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Antitumorigenic Effects of ZAKβ, an Alternative Splicing Isoform of ZAK

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Abstract

Sterile alpha motif (SAM)- and leucine-zipper-containing kinase (ZAK) plays a role in the regulation of cell cycle progression and oncogenic transformation. The ZAK gene generates two transcript variants, ZAKα and ZAKβ, through alternative splicing. In this study, we identified that ZAKα proteins were upregulated in tumor tissues, whereas ZAKβ proteins were mostly expressed in corresponding normal tissues. The ectopically expressed ZAKβ proteins in cancer cells inhibited cancer cell proliferation as well as anchorage-independent growth. The ZAKβ:ZAKα protein ratio played a role in the regulation of the cyclic adenosine monophosphate (cAMP) signaling pathway, whereas high ZAKβ protein levels led to the activation of cAMP response element binding protein 1 (CREB1) and exerted antitumor properties. Overexpression of ZAKβ or CREB1 cDNAs in cancer cells inhibited anchorage-independent growth and also reduced the levels of cyclooxygenase 2 (Cox2) and β-catenin proteins. Cancer cells treated with doxorubicin (Doxo) resulted in the switching from the expression of ZAKα to ZAKβ and also inhibited cancer cell growth in soft agar, demonstrating that pharmacological drugs could be used to manipulate endogenous reprogramming splicing events and resulting in the activation of endogenous antitumorigenic properties. We showed that the two ZAK transcript variants, ZAKα and ZAKβ, had opposite biological functions in the regulation of tumor cell proliferation in that ZAKβ had powerful antitumor properties and that ZAKα could promote tumor growth.

Key Words: alternative splicing, β-catenin, CREB1, NFκB, ZAKα, ZAKβ

Introduction

Sterile alpha motif (SAM)- and leucine-zipper-containing kinase (ZAK) belongs to a family of mixed-lineage kinases (MLKs) and acts as a serine/threonine kinase. ZAK proteins are classified as mitogen-activated protein 3 kinases (MAP3Ks), which can lead to the activation of the mitogen-activated protein kinases (MAPKs) cascades (8). MAP3Ks phosphorylate and activate MAP2Ks, which in turn activate classical downstream MAPKs, such as c-JUN N-terminal kinase (JNK), p38, extracellular signal-regulated kinase (ERK) 1/2, and ERK5. Under numerous extracellular stimulations, MAPKs transduce and integrate large numbers of extracellular signals to several biological responses including cell growth, proliferation, migration, differentiation and death (28). MLK family proteins are MAP3Ks and activate downstream

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pathways mainly through the JNK and p38 pathways; some MLKs may regulate the ERK5 pathway, and others can activate the ERK pathway (3, 10). MLKs have been implicated in multiple diseases, including neurodegenerative diseases, metabolic disorders, inflammation and cancers (5). Recent reports have suggested that some MLK members are involved in cancer progression *via* participating in the regulations of cell proliferation, differentiation, cell cycle, apoptosis, cell migration and invasion. Thus, cumulating evidences have presented that MLKs play some roles in tumorigenesis and acquisition of malignant phenotype, which may imply that MLKs are potential targets for cancer therapy (1, 22).

ZAK regulates many biological responses including cardiac hypertrophy and cell cycle progression. ZAKα activates signal transduction cascades through MAP kinase kinase 7 (MKK7), which in turn activates JNK1/2 to mediate several cellular responses, such as cell survival and inflammation. The human ZAK gene is able to produce two protein isoforms, ZAKα and ZAKβ, which are transcript splicing variants. These two proteins share the N-terminal kinase and leucine zipper domains. ZAKα has a long C-terminal region and a SAM, whereas ZAKβ is composed of a unique 124-amino acid C-terminal and lack of the SAM motif. In previous studies, we reported that ZAKα kinase activity regulates the proliferation of Rat6 embryo fibroblast cells (29). Both ZAKα and ZAKβ modulate cancer cell migration in a kinase-dependent manner (23).

Cancer is thought to be a complex genomic disease. During tumorigenesis, the genome of cancer cells exhibits dynamic exchanges to elicit cancer phenotypes. All cancers acquired six common capabilities, including sustaining proliferation signaling, evading growth suppressors, resisting cells death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis (11, 12). Beside genomic mutations, there are other biochemical alterations within cancer cells, such as changes in the processing of primary transcripts *via* alternative splicing, which is a regular posttranscriptional process for most human genes. Aberrant alternative splicing is another hallmark of cancer (9); however, the causes and consequences of aberrant alternative splicing remain unknown. Some cancer-associated genes express various spliced isoforms with different or even opposing characteristics. For instance, several apoptotic regulators express transcript isoforms that show pro- or anti-apoptotic properties, and the vascular endothelial growth factor (VEGF)-A gene also expresses both pro- or anti-angiogenic isoforms (13, 27). A variation in the ratio between these cancer-associated spliced isoforms can either promote or inhibit tumorigenesis. Aberrant alternative splicing can also affect epithelial to mesenchymal transition (EMT) that is also correlated with cancer progression (4).

To understand the mechanisms of ZAK isoforms in cancer progression, the functions of ZAKα or ZAKβ in cancer cells and their downstream signaling were investigated in this work. The data showed that ZAKα was essential for tumor progression whereas ZAKβ inhibited tumor growth. It was also demonstrated here that there were differential expression levels between these ZAK isoforms in tumor tissues or their normal adjacent tissues during tumor development. Our data further suggested that the switching of ZAK isoforms in cancer cells regulated the downstream cyclic adenosine monophosphate (cAMP) signaling pathway for neoplastic cell transformation.

Materials and Methods

Cell Culture and Generation of Stable Cell Lines

The nasopharyngeal carcinoma cell line, KB, and human osteosarcoma cell line, MG-63, were cultured at 37°C in humidified atmosphere of 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Invitrogen Corporation, NY, USA) supplemented with 10% fetal bovine serum (FBS) and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin). In the anchorage-independent cell cultures, cells were maintained in the complete DMEM with 1.3% methylcellulose. Transfection was performed using the Ca-PO_4 method. The short hairpin RNA (shRNA) plasmids were constructed using the method described in Chang (2).

Soft Agar Assay

Cells were suspended in DMEM supplemented with 10% FBS and 0.3% Agar Noble (BD, Difco, MD, USA) and plated on a layer of 0.5% Agar Noble. Experiments were performed in 6-well plates with approximately 2,500-10,000 cells per well and in triplicates. Colonies were stained with iodonitrotetrazolium chloride (INT) solution and counted manually after 2-4 weeks of incubation at 37°C and 5% CO_2. Colonies were photographed and quantified manually. The number of colonies was counted under an inverted light microscope at ×40 magnification. The data are shown as mean number of colonies ± standard error (SE) from six fields of three independent wells.

Immunoprecipitation and Detection of ZAK Kinase Activity

The cells were harvested in lysis buffer (50 mM

Tris-HCl at pH 8.0, 250 mM NaCl, 1% NP-40, and 2 mM ethylenediaminetetraacetic acid, EDTA) containing 1 mM phenylmethane sulfonyl fluoride (PMSF), 10 ng/ml leupeptin, 50 mM sodium fluoride (NaF) and 1 mM sodium orthovanadate. ZAK kinase activity was determined as previously reported (14). In brief, cell lysates were subjected to immunoprecipitation analysis using an anti-ZAKα polyclonal antibody. Protein kinase assays were performed using a GST-RhoGDI fusion protein as the substrate.

siRNA Knockdown

The siRNA knockdowns were performed as described previously (14). In brief, KB and MG-63 cell lines were transfected with pCDNA-HU6-shRNA plasmids and a pBABE-puro plasmid as a selection marker. The oligonucleotides used for shRNA were as follows: ZAKβ1338iF (GATCCGGAGGATAATGACATGGATAATTCAAGAGAT), ZAKβ 1338iR (AGCTTAAAAAGGAGGATAATGACATGGATAATCTCTTGAAT), CREB755iF (GATCCGAGAGAGGTCCGTCTAATGTTCAAGAGAC), CREB755iR (AGCTTAAAAAGAGAGAGGTCCGTCTAATGTCTCTTGAAC) and ZAK460iF, ZAK460iR, ZAK1712iF, and ZAK1712iR as reported previously (16).

Analysis of ZAK Transcript

mRNAs from various human tissues (Clontech, Mountain View, CA, USA) were probed with the 5'-end sequence of ZAKα cDNA labeled with ^{32}P using the random priming method and hybridization was performed as described previously (30).

Mouse Models of Tumor Growth

Six week-old nude mice were purchased from the National Applied Research Laboratories, Taiwan. The mice were maintained under standard conditions and cared for according to the institutional guidelines for animal care. An aliquot of 2.5×10^5 cells suspended in complete medium was injected subcutaneously into both flanks of the mice. The mice were maintained under routine conditions for about five weeks and all animals were euthanized at the end of the study. Tumors were removed and weighed. Tumor weight of each animal was calculated as mean weight ± standard deviation (SD) from each site of four animals. All animal experimental procedures were performed with the approval of the Institutional Review Board of Taichung Veterans General Hospital (IRBTCVGH No: 950727/C06134).

Statistical Analysis

Independent experiments were performed at least three times with similar results. For *in vivo* experiments, the results were analyzed with the use of SigmaStat. Statistical analyses were performed using *t*-test for comparison of tumor weights. Values are expressed as mean ± SD. *P*-values < 0.05 were considered significant.

Results

ZAKα Kinase Activity is Required for Anchorage-Independent Growth

To determine whether the overexpression of ZAKα affects cancer cell proliferation, pEGFPC1-ZAKα or kinase-dead ZAKα (ZAKαdn) cDNA was transfected into KB cells, a nasopharyngeal carcinoma cell line, and the ZAKα- or ZAKαdn-overexpressing stable cells were cloned. To determine the role of ZAKα in neoplastic transformation, a soft agar assay was performed to characterize the growth ability of the neoplastic cells. The number of colonies of stable cells transfected with pEGFPC1-ZAKα was slightly larger than that of mock vector cells; however, the ZAKαdn cells lost the potential of anchorage-independent growth (Fig. 1A). The stable clones expressed the EGFPC1-ZAKα or -ZAKαdn proteins in addition to the endogenous ZAKα proteins (Fig. 1B). Studies have shown that many cellular proteins, such as cyclooxygenase 2 (Cox2), affect the development of many cancers (25). Hence, we investigated whether ZAKα-overexpressing cells induced the expression of Cox2. The results showed that ZAKα cells expressed more Cox2 protein than did the mock cells, whereas the ZAKαdn had less effect on Cox2 expression under non-adhesion conditions (Fig. 1B). These data showed that ZAKα positively regulates the Cox2 expression and this regulation is dependent on ZAKα kinase activity. Studies on Wnt signaling have also indicated that deregulation of Wnt/β-catenin signaling is associated with several cancers (20). The Wnt pathway is activated in numerous ways and its signaling is required for proliferation of some cancer cells (6). Our data showed that the β-catenin expression was also ZAKα kinase activity-dependent (Fig. 1B), suggesting that ZAKα kinase activity plays a role in neoplastic transformation and also in the regulation of the expression of Cox2 and β-catenin.

To determine whether ZAKα is involved in cancer cell proliferation, the growth rates of cells expressing ZAKα or ZAKαdn under non-adhesion growth conditions were investigated. Both the ZAKα and mock cells had a similar growth rate, whereas the ZAKαdn cells had a slower proliferation rate under anchorage-independent conditions (Fig. 1C). The data suggested that ZAKα was involved in cancer

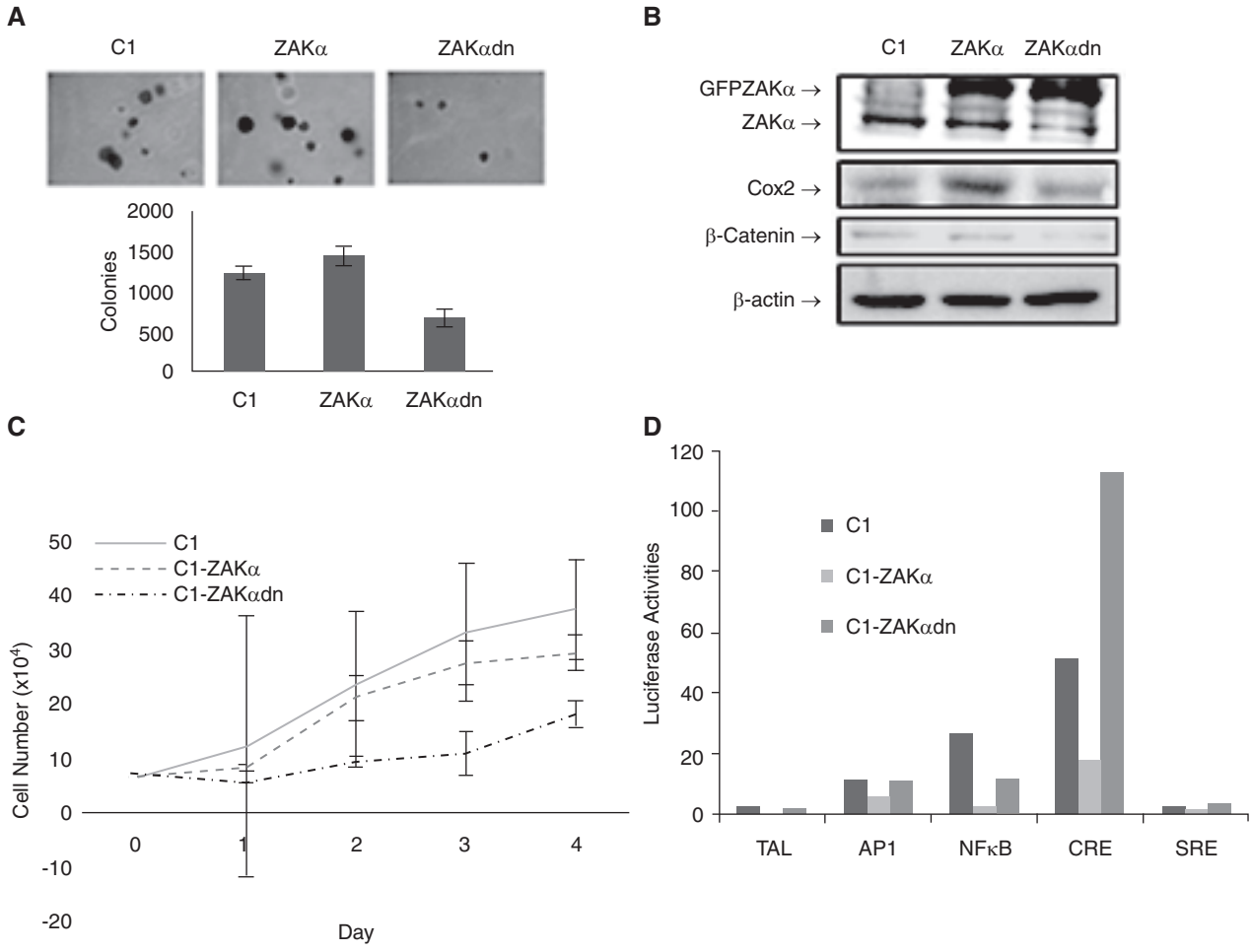

Fig. 1. Effects of ZAKα in KB cells. (A) ZAKα kinase activity is required for anchorage-independent growth. pEGFC1 vector (C1), pEGFPC1-ZAKα or kinase-dead ZAKα (ZAKαdn) cDNA-expressing KB cells were subjected to soft agar assays (top panels). Quantification of the number of colonies observed is shown in the bottom panels. (B) Effects of ZAKα on the expression of Cox2 and β-catenin under non-adhesion growth conditions. KB cells expressing C1, ZAKα, or ZAKαdn were growing in non-adhesion condition and cell lysates were harvested for western blot analysis. (C) ZAKα kinase activity is required for cell growth under non-adhesion conditions. Stable KB cells expressing EGFPC1, EGFPC1-ZAKα, or pEGFPC1-ZAKαdn were cultured in methylcellulose-containing medium and the cell numbers were counted at the indicated time points. (D) KB cells transiently transfected with multiple signaling pathways in a luciferase reporting system and cultured in non-adhesion condition for 48 h. Cell lysates were harvested and luciferase activities were determined.

cell proliferation under non-adhesion conditions. Our previous study revealed that ZAKα overexpression upregulated nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP1) signals during HepG2 cell growth under adherent conditions (18). Hence, we next determined the signaling pathways induced by ZAKα- or ZAKαdn-overexpression in KB cells when the cells were grown under anchorage-independent conditions. ZAKα-expressing cells were found to downregulate NFκB, AP1 and CRE activities (Fig. 1D), and the levels of CRE were robustly increased in ZAKαdn cells. CRE activity was inversely correlated with anchorage-independent growth suggesting that cAMP signaling was the downstream signals of ZAKα during neoplastic transformation.

Relative Expression Levels of ZAK Isoforms Affect Cancer Cell Proliferation

To confirm that ZAKα proteins are involved in neoplastic transformation, ZAKα was depleted by using two shRNAs 460i and 1712i. Cells transfected with both shRNAs showed substantially less ZAKα protein compared to the control parental cells (Fig. 2B). The ZAKα-depleted cells were then seeded in soft agar and the growth ability was tested. The two shRNA sets of ZAKα-depleted cells exhibited different growth abilities in the soft agar. The 460i-transfected cells exhibited robust colony formation, whereas the

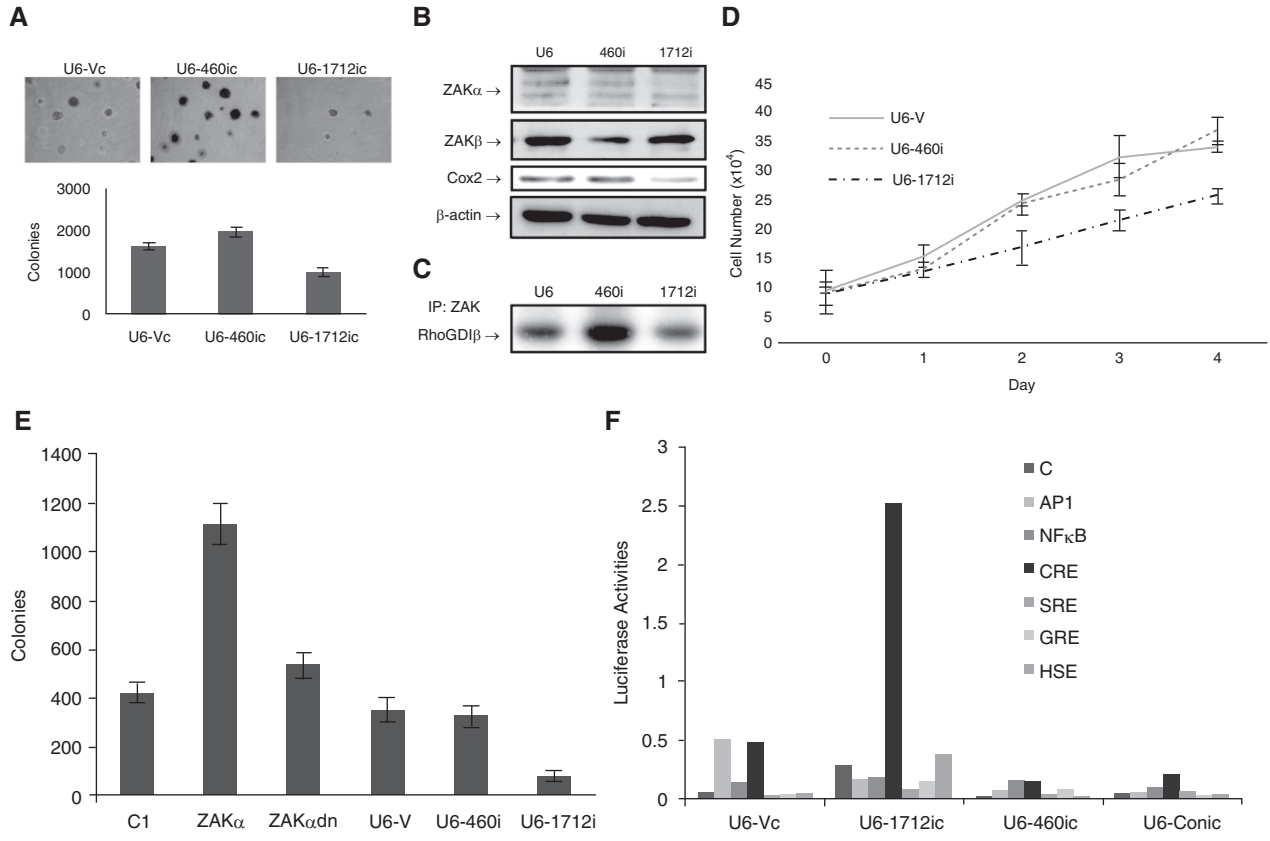

Fig. 2. ZAKα proteins are required for tumor cell proliferation. (A) ZAK isoform expression levels affect KB cell proliferation. ZAKα expression was silenced by using two different shRNAs, U6-460i or U6-1712i, constructed in an expression vector pCDNA-HU6, and the anchorage-independent growth ability was determined by soft agar assay. The U6-Vc represents the vector control cells. (B) Cox2 protein levels correlated with decreased ZAKα protein levels, shown in western blots after transfection with the U6-460i and U6-1712i shRNAs. (C) In vitro kinase assay indicated that ZAKα kinase activities were suppressed in the 1712i cells. RhoGDIβ was used here as a substrate for in vitro kinase assay. (D) Cells with the higher ratio of ZAKα to ZAKβ proteins (B, 460i) show higher cell growth rate under non-adhesion conditions. (E) ZAK isoforms regulate anchorage-independent growth of MG-63 cells. ZAKα expression was silenced by using shRNAs, U6-460i or U6-1712i, and the anchorage-independent growth ability was determined by soft agar assay. (F) ZAKα silences KB cells (U6-460ic and U6-1712ic) and control cells (U6-Vc and U6-conic) transfected with signaling pathway-luciferase reporting systems and cultured in non-adhesion condition for 48 h. Cell lysates were harvested and luciferase activities were determined. The U6-Vc represents as vector control cells and U6-conic represents scramble shRNA control cells.

1712i cells lost their anchorage-independent growth ability (Fig. 2A). On careful examination of both shRNA sequences, it was observed that the shRNA 460i targeted both the ZAKα and ZAKβ isoforms, whereas shRNA 1712i targeted only ZAKα. To verify the importance of the shRNA sequences, the ZAKβ protein expression levels were determined and it was confirmed that ZAKβ protein levels were decreased in the cells that expressed shRNA 460i. Moreover, Cox2 protein levels were decreased in the 1712i cells, which also correlated with the dramatically decreased ZAKα protein levels (Fig. 2B). The RhoGDIβ protein was further used to determine the activity of ZAKα (15). The data confirmed that the kinase activity of ZAKα was decreased in the 1712i cells (Fig. 2C). Therefore, ZAKα may be required in neoplastic transformation and ZAKβ may act as a tumor suppressor. The data also suggest that the ratio of the ZAKα and ZAKβ proteins in cells might determine the result of neoplastic transformation.

To determine whether ZAKα and ZAKβ each plays a role in regulating cancer cell proliferation, the growth rates of the shRNA-knockdown cells under non-adhesion growth conditions were determined. The growth rate of the 1712i cells was slower than that of the 460i or mock vector-transfected cells under non-adhesion conditions (Fig. 2D). Thus, the higher ratio of ZAKβ to ZAKα protein levels in the 1712i cells had a slower growth rate. These data suggested that

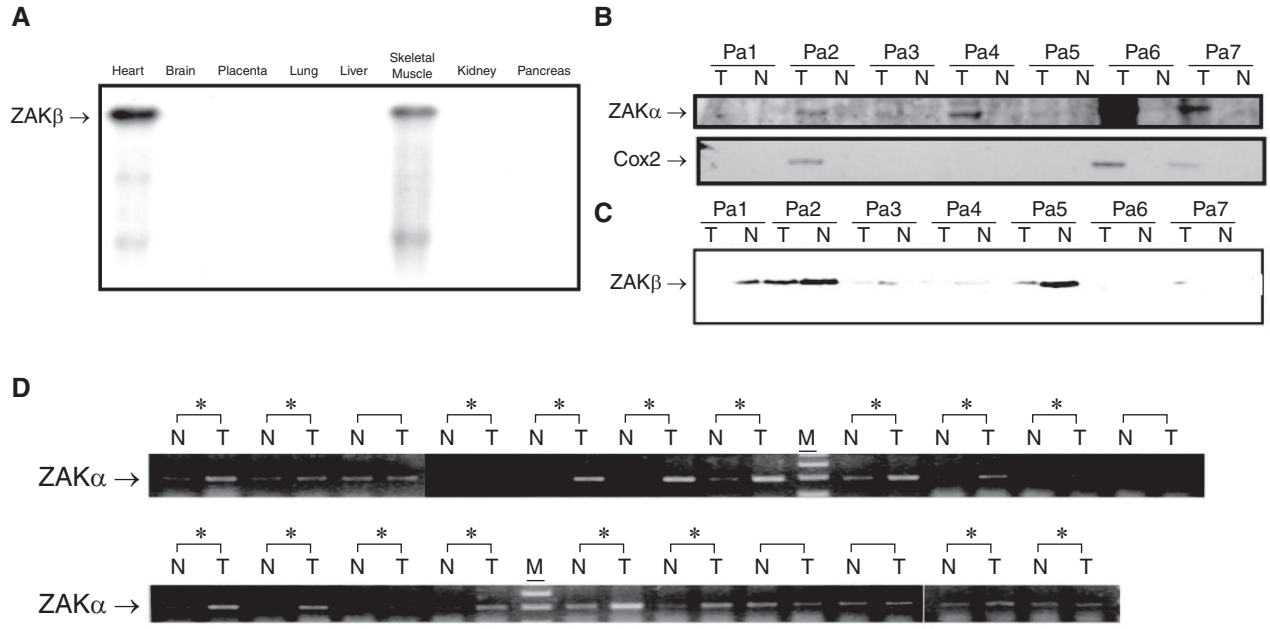

Fig. 3. The expression patterns of ZAKα and ZAKβ in normal tissues and in tumors. (A) ZAKβ mRNA expression is most abundant in heart and skeleton muscle. ZAK mRNA was detected by Northern blot analysis using ZAK cDNA labeled with α [^{32}P] dCTP as a probe. A multiple human tissue blot containing poly (A) RNA was probed with a 5'end ZAK cDNA. (B, C) Differential expression of ZAK isoforms between normal and oral cancer tissues. Proteins were extracted from oral cancer tissues (T) or respective normal adjacent tissues (N) and ZAKα, ZAKβ, and Cox2 protein levels were detected by western blot analysis. (D) The expression of ZAKα mRNA was more abundant in gastric cancer tissues than in normal tissues among 16 of 21 patients as determined by semi-quantitative RT-PCR.

the ratio of ZAKα to ZAKβ protein levels in cells might determine cell proliferation rate, echoing the association of ZAKα:ZAKβ ratio with neoplastic transformation above.

To study whether this phenomenon is unique in nasopharyngeal carcinoma cells, similar pattern of anchorage-independent growth pattern was reproduced in MG-63 cells, a human osteosarcoma cell line (Fig. 2E). The data supported that ZAK isoform usage could have strong mechanistic association with tumorigenesis in general. Signal transduction pathways in the specific ZAK shRNA-knockdown cells were next tested. Only the 1712i cells, which had a higher ZAKβ:ZAKα protein ratio (Fig. 2B), induced the cAMP pathway (Fig. 2F), suggesting that the higher ZAKβ:ZAKα protein ratio upregulated the cAMP signal pathway to result in the suppression of cancer cell proliferation.

The ZAKα:ZAKβ Expression Ratio Determines Cell Fate

To determine the ZAK isoform status in other tissues, the expression of the ZAKβ transcripts was examined in human heart and skeleton muscle tissues by using a 5'end sequence of the ZAK cDNA as a probe in Northern blot analysis. The data showed that the ZAKβ transcript was abundantly expressed in the heart and skeleton muscle tissues (Fig. 3A). Because the ZAKβ protein has the potential to suppress tumor growth, abundant expression of ZAKβ may be associated with why cancers rarely develop in the heart and muscle tissues. ZAK isoforms have different biological activities; thus, the expression patterns of ZAK isoforms in oral cancer tissues were validated by western blot analysis. The ZAKα protein level was upregulated in the tumor tissues compared with the normal adjacent tissues (Fig. 3B), whereas the ZAKβ protein was abundant in the normal tissues and was decreased in the tumors (Fig. 3C). These data suggested that the development of malignancy was associated with the switch of the expression profile from ZAKβ to ZAKα, and that this switching was likely regulated through alternative splicing. Furthermore, expression of the Cox2 protein was increased in the tumor tissues, correlating with the ZAKα expression level (Fig. 3B). To determine whether the upregulated ZAKα in cancer cells was unique or not in specific cancers, the ZAKα transcript levels in gastric cancer tissues were further examined by reverse transcription-polymerase chain reaction (RT-PCR) and compared with its level in the normal adjacent tissues. Sixteen (76.2%) out of 21 gastric cancer tissues showed increased ZAKα transcript levels compared to the corresponding normal tissues (Fig.

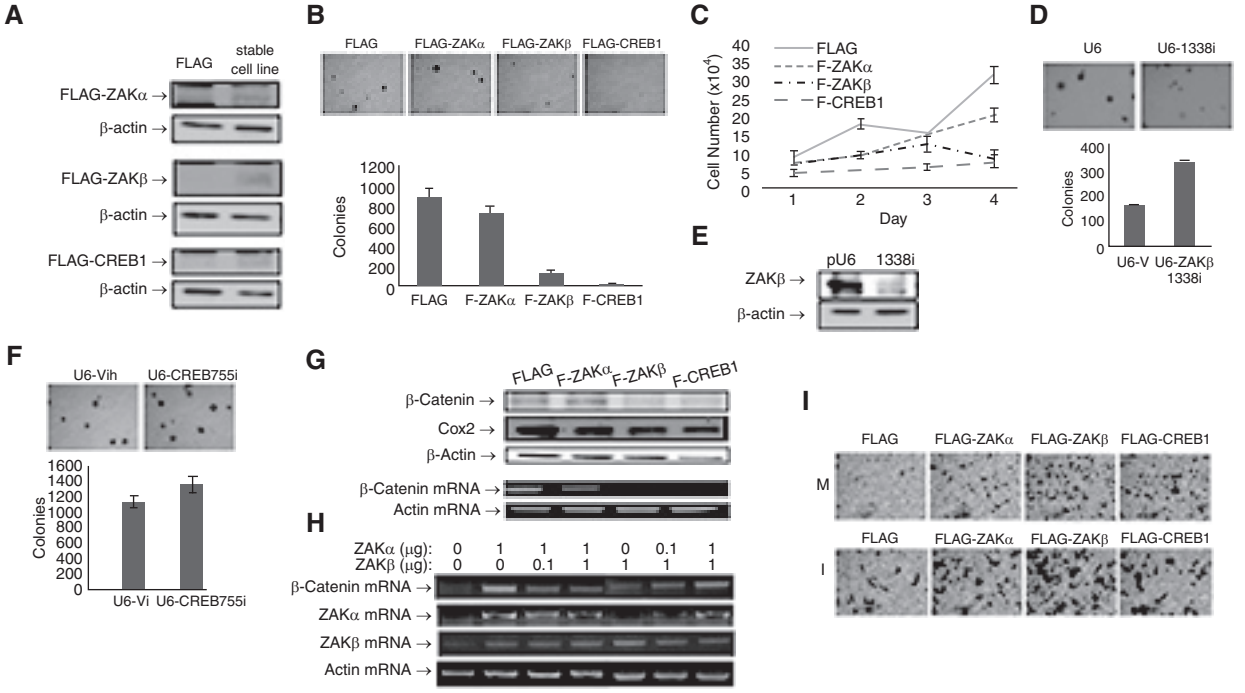

Fig. 4. Effects of ZAK alternative splicing on cancer cells. (A) Stable KB cells expressing ZAKα, ZAKβ or CREB1 proteins. (B) ZAKβ- and CREB1-overexpressed cells lost their anchorage-independent growth ability as determined in soft agar assays. (C) The growth rate in ZAKα-, ZAKβ- or CREB1-expressed cells under anchorage-independent conditions. (D) Silencing the ZAKβ expression restored cancer cell growth in soft agar. (E) ZAKβ expression was knocked down by a specific ZAKβ shRNA, 1338i. (F) Silencing the CREB1 (U6-CREB755i) expression restored cell growth in soft agar. (G) β-catenin was upregulated in ZAKα-overexpressed cells and downregulated in ZAKβ- and CREB1-overexpressed cells. The upper panel shows β-catenin or Cox2 protein levels regulated by ZAKα, ZAKβ or CREB1. The lower panel shows β-catenin mRNA levels regulated by ZAKα, ZAKβ or CREB1. (H) ZAKβ: ZAKα expression ratio determines β-catenin expression levels. HEK293 cells transfected for 48 h with different combinations of ZAKβ and ZAKα cDNAs followed by β-catenin mRNA quantification by semi-quantitative RT-PCR. (I) ZAKβ- and CREB1-overexpressed cells revealed high migration (upper panel) and invasion (lower panel) rates through the boyden chamber assay.

3D), suggesting a possible role of ZAKα in neoplastic transformation.

To confirm that the ZAK isoforms play different roles in regulating the cAMP pathway and cell proliferation, ZAKα, ZAKβ and the human CREB1 were next overexpressed in KB cells (Fig. 4A). Control and ZAKα-overexpressed cells were able to grow in the soft agar, whereas ZAKβ- and CREB1-overexpressed cells lost their anchorage-independent growth ability (Fig. 4B). The growth rate of the transfected cells under non-adhesion conditions was next measured, and it was found that the control and ZAKα-expressed cells showed high proliferation rates, whereas ZAKβ and CREB1 cells did not proliferate over the four-day culture period (Fig. 4C), indicating that ZAKβ and CREB1 could have antitumorigenic properties. If ZAKβ is involved in the suppression of tumorigenesis, the knocking down of ZAKβ can at least maintain anchorage-independent growth in cancer cells. A ZAKβ-specific shRNA, designated as 1338i, was designed to silence the expression of ZAKβ. The ZAKβ-silenced cells maintained the ability to grow in the soft agar and the growth was better than that of the control cells (Figs. 4D and 4E). Thus, the absence of the ZAKβ protein was associated with the loss of anti-tumorigenicity properties. The experiments also indicated that CREB1 might be regulated by both the ZAK isoforms to suppress cancer cell proliferation. For further validation, when a specific shRNA, 755i, was used to knockdown CREB1 expression, the 755i-transfected cells were shown to be able to grow in soft agar as in the control cells (Fig. 4F).

There are numerous genes with the potential to drive tumorigenesis. Therefore, it is important to determine which candidate genes can be regulated by ZAKα in neoplastic transformation. The results showed that β-catenin was upregulated in ZAKα-overexpressed cells and downregulated in ZAKβ- and CREB1-overexpressed cells (Fig. 4G). Thus, the changing ZAK expression patterns, mediated through CREB1, play a critical role in the regulation of β-catenin expression.

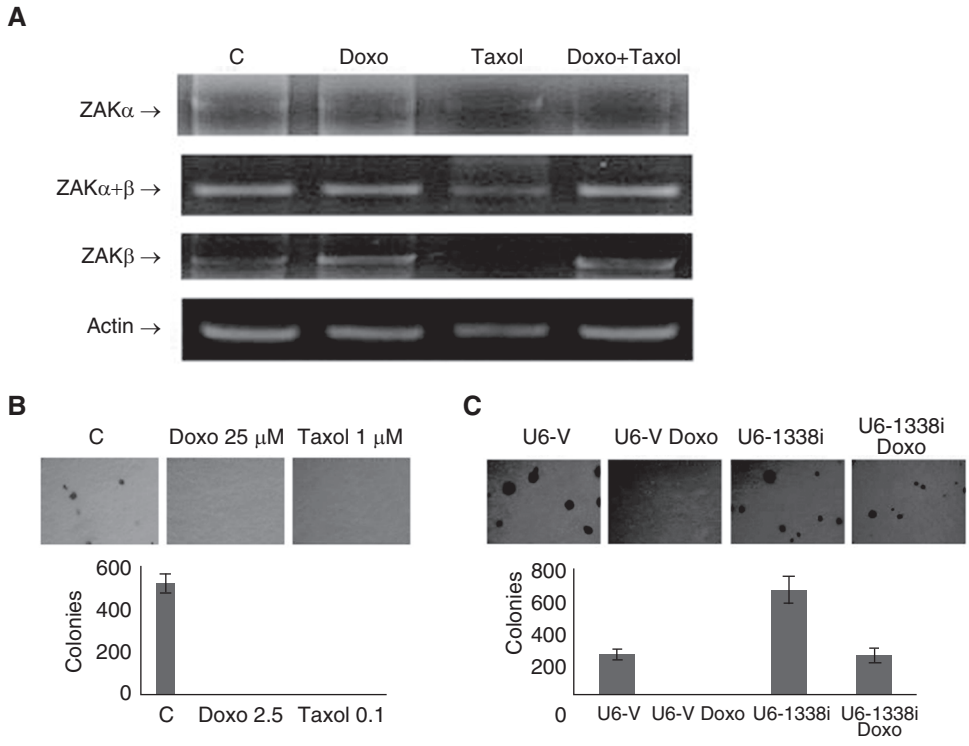

Fig. 5. Doxo regulates ZAKα or ZAKβ switching in cancer cells. (A) KB cells were incubated with 2.5 μM Doxo, 0.1 μM Taxol, or 2.5 μM Doxo + 0.1 μM Taxol for 24 h and the ZAKα, ZAKβ or total ZAK (ZAKα+β) transcripts were quantified by RT-PCR. (B) KB cells treated with either Doxo (25 or 2.5 μM) or taxol (1 or 0.1 μM) effectively suppressed anchorage-independent growth. C, untreated control cells. (C) Doxo at 2.5 μM was able to suppress anchorage-independent growth in control KB cells (U6-V) and ZAKβ-silenced KB cells (U6-1338i) partially restoring anchorage-independent growth when treated with 2.5 μM Doxo to a level similar to that of U6-V control cells without treated with Doxo.

We propose that the ZAKβ:ZAKα expression ratio regulates β-catenin expression. To confirm this hypothesis, HEK293 cells were transiently transfected with different combinations of ZAKβ and ZAKα cDNAs and the β-catenin expression levels were determined by RT-PCR. It was found that the expression levels of β-catenin and ZAKβ were consistently inverse to each other (Fig. 4H).

Most neoplastic cells have high migration and invasion rates, as found for ZAKα-overexpressed cells. Although ZAKβ- and CREB1-overexpressed cells had low proliferation rates (Fig. 4C), however these cells showed high migration and invasion rates (Fig. 4I) Thus, our data shown here is consistent with the notion that migration and invasion might be dissociated from cancer cell proliferation.

ZAKβ Participates in Doxorubicin (Doxo)-Induced Inhibition of Cell Proliferation

Doxo, an anthracycline drug, is one of the most effective and widely used anticancer drugs for cancer treatment. Doxo-treated KB cells expressed more ZAKβ transcript variants than taxol-treated KB cells (Fig. 5A). Cancer cells treated with either Doxo or taxol failed to grow in soft agar, indicating that both anticancer drugs effectively suppressed tumor cell proliferation (Fig. 5B). Doxo targeted many biomolecules in cells to suppress tumor cell proliferation. Our data suggested that expression of the ZAKβ transcript might be one of the mechanisms targeted by Doxo. ZAKβ-silenced cells were partially able to restore anchorage-independent growth to a level similar to that in the mock cells without Doxo treatment (Fig. 5C). Thus, ZAKβ may play a role in the action of Doxo on cancer cell proliferation.

ZAKβ Suppresses Tumor Cell Growth in Vivo

To validate the effects of the ZAK isoforms in cancer cell proliferation *in vivo*, ZAKα- or ZAKβ-overexpressed KB cells were transplanted into the flank of immunocompromised nude mice through subcutaneous inoculation. Analysis of the weights of the primary tumors obtained revealed significant smaller tumors in the ZAKβ mice compared with those of the control or ZAKα-overexpressed group (Fig. 6). ZAKβ-overexpressing cells reduced the tumor weight by

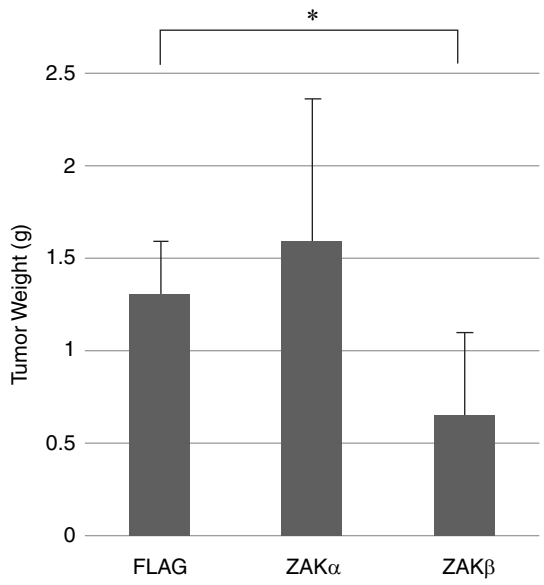

Fig. 6. Effects of ZAK isoforms in cancer cell proliferation *in vivo*. ZAKα- or ZAKβ-overexpressed KB cells were transplanted into the flanks of nude mice through subcutaneous inoculation and the weights of primary tumors were analyzed on day 35 post-injection. Tumor weight of each animal was calculated as mean weight ± SD from each flank of four animals, with a total of eight samples. FLAG, blank vector.

more than 50% ($P < 0.01$), confirming the *in vitro* data of soft agar assays (Fig. 4A) that ZAKβ possesses antitumorigenic properties.

Discussion

Human genes can generally produce multiple distinct protein isoforms through organ-, tissue- or cell type-specific alternative splicing. Splicing defects are often correlated with human diseases, including cancer. A cancer-associated gene may also express different spliced isoforms that either promote or suppress cancer cell growth. For example, the signal transducer and activator of transcription (STAT) gene produces two predominant isoforms one of which is the full form of STAT5B and is regarded as a proto-oncogene, and the other is a naturally occurring truncated isoform, STAT5ΔB, that acts as a tumor suppressor (24). We report here that the ZAK gene is able to produce two transcripts, ZAKα and ZAKβ, through alternative splicing, and our data show that these two isoforms regulate distinct biological activities. ZAKα may be necessary during neoplastic transformation in a kinase-dependent manner, whereas ZAKβ may act as a tumor suppressor. In this study, we discovered that the ZAKα transcript was induced in gastric cancer tissues compared with the corresponding normal tissues (Fig. 3D). The ZAKα protein was also upregulated in oral cancer tissues (Fig. 3B) whereas overexpressed kinase-dead (ZAKαdn) protein, or depletion of ZAKα in KB cells, resulted in the inhibition of cell proliferation (Figs. 1A, 1C, 2A and 2D). These results implied that ZAKα and/or its kinase activity might be involved in the development of neoplastic transformation. However, the ZAKβ protein was predominantly expressed in the normal tissues (Figs. 3B and 3C). The upregulation of ZAKα was observed in gastric tumor cell lines, colorectal cancers, bladder cancers, and breast cancers (17) and we are the first to demonstrate here that ZAKβ probably acts as a tumor suppressor. The switching between the ZAKα isoforms might be associated with cancer cell proliferation or tumor suppression through regulation of the cAMP pathway.

This work also found that ZAKα inhibited CREB1 activity and subsequently CREB1 transcription activity (Fig. 1D). How does ZAKα regulate the function of the CREB? CREB1 is a member of the basic region leucine zipper (bZIP) protein family and forms as a homodimer to bind to the CRE. ZAKα has a leucine zipper domain next to the kinase domain. We speculate here that ZAKα might sequester CREB1 through the leucine zipper dimerization. Loss of ZAKα function was inversely correlated with the activation of cAMP signaling (Fig. 1D). The 1712i cells, but not the 460i cells, had a higher ZAKβ:ZAKα protein ratio and induced CRE activity (Fig. 2F) and resulted in the inhibition of anchorage-independent growth. The higher ZAKβ:ZAKα protein ratio in the cells led to the activation of the cAMP pathway, which suppressed cancer cell proliferation (Fig. 4A). On the other hand, cancer cells with a higher ZAKα:ZAKβ protein ratio might result in suppressing the cAMP pathway and supporting cancer cell proliferation. The alternative splicing mechanism is proposed here to modulate the ZAKβ:ZAKα protein ratio and plays a role in the regulation of the cAMP pathway to subsequently regulate cancer cell proliferation.

Aberrant alternative splicing could also affect EMT and EMT triggered by a variety of mechanisms including the Wnt pathway, transforming growth factor-β, epidermal growth factor and hypoxia inducible factor (19). β-catenin, a transcription co-factor that controls the key development of gene expression programs, is critical in the canonical Wnt signaling pathway (7). Constitutively activated β-catenin signaling results in excessive cell proliferation and is critical to tumorigenesis (21). We observed in this report that higher ZAKβ protein expression led to the activation of the cAMP pathway and suppressed β-catenin transcription and cancer cell proliferation (Fig. 4G).

Our data showed that the two ZAK transcript variants, ZAKα and ZAKβ, had opposite biological functions in the regulation of tumor cell proliferation; it was further found that the cancer therapeutic drug, Doxo, might play a regulatory role in ZAK alternative splicing mechanism. We identified that ZAKβ had powerful antitumor properties and that ZAKα could promote tumor growth. In conclusion, the manipulation of the expression of ZAKα and ZAKβ through alternative splicing could affect neoplastic transformation. The unique ZAKβ signature increased activation of the cAMP pathway and suppression of β-catenin expression to suppress cell proliferation. Pharmacological manipulation of ZAK splicing events can manifest antitumorigenic properties, and is a possible novel therapeutic opportunity.

Conflict of Interest

The authors declare that they have no competing interests.

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