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ZAK inhibits human lung cancer cell growth via ERK and JNK activation in an AP-1-dependent manner

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Novel mixed-lineage kinase protein zipper sterile-α-motif kinase (ZAK) was first cloned by our laboratory. Lung cancer is the leading cause of cancer-related death in the world, including in Taiwan. Here, we wanted to investigate whether ZAK plays a potential role in lung cancer development. First, Western blot analysis results demonstrated that four cell lines expressed high levels of ZAK from among a panel of 10 lung cancer cell lines, and two of three normal lung cells expressed ZAK. ZAK gene expressions were down-regulated in lung cancers by real-time PCR analysis. Overexpression of ZAK suppressed cell proliferation in parallel with increased phosphorylated levels of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). In contrast, ZAK silencing cells inhibited the expressions of phosphorylated ERK and JNK without affecting the expression of phosphorylated p38. The effect of the decreased cell growth rate was significantly but incompletely reversed when ZAK-overexpressing cells were treated with a specific ERK or JNK inhibitor. Moreover, c-Fos and c-Jun, the major downstream components of MAPKs, were up-regulated by ERK and JNK, respectively. When ZAK-overexpressing cells introduced with c-Jun RNA interference (RNAi), the activator protein-1 (AP-1) transcription activity detected by a secreted alkaline phosphatase (SEAP) assay was suppressed and the decreased cell number was reversed compared with the control RNAi-treated group. More importantly, ZAK significantly depressed tumor growth in *in vivo* study. Taken together, results from both *in vitro* and *in vivo* studies indicated that the decrease of lung cancer cell proliferation by ZAK may involve the ERK and JNK pathways via an AP-1 transcription factor. (*Cancer Sci* 2010; 101: 1374–1381)

The mixed-lineage kinases (MLKs) are a family of serine/threonine protein kinases that function as mitogen-activated protein kinase (MAPK) kinase kinases (MAPKKKs).[1] The MLKs cluster into three subgroups based on the domain arrangements and sequence similarity within their catalytic domains: the MLKs, the dual-leucine-zipper-bearing kinases, and zipper sterile-α-motif kinase (ZAK). We are the first group to clone ZAK in 2000[2] (GenBank accession number: AF238255). This cDNA has 2456 bp and encodes a protein of 800 amino acids that contains a kinase catalytic domain, a leucine zipper, and a sterile-α-motif (SAM). Our ZAK is also known as ZAK-α or MLK-like MAP triple kinase-α (MLTK-α).[1,3] ZAK-β is an alternative splicing product and is also referred to as MLTK-β or MLK7. ZAK-β is identical to ZAK-α from the amino terminus to the zipper domain, but then diverges and terminates shortly thereafter, so it lacks a SAM domain.[1,3] Similar to other MLK family members, the MLTKs regulate signaling of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase.[4]

The transcription factor activator protein-1 (AP-1) regulates a wide range of cellular processes, including cell proliferation, apoptosis, survival, and differentiation.[5] The mammalian AP-1 proteins are homodimers and heterodimers composed of basic region-leucine zipper proteins that belong to the Jun, Fos, Maf, and activating transcription factor (ATF) families.[5] AP-1 transcription activity can be regulated by several mechanisms, including via the activation of the MAPK pathways.[6]

References in the role of MLKs in cancer development are limited. It has been reported that overexpression of wild-type MLK3 results in the transformation of NIH-3T3 fibroblasts and anchorage-independent growth.[7] The cell proliferation of the HCT15 colon cancer cells was substantially impaired by MLK3 RNA interference.[8] However, Kim *et al.* showed that MLK3 is the mediator of transforming growth factor-β1 (TGF-β1)-induced apoptosis signaling in Hep3B hepatoma cells.[9] Also, Hong and Kim demonstrated that genipin signaling to apoptosis of PC3 prostate cancer cells is mediated via activation of MLK3.[10] Overexpression of MLTK-α effectively induces proliferation and malignant cell transformation in JB6 C141 skin epidermal cells.[11] In addition to these actions, MLTK-α-overexpressing cells formed fibrosarcomas when injected subcutaneously into athymic mice. Nevertheless, our previous studies indicated that overexpression of ZAK induced cell death of Hep3B hepatoma cells[2] and inhibited cell proliferation of Rat6 embryonic fibroblasts.[12]

Lung cancer is the most common malignant neoplasm worldwide and represents the leading and second leading cause of cancer death of females and males, respectively, in Taiwan.[13] The purpose of the present study was to determine whether the *ZAK* gene can decrease human lung cancer cell growth and to determine the necessity of ERK, JNK, and AP-1 activation in any such decreases in lung cancer cell growth.

Materials and Methods

Cell culture and reagents. Human lung cancer cell lines A549, Calu-1, H23, H358, H441, H460, H1299, and H1688 were purchased from the American Type Culture Collection (Rockville, MD, USA). BEAS-2B, IMR-90, and MRC-5 were also purchased from the American Type Culture Collection. IMR-90 and MRC-5 are normal human lung fibroblasts; BEAS-2B is one of the normal human bronchial epithelial cells. H226 and H1355 were kindly provided by Dr Chun-Ming Tsai (Taipei Veterans General Hospital, Taipei, Taiwan). The human lung cancer cell lines A549, Calu-1, H358, H460, and H1299 were cultured in DMEM medium (Gibco BRL, Grand Island, NY, USA); H23, H226, H441, H1355, and H1688 were cultured in RPMI-1640 medium (Gibco BRL). IMR-90 and MRC-5 were cultured in MEM medium (Gibco BRL). All media were supplemented with 2 mM glutamine, 100 μM sodium pyruvate, 100 μM

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nonessential amino acids, 1% penicillin–streptomycin, and 10% fetal bovine serum (Gibco BRL). BEAS-2B was cultured in LHC-9 medium (Invitrogen, Carlsbad, CA, USA). Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C.

Quantitative real-time PCR. This study enrolled 40 lung cancer patients including 30 males and 10 females. Their median age was 67 years old (range, 39–87 years). The histologic types of lung cancers were as follows: 20 patients (50%) with adenocarcinoma; 19 patients (47.5%) with squamous cell carcinoma; and one patient (2.5%) with bronchioloalveolar adenocarcinoma. The pathologic stage of the lung tumors was as follows: 22 patients (55%) with stage I; 7 patients (17.5%) with stage II; and 11 patients (27.5%) with stage III. The study was conducted after human experimentation review by the Institutional Review Board of Chung Shan Medical University Hospital, and informed consent was obtained for every examined specimen. Before extracting RNA from lung tissue blocks, microdissection of tissue sections was performed to ensure at least 60% section areas were tumor cells. cDNA derived from human lung tumor specimens were kindly provided by Dr Huei Lee (Chung Shan Medical University, Taichun, Taiwan). Quantitative PCR was performed using SYBR Green PCR master mix (Protech Technology, Taipei, Taiwan) and the Prism 7000 Sequence Detection System instrument (Applied Biosystems, Foster City, CA, USA). PCR amplification began with a 10-s denaturation step at 95°C and then 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. The samples were analyzed in duplicate, and the ZAK levels were normalized to the corresponding 18S rRNA levels. The PCR primer sequences used were as follows: ZAK sense primer ACTTTGGTGCCTCTCGGT and antisense primer TTCTGGGGCAACTGCTT; 18S rRNA sense primer TCGGAACTGAGGCCATGA and antisense primer CCGGTCGGCATCGTTTA. The relative amounts of the *ZAK* gene, standardized against the amount of 18S rRNA, were expressed as ΔC_t (threshold cycle) = $C_{t(ZAK)} - C_{t(18S\ rRNA)}$. The ratio of ZAK mRNA copies to 18S rRNA copies was then calculated as $2^{-\Delta Ct} \times K$ ($K = 10^6$, a constant).

Transfection of pcDNA3-HA-ZAK and ZAK RNA interference. Construction of pcDNA3-HA-ZAK has been described in our previous studies.[2] pcDNA3-HA-ZAK containing the full length of the *ZAK* gene was stably expressed in H460 cells, which had a very low ZAK expression level in tested cancer cell lines. RNA interference is a powerful technique used to block gene expression in eukaryotes. A short-hairpin RNA (shRNA) construct targeting ZAK mRNA was stably expressed in A549 cells, which had a higher ZAK expression level in tested cell lines. The shRNA template was constructed by two complementary oligonucleotides that, when partially annealed, created a loop region with complementarity to ZAK mRNA. The forward and reverse primers, containing 21 nucleotides of the ZAK sequence, were as follows: forward primer, 5′-GATCCGCCTCTCGGTTCCATAACCATTTCAAGAGAA-3′; reverse primer, 5′-AGCTTAAAAAGCCTCTCGGTTCCATAACCATTCTCTTGAAA-3′. The shRNA template was cloned into the vector pcDNA-HU6. The pcDNA3-HA-ZAK construct or ZAK-specific shRNA construct (2 μg) was then mixed with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and added to H460 cells and A549 cells, respectively. After 24-h transfection, stable transfectants were selected with 500 μg/mL of gentamycin (Promega, Madison, WI, USA). Thereafter, the selection medium was replaced every 3 days for several weeks. c-Jun RNAi and control RNAi were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Various amounts of RNAi (10 μM) were transfected into tumor cells for 2 days, then the whole cell lysates were harvested. Effect of gene knockdown or overexpression was confirmed by Western blotting.

Western blot analysis. Harvested cells were dissolved for 30 min in 50 mL RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 2 mM EDTA, and 50 mM Tris [pH 7.4]) containing phosphatase and protease inhibitors. Cell lysates were separated by 10% to 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and were then transferred to PVDF membranes (Pall, Pensacola, FL, USA). These membranes were incubated overnight at 4°C with primary antibodies. ZAK polyclonal antibodies were produced at the animal center of Chung Shan Medical University (Taichung, Taiwan). β-actin antibodies were purchased from Santa Cruz Biotechnology. c-Fos, ERK, JNK, p38, phosphorylated ERK (Thr202/Tyr204), phosphorylated JNK (Thr183/Tyr185), phosphorylated p38 (Thr180/Tyr182), phosphorylated c-Jun (Ser63), and phosphorylated Hsp27 (Ser82) antibodies were purchased from Cell Signaling Techology (Danvers, MA, USA). Membranes were then washed with PBS-Tween20 and incubated with the appropriate secondary antibodies. An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, USA) was used to detect the target proteins.

Cell proliferation assay. H460 and A549 cells were seeded at an initial density of 5×10^4 cells per well in six-well plates and then cultured and harvested at several time points. Cell number determination was performed using a hematocytometer under a microscope. Growth curves were plotted. The doubling times were calculated at the exponential phase of the growth cycle. Cell viability also was conducted by an MTT [3-(4, 5-dimethylthiazol-2-yl)-25-diphenyltetrazolium bromide] assay. 8×10^3 of H460 cells were seeded per well in a 24-well plate and then cultured and harvested at several time points. When each time point was reached, MTT was added into each well at a final concentration of 0.5 mg/mL. The insoluble formazan was collected and dissolved in dimethylsulfoxide and the optical density (OD) value was measured with a scanning spectrophotometer at a wavelength of 535 nm. Moreover, cell proliferation was detected by cell colony formation assay. H460 cells were seeded in a 6-cm dish at a density of 5000 cells, and allowed to grow for 7 days. At the end of incubation, cell colony formation was observed with 20% Geimsa staining. The size of cell colonies >1 mm were scored.

AP-1 secreted alkaline phosphatase (SEAP) assay. AP-1 SEAP assay was performed with a Great EscAPe chemiluminescent SEAP kit (ClonTech, Mountain View, CA, USA). The initial cell number at 5×10^5 was seeded in a 6-cm plate, then transfected with AP-1 SEAP reporter plasmid (1 μg) for 3 days. After that incubation, 25 μL of cell-cultured medium was placed in a microcentrifuge tube mixed well with 75 μL of 1× dilution buffer. Samples were heated to 60°C using a heating block for 30 min, and cooled to room temperature by placing on ice for 3 min, then equilibrated to room temperature for 10 min. One hundred microliter of assay buffer was added to each sample and samples were incubated for 5 min at room temperature. Finally, 100 μL SEAP substrate was added to each sample and samples were incubated for 30 min at room temperature. Thereafter, the chemiluminescent signal from each sample was read by a luminometer.

Mouse model of tumor xenograft growth. Specific pathogen-free severe combined immunodeficient (SCID) CB17 mice (obtained from the Animal Center of Taiwan University, Taipei, Taiwan) were kept in individual ventilated cages at the Animal Center of Chung Shan Medical University for tumor study. Tumors were induced in SCID mice by subcutaneous (s.c.) injection of 5×10^6 H460 tumor cells in 200 μL of PBS. Tumor size was measured with calipers every 3 days throughout the study. Tumor volume was calculated as: tumor volume = length × $(\text{width})^2/2$. On Day 34 after tumor cell inoculation, all mice were sacrificed, and tumor mass was

weighed. Animal care and use were in accordance with the policies of the Institutional Animal Care and Use Committee of Chung Shan Medical University.

Statistical analysis. Data were analyzed by ANOVA followed by Dunnett's test to evaluate the significance of differences between the vector control group and the other groups (*P*-values determined by Dunnett's test are indicated in the Figures). In addition, the difference between two groups was assessed by ANOVA followed by the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Expression profile of ZAK in human lung cancer cell lines and human lung tumor specimens. In order to investigate whether ZAK plays a role in lung cancer development, we examined ZAK expression profile using a panel of 10 human lung cancer cell lines. Western blotting results demonstrated that A549, H23, H358, and H1355 cells expressed high levels of ZAK; the other lung cancer cell lines showed moderate or little expression of ZAK (Fig. 1a). Normal human lung cells BEAS-2B, and IMR-90 expressed moderate levels of ZAK, whereas MRC-5 showed little expression of ZAK. Human lung tumor specimens were collected to observe the ZAK expression pattern. We performed quantitative real-time PCR analysis to examine the expression of ZAK in lung tumor specimens. After normalization with housekeeping gene *18S* rRNA, markedly decreased expression of ZAK was observed in 10 of the 40 lung tumor parts, whereas ZAK expression was almost equal in three pairs of tissue specimens (Fig. 1b). Our real-time-PCR results indicated that ZAK was expressed more in the adjacent normal part than in tumor part.

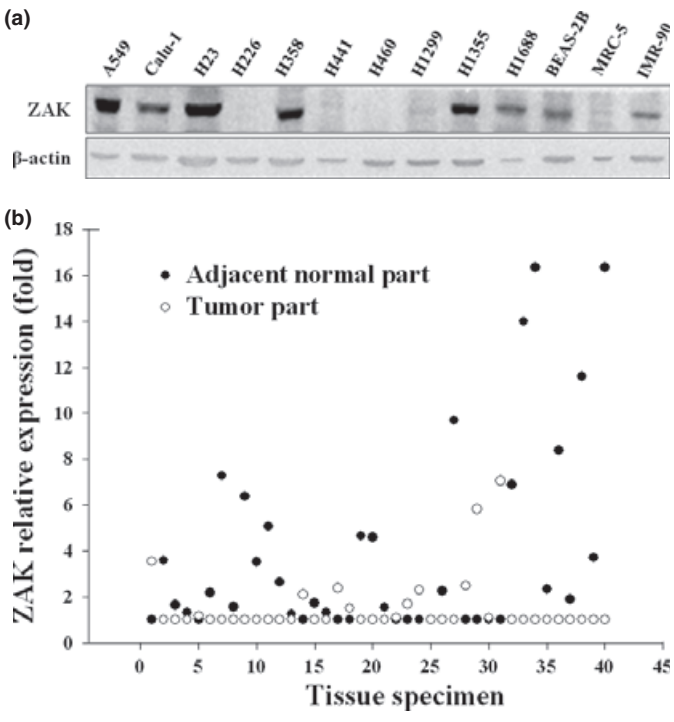

Fig. 1. Expression profile of zipper sterile-α-motif kinase (ZAK) in human lung cancer cell lines and human lung tumor specimens. (a) Expression pattern of ZAK in lung cancer cell lines was detected by Western blotting. β-Actin was used as an internal control. (b) Expression of ZAK in lung cancer tissues was measured by quantitative real-time PCR. 18S rRNA was used as an internal control. The relative amounts of the *ZAK* gene, standardized against the amount of 18S rRNA, were expressed as ΔC_t (threshold cycle) = $C_{t(ZAK)} - C_{t(18S\ rRNA)}$. The clinical samples were analyzed in duplicate.

ZAK overexpression decreased cell proliferation. To assess whether ZAK might play a role in growth or survival of lung cancer cells, we overexpressed the wild-type *ZAK* gene into H460 cells, because endogenous ZAK expression almost cannot be detected in H460 cells. Stable clones overexpressing ZAK in H460 cells (referred to as H460/ZAK cells) or a vector control (termed H460-C) were isolated following selection with G418. Gene expression of ZAK was validated by Western blot analysis. Clones 4 (H460/ZAK C4) and 5 (H460/ZAK C5), which showed strong expressions of ZAK compared with vector control or parental cells (Fig. 2a), were chosen for the following experiments. Proteins extracted from Hep3B hepatoma cells were used as a ZAK-positive control.[2] To clarify the effect of ZAK on lung cancer cell growth, we performed cell proliferation experiments by cell counting, MTT, and cell colony formation assays. In the cell counting assay, the seeded cell numbers at the initial time points of H460, H460-C, and H460/ZAK were the same, and then culturing continued for a period of time. With the overexpression of ZAK in H460 cells, the cell growth rate was suppressed at a statistically significant level compared with vector control or parental cells (Fig. 2b, upper panel). The population doubling times for H460, H460-C, H460/ZAK C4, and H460/ZAK C5 were approximately 17, 18.1, 22.4, and 23.7 h, respectively, with statistically significant difference ($P < 0.05$) between the H460-C and H460/ZAK groups. In order to rule out that the antiproliferative effect induced by ZAK is cell-type specific, we used another cell line, H1299, which had a lower expression of ZAK by cell counting assay. H1299 cells were transiently transfected with vector, 2 μg (+), and 4 μg (++) of the wild-type *ZAK* gene, respectively. After 3 days' incubation, the cells were harvested for cell counting. As demonstrated in the lower panel of Figure 2(b), ZAK decreased the cell proliferation of H1299 cells in a dose-dependent manner. Similar cell proliferation results for H460 were confirmed by a MTT assay (Fig. 2c). In addition, colony formation assay revealed that the size and density of colonies derived from H460/ZAK cells were generally smaller than those of the vector control or parental cells (Fig. 2d, upper panel). The colony number was quantified as shown in the lower panel of Figure 2(d). In contrast, we used a shRNA strategy to knockdown endogenous ZAK expression in A549 cells. Clones 3 (A549/shRNA ZAK C3) and 4 (A549/shRNA ZAK C4) showed little expression of ZAK compared with vector control or parental cells (Fig. 2e). The ZAK-silencing A549 cells significantly enhanced cell proliferation in comparison with vector control or parental cells (Fig. 2e). The population doubling times for A549, A549-C, A549/shRNA ZAK C3, and A549/shRNA ZAK C4 were approximately 22, 22.6, 18.1, and 17.9 h, respectively, with a statistically significant difference ($P < 0.05$) between the A549-C and A549/shRNA ZAK groups. These results demonstrated that overexpression of ZAK inhibited cell proliferation in lung cancer cells *in vitro*.

Enforced expression of ZAK induced ERK and JNK activation. Our previous studies indicated that overexpression of ZAK in murine fibroblasts decreased cell proliferation via activating the JNK pathway.[12] In this study, we wanted to investigate whether ZAK plays a similar role in lung cancer cells. ZAK-overexpressing H460 cells enhanced phosphorylated ERK and JNK expressions but did not affect expression of phosphorylated p38 compared with vector control or parental cells (Fig. 3a). To clarify the activities of MAPKs, expressions of their major downstream substrates were performed. ZAK-overexpressing H460 cells enhanced c-fos and phosphorylated c-Jun expressions, but did not affect expression of phosphorylated

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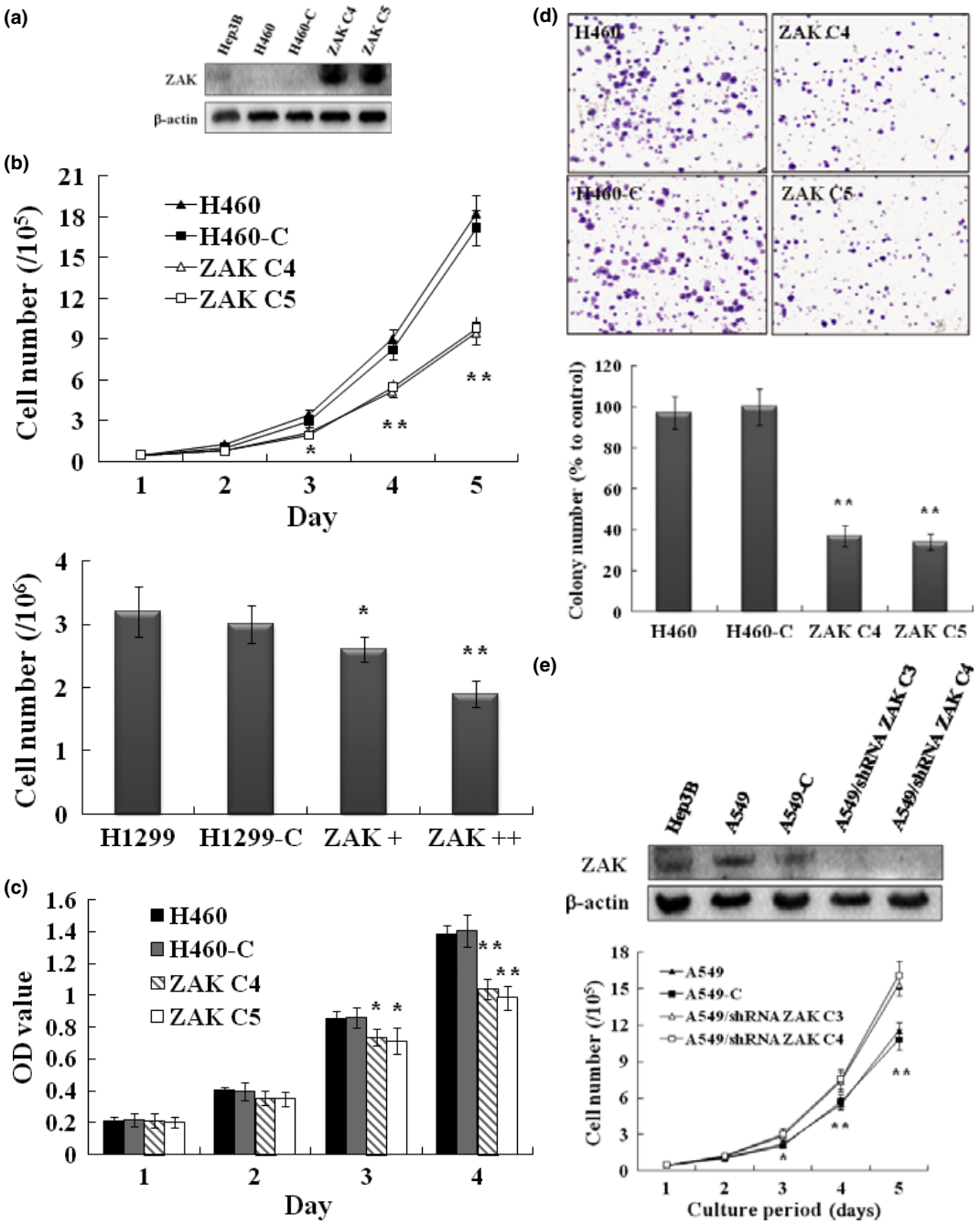

Fig. 2. Zipper sterile-α-motif kinase (ZAK) overexpression decreased cell proliferation. (a) H460 cells were stably transfected with wild-type *ZAK* gene. H460/ZAK C4 and H460/ZAK C5 showed strong expression of ZAK compared with vector control. (b) Cell growth curves of H460/ZAK and H460-C cells were examined by a cell counting assay (upper panel). The result of cell numbers of H1299/ZAK and H1299-C is shown in the lower panel. (c) Cell proliferation of H460 cells also was examined by a MTT assay. OD, optical density. (d) Images of cell colonies are shown in the upper panel. In the lower panel, colony numbers are presented as a percentage of vector control for H460/ZAK cells. (e) Clones 3 (A549/shRNA ZAK C3) and 4 (A549/shRNA ZAK C4) showed little expression of ZAK compared with vector control or parental cells. Cell growth curves of A549/shRNA ZAK and A549-C cells were examined by a cell counting assay. Values shown are means ± SD. Values significantly different from the vector control were determined with ANOVA followed by Dunnett's test and are indicated by $*P < 0.05$ or $**P < 0.01$. Results are representatives of three independent experiments.

Hsp27 compared with vector control or parental cells (Fig. 3a). This result indicated that ZAK-overexpressing cells may increase the activities of ERK and JNK via up-regulation of c-Fos and phosphorylated c-Jun expressions, respectively. However, in the loss of ZAK function experiments, ZAK-silencing A549 cells inhibited the expressions of phosphorylated ERK and JNK without affecting the expression of phosphorylated p38 compared with vector control or parental cells (Fig. 3b). In order to see whether the decreased proliferation rate of ZAK-overexpressing cells was due to ERK and JNK activation, H460/ZAK cells were treated with a specific ERK inhibitor (PD98059, 10 μM) and/or JNK inhibitor (SP600125, 10 μM) for 3 h, and

were then washed twice and further cultured for 3 days to count their proliferation rates. In H460-C cells, treatment with ERK and JNK inhibitors slightly affected cell proliferation compared with untreated H460-C cells (Fig. 3c). But in H460/ZAK cells, treatment with either ERK inhibitor or JNK inhibitor enhanced cell proliferation in comparison with untreated H460/ZAK cells (Fig. 3c).

Overexpressed ZAK induced AP-1 transcription activity. Because ERK and JNK are upstream signaling for AP-1 activation, we speculated that AP-1 activity could be enhanced in ZAK-overexpressing cells. As expected, AP-1 activity detected by a SEAP assay was substantially raised in ZAK-overexpressing H460 cells compared with vector control or parental cells (Fig. 4a, upper panel). To evaluate whether this AP-1 activity result was reproducible in other lung cancer cells, the same number of H1299 cells was transiently transfected with AP-1 SEAP reporter plasmid (1 μg) and vector (4 μg) or 2 μg (+) or 4 μg (++) wild-type *ZAK* gene. As shown in the lower panel of Figure 4(a), ZAK significantly increased AP-1 activity proportional to transfected gene amounts. The c-Jun RNAi strategy was employed to evaluate whether AP-1 activity would be affected. The result showed that 10 μL (++) but not 5 μL (+) of c-Jun RNAi was effective in diminishing c-Jun protein expression (Fig. 4b). Moreover, the AP-1 transcription activity was significantly attenuated in ZAK-overexpressing cells treated with a high dose (10 μL) of c-Jun RNAi (Fig. 4c). This result suggested that c-Jun silencing may decrease AP-1 activity by impairing AP-1 composition. As a sequence of c-Jun silencing, the reduced number of ZAK-overexpressing cells was significantly reversed compared with the control RNAi group (Fig. 4d).

Ectopic ZAK resulted in impaired tumor growth. To determine their relative tumorigenic abilities *in vivo*, H460-C and H460/ZAK cells were subcutaneously injected into CB17-SCID mice, and tumor formation was monitored for 34 days. ZAK-overexpressing cells significantly inhibited tumor growth, as proved by the increase of both tumor volume (Fig. 5a) and tumor weight (Fig. 5b). The subsequent ZAK immunoblots of the representative tumors derived from ZAK-overexpressing cells and vector control cells were validated (Fig. 5c). Proteins extracted from A549 cells were used as a ZAK-positive control. Also, proteins extracted from representative tumor tissues were subjected to Western blotting to confirm c-Fos and phosphorylated c-Jun expressions. As shown in Figure 5(d), tumor tissues that expressed more ZAK also expressed more c-Fos and phosphorylated c-Jun. Proteins extracted from H460/ZAK C5 cells were used for c-Fos and phosphorylated c-Jun positive controls. These results matched those acquired from H460 cell extracts.

Discussion

Our results indicated that about half the lung cancer cell lines expressed ZAK (Fig. 1a). ZAK expressions were also found in two normal lung cells (Fig. 1a). Moreover, overexpression of ZAK in H460 and H1299 cells retarded cell proliferation in *in vitro* studies (Fig. 2b). On the other hand, silencing of ZAK by shRNA in A549 cells enhanced cell proliferation compared with vector control cells (Fig. 2e). Furthermore, ZAK suppressed lung tumor growth in a xenograft transplantation mouse model (Fig. 5a,b). Finally, most expression levels of ZAK were lower in tumor tissues compared with their cognate normal tissues collected from 40 lung tumor specimens (Fig. 1b). The current findings suggest that ZAK may act as a potential tumor suppressor gene in lung cancers by regulating cell proliferation. The study of more lung tumor specimens for ZAK expression profile is required to support this hypothesis.

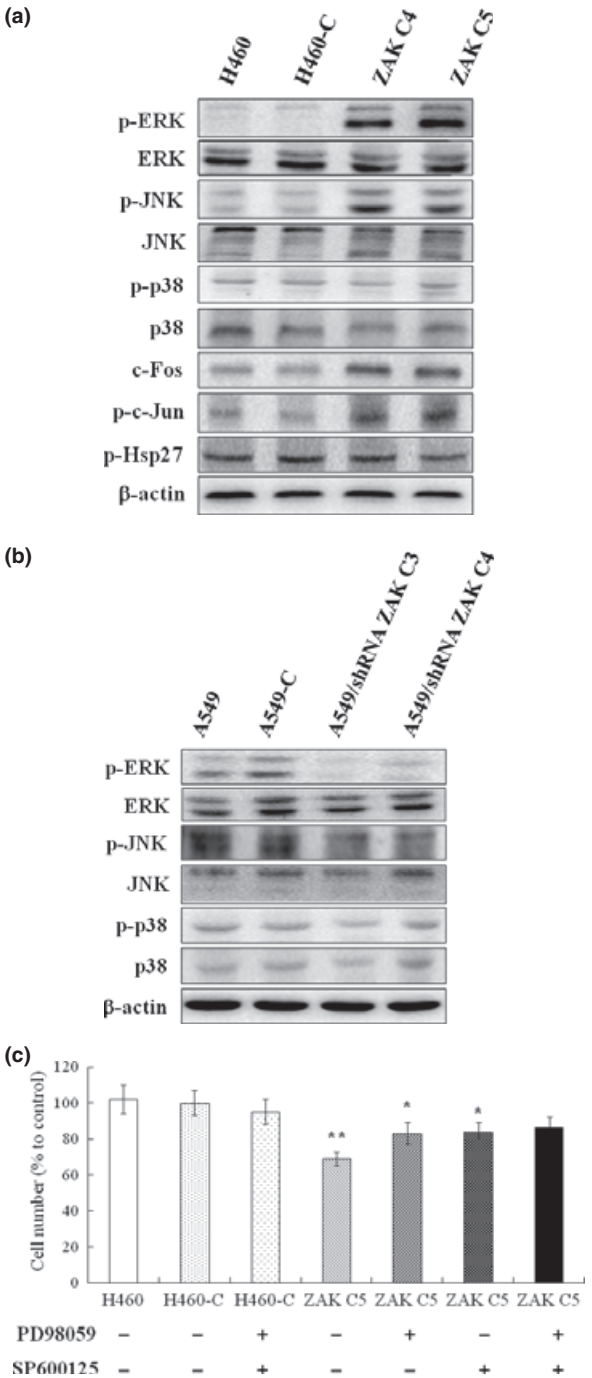

Fig. 3. Enforced expression of zipper sterile-α-motif kinase (ZAK) induced ERK and JNK activation. (a) Protein levels from H460, H460 vector control, and H460/ZAK cells were subjected to Western blot analysis. Total and phosphorylated ERK, JNK, and p38 protein levels were detected by Western blotting. c-Fos, c-Jun, and Hsp27 were the downstream targets of ERK, JNK, and p38, respectively. Their expression levels were also detected by Western blots. (b) Protein levels from A549, A549 vector control, and A549/shRNA ZAK cells were subjected to Western blot analysis. Total and phosphorylated ERK, JNK, and p38 protein levels were detected by Western blotting. (c) Cells were treated with a specific ERK inhibitor (PD98059, 10 μM) and/or JNK inhibitor (SP600125, 10 μM) for 3 h, and then were washed and further cultured for 3 days to do cell counting. Values significantly different from the H460 vector control (untreated) were determined with ANOVA followed by Dunnett's test and are indicated by $*P < 0.05$ or $**P < 0.01$. Each experiment was repeated twice for reproducibility.

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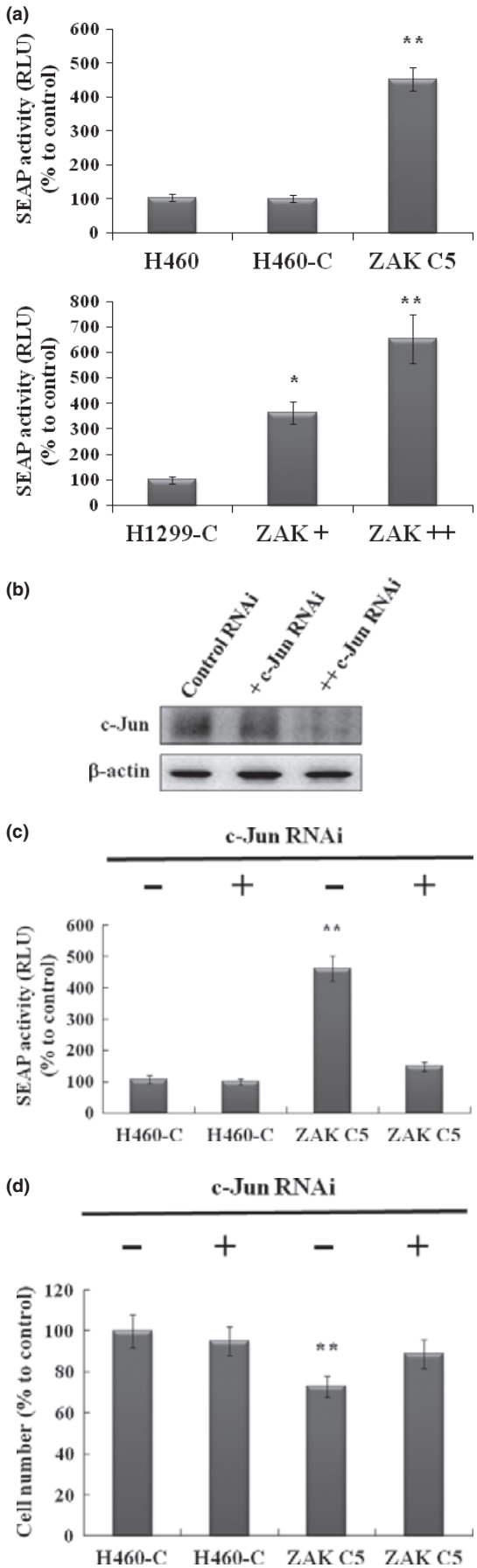

Fig. 4. Overexpressed zipper sterile-α-motif kinase (ZAK) induced activator protein-1 (AP-1) transcription activity. (a) The results of AP-1 transcription activity in H460 and H1299 cells are shown in the upper and lower panels, respectively. RLU, relative luminescent unit; SEAP, secreted alkaline phosphatase. (b) ZAK-overexpressing H460 cells were transiently transfected with c-Jun RNAi (10 μM) for 2 days. c-Jun protein levels were suppressed when cells were treated with 10 μL (++) but not 5 μL (+) of c-Jun RNAi. (c) ZAK C5 was treated with AP-1 SEAP reporter plasmid (1 μg) and c-Jun RNAi (10 μL) for 3 days, and AP-1 transcription activity was detected. (d) Under the same condition as the experiment described in (c), at the same time as per SEAP assay, the cell counting assay was performed. Values significantly different from the H460 vector control (untreated) were determined with ANOVA followed by Dunnett's test and are indicated by $*P < 0.05$ or $**P < 0.01$. Each experiment was repeated twice for reproducibility.

Our previous studies indicated that ZAK has been implicated in inhibiting cell proliferation through the activation of JNK in fibroblast cells.[12] Also, transient expression of ZAK triggered apoptosis of hepatoma cells via the activation of JNK.[2] This prompted us to evaluate the signal transduction pathways regulated by ZAK in lung cancer cells. Besides the JNK pathway, ZAK also strongly activates the ERK pathway as determined by phosphorylation status (Fig. 3a). Also, the effect of decreased cell growth rate was significantly but incompletely reversed when ZAK-overexpressing cells were treated with a specific ERK or JNK inhibitor (Fig. 3c). ERK cascade is thought to play key roles in the proliferative process and cell survival, whereas the JNK pathway is stimulated for apoptosis signal.[14,15] A growing number of reports suggest that activation of ERK may suppress cell proliferation[16–19] or induce cell death.[20–25] Not only the duration, but also the magnitude of the ERK signal, may play a key role in determining the final cellular outcome of this pathway. Rapid and transient activation of ERK is usually associated with cell proliferation. However, several studies have shown pro-apoptotic and growth inhibitory effects that were dependent on strong and sustained activation of ERK.[18–25] In addition, there are several lines of evidence indicating that both ERK and JNK signaling pathways are involved in apoptotic process.[26–29] In some cases, a decreased cell proliferation rate could be due to cell apoptosis. The above reports are, to a certain degree, in agreement with our results. The ERK pathway was highly activated in ZAK-overexpressing lung cancer cells. However, the ERK pathway was not detected in fibroblast and hepatoma cells introduced with ZAK.[2,12] The activation of such a pathway might be cell-type specific. Moreover, this differential phenomenon might contribute to distinct regulation mechanisms by ZAK of cell proliferation between lung cancer cells and other cells.

In contrast to our results, ectopically expressed MLTK-α (or ZAK-α) effectively induces proliferation, and malignant cell transformation in JB6 C141 skin epidermal cells has been documented.[11] Very importantly, MLTK-α-overexpressing cells formed fibrosarcomas when injected subcutaneously into athymic mice. Why does the same gene working in different cells lead to such a reverse effect? The reason is unknown. Previous studies have pointed out that decreased cell growth rate or trigger of apoptosis by ZAK may mediate the JNK pathway.[2,12] The present study indicates the involvement of the JNK and ERK pathways in suppression of lung cancer cell growth. It is important to note that MLK7 (or ZAK-β) is involved in JNK and p38 activation in response to apoptotic stimuli.[30] However, expression of activating transcription factor-2 (ATF-2) was enhanced but not that of c-Jun and Elk-1 in MLTK-α-overexpressing skin epidermal cells without any stimulation.[11] Also, that ATF-2 protein

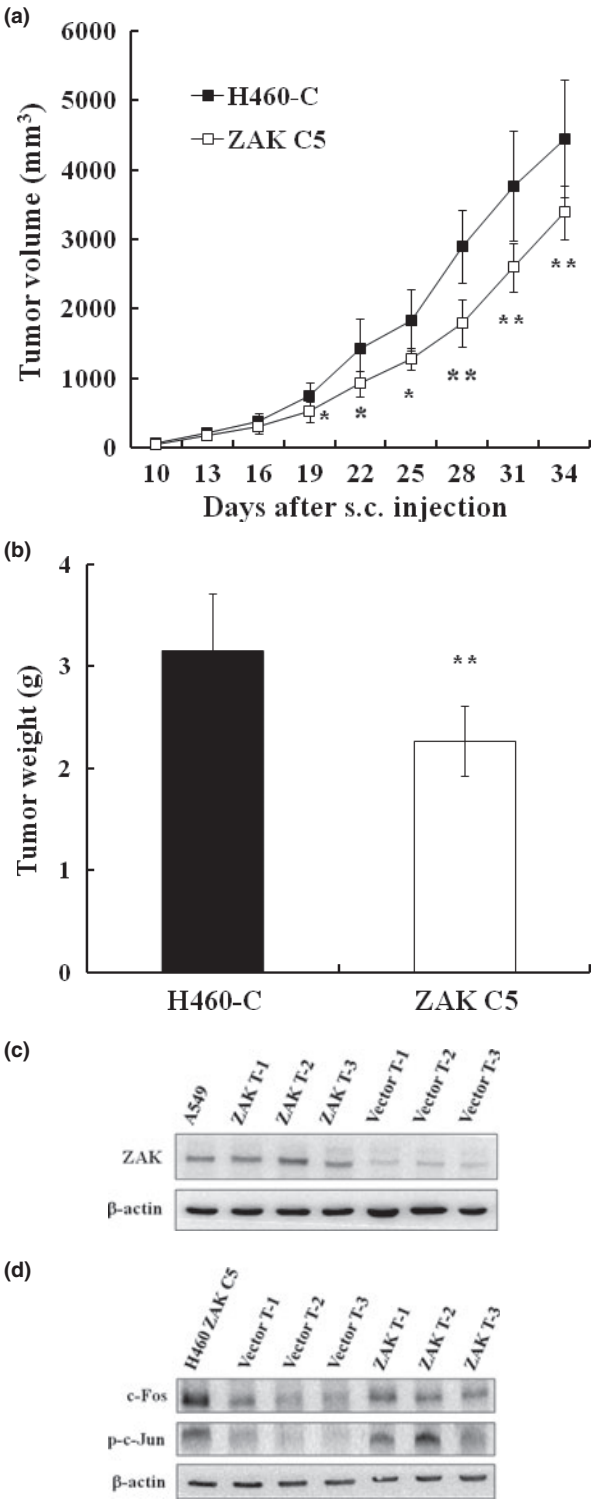

Fig. 5. Ectopic zipper sterile-α-motif kinase (ZAK) resulted in impaired tumor growth. (a) Tumor volume was measured at the indicated days. (b) Tumor weight was evaluated at the end of experiment. (c) Proteins extracted from representative tumor tissues were subjected to Western blotting to confirm ZAK expression. (d) Proteins extracted from representative tumor tissues were subjected to Western blotting to validate c-Fos and phosphorylated c-Jun expressions. Values shown are means ± SD ($n = 6$ mice per group). Values significantly different from the vector control were determined with ANOVA followed by Student's t-test and are indicated by $^*P < 0.05$ or $^{**}P < 0.01$.

correlates with malignant phenotypes in mouse skin carcinogenesis has been demonstrated by Zoumpourlis *et al.*[31] To this end, ZAK may activate distinct MAPK pathways in different cell types leading to an opposite effect. It is worth noting that accumulation of MLTK-α in the nucleus might be important in cell proliferation and cell transformation in skin epidermal cells. However, our results demonstrated that ZAK is mainly located in the cytoplasm (data not shown). Even the same gene located in different places might play a distinct role in cell proliferation.

The necessity of ERK and JNK activation for ZAK-induced cell proliferation was demonstrated by significantly but partially blocking the effect of ZAK with a specific ERK or JNK inhibitor (Fig. 3c). The levels of c-Fos and phosphorylated c-Jun were up-regulated as a sequential effect of ERK and JNK activation (Fig. 3a). Also, the transcription activity of AP-1 was tremendously enhanced in ZAK-overexpressing cells (Fig. 4a). However, the cell growth rate was significantly increased in ZAK-overexpressing cells in addition to c-Jun RNAi in parallel with a decreased AP-1 activity (Fig. 4d). Some of the following references may partly provide evidence to support our observations. One report indicated that genistein (one kind of isoflavonoid) can induce G_2/M cell cycle arrest via activation of the ERK pathway leading to c-Fos and c-Jun up-regulation in breast cancer cells.[18] This accounts for the necessity of inducement of c-Fos and c-Jun by ERK for reduced cell proliferation. Activation of the ERK and JNK pathways resulting in increased protein levels of Egr-1, Elk-1, c-Fos, and/or c-Jun were documented in some chemically treated cells.[26,27,29] Also, the apoptotic effect was accompanied by increased transcription activity of Egr-1 and/or AP-1 which are downstream effectors of the ERK and JNK pathways.

The AP-1 transcription factor is assembled as homodimers or heterodimers from Jun-Jun, Jun-Fos, or Jun-ATF family proteins.[5] MAPKs and c-Fos-regulating kinase (FRK) have been reported to regulate AP-1 activity.[32,33] Also, the regulation of AP-1 activity is critical for the cell fate decision and occurs at various levels including dimer-composition.[34] Our data demonstrated that knockdown of c-Jun by RNAi can substantially suppress AP-1 transcription activity and significantly reverse the decreased cell growth in ZAK-overexpressing cells. c-Fos and c-Jun were up-regulated, and other undetected members of the Jun, Fos, and ATF families may be regulated by ZAK to affect AP-1 activity. At least, c-Jun is a very important component for AP-1 dimerization in our case. The real composition of AP-1 in this study needs to be defined in the further research. Our previous results have shown that nuclear factor-kappa B (NF-κB) transcription activity was activated by ZAK in hepatoma cells;[2] whether NF-κB or other pathways might be activated by ZAK in lung cancer cells will be under investigation in future study.

In summary, the present results are the first to indicate that ZAK may function as a tumor suppressor gene by inhibiting human lung cancer growth. The current study may provide a foundation to further explore the role of ZAK in lung cancer development and suggest a strategy for the development of rationally designed therapeutics using ZAK as a target. To explore whether ZAK expression has clinical relevance by serving as a prognostic marker in lung cancer patients, further studies are needed using lung tumor tissue arrays to correlate ZAK expressions and the overall survival rate in lung cancer patients.

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