Mice lacking a transcriptional corepressor Tob are predisposed to cancer

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Tob is a member of antiproliferative family genes. Mice lacking tob are prone to spontaneous formation of tumors. The occurrence rate of diethylnitrosamine-induced liver tumors is higher in tob−/− mice than in wild-type mice. tob−/−p53−/− mice show accelerated tumor formation in comparison with single null mice. Expression of cyclin D1 mRNA is increased in the absence of Tob and is reduced by Tob. Tob acts as a transcriptional corepressor and suppresses the cyclin D1 promoter activity through an interaction with histone deacetylase. Levels of tob mRNA are often decreased in human cancers, implicating tob in cancer development.

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There is accumulating evidence that genes involved in the negative control of cell growth can function as tumor suppressors. In humans, tob, tob2, ana, pc3b, btg1, and btg2 comprise a family {tob family} of antiproliferative genes [Bradbury et al. 1991; Fletcher et al. 1991; Rouault et al. 1992; Matsuda et al. 1996; Guehenneux et al. 1997; Yoshida et al. 1998; Ikematsu et al. 1999; Buanne et al. 2000]. Exogenous expression of Tob family proteins suppresses growth of NIH-3T3 cells by inhibiting G1 progression of the cell cycle (Yoshida et al. 1998; Ikematsu et al. 1999; Guardavaccaro et al. 2000; Maekawa et al. 2002; Suzuki et al. 2002). We showed previously that Tob is a substrate of Erk MAPK, and unphosphorylated Tob suppresses cell-cycle entry of quiescent cells. Erk phosphorylation of Tob blocks the antiproliferative activity [Maekawa et al. 2002; Suzuki et al. 2002], which, at least in part, describes the importance of Erk activation in the cells stimulated by growth factors. When Tob is depleted, Cyclin D1 continues to be expressed and readily progress into S phase during serum starvation [Suzuki et al. 2002]. In addition, the antiproliferative activity of Tob is impaired in the presence of exogenously coexpressed Cyclin D1 [Suzuki et al. 2002]. These data suggest that tob functions as a tumor suppressor. However, possible involvement of Tob in tumorigenesis and roles of Tob in the control of cyclin D1 expression are unclear.

Tob family proteins associate with transcription factors. Virtually all of the Tob family members interact with Cafl [Rouault et al. 1998; Ikematsu et al. 1999; Yoshida et al. 2001], whose yeast homolog is a component of the CCR4–NOT transcriptional complex [Albert et al. 2000]. The CCR4–NOT complex participates in the control of specific sets of genes such as those involved in the late mitotic phase of the cell cycle [Liu et al. 1997]. Both BTG1 and BTG2 associate with HoxB9 and estrogen receptor η, and modulate their transcription activity [Prevot et al. 2000, 2001]. Tob associates with Smads transcription complex and affects Smad-mediated gene expression [Yoshida et al. 2000; Tzachanis et al. 2001]. This suggests that Tob family proteins are regulators of gene transcription, functioning as either coactivators or corepressors.

Here, we report that mice lacking tob are prone to spontaneous formation of tumors in various tissues. Intriguingly, we find that levels of tob mRNA are often decreased in human cancers. We further show that Tob is a transcriptional corepressor and suppresses the promoter activity of genes, such as cyclin D1, relevant to cell growth control through an interaction with histone deacetylase.

Results and Discussion

Tumor development in mice lacking tob

Tob−/− mice show no apparent phenotypic abnormalities in their early lives except development of osteopetrotic phenotype [Yoshida et al. 2000]. Therefore, we conducted a long-term study of spontaneous tumor development in wild-type and tob−/− mice. By 18 mo of age, 16% (5/31) of wild-type mice developed tumors, primarily malignant lymphomas and lung adenomas, whereas 77% (20/26) of tob−/− mice had developed a variety of tumors, including hemangiosarcomas, lung carcinomas, and hepatocellular adenomas. The spectrum of tumors observed in tob−/− mice between 6 and 22 mo is shown in Table 1. Typical histopathological findings for some of these tumors are shown in Figure 1A–I. Despite extensive systematic studies of tumor suppressor genes, there are presently no mouse models of increased susceptibility to liver tumors. Because tob−/− mice often developed liver tumors, we further assessed tumor susceptibility of tob−/− mice by treatment with a liver-specific carcinogen, diethylnitrosamine [DEN]. Intrapertioneal injection
of DEN into 2-week-old tob−/− mice led to earlier onset of liver tumors; at 6–9 mo after DEN administration, liver tumors were observed more frequently in tob−/− mice than in wild-type mice [Fig. 1J–K]. These findings suggest that tob−/− mice are predisposed to cancer and may serve as a model of liver cancer.

Because mutations in the p53 tumor suppressor gene are the most frequently observed genetic lesions in human cancers, we investigated the relation between tob and p53 in tumorigenesis by generating mice carrying null mutations of both genes. Eight percent (3/39) of tob−/− mice and 59% (17/29) of p53−/− mice showed tumor development within 6 mo, but the tumor incidence increased to 81% (30/37) in tob−/− p53−/− mice. The difference between tob−/− mice and tob−/− p53−/− mice was statistically significant (χ² test, p < 0.05). As in p53−/− mice, malignant lymphomas were found most frequently (80%, 24 of 30 tumors) in tob−/− p53−/− mice. However, several pathological changes, such as a glioblastoma, were uniquely observed in tob−/− p53−/− mice (see Supplemental Material). These data suggest that tob and p53 contribute synergistically to tumor suppression.

Growth aberration of tob−/− MEFs

Primary mouse embryonic fibroblasts [MEFs] of tob−/− animals were morphologically indistinguishable from wild-type MEFs and grew at a rate similar to that of wild-type MEFs. Upon successive passages with the defined 3T3 protocols, wild-type and tob−/− MEFs initially underwent approximately four population doublings with each passage. Growth virtually ceased around passages 5–7 due to senescence crisis [Fig. 2A]. tob−/− MEFs weathered the senescence crisis around passage 10, whereas wild-type MEFs did so after passage 15. Furthermore, established tob−/− MEFs had a shorter doubling time than did wild-type cells [Fig. 2B]. The saturation densities of the established tob−/− MEF lines were higher than those of wild-type cells, suggesting that contact inhibition of cell growth was hampered, at least in part, in the absence of Tob. MEFs lacking a tumor suppressor gene, such as p19ARF, proliferate continuously and never undergo an obvious senescence crisis [Harvey et al. 1993; Kamijo et al. 1997]. Secondary genetic alterations might have occurred in the established tob−/− MEF lines, and the Tob deficiency could contribute to such alterations. Supportingly, tob−/− MEFs at passage 4 displayed an increased number of chromosome aberrations (χ² test, p < 0.005; Fig. 2C). The number of chromosome aberrations in DEN-treated MEFs were increased [approximately twofold] in the absence of Tob [Fig. 2D]. MEFs lacking DNA repair genes XRCC4 and Ku80 show marked genomic instability [Difilippantonio et al. 2000;
Gao et al. 2000). Because expression of tob is induced in response to DNA damage, such as that caused by adriamycin treatment or γ-irradiation exposure (Cortes et al. 2000), Tob may contribute to genome stability.

Involvement of Tob in regulation of cyclin D1 transcription

Tob family proteins are involved in transcriptional regulation. To identify target genes whose transcription might be regulated by Tob, we examined transcripts that were affected by exogenous Tob expression [gain of function] and by depletion of Tob [loss of function]. Microarray analyses of ~500 cancer-related genes revealed that expression of several genes appeared to be regulated either directly or indirectly by Tob (data not shown). The genes whose expression was suppressed to approximately half or less by gain-of-function and induced more than twofold by loss-of-function include the cyclin D1, E2F5, Rala, and RalBP1 genes.

The cyclin D1 gene is relevant to G1 progression, and expression of the gene is often abrogated in human tumors (Prober and Edgar 2001). Because partial hepatectomy provides an in vivo model for the study of G0 progression, RNAs prepared from partially hepatectomized liver of 10-week-old tob−/− and wild-type mice were analyzed for cyclin D1 expression. As shown in Figure 3A, expression of cyclin D1 mRNA in both untreated and partially hepatectomized liver was increased in the absence of Tob, suggesting that Tob suppresses cyclin D1 expression in both resting and growing cells. The level of cyclin D1 mRNA was reduced in 293T cells that overexpress Tob [Fig. 3B]. These observations are consistent with our previous findings that significant levels of Cyclin D1 are present in serum-starved tob−/− MEFs, and the levels are reduced by re-expression of Tob (Suzuki et al. 2002). Luciferase assay with a reporter plasmid containing the promoter region of the cyclin D1 gene [Matsumura et al. 1999] revealed that overexpression of Tob suppressed activity of the cyclin D1 promoter [Fig. 3C]. Interestingly, the Tob-mediated repression of transcrip-

Figure 2. Characterization of tob−/− MEFs. (A) Cell proliferation on a 3T3 protocol. At 3-d intervals, the total numbers of cells per culture were determined prior to dilution of the cells to 7.5 × 10⁴ cells per 10-cm dish for repassage. (B) Growth properties of established tob−/− cells. Cells from wild-type and tob−/− mice at passage 20 were seeded at 1 × 10⁵ cells per culture in 6-cm dishes. Duplicate dishes were harvested at daily intervals, and the total numbers of cells per culture were determined. (C) Typical chromosome aberrations found in tob−/− MEFs at passage 4. [Left] A representative chromosome spread from tob−/− MEFs. chromatid gap [ctg, top, left]; chromosom e gap [ctg, top, right]; chromatid break [ctb, bottom, left]; and chromatid exchange, quadriradial [cte, qr; bottom, right]. (D) Percentage of cells with chromosome aberrations. Wild-type and tob−/− MEFs at passage 4 were treated with or without DEN, and each metaphase spread was assessed for the frequency of chromosome abnormalities. The total number of cells counted were as follows: Untreated, 624 for wild-type and 726 for tob−/− MEFs, DEN-treated, 365 for wild-type and 372 for tob−/− MEFs.

Figure 3. Inhibition of cyclin D1 promoter activity by Tob. (A) Increased expression of cyclin D1 mRNA in the absence of Tob. RNAs were prepared from liver cells of wild-type and tob−/− mice before (Liver) and 3.5 h after partial hepatectomy (PH3.5). [B] Suppression of cyclin D1 mRNA by Tob. RNAs were prepared from 293T cells transfected with pME18S (vector) or pMETob-Flag (Tob) and incubated for 24 h. In A and B, RNAs were subjected to Northern blot hybridization with the cyclin D1 cDNA probe. [C] Inhibition of Tob on cyclin D1 promoter activity. HeLa cells were transfected with increasing amounts (0.025–0.25 μg DNA/well in 12-well tissue culture plates) of pMETob-Flag together with pRL-TK and –1745-CD1-Luc. Six hours after transfection, TSA was added at the indicated concentration, incubated for another 18 h, and luciferase activity was measured. [D] Tob is associated with histone deacetylase activity. The 293T cells were transfected with pME18S or pMETob-Flag. Twenty-four hours after transfection, proteins in the lysates were immunoprecipitated with anti-Flag antibody. The HDAC activity was measured. [E] Interaction between Tob and HDAC1. COS-7 cells were transfected with pMETob-Flag, pRL-TK, and –1745-CD1-Luc. Six hours after transfection, TSA was added at the indicated concentration, incubated for another 18 h, and luciferase activity was measured. [F] Interaction between Tob and HDAC1. COS-7 cells were transfected with pMETob-Flag, pRL-TK, and –1745-CD1-Luc. Six hours after transfection, TSA was added at the indicated concentration, incubated for another 18 h, and luciferase activity was measured. [G] Interaction between Tob and HDAC1.
tion from the *cyclin D1* promoter was reduced significantly by increasing concentrations of trichostatin A (TSA), an inhibitor of HDAC activity [Fig. 3D]. The results suggested that HDAC is involved in Tob-mediated repression of transcription. Anti-Flag immunocomplexes prepared from lysates of Flag-Tob-transfected cells contained significantly higher HDAC activities than those from control cells [Fig. 3E], indicating that Tob complexes contain HDAC proteins. The interaction of Tob with HDAC1 was confirmed by coimmunoprecipitation experiments with COS7 cells transfected with the 6Myc-Tob construct and an expression plasmid encoding Flag-HDAC1 protein [Fig. 3F]. Therefore, overexpression of Tob could suppress transcription of the *cyclin D1* gene via recruitment of HDAC1. Analysis of the *cyclin D1* promoter by chromatin immunoprecipitation assay showed that lower levels of the acetylated histones, acetyl H3 and acetyl H4, were associated with the promoter in Tob-transfected HeLa cells than in control cells [Fig. 3G]. Again, the data suggest that Tob recruits HDAC to the *cyclin D1* promoter region. Analysis of a series of deletion mutants of the *cyclin D1* promoter suggested that the Tob/HDAC complex acted through several cis-acting elements in the promoter region (data not shown).

In addition, Tob is likely to be involved in suppression of expression of other genes such as *Rala*, *E2F5* [see above], and *IL-2* [Tzachanis et al. 2001], as well as the genes regulated by Smad downstream of bone morphogenetic protein (Yoshida et al. 2000). Taken together, these data indicate that Tob may recruit HDAC to different transcription factors.

**Suppression of *tob* in human cancers**

Analysis of more than 50 human tumors did not reveal any point mutations or gross aberrations of the *tob* gene. In contrast, reverse transcriptase PCR (RT–PCR) analysis revealed that the level of *tob* mRNA was decreased to 4.7%–87.3% [mean, 30.1%] of the normal level in 13 of 18 human lung cancers [Table 2]. The decrease was not related to the type of lung cancer. The data suggest that suppression of Tob expression contributes to tumor progression. It is theoretically possible that Tob expression was suppressed in the lung tumors as a consequence of growth stimulation. However, levels of *tob* transcript in an EBC1 human lung tumor cell line, in which the level of endogenous *tob* mRNA expression is low, were increased upon treatment of the cells with 5-aza-2'-deoxycytidine and/or TSA [data not shown]. Further analysis of the methylation and acetylation of the *tob* promoter might clarify this issue.

**Conclusion**

Tob family proteins associate with transcription factors that could interact with HDAC [Yoshida et al. 2000; Prevo et al. 2001] and protein arginine methyltransferase that could regulate transcription through methylation of histones [Tirone 2001], suggesting that the Tob family proteins function as transcriptional coregulators. Consistently, we show here that Tob negatively regulates the *cyclin D1* gene by recruiting HDAC to *cyclin D1* promoter. Amplification and overexpression of *cyclin D1* have been reported in various human tumors, including non-small-cell lung carcinomas, and hepatocellular carcinomas [Betticher et al. 1996; Joo et al. 2001]. Recent evidence suggests that overexpression of Cyclin D1 is an early, causative event in hepatocarcinogenesis [Joo et al. 2001]. A constitutive enhancement of *cyclin D1* expression observed in *tob−/−* mice may lead to development of cancers. In addition, increased susceptibility to the alkylation agent DEN of *tob−/−* mice and *tob−/−* MEF strongly suggests that Tob contributes to genome stability in vivo. Because *tob* expression is often decreased in human cancers [Table 2; M. Komoda, M. Suganuma, K. Iwanaga, N. Sueoka, E. Sueoka, T. Suzuki, Y. Yoshida, and T. Yamamoto, unpubl.], depletion and/or epigenetic suppression of *tob* may contribute to development of human cancers.

**Materials and methods**

**DEN treatment of mice**

Mice were maintained under standard specific-pathogen-free conditions. On day 15 after birth, offspring received a single intraperitoneal injection of DEN [20 μg/g body weight] in PBS. DEN-treated and untreated mice were sacrificed at 6 or 9 mo, and were analyzed for macroscopically visible tumors. Experiments with animals were carried out following guidelines for animal use issued by the Committee of Animal Experiments, Institute of Medical Science, University of Tokyo.

**Generation of *tob−/−p53−/−* mice**

To generate *tob−/−p53−/−* mice, *tob−/−p53−/−* mice were crossed. Wild-type, *p53−/−*, and *tob−/−* mice of the same background were used for comparisons. These mice were maintained in a hybrid C57BL/6J/129 SV background (75%/25%, respectively).

**Cells and culture**

MEFs were obtained from 14.5-day-old embryos by an established procedure [Todaro and Green 1963]. MEFs, HeLa cells, and 293T cells were maintained in DMEM containing 10% FBS, 50 μM β-mercaptoethanol, and antibiotics. Growth rates of the cells at the twentieth passage were determined by plating triplicate cultures of 1 × 10⁶ cells in 60-mm dishes. DEN [1 mg/mL] treatment was performed for 48 h after the third passage. The DEN-treated cells were washed and then cultured for another 12 h in fresh medium without DEN for the cytogenetic examination. EBC1 cells of a human lung cancer cell line (American Type Culture
Collection were cultured in RPMI 1640, supplemented with 10% FBS and antibiotics.

Chromosome analysis
MEFs, after four passages, were exposed to colcemid (0.02 μg/mL) for 2 h. Mitotic chromosome spreads were prepared by standard procedures and stained with 4',6'-diamidino-2-phenylindole. At least 300 metaphase spreads were subjected to the analysis.

Northern blot analysis
Total RNAs were isolated with ISOGEN (Nippon Gene) per the manufacturer's instructions. Total RNAs were prepared from mouse livers before and 3.5 h after partial hepatectomy and from 293T cells transfected with pME18S (Ikematsu et al. 1999) or pMETob-Flag (Tob, Yoshida et al. 2000). RNA samples (20 μg) were subjected to Northern blot hybridization using cyclin D1 cDNA labeled with [α-32P]dCTP by random priming as described previously [Ikematsu et al. 1999].

Immunoprecipitation and immunoblotting
Immunoprecipitation and immunoblotting were performed as described previously [Yoshida et al. 2000]. Antibodies used for blotting were anti-Myc monoclonal antibody (Santa Cruz Biotechnology), anti-Flag monoclonal antibody (Sigma) and anti-Tob antibodies [Matsuda et al. 1996].

DNA microarray analysis
Recombinant adenovirus vectors, Ad-Tob and Ad-LacZ, were constructed using homologous recombination between the expression cosmid and the parental virus genome as described [Miyake et al. 1996]. EBC1 cells, which show a low level of Tob expression (Yanagie et al. 2001), were transiently transfected with various combinations of the following plasmids by lipofection: pME18S, pMETob-Flag, pRL-TK, and −1745-CD1-Luc (Matsumura et al. 1999). Forty-eight hours after transfection, cell extracts were analyzed for luciferase activity with a Dual-Luciferase Reporter System (Promega). Transfection efficiency was standardized with an internal control plasmid, pRL-TK.

HDAC assay
HDAC activity was measured as described [Nomura et al. 1999]. Lysates were prepared from 293T cells transfected with pMETob-Flag or empty pME18S vector and were immunoprecipitated with anti-Flag antibody. Immunocomplexes were incubated for 5 h at 37°C with 1500 cpm of acid-soluble 3H-labeled histones.

ChIP assay
HeLa cells stably transfected with pMETob-Flag and parental cells were fixed with 1% HCHO. After fixation, chromatin were prepared from the cell lysates and subjected to ChIP assay [Upstate Biotechnology]. The average size of the DNA fragments was ~300 bp. The same amount of chromatin was used for immunoprecipitation with specific antibodies. The presence of the cyclin D1 promoter was analyzed by quantitative PCR with the primer specific primer pair 5'-GCGATGTCTTCTATGA-3' (forward) and 5'-CAGACTCTCTGATGCTGCTCT-3' (reverse).

RT-PCR
RNAs were prepared from human lung cancers and subjected to semi-quantitative RT–PCR. Briefly, the cDNAs were amplified by PCR using specific primers for Tob (F, 5'-CACAGGATCTTGTGTTTCTGATCGA-3'; R, 5'-TTTTCTTCAATGTTGAGCCGCAACT-3') for 24 cycles and for actin (F, 5'-CAGAGATGGCCACCGCTGCT-3'; R, 5'-TCTCTCTGCATCCTGCGGACA-3') for 19 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec in the presence of [α-32P]dCTP. PCR products were analyzed by 5% polyacrylamide gel electrophoresis. Radioactivity of PCR product was determined by BAS 2000 Bioimage Analyzer (Fuji Photo Film). Expression of actin mRNA was used as a control. Informed consent was obtained from all patients. Experiments with human mate-
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