kizu et al. 2003). Induction of c-Jun depends on the medication of AhR through activation of p38 MAPK signaling pathway (Weiss et al. 2005). Therefore, B(a)P might enhance c-Jun activity by AhR-dependent mechanism, which is also involved in metabolic activation of B(a)P. Since B(a)P is identified as a complete carcinogen in animal carcinogenesis models, our results strongly suggest that c-Jun activation is one of the events necessary for the tumor promotion of B(a)P.

c-Jun, also referred to as downstream transcription factor, is regulated in a given cell by a various of signaling pathways including ras/ MAPK (Davis, 2000; Hibi et al, 2003), PI-3K/ Akt (Shahabi et al. 2006), IKK/NF- $\kappa$ B (Fujioka et al, 2004) and p53/p21 (Huang et al, 2003). Here, we found that PI-3K/Akt has been implicated in c-Jun activation. Using dominant negative mutants of either PI-3K ( $\Delta$ p85) or Akt substantial inhibited c-Jun phosphorylation elicited by B(a)P This is consistent with our recently observation that PI-3K/Akt/p70<sup>S6k</sup> is involved in AP-1 transactivation by B(a)P (Gao et al, 2007). PI-3K signaling pathway also plays a crucial role during tumorigenesis. Compelling data have shown that PI-3K activation occurs in the development of many cancers (Phillip, 2006). Studies from our group have confirmed that PI-3K/Akt/p70<sup>S6k</sup> pathway mediates B(a)P-induced cell cycle alternations (Gao et al, 2007), clearly indicating that PI-3K signal pathway is associated with the carcinogenic effects of B(a)P.

The link between PI-3K and MAPK in response of cells to B(a)P has been suggested in many experimental models. Previous studies have demonstrated that PI-3K/Akt regulates B(a)PDE-induced AP-1 transactivation specifically through JNK-dependent pathway (Li et al, 2004; Huang et al. 2005). In the current work, we found that PI-3K/Akt pathway mediated c-Jun phosphorylation selectively through activation of ERK, but not JNK. It is well known that serum and growth factors activate c-Jun transcriptional activity mainly through ERK, whereas cytokines and genotoxic stress is mostly through activating JNK cascades (Reddy and Mossman, 2002). In light of our observation that B(a)P exposure induce cell proliferation, our results indicate that in this context, B(a)P might act as a proliferative signal, inducing c-Jun phosphorylation through the activation of ERK pathway. Nonetheless, the PI-3K-dependent activation of c-Jun by enhancing ERK activity, might be an incomplete picture of PI-3K interaction with MAPK. Analogous to the foregoing observations, one might postulate that PI-3K/Akt pathway phosphorylates and activates other member of MAPK, such as JNK and p38, defined by different cell type and/or extracellular stimuli. The correlation between PI-3K and MAPK is still under investigation in our laboratory.

Transcription factor p53 is one of the classic tumor suppressor genes that interfere with cancer development (Appella, 2001). It is known that p53 is the most commonly (>50%) mutated gene associated with human tumors (Reid et al, 2004). Previous findings show that p53 can even induce expression of AP-1 components such as Fos (Elkeles et al., 1999), suggesting that p53 activity can act upstream of AP-1 signaling. Similarly, our results from

the pifithrin- $\alpha$ , to certain degree, suggests that p53 might provide an inhibitory signaling for B(a)P-induced c-Jun activation, although it has been shown that pifithrin- $\alpha$  not only has anti-p53 function (Komarova and Gudkov, 2000; Chramostova et al, 2004), but also in some cases has inhibitory effect towards cytochrome P450 1 isoforms (Solhaug et al, 2005; Sparfel et al, 2006). Furthermore, our data shown here indicate that inhibition of p53 by its siRNA induces the significant increases in the phosphorylation of c-Jun, Akt and ERK. Taken together with the evidence that PI-3K and MAPK signaling pathways play critical roles in tumorigenesis (Dai et al, 2005; Hay, 2005; Johnson and Nakamura, 2007), these results further substantiate that inhibitory effect of p53 on c-Jun activation might be due to its specific inhibition of PI-3K/Akt/ERK pathway.

Considerable studies support a crucial role of c-Jun in cell cycle regulation (Eferl et al. 1999; Schreiber et al. 1999). In our previous studies, we found that exposure of cells to B(a)P induced cell cycle alternations accompanied with AP-1 transactivation (Gao et al. 2006b), suggesting that c-Jun might be involved in B(a)P-induced cell cycle alternations in AP-1-dependent manner. However, since c-Jun has been indicated to be involved in pro-survival or pro-apoptosis under different circumstances, and the function of c-Jun varies according to the cellular or microenvironment, in a certain context c-Jun might complement or oppose, even might act as a bystander with no direct effects on B(a)P-induced cell cycle alternations in HELF cells, the actual role of c-Jun in B(a)P-induced cell cycle alternations has not been ascertained. To answer this question, the cells stably expressing dominant negative mutant c-Jun (TAM67) were established, termed as HELF-cyclin D1-TAM67. Consistent with numerous observations in a wide variety of cell types, we found that TAM67 leads to a marked inhibition of AP-1 transactivation in B(a)P-stimulated cells (data not shown). The expression of TAM67 was well tolerated in untreated cells, and most of the cell lines generated displayed normal growth kinetics and essentially unaltered basal cyclin D1 transactivity. HELF-cyclin D1-TAM67 cell lines therefore provided a comparatively stable model system in which to investigate the role and regulatory mechanism of c-Jun in B(a)P-induced cell cycle alternations.

The present study indicated that exposure of HELF cells to 0.5~2  $\mu\text{mol/L}$  B(a)P evoked the increase in the percentage of cells in S phase, and induced cell proliferation. Inconsistent with this notion, 1 mmol/L B(a)P is able to induce S-phase delay in HELF cells, which is related to formation of DNA adducts (Binkova et al, 2000). Additionally, it has been reported that 2  $\mu\text{mol/L}$  B(a)P induces G<sub>1</sub> arrest in Swiss 3T3 cells (Khan and Dipple, 2000), whereas the same concentration elicits significant apoptosis in human carcinoma cells (Yoshii et al. 2001). These apparent contradictions might be primarily due to different cell types and/or stimuli concentration. Anyway, in this context, B(a)P induced an increase in G<sub>1</sub>-S transition. Overexpression of TAM67 was able to significantly block G<sub>1</sub>-S transition after treatment with B(a)P for 24 h. However, a slight alternation was observed at 48 h in TAM67-expressing cells after B(a)P treatment. Consistent with this result, TAM67 was able to inhibit cell proliferation when TAM67-transfected cells were incubated with B(a)P for 24 hours but only showing a little effect at 48 hours after B(a)P exposure. Moreover, similar results were obtained when the effect of TAM67 on cell cycle regulators, cyclin D1, E2F1 and Rb, was analyzed by Western blot. These observations suggest that the effect of TAM67 might be adapted by HELF-cyclin D1-TAM67 cells after long-term of B(a)P exposure, or there might be some other pathways involved in cell cycle alternations stimulated by B(a)P.

It is noteworthy that numerous genes coding for cell cycle machinery (cyclin, CDKs, etc) contain putative c-Jun/AP-1 binding sites in their promoters (Wisdom et al, 1999). cyclin D1 is a prototype of such a gene that is implicated in G<sub>1</sub> progression (Fu et al, 2004). Thus, in this experiment using the cyclin D1 luciferase reporter, transactivation of cyclin D1 was stimulated after exposure HELF-cyclin D1 cells to B(a)P for 12 h. In contrast, in HELF-



cyclin D1-TAM67 cells, the effect of B(a)P on cyclin D1 was completely blocked after treatment with B(a)P for 12 h. The role of TAM67 was further checked by applying western blot and immunofluorescence assay. Paralleled the aforementioned results of cell cycle, B(a)P-induced cyclin D1 overexpression was recovered in HELF-cyclin D1-TAM67 cells exposed to B(a)P for 48 h. TAM67 also showed similar inhibitory effects on hyperphosphorylation of pRb and overexpression of E2F1 induced by B(a)P. These findings clearly show that c-Jun is essential for the activation of regulators of G<sub>1</sub> phase, including cyclin D1, E2F1 and pRb, and consequently promote G<sub>1</sub>-S transition.

In summary, we presented previously data supporting the hypothesis that PI-3K signaling pathway was necessary for AP-1 transactivation and cell cycle alternations induced by B(a)P (Gao et al, 2007). We now extend this hypothesis to suggest that, collectively, c-Jun activation by p53-dependent PI-3K/Akt/ERK pathway contributes to B(a)P-caused increased G<sub>1</sub>-S transition via cell cycle regulators such as cyclin D1, E2F1, and pRb in HELF cells. In light of the key role of alterations of cell cycle machinery in tumorigenesis, these findings will help us to understand the signal transduction mechanisms involved in the carcinogenic effects of B(a)P.

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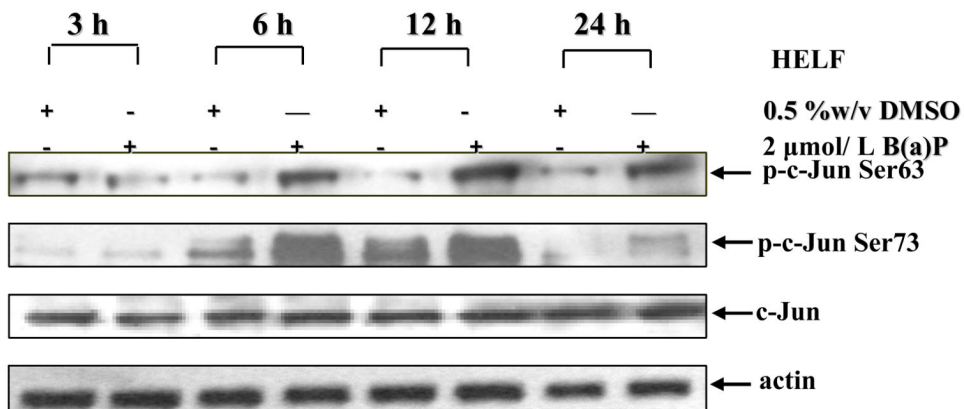
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## Abbreviations

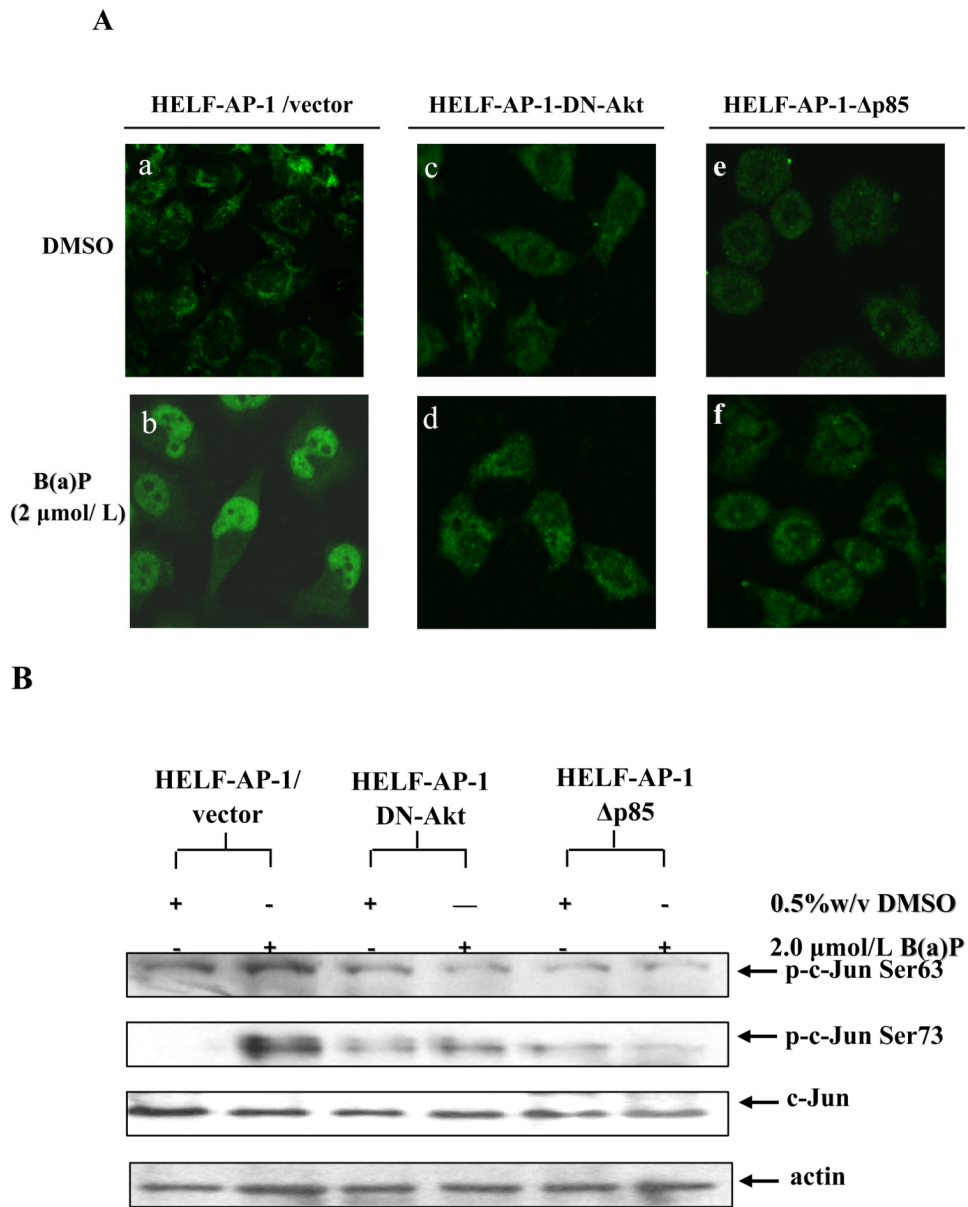
<b>B(a)P</b>	Benzo(a)pyrene
<b>DMSO</b>	dimethyl sulfoxide
<b>HELF</b>	human embryo lung fibroblast
<b>HELF-AP-1/vector</b>	human embryo lung fibroblast transfected with AP-1 luciferase reporter plasmid
<b>HELF-AP-1-DN-Akt</b>	human embryo lung fibroblast cotransfected with AP-1 luciferase reporter plasmid and dominant negative mutant plasmid of Akt

<b>HELFL-AP-1-DN-<math>\Delta</math>p85</b>	human embryo lung fibroblast stably cotransfected with AP-1 luciferase reporter plasmid and dominant negative mutant plasmid of PI-3K ( $\Delta$ p85)
<b>HELFL-cyclin D1/vector</b>	human embryo lung fibroblast stably transfected with cyclin D1 luciferase reporter plasmid
<b>HELFL-cyclin D1-TAM67</b>	human embryo lung fibroblast stably cotransfected with cyclin D1 luciferase reporter plasmid and dominant negative mutant plasmid of c-Jun (TAM67)
<b>HELFL-sip53 cells</b>	human embryo lung fibroblast stably transfected with p53 siRNA
<b>p-Akt</b>	phosphorylation of Akt
<b>p-c-Jun</b>	phosphorylation of c-Jun
<b>p-ERK</b>	phosphorylation of ERK
<b>p-JNK</b>	phosphorylation of JNK
<b>pRb</b>	phosphorylation of retinoblastoma protein



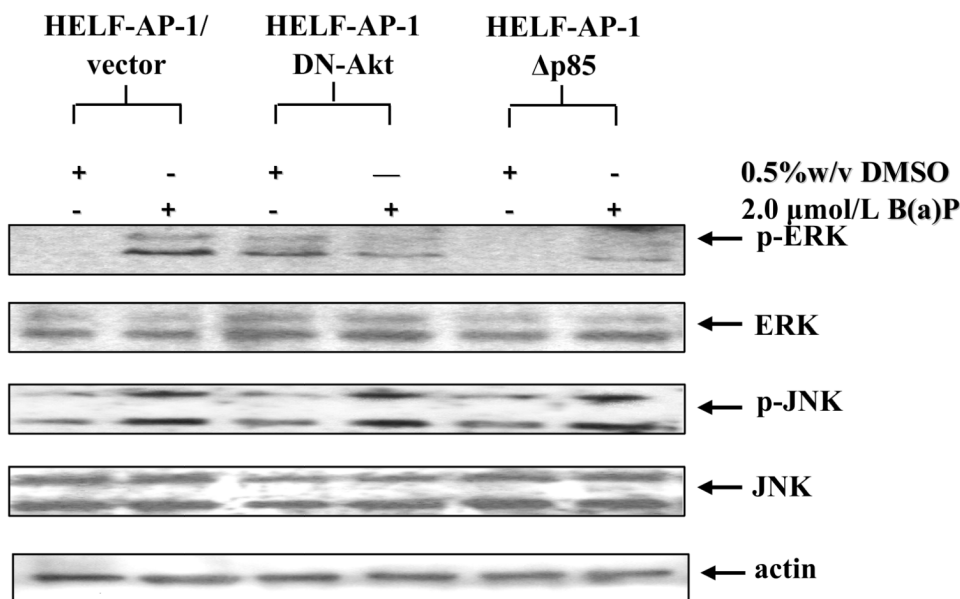
**Fig. 1. B(a)P enhanced c-Jun phosphorylation in HELF cells**  
 HELF cells were cultured until subconfluent (80-85 %) in 75-cm<sup>2</sup> culture flasks with RPMI1640 containing 10% FBS, and then replaced with 0.5% FBS RPMI-1640. After being cultured for 24 h, the cells were exposed to 2 μmol/L B(a)P for various time points as indicated. The cells were then washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with specific antibodies as indicated, and then detected with secondary antibody as indicated. The results were one representative data from three independent experiments.

**Fig.2**



**Fig. 2. PI-3K/Akt pathway mediated B(a)P-induced c-Jun activation**

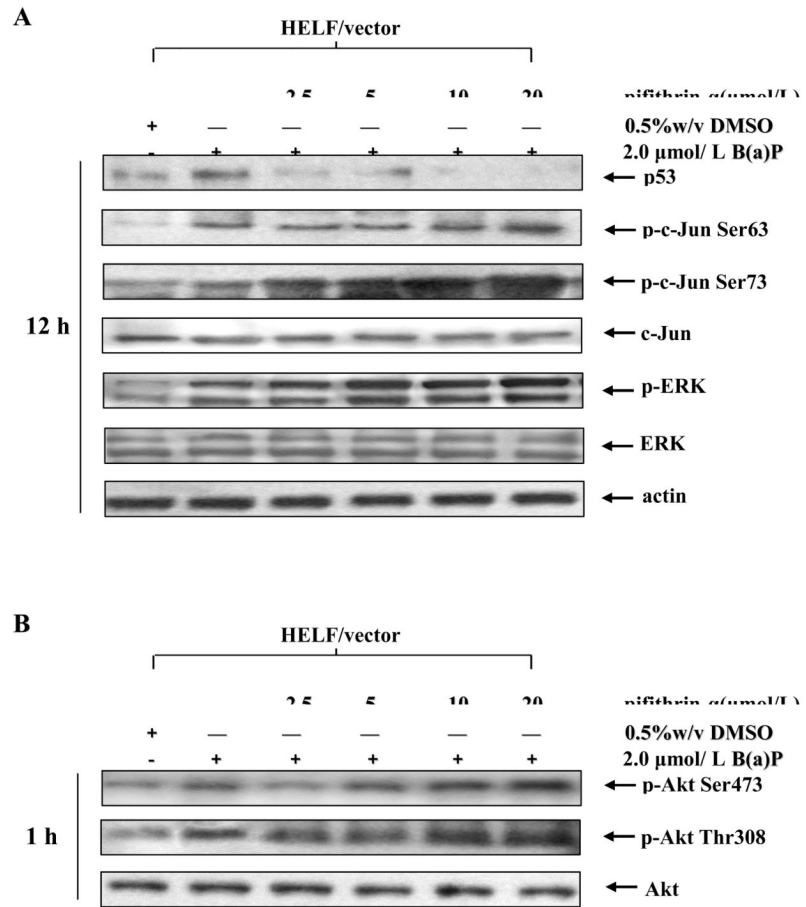
A, HELF-AP-1/vector (a and b), HELF-AP-1-DN-Akt (c and d) and HELF-AP-1- DN-Δp85 (e and f) were seeded into six-well plates respectively and cultured in 10% FBS RPMI 1640 medium at 37°C overnight. After being serum-starved for 24 h, cells were untreated (a, c and e) or treated (b, d and f) with 2 μmol/L B(a)P for 6 h, and then fixed. The fixed cells were subsequently blocked with 5% goat serum, incubated with phospho-specific c-Jun at Ser63 antibody, and detected by immunofluorescence assay with FITC-conjugated second antibody (*green*). B, The aforementioned cells were incubated with 2 μmol/L B(a)P for 12 h, and then extracted, sequentially detected by Western blot with specific antibodies as indicated.



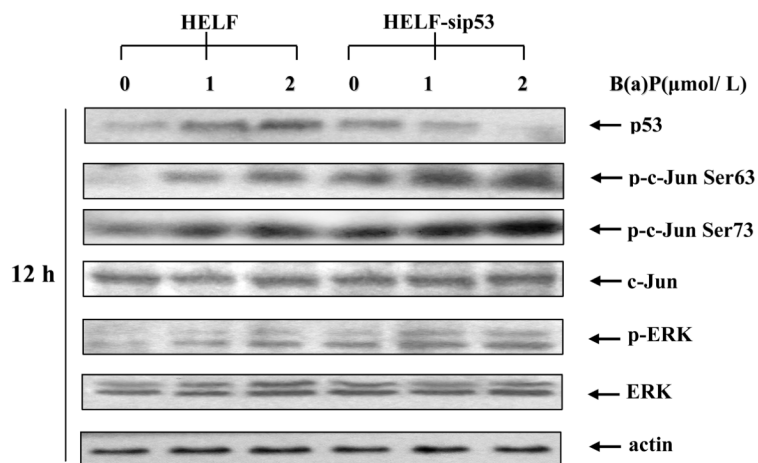
**Fig. 3. ERK activation was essential for PI-3K/Akt pathway mediating B(a)P-induced c-Jun activation**  
 HELF-AP-1/vector, HELF-AP-1-DN-Akt and HELF-AP-1-DN-Δp85 cells were treated with or without 2 μmol/L B(a)P for 12 h. The cells were then washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with specific antibodies as indicated, and then detected with secondary antibody. The results were one representative data from three independent experiments.



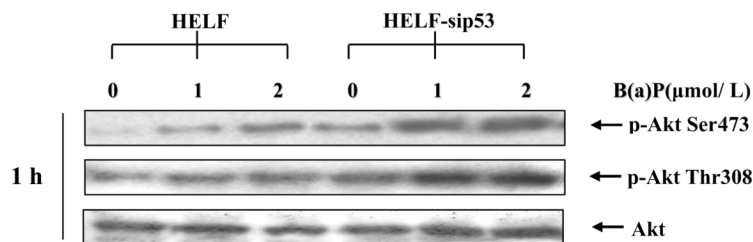
**Fig. 4**



C

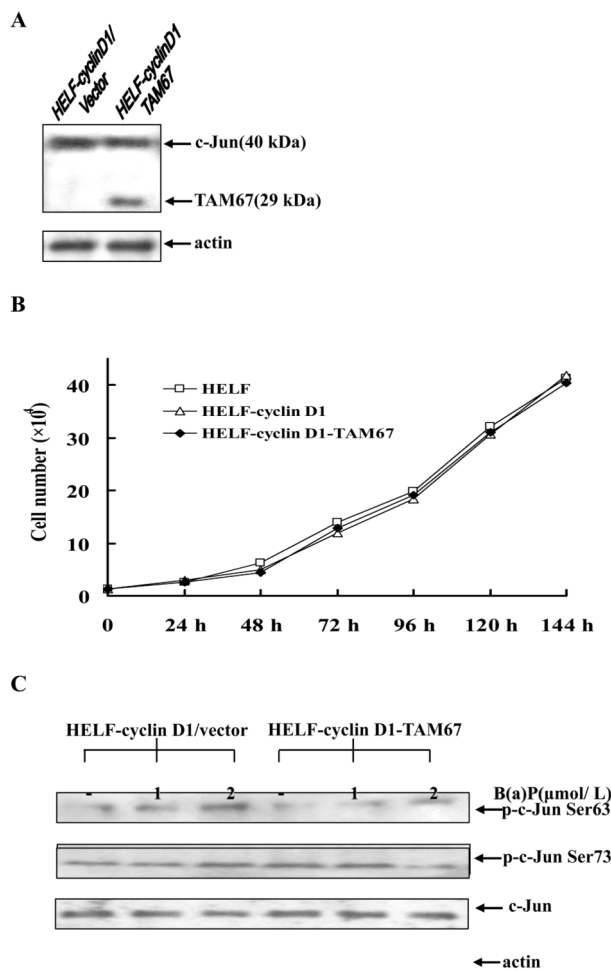


D



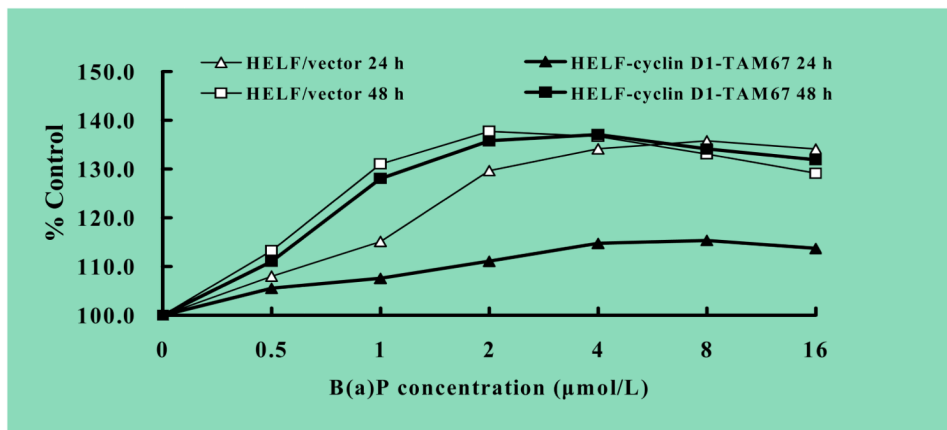
**Fig. 4. p53 inhibition increase B(a)P-induced c-Jun phosphorylation through the activation of PI-3K/Akt/ERK pathway**

*A, B*, After pretreatment with the various concentrations of p53 inhibitor pifithrin- $\alpha$  for 30 min, HELF cells were incubated with or without 2  $\mu\text{mol/L}$  B(a)P for 12 h (*A*) and 1 h (*B*). The cells were then extracted with SDS sample buffer and separated on polyacrylamide-SDS gels, and detected with specific antibodies as indicated. *C, D*, HELF and HELF-sip53 cells were stimulated with several concentrations of B(a)P for 12 h (*C*) or 1 h (*D*). The cells were then extracted and analyzed by Western blot with specific antibodies as indicated. The results were one representative data from three independent experiments.



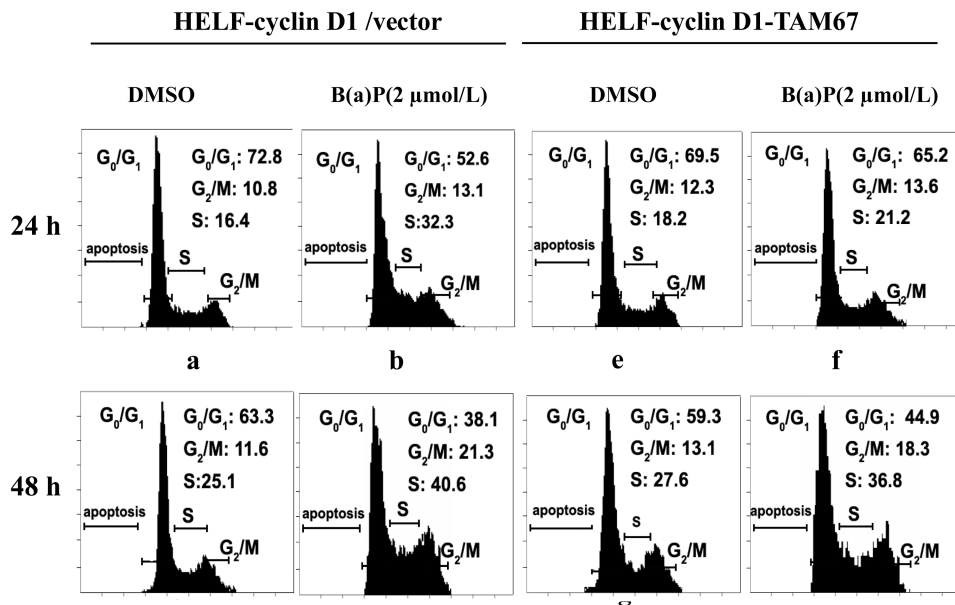
**Fig. 5. Characteristics of TAM67-transfected cells**

A, HELF cells were stably transfected with TAM67 as described in *Materials and Methods*, and extracts were subjected to immunoblotting with an antibody to the C terminus of c-Jun to detect TAM67 at 29 kDa (*left*). Endogenous c-Jun proteins appeared at the upper region of the gel at about 40 kDa. B, TAM67 stable transfectant (HELF-cyclin D1-TAM67 cells), vector transfectant (HELF-cyclin D1 cells) and parent HELF cells were seeded in 6-well plates ( $1 \times 10^4$ /well). Cell viability was measured at daily intervals by counting the number of trypan blue-excluding intact cells. Cell number was determined daily in triplicate for 6 d, and mean values were plotted. C, HELF-cyclin D1/vector or HELF-cyclin D1-TAM67 cells were stimulated with several concentrations of B(a)P for 12 h as indicated. The cells were then extracted and analyzed by Western blot with specific antibodies.



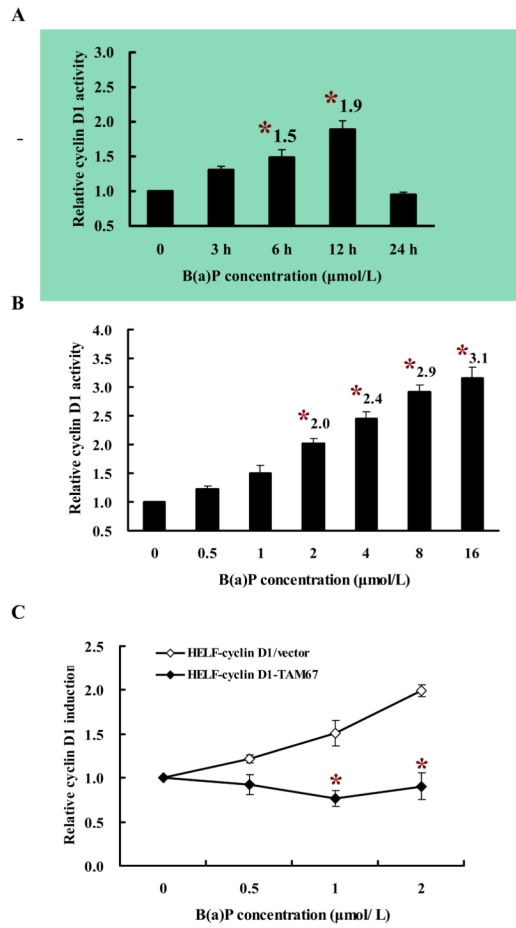
**Fig. 6. B(a)P induced cell proliferation in HELF-cyclin D1/vector but not in TAM67-expressing cells**

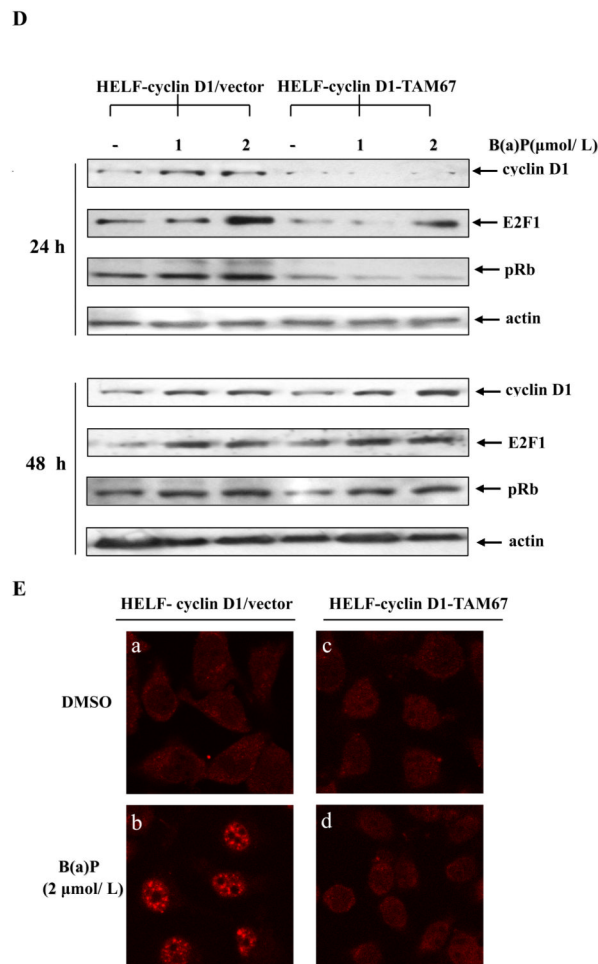
HELF-cyclin D1/vector (open) and HELF-cyclin D1-TAM67 (filled) cells were seeded in 96-well plates ( $5 \times 10^3$ /well) at 37°C overnight, and then exposed to B(a)P at various concentrations of 0.5-16  $\mu\text{mol/L}$ . Following treatment for 24 h (triangles) and 48 h (squares), MTT assay was performed as described in materials and methods respectively. All experiments were done in triplicate, and proliferation rate was expressed as a percentage of the control.



**Fig. 7. Overexpression of TAM67 abrogated B(a)P-induced cell cycle alternations**  
 HELF-cyclin D1/vector and TAM67-transfected cells were cultured in 75-cm<sup>2</sup> culture flasks with RPMI1640 containing 10% FBS at 37°C overnight. After being cultured in 0.5% FBS RPMI1640 for 24 h, cells were unstimulated or stimulated with 2 μmol/L B(a)P, as indicated, for 24 h and 48 h. Cells were sequentially fixed with 70% ice-cold ethanol overnight at 4°C, and then incubated with RNase A (1 mg/ml) for 30 min at room temperature. DNA was stained with propidium iodide (50 μg/mL) at 4°C for 1 h. Cell cycle distribution was determined by flow cytometry. The results were one representative data from three independent experiments.

Fig. 8





**Fig. 8. Requirement for c-Jun activation for up-regulation of G<sub>1</sub>regulators in HELF cells**  
**A**, HELF-cyclin D1/vector cells ( $2 \times 10^5$ ) were seeded in each well of a 6-well plate. After being serum-starved for 24 h, the cells were exposed to 2 μmol/L B(a)P for the time points as indicated, The luciferase activity was then measured, and the results are presented as relative cyclin D1 induction. Columns, means of triplicate assay wells; bars, standard deviation. The asterisk indicates a significant increase from medium control ( $P < 0.05$ ). **B**, HELF-cyclin D1/vector cells were treated with various concentrations of B(a)P as indicated for 12 hours. The luciferase activity was measured as described in *Materials and Methods*. **C**, HELF-cyclin D1/vector (open) and HELF-cyclin D1-TAM67 (filled) cells were subjected to reporter gene assay after treatment with the indicated concentrations of B(a)P for 12 h. All treatments were performed in triplicate and the results were expressed as cyclin D1 induction relative to control. The asterisk indicates a significant decrease from HELF-cyclin D1/vector cells ( $P < 0.05$ ). **D**, HELF-cyclin D1/vector cells and HELF-cyclin D1-TAM67 cells were untreated or treated with the indicated concentrations of B(a)P for 24 h and 48 h, and then extracted and detected by Western blot with special antibodies as indicated. **E**, HELF-cyclin D1/vector (a and b) or HELF-cyclin D1-TAM67 cells (c and d) were subjected to incubate with special anti-cyclin D1 antibody and detect by immunofluorescence assay with TRITC-conjugated second antibody (*red*).