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c-MYC overexpression overrides TAK1 dependency in efficient tumorigenicity of AKT-transformed cells

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ABSTRACT

Transforming growth factor activated kinase 1 (TAK1) provides prosurvival signals in various types of cells, and emerging evidence indicates that targeting TAK1 is a promising means to eliminate certain types of cancer cells. Here, we show that TAK1 is required for efficient tumorigenicity of AKT-transformed cells. TAK1 inhibition accelerates cell apoptosis of AKT-transformed cells in anchorage-independent cell growth accompanying by the downregulation of Mcl-1 and Bcl-2 expression. On the contrary, the tumorigenicity of c-Myc-transformed cells is not significantly affected by TAK1 inhibition. Moreover, AKT-transformed cells with c-Myc overexpression tolerate TAK1 inhibition in anchorage-independent growth and tumorigenicity *in vivo*. Together, our results provide evidence that TAK1-dependency in the tumorigenicity of AKT-transformed cells can be alleviated by c-Myc overexpression. These findings suggest that dual-targeting TAK1 and c-Myc might be a rational therapeutic strategy for treatment of certain types of cancer.

1. Introduction

Transforming growth factor activated kinase 1 (TAK1), one of the MAP3K members, can integrate multiple upstream signals and regulate various cellular processes [1]. The role of TAK1 in tumorigenesis is complex, depending on cell type and biological context. TAK1 can promote tumor angiogenesis and metastasis [2–4]. The inhibition of TAK1 promotes cell death in mantle cell lymphoma [5], and a small-molecule inhibitor targeting TAK1 can significantly reverse the intrinsic chemoresistance of pancreatic cancer [6]. In addition, TAK1 is required for R-Ras-mediated transformation of mammary epithelial cells [7]. Moreover, targeting TAK1 has been suggested as a potential therapeutic approach for cancer treatment. For example, TAK1 inhibition in colon tumors with aberrant K-Ras and Wnt activity [8] and skin tumors [9] leads to tumor regression. On the other hand, TAK1 also displays a suppressive function under some conditions. It has been reported that the deletion of TAK1 is associated with and contributes to the progression of prostate cancer [10]. The implication of atypical heat shock protein H11/HspB8 in experimental tumor therapy reveals that activation of TAK1-dependent death pathways may be a useful strategy for melanoma therapy [11].

The aberrant regulation of v-akt murine thymoma viral oncogene homologue (AKT) has been documented extensively in a variety of human tumors [12,13]. Hyperactivation of AKT is sufficient to convert immortalized cells into tumorigenic cells [14–16]. To date, the underlying mechanisms for AKT-mediated transformation and -dependent tumorigenesis are mainly related to the regulation of its downstream events, such as activation of mTOR, phosphorylation of FOXO3a and GSK-3β [17]. However, whether and which additional events are required for AKT-transformed cells to adapt to environmental stress or checking machinery and thereby to acquire tumorigenic potential *in vivo*, are still poorly defined. Moreover, since the "non-oncogene addiction (NOA)" [18] hypothesis is becoming prevalent on the basis of heavy reliance of tumor cells on certain non-oncogenes, identification of NOA-related genes and pathways using a defined oncogenic transformation model may provide important targets for antitumor therapies.

In the present study, we addressed the role of TAK1 in AKT1-mediated tumorigenesis *in vivo*. We show that TAK1 inhibition significantly attenuates the tumor growth of AKT1-transformed mouse liver progenitor cells and NIH3T3 fibroblast cells. The requirement of TAK1 in AKT-dependent tumor formation involves FADD-independent mechanisms. More importantly, we

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demonstrate that c-Myc overexpression significantly alleviates the dependency on TAK1 in the tumor growth of AKT-transformed cells. Our findings suggest that TAK1 confers AKT-transformed cells the competent fitness for tumor growth *in vivo*. Dual-targeting TAK1 and c-Myc may be an efficient strategy for treatment of cancers with AKT hyperactivation.

2. Materials and methods

2.1. Cell culture

Mouse fetal liver progenitor cells (LPCs) were isolated from p53$^{-/-}$ mice as described previously by Zender et al. [19,20] and cultured in DMEM/F12(1:1) medium (GIBCO, Grand Island, NY, USA). NIH3T3 murine fibroblast cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in DMEM medium (GIBCO). Both cultures were supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO) and cultured at 37 °C in a humidified atmosphere consisting of 5% CO_2.

2.2. Preparation of retroviruses and lentiviruses

Human myristoylated-AKT1 was inserted into MSCV-IRES-GFP retroviral vector. Mouse mutant TAK1 (K63W) was cloned, fused with a Flag tag, into the retroviral transfer plasmid MSCV-puro. For retroviral expression of the dominant negative form of FADD, the cDNA corresponding to amino acids 80-205 of mouse FADD, tagged with Flag, was introduced into MSCV-IRES-dsRed. c-Myc was amplified from MSCV-HAM-Myc-puro (a gift of Scott Lowe, Addgene plasmid 18775) and inserted into MSCV-IRES-dsRed. For generation of retroviruses and cell lines, plasmids were transfected into phoenix packaging cells by using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Lentivirus preparation was carried out by introducing pPRIME-CMV-dsRed-FF3 (Addgene plasmid 11664) with psPAX.2 packaging vector and pMD2.G envelope vector into 293T cells using Lipofectamine™ 2000 reagent. Supernatant was harvested at 24 h and 48 h after transfection, mixed with 6 μg/ml polybrene (Sigma–Aldrich, St. Louis, MO, USA), and then used to infect cell lines. The GFP-positive and dsRed-positive cells were sorted by FACS Vantage flow cytometry (Becton–Dickinson, San Jose, CA, USA). The puro-positive cells were selected with 5 μg/ml of puromycin (Sigma–Aldrich) for 1 week.

2.3. Reagents and antibodies

TNFα and TRAIL were purchased from PeproTech (Rocky Hill, NJ, USA). super-FasL was purchased from Alexis Biochemicals (San Diego, CA, USA). The antibodies were as follows: anti-phospho-p65(Ser536), anti-p65, anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-ERK1/2(Thr202/Tyr204), anti-ERK, anti-phospho-JNK1/2(Thr183/185), anti-JNK1/2, anti-phospho-AKT(Ser473), anti-AKT, anti-cleaved caspase3, anti-cleaved caspase9, anti-cleaved PARP, anti-c-Myc, anti-Mcl-1, anti-Bcl-2, anti-survivin (all from Cell Signaling Technology; CST, Danvers, MA, USA); anti-Flag (Sigma–Aldrich); anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4. Immunoblotting

Cell lysates were prepared with RIPA lysis buffer (Beyotime, Nantong, China) with Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Cell extracts were separated on 10% or 12% sodium dodecyl sulfate-PAGE, transferred to nitrocellulose membranes (Pall, Glen Cove, NY, USA), and blocked with 5% non-fat milk/PBS, 0.05% Tween (PBST). The membranes were immunoblotted with various antibodies, and the bound was detected with the ChemiDoc™ XRS system (Bio-Rad, Hercules, CA, USA).

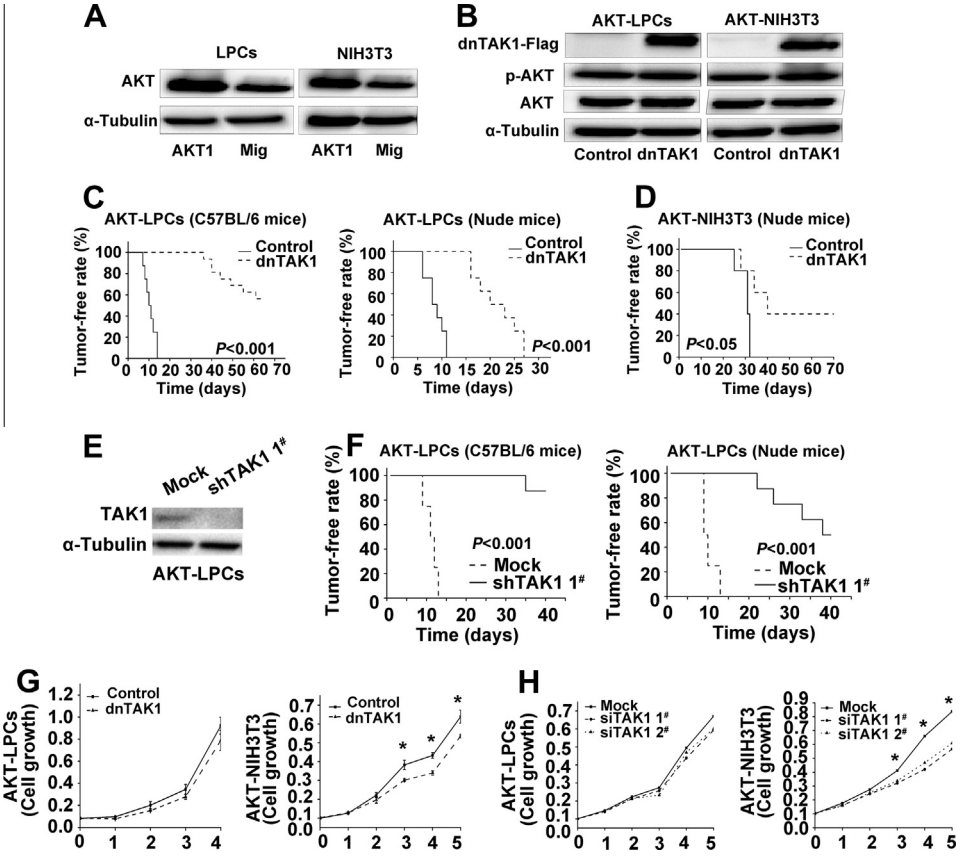

Fig. 1. TAK1 inhibition attenuates tumorigenicity of AKT-transformed cells. (A) Liver progenitor cells (LPCs) from p53-/- mice and NIH3T3 cells were transduced with empty retroviral vector Mig-GFP or activated myristoylated AKT1, and subjected to immunoblotting with the indicated antibodies. (B) AKT-transformed cells were infected with retro-puro (control) or retro-dnTAK1, and subjected to immunoblotting with the indicated antibodies. (C) Kaplan–Meier analysis of tumor onset in mice of the indicated types. AKT-LPCs with or without dnTAK1 (2×10^6) were subcutaneously injected into immunocompetent C57/BL6 or nude mice. Significant differences of tumor onset were observed in both C57/BL6 ($n = 16$; $P < 0.001$) and nude mice ($n = 8$; $P < 0.001$). (D) Nude mice were injected s.c. with AKT-NIH3T3 cells expressing dnTAK1 or empty vector (4×10^6), and tumor latency was monitored ($n = 5$; $P < 0.05$). (E) The efficiency of TAK1 knock down is verified by western blotting. (F) Kaplan–Meier analysis of tumor onset in mice of the indicated types ($n = 8$). (G and H) AKT-LPCs and AKT-NIH3T3 cells were grown for the indicated time in the presence of (G) dnTAK1 expression or (H) siRNA transduction for TAK1 downregulation, and cell proliferation was measured using MTT assay. The values show means and standard error of the mean (SEM) ($n = 3$; $^*P < 0.05$).