Deficiency of antiproliferative family protein Ana correlates with development of lung adenocarcinoma

Mitsuhiro Yoneda,[1] Toru Suzuki,[1] Takahisa Nakamura,[1,6] Rieko Ajima,[1,4,7] Yutaka Yoshida,[1,8] Shigeru Kakuta,[2] Katsuko Sudo,[2,9] Yoichiro Iwakura,[2] Makoto Shibutani,[3] Kunitoshi Mitsumori,[3] Jun Yokota[4] and Tadashi Yamamoto[1,5]

[1]Division of Oncology, Department of Cancer Biology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639; [2]Laboratory Animal Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639; [3]Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509; [4]Biology Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan

(Received August 20, 2008/Revised October 15, 2008/Accepted October 17, 2008/Online publication December 8, 2008)

The abundant in neuroepithelium area *(ana)* gene was originally identified as a member of the *tob*/*btg* family of antiproliferative genes. Like the other family members, Ana inhibits growth of NIH3T3 cells when overexpressed. However, whether or not Ana is involved in tumor progression has been elusive. Here, we show that expression of *ana* is relatively high in the lung, the expression being restricted in type II alveolar epithelial cells. We further show that *ana* expression is reduced in 97% of the human lung cancer cell lines examined (61/63) and 86% of clinical samples from lung adenocarcinoma patients (36/42). Long-term observation of *ana*-deficient (*ana*$^{-/-}$) mice reveals that 8% of them develop lung tumors (5/66) by 21 months after birth, while 0% of wild-type mice (0/35) develop the same type of tumors. We also show that exogenously expressed *ana* gene product suppresses the levels of matrix metalloproteinase-2 (*MMP-2*) and plasminogen activator inhibitor-1 (*PAI-1*) expression in lung cancer cells. Taken together, we propose that *ana* functions as a tumor suppressor and that its product inhibits tumor progression as well by suppressing angiogenesis, invasion, and metastasis. (*Cancer Sci* 2009; 100: 225–232)

Lung cancer is a conglomeration of diseases of diverse etiology, divided into non-small-cell lung cancer (NSCLC) accounting for 80% of lung cancers, and small-cell lung cancer (SCLC) accounting for 20% of lung cancers. NSCLC comprises diverse histological subtypes including adenocarcinomas, squamous-cell carcinomas, large-cell carcinomas, and adenosquamous carcinomas. Accumulating evidence shows that the most frequent alterations detected in lung cancers are *p53* mutations, being detected in ~90% of SCLC and in ~50% of NSCLC.[1] The *Rb* gene is also inactivated in ~90% of SCLC, but in only 15% of NSCLC.[1] In contrast, the *p16* gene, whose product inhibits Rb phosphorylation, is inactivated in ~70% of NSCLC, but rarely in SCLC.[1] Importantly, neither *p53* nor *Rb*/*p16* is inactivated in certain numbers of lung cancer patients. Therefore, other genes whose inactivation is responsible for lung carcinogenesis remain to be clarified.

The *ana* gene was initially identified as a novel antiproliferative *tob*/*btg* family gene basing on its homology to the *tob* gene.[2] The *tob*/*btg* family consists of *tob*, *tob*2, *btg1*, *btg2*/*tis21*/*pc3*, *ana*/*btg3*, and *pc3b*. All the protein products of the family genes suppress cell growth when overexpressed.[2–6] In addition, *tob*-deficient mice are highly prone to spontaneous tumor formation, partially due to elevation of cyclin D1.[7] In clinical samples, Tob expression was reduced in lung cancer and anaplastic carcinoma of the thyroid.[7–9] Moreover, the Btg2 expression level was decreased in breast cancer and renal cell carcinoma.[10,11] Although Ana exhibits antiproliferative activity in NIH3T3 cells similar to the other Tob/Btg family proteins,[2] the role of Ana in cancer development has been poorly understood.

In the present study, by analyzing expression of *ana* in lung cancer tissues and cell lines and by examining *ana*$^{-/-}$ mice pathologically, we show that Ana plays a part in the development of lung cancer. In addition, we provide evidence suggesting that Ana is also involved in regulation of angiogenesis, invasion, and metastasis.

Materials and Methods

Patients. Primary lung cancers and corresponding noncancerous tissues were obtained at surgery from 54 patients who were treated at the National Cancer Center Hospital (Tokyo, Japan). The materials to be analyzed were selected by a pathologist to ensure that the samples were macroscopically entirely cancerous and chosen from an area devoid of necrotic tissue. Cancerous and noncancerous tissues were stored at –70°C until RNA extraction. This study was undertaken under the approval of the Institutional Review Board of National Cancer Center of Japan and was conducted in accordance with the Declaration of Helsinki.

Lung cancer cell lines. The 63 lung cancer cell lines subjected to real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis were 19 small-cell lung carcinoma cell lines, Lu134, Lu135, Lu139, NCI-H69, NCI-H82, N417, NCI-H526, NCI-H209, NCI-H841, SBC-5, Ms-18, HCC 33, NCI-H1184, NCI-H2107, NCI-H2171, NCI-H2141, NCI-H128, NCI-H2195, and NCI-H1963; and 44 NSCLC cell lines that included 27 adenocarcinomas, A427, A549, NCI-H23, NCI-H322, NCI-H441, PC3, PC7, PC9, PC14, RERF-LCOK, VMRC-LCD, ABC-1, RERF-LCMS, Ma17, Ma29, HCC44, HCC78, HCC515, NCI-H1395, NCI-H2009, NCI-H2087, NCI-H2122, NCI-H1437, NCI-H1648, NCI-H1703, NCI-H2126, and NCI-H2347; 10 squamous-cell carcinomas, NCI-H157, NCI-H520, PC10, EBC-1, LC1-Sq, SQ-5, LK-2, Sq-1, SK-MES-1, and NCI-H1869; two adenosquamous carcinomas, NCI-H596 and HCC366; and five large-cell carcinomas, Lu65, Lu99, NCI-H1155, PC13, and NCI-H1299. These cell lines were provided by Drs John D. Minna, Y. Hayata, T. Terasaki, S. Hirohashi, and M. Takada. Cell lines were also obtained from the American Type Culture Collection (Manassas, VA, USA), the Japanese Collection of

[5]To whom correspondence should be addressed. E-mail: tyamamot@ims.u-tokyo.ac.jp
[6]Present address: Department of Genetics and Complex Diseases, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115, USA
[7]Present address: Cancer and Developmental Biology Laboratory, National Cancer Institute, National Institutes of Health, Frederick, Maryland 21702-1201, USA
[8]Present address: Division of Developmental Biology, Cincinnati Children's Research Foundation, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA
[9]Present address: Animal Research Center, Tokyo Medical University, Shinjuku-ku, Tokyo 160-8402, Japan

doi: 10.1111/j.1349-7006.2008.01030.x

Research Bioresources (Tokyo, Japan), and the RIKEN BioResource Center (Tsukuba, Japan). Detailed information on these cell lines can be obtained upon request. Poly $(A)^+$ RNA was prepared from 63 lung cancer cell lines, a primary culture of normal human small airway epithelial cells (Clonetics, Walkersville, MD, USA), NHBE (Clonetics), and a lung fibroblast cell line (WI-38) by the Fast Track mRNA isolation kit (Invitrogen, Carlsbad, CA, USA). Poly $(A)^+$ RNAs from human normal tissues were purchased from Clontech Laboratories Japan. (Tokyo, Japan). Randomly primed cDNAs were reverse-transcribed from 0.5-μg mRNAs or 10-μg total RNAs by using SuperScriptII RNase H Reverse Transcriptase (Life Technologies, Rockville, MD, USA) following the supplier's protocol.

Generation of *ana*$^{-/-}$ mice. The 14-kbp genomic DNA fragment of *ana* was subcloned into pBluescript. An exon that contains the first methionine and the second exon were replaced with *lacZ* and a *PGK1neo* poly (A) fragment flanked by *lox*P sites to generate the targeting vector. The replacement was done so that the *lacZ* coding sequence was inserted in-frame with the first methionine of Ana. The *diphtheria toxin A* (*DT-A*) fragment was ligated to the 5′ end of the targeting vector for negative selection. E14.1 embryonic stem cells were electroporated with linearized targeting vector and selected with neomycin. *ana*-targeted clones were identified by Southern blot hybridization with the 549-bp DNA probe shown in Fig. 3(a), and were aggregated with two C57BL/6 J eight-cell stage embryos as described previously,[12] and mated chimeric offspring with C57BL/6 J mice. Heterozygous F1 mice were crossed to produce homozygous *ana*$^{-/-}$ mice. Wild-type and *ana*$^{-/-}$ mice were maintained under standard specific-pathogen-free conditions. 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.

Antibodies. For immunoblotting, anti-Ana antibodies, anti-ERK1 antibodies C-16 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. The anti-Ana antibodies were prepared by immunizing the *Escherichia coli*-produced GST-fusion protein containing C-terminal amino acid residues (129–231) of mouse Ana.

Plasmid constructs. Human and mouse *ana* cDNAs with Flag sequence at the 3′ end were inserted into the pME-18S mammalian expression vector. Mouse *ana* cDNA was also inserted into a pMXs-puro,[13] retroviral vector, containing a *puromycin resistance* marker. The retroviral vector and the packaging cell line Plat-A (based on the 293T cell line) were gifts from T. Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). After infection of the *ana* virus into HCC366 cells, selection was initiated with 2.0-μg/mL puromycin. For anti-Ana antibodies production, mouse *ana* cDNA (amino acids 129–231) was inserted into a pGEX-6P-3 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Integrity of all constructs was confirmed by sequencing.

X-gal staining. Lung tissues were fixed for 3–4 h in 4% paraformaldehyde at 4°C, and cryoprotected in 30% sucrose in phosphate-buffered saline overnight. Sections were fixed with 1% paraformaldehyde at 4°C and stained overnight at 37°C followed by counter-staining with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan).

Histological analysis. Lung tissues were fixed in phosphate-buffered 10% formalin (pH 7.4), embedded in paraffin and cut into 4-μm sections. Sections were dewaxed and stained with hematoxylin–eosin.

Cell cultures. HCC366 cells were cultured in RPMI-1640 (Wako Pure Chemicals, Osaka, Japan) containing 10% fetal bovine serum and antibiotics.

Semi-quantitative real-time RT-PCR analysis. Real-time RT-PCR analysis was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA).

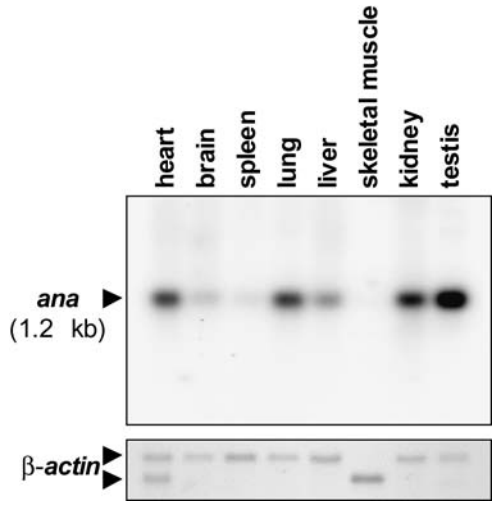

Fig. 1. *ana* mRNA expression in mouse tissues. Northern blot hybridization was performed using the mRNA blot from Clontech. The entire coding sequence of the mouse *ana* cDNA was used as a probe (upper panel). The RNA loading was justified by hybridizing the same blot with the β-actin probe (lower panel).

Primer sequences and TaqMan probes for the *ana*, *GAPDH*, and *HPRT* genes are shown in Table S1. *GAPDH* was used as an internal control for SYBR Green PCR. SYBR Green PCR was performed in a single tube in triplicate, and the results were expressed as the representative average of three separate experiments. TaqMan PCR for each target gene and the reference gene *HPRT* was performed in a single tube in duplicate, and the results were expressed as the average of the two independent tests.

Statistical analysis. Levels of significance for comparison between samples were determined by the Student's *t*-test. *P*-values of <0.05 were considered to be statistically significant.

Results

Decreased expression of *ana* in lung cancer specimens. We investigated *ana* expression in various murine tissues. *ana* mRNA was ubiquitously expressed in adult mice, the level being relatively high in the heart, lung, kidney, and testis, but low in the spleen and skeletal muscle (Fig. 1). A previous report showed that *ana* expression is also high in the human adult lung and is not grossly altered in several cancer cell lines examined.[2] Nevertheless, because the *ana* gene is homozygously deleted in two lung cancer cell lines,[14,15] we thought that silencing of *ana* expression might be relevant to lung tumor development. Then, we extensively examined the level of *ana* expression in human lung cancer by applying real-time RT-PCR to a relatively large number of clinical samples. A total of 54 NSCLC cases (42 adenocarcinomas, 10 squamous-cell carcinomas, and two large-cell carcinomas) were studied. Patient characteristics are shown in Table 1, with ages ranging from 30 to 81 years (median age, 62 years) and stages I to III. In each patient, the level of *ana* expression normalized by *GAPDH* expression in cancerous tissue was compared with that in noncancerous tissue. In 85.2% (46/54) of specimens, *ana* expression was reduced in cancerous tissues compared to noncancerous tissues (Fig. 2a,S1). Note that *ana* expression levels were low in 85.7% (36/42) of clinical samples from lung adenocarcinoma patients (Fig. 2a). As shown in Fig. 2(b), the reduction was statistically significant ($P = 0.00043$, Student's *t*-test). By the criteria described in the figure legend, average expression level of *ana* in adenocarcinomas was

doi: 10.1111/j.1349-7006.2008.01030.x

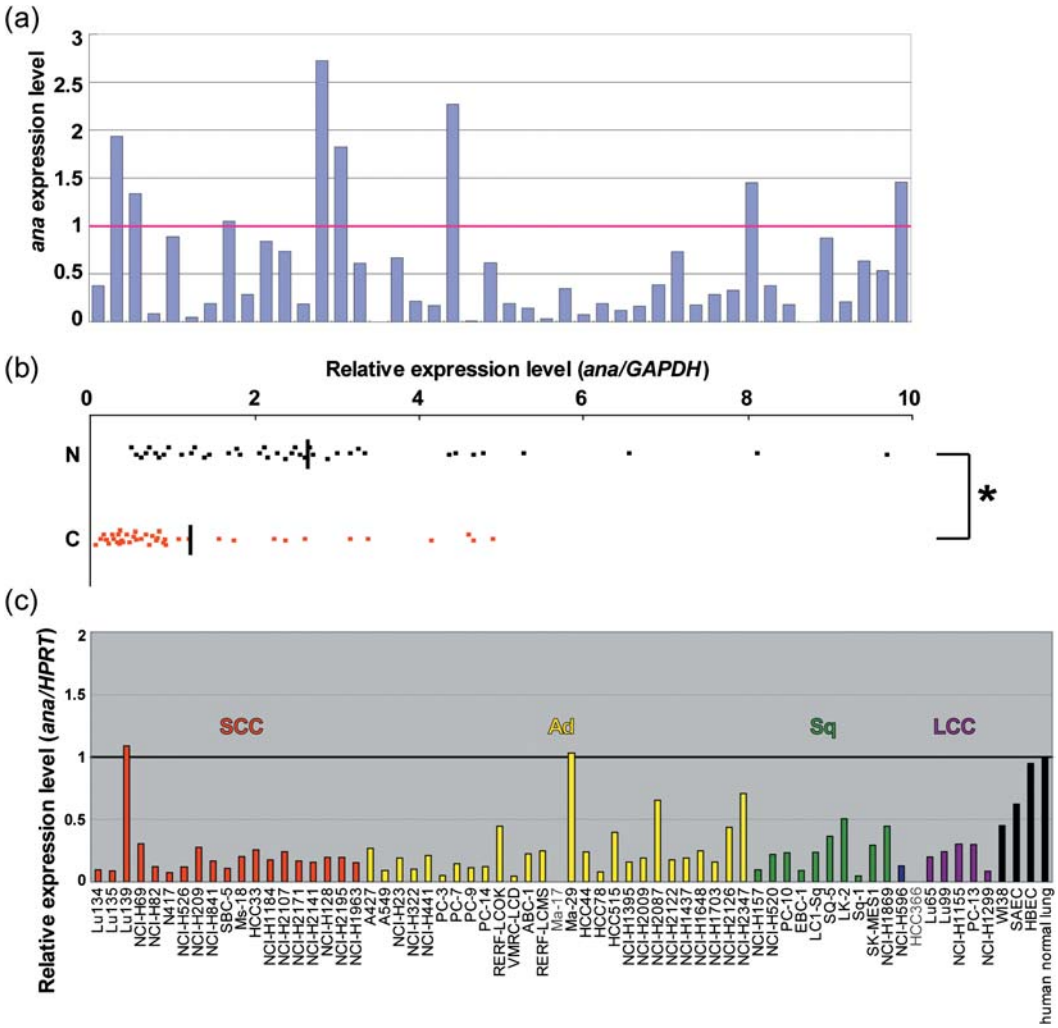

Fig. 2. *ana* mRNA expression in lung cancer. (a) The levels of *ana* mRNA expression in 42 human primary lung adenocarcinomas were evaluated by real-time reverse transcription–polymerase chain reaction and normalized by *GAPDH* mRNA expression. The data shown were the ratio of *ana* expression in each cancerous tissue to that of corresponding noncancerous tissue. (b) Expression level of *ana* in each cancerous tissue (C) was compared to that of corresponding noncancerous tissue (N). The level of *ana* mRNA expression in normal lung tissue purchased from Clontech was set to 1. Asterisk (*) indicates a statistical difference between experimental groups ($P < 0.05$, Student's *t*-test). (c) The levels of *ana* mRNA in cancer cell lines. The level of *ana* mRNA in normal lung tissue purchased from Clontech was set to 1. *ana* expression level was normalized by *HPRT*. SCC (red), small-cell carcinoma cell lines; Ad (yellow), adenocarcinoma cell lines; Sq (green), squamous-cell carcinoma cell lines; LCC (purple), large-cell carcinoma cell lines. NCI-H596 (blue graph) and HCC366 cells are adenosquamous carcinoma cell lines. The cells written in red lacked *ana* expression.

Table 1. Patient characteristics of human lung cancer

	Adenocar cinoma (Ad)	Squamous-cell carcinoma (SCC)	Large-cell carcinoma (LCC)
	(*n* = 42)	(*n* = 10)	(*n* = 2)
Age			
Range (years)	30–76	49–80	69, 81
Median (years)	62	62	75
Sex			
Male	26	8	1
Female	16	2	1
Differentiation			
Well	15	5	
Moderately	22	5	
Poorly	5		
Stage			
I	22		
II	9		
III	11		

determined to be 1.24 and that in corresponding non-cancerous tissues 2.67. Multivariate analysis showed that there was no significant correlation between the degree of *ana* suppression and the status of the lung tumors in our present results. However, because the number of specimens we analyzed is too small to evaluate the relation, future analysis with much large number of specimens is needed.

The level of *ana* expression was also evaluated by real-time RT-PCR analysis in 63 lung cancer cell lines, 19 SCLC cell lines, and 44 NSCLC cell lines, including 27 adenocarcinomas, 10 squamous-cell carcinomas, two adenosquamous carcinomas, and five large-cell carcinomas. Since the expression level of *GAPDH* in some cell lines was very low compared to normal lung tissue (Fig. S2), the *ana* expression level was also normalized by *HPRT* expression. As shown in Fig. 2(c), *ana* expression was reduced in 61 cell lines compared to normal lung tissue. Therefore, we hypothesized that loss of *ana* expression might contribute to the development of human lung cancer.

Development of lung tumor in *ana*$^{-/-}$ mice. To examine the causative role of *ana* in lung tumor formation, we generated

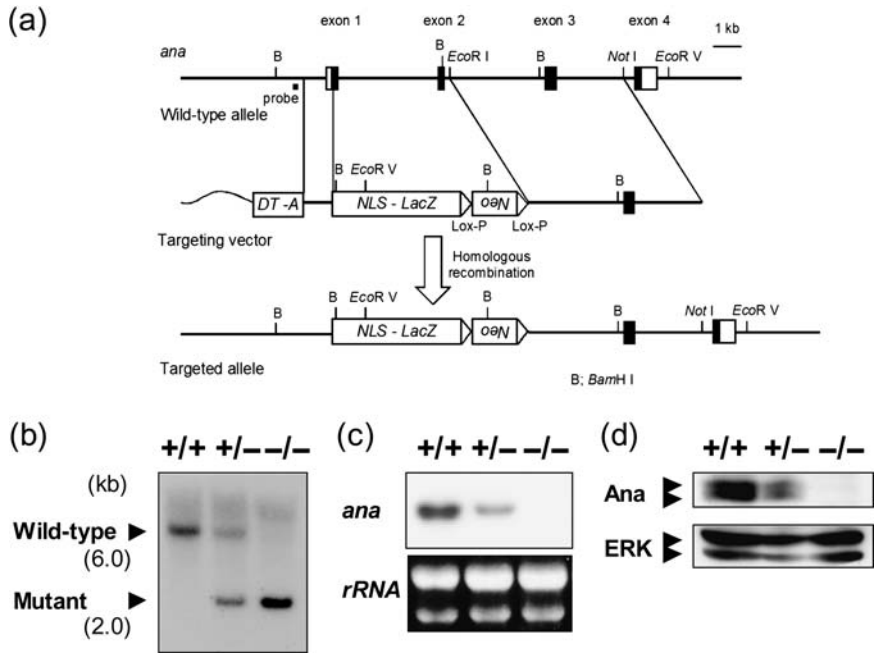

Fig. 3. Targeted disruption of *ana*. (a) The 1st exon which contains the first methionine and 2nd exon of *ana* were replaced with *lacZ* and neomycin resistance genes. The *lacZ* gene was inserted in-frame with the first methionine of Ana. (b) Tail DNA from F1 progeny of two heterozygote intercrosses were digested with *Bam*HI and subjected to Southern blot hybridization using the sequence upstream of the 1st exon as a probe (indicated as probe in panel [a]). The genotype for the *ana* locus was indicated above each lane. Sizes of the DNA fragments were indicated on the left in the parenthesis. (c) Northern blot analysis of total RNAs from primary chondrocytes of wild-type (+/+), *ana* hetero-deficient (+/–), and *ana*-deficient (–/–) mice was performed using the entire coding sequence of the mouse *ana* cDNA as a probe. *rRNA* in the agarose gel was visualized to monitor RNA loading. (d) Western blot analysis of protein extracts from primary chondrocytes was performed with the anti-Ana antibodies described in 'Materials and Methods'. Blotting with anti-Erk antibodies was to check protein loading.

ana$^{-/-}$ mice in which the 1st and 2nd exons of *ana* were replaced with the *lacZ* reporter gene by homologous recombination (Fig. 3a). Southern blot, northern blot, and western blot analyses revealed disruption of the wild-type *ana* alleles (Fig. 3b–d) in *ana*$^{-/-}$ mice. *ana*$^{-/-}$ mice were alive at birth and born at the expected Mendelian frequency. Adult *ana*$^{-/-}$ mice appeared to be normal in both size and behavior.

Taking advantage of the *lacZ* gene replacement in *ana*-mutant mice, we explored the *ana* gene expression in *ana* hetero-deficient (*ana*$^{+/-}$) mice by examining cells positive for the chromogenic substrate 5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Lung epithelium comprises diverse histological subtypes, including bronchial epithelial cells and alveolar epithelial cells. Alveolar epithelial cells are formed by two cell types, squamous type I cells and surfactant-producing cuboidal type II cells. At least 5 weeks after birth, X-gal staining revealed that *ana* was highly expressed in type II alveolar epithelial cells, but not in type I alveolar epithelial cells or bronchial epithelial cells (Fig. 4a,S3). LacZ expression could not be detected in bronchial epithelial cells at a late embryonic stage (E18.5) (Fig. S3). Since the lung adenocarcinoma is thought to be mainly derived from type II alveolar epithelial cells,[16,17] we speculated that there is a correlation between suppression of *ana* expression and lung adenocarcinogenesis.

Nevertheless, *ana*$^{-/-}$ mice showed no apparent phenotypic abnormalities in their early lives. Histological analysis of 9-month-old wild-type and *ana*$^{-/-}$ mice revealed no obvious tumor formation either (data not shown). Therefore, we conducted a long-term study of spontaneous tumor development in wild-type and *ana*$^{-/-}$ mice. By 21 months of age, 7.6% (5/66) of *ana*$^{-/-}$ mice developed lung tumors as compared to 0% (0/35) of wild-type mice. A typical example is shown in Fig. 4(b). Histologically, three lung tumors developed in two *ana*$^{-/-}$ mice were moderately differentiated alveolar/bronchiolar carcinomas with neoplastic cells which formed papillary clusters showing increased nucleus/cytoplasm (N/C) ratio and nuclear pleomorphism (Fig. 4c). Lung tumors developed in other *ana*$^{-/-}$ mice were one well-differentiated alveolar/bronchiolar carcinoma and two alveolar/bronchiolar adenomas. Additionally, infiltration of cancer cells into the bronchioles was observed in all four carcinomas (Fig. 4c). Sixteen *ana*$^{-/-}$ mice and 15 wild-type mice were analyzed histologically in systemic tissues by 21 months of age. Although *ana*$^{-/-}$ mice developed lymphomas, hepatocellular carcinomas, an angiosarcoma of the spleen, a giant cell sarcoma of the spleen, and lung tumors, most of the tumors were also observed in wild-type mice (Table S2). These data suggest that deregulation of *ana* gene may not be related to tumor development. Nonetheless, because the lung tumors were not seen in the wild-type mice, the possibility that *ana* depletion is related to the lung tumor development remains.

Effect of Ana on growth of lung cancer cells and on expression of invasion- and metastasis-related genes. To confirm the suppressive role of Ana in lung cancer development, we infected retroviruses carrying *ana* cDNA into human lung cancer cell lines (A549 adenocarcinoma, NCI-H322 adenocarcinoma, and NCI-H1299 large-cell carcinoma) in which the *ana* expression level is very low, or into HCC366 adenosquamous carcinoma in which the *ana* gene is homozygously deleted. Comparison of the growth rate in soft agar or under low serum conditions between the Ana-expressing cells and control cells showed that *ana* expression did not affect either the colony formation rate or cell proliferation rate (Fig. S4). The observation was unexpected, because Ana is supposed to suppress the cell growth. However, we reasoned that this was probably due to genetic or epigenetic alterations occurring on multiple cancer-related genes other than *ana* in these cancer cell lines. We also examined the expression of invasion- and metastasis-related genes, which promote tumor invasion and metastasis,[18,19] in HCC366 cells transfected with a recombinant retroviral vector carrying the *ana* gene or the vector alone. Real-time RT-PCR analysis revealed that the expression levels of *MMP-2* and *PAI-1* were decreased in Ana-expressing cells as compared with the control cells (Fig. 5). Similarly, the expression levels of *MMP-2* and *PAI-1* were also decreased in Ana-expressing A549 cells (data not shown). We also examined the effect of Ana on the gelatinase activity of MMP-2. The gelatin zymography showed that the gelatinase activity of MMP-2 in Ana-transfected cells was about 50% of that in control cells (data not shown), which is consistent with the decrease of *MMP-2* mRNA level in Ana-transfected cells as compared with the control cells (Fig. 5). These data suggest that Ana affects the *MMP-2* mRNA expression level but not the enzymatic activity of MMP-2.

doi: 10.1111/j.1349-7006.2008.01030.x

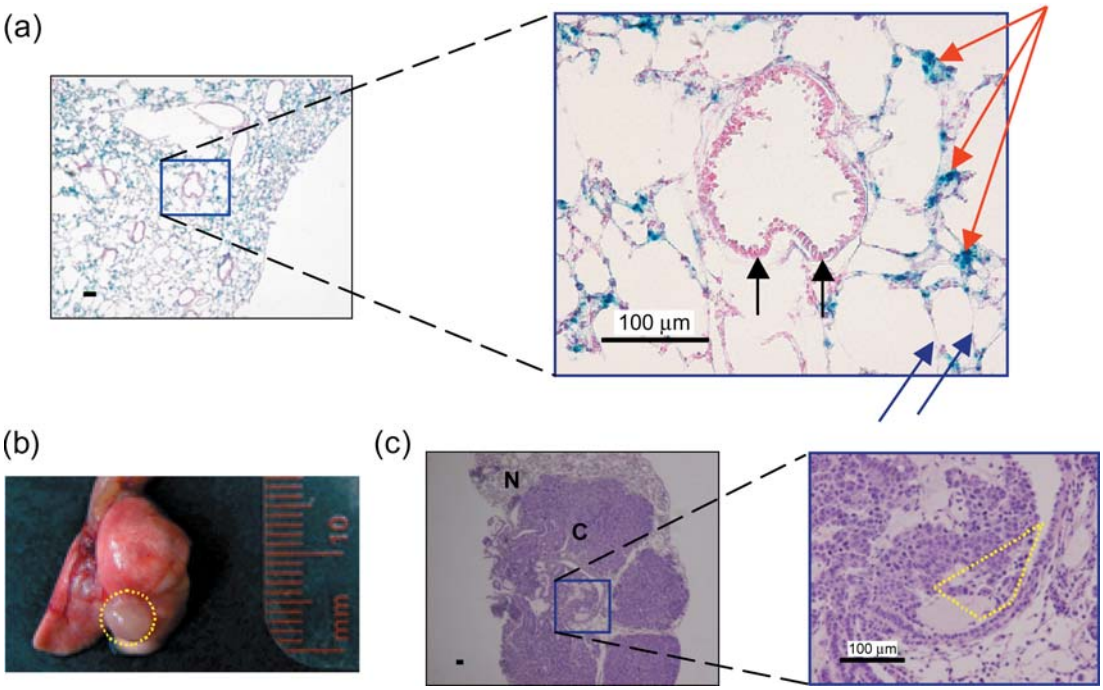

Fig. 4. Development of lung alveolar/bronchiolar carcinomas in *ana*-deficient mice. (a) Expression of *ana* was monitored by X-gal staining of the lung from 5-week-old *ana* hetero-deficient mice. X-gal positive cells were stained blue. *ana* expression was detected in type II alveolar epithelial cells (red arrows), but hardly in bronchial epithelial cells (black arrows), or type I alveolar epithelial cells (blue arrows). Sections were counterstained with Kernechtrot stain solution (to stain DNA; red). Original magnification: ×40 (left) and ×200 (right). (b) A representative lung tumor developed in *ana*-deficient mice (shown within the yellow dotted circle). (c) A representative lung alveolar/bronchiolar carcinoma developed in *ana*-deficient mice was examined histologically with hematoxylin–eosin staining. Cancerous (C) and noncancerous (N) lung tissues were shown (left). Infiltration of cancer cells into bronchiole was depicted by the yellow dotted line (right). Original magnification: ×40 (left) and ×200 (right). These mice were maintained in a hybrid C57BL/6 J/129Ola background (50% and 50%, respectively).

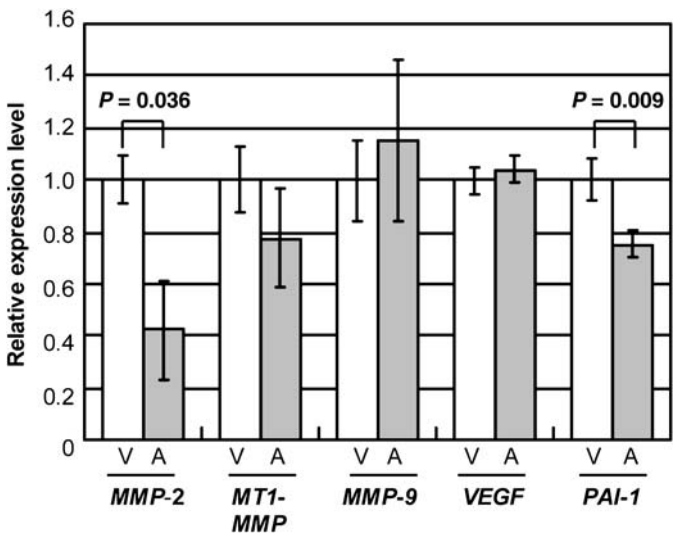

Fig. 5. The expression levels of *MMP-2* and *PAI-1* are decreased in Ana-overexpressing lung cancer cells. Expression of invasion- and metastasis-related genes in HCC366 lung adenosquamous carcinoma cells which have been transfected with the retrovirus carrying *ana* cDNA (A) or with control retrovirus (V). Expression level of each gene in *ana* virus-infected cells was measured by real-time reverse transcription–polymerase chain reaction and was normalized with *GAPDH* and compared to control virus-infected cells. Error bars represent ± SE.

Discussion

The Tob/Btg family proteins negatively regulate cell proliferation,[2–6] and are involved in tumor development.[7–9,11,20] Here, we showed that expression of one of the *tob/btg* family gene *ana* was suppressed in most specimens derived from lung tumors. Those tumors in which *ana* expression was reduced were adenocarcinomas of NSCLC whose origin is thought to be mainly type II alveolar epithelial cells. Importantly, by analyzing the *lacZ* expression of the $ana^{+/-}$ mice in which the *lacZ* gene replaces *ana*, we found that *ana* was prominently expressed in type II alveolar epithelial cells. Furthermore, we showed that $ana^{-/-}$ mice developed lung tumors, which are most likely derived from type II alveolar epithelial cells and phenotypically resemble human adenomas and adenocarcinomas, although the onset was late. Therefore, we propose that Ana suppresses development of tumors, especially lung tumors. In support of this, a previous report showed that the *ana* gene, located on chromosome 21q11.2-21.1, is homozygously deleted in Ma17 human lung adenocarcinoma cells.[14] HCC366 human lung adenosquamous carcinoma cells also harbor a homozygous deletion which eliminates part of the coding portion of the *ana* gene.[15] Furthermore, loss of heterozygosity on human chromosome 21q11.2-21.1 is observed in esophageal squamous-cell carcinoma, gastric adenocarcinoma, breast cancer, and lung cancer,[14,21–23] suggesting that *ana* could be involved in the development various types of tumors in addition to lung adenocarcinoma. It may be worthy to mention that the level of *ana* mRNA is decreased in 89% of the oral squamous-cell carcinoma cell lines examined (8/9) and 60% of clinical samples from oral squamous-cell carcinoma patients (12/20).[24] Analysis

of *ana*$^{-/-}$ mice suggested that deregulation of *ana* gene may not be related to tumor development in tissues other than lung, probably due to the functional redundancy of Tob/Btg family members (see below).

Epigenetic mechanisms are relevant to the transcriptional silencing of various genes such as tumor suppressor genes. To address whether epigenetic mechanisms are involved in *ana* down-regulation, we treated the three human lung cancer cell lines (A549 adenocarcinoma, NCI-H322 adenocarcinoma, and NCI-H1299 large-cell carcinoma), in which the *ana* expression level is very low, with the demethylating agent 5-aza-2′-deoxycytidine or the histone deacetylase inhibitor tricostatin A(TSA). *ana* mRNA expression did not increase even after the treatment, suggesting that neither the methylation nor histone deacetylase of *ana* promoter region might be involved in decrease of *ana* expression in the tumor cells (data not shown). Because the number of tumor cell lines we analyzed was limited, further analysis with a large number of cell lines is needed to clarify whether or not epigenetic changes of *ana* are involved in lung tumor development.

Other Tob/Btg family proteins, such as Tob and Btg2, are expressed in the lung and have been reported to express in bronchial epithelial cells.[8,25] We further showed that *tob*2 was also expressed in bronchial, type I alveolar, and type II alveolar epithelial cells (Fig. S5) by X-gal staining of *tob2*$^{-/-}$ mice in which the *tob2* coding sequence was replaced with the *lacZ* gene.[26] Therefore, depletion of *ana* in type II alveolar epithelial cells might have been functionally complemented by *tob*2, which could explain, at least in part, the late onset of the tumors. Additional alteration of *tob*2 and/or other genes of the same family possibly expressed in type II alveolar epithelial cells may be required to induce lung tumors derived from those cells. To examine whether *tob*/*btg* family genes are functionally redundant in tumor suppression, we analyzed expression of other *tob*/*btg* family genes in most of the lung cancer cell lines we examined by real-time RT-PCR analysis. The expression of *tob*, *tob*2, and *btg2* was similarly decreased in the lung cancer cells as compared to normal lung tissue (data not shown). It is therefore possible that the family genes are, at least in part, functionally redundant in suppressing lung tumor development. The functional redundancy in tumor suppression among Tob/Btg family may be related to the late-onset of tumor development in *ana*$^{-/-}$ mice.

Various genetic altertions have been shown to be involved in lung cancer development.[1,27] Because *p53* mutations are the most frequent altertions detected in lung cancers and Ana reportedly functions downstream of p53,[1,28–30] we analyzed whether or not p53 inactivation is relevant to down-regulation of the *ana* gene in lung carcinogenesis. Among 63 lung cancer cell lines we examined for *ana* expression, 53 cell lines have mutations in *p53* gene whereas nine cell lines have functional p53. There is no information about *p53* state in the other one cell line. Apparently, the level of *ana* expression (Fig. 2c) is not correlated with the state of *p53*. However, to be more conclusive much large number of lung cancer specimens need to be analyzed. Whether expression level of the *ana* gene correlates with the status of lung cancer-related genes other than *p53* should also be addressed in future studies.

The molecular mechanisms underlying the antiproliferative activity of Tob/Btg family proteins have not been well addressed. Some reports show that they suppress *cyclin D1* expression, acting as cofactors of transcriptional regulation.[7,20] It is also reported that Btg1 and Btg2 regulate ER-α-mediated transcription.[31] The present results showed that the mRNA levels of *MMP-2* and *PAI-1* were suppressed by exogenously expressed Ana, suggesting that Ana regulates transcription of these genes by acting as a transcriptional repressor. Luciferase assay was performed to examine whether Ana affects transcriptional activity on the *PAI-1* promoter with plasmid p800LUC containing *PAI-1* promoter.[32] Ana repressed transcriptional activity on the *PAI-1* promoter (Fig. S6). A recent report showed that *ana*/*btg3* is a transcriptional target of p53, and Ana/Btg3 in turn binds to and inhibits E2F1-mediated transcription.[30] These data imply that Tob/Btg family proteins, including Ana, function as regulators of the transcription machinery. The question of how Ana regulates the promoter activity of the *MMP-2* gene remains to be addressed. It should also be noted that another Tob/Btg family member Btg2 enhances cellular deadenylase activity by binding to Cnot7 deadenylase, although the precise mechanism remains to be clarified.[33] Since Ana interacts with Cnot7,[34] exogenously expressed Ana may stimulate Cnot7 to unusually shorten the poly (A) tails of *MMP-2* and *PAI-1* mRNAs, resulting in their destabilization and the decrease of the levels of MMP-2 and PAI-1 proteins. In lung cancer in which *ana* expression is suppressed, Cnot7 deadenylase activity may be decreased, resulting in stabilization of the *MMP-2* and *PAI-1* mRNAs, and leading to accumulation of MMP-2 and PAI-1 proteins.

MMP-2 promotes invasion, metastasis, and angiogenesis, and PAI-1 is needed for the optimal function of the urokinase-type plasminogen activator (u-PA) system that regulates tumor invasion and metastasis.[35–37] Furthermore, PAI-1 possesses both pro- and anti-angiogenic effects depending on the PAI-1 concentration.[38] The observations that PAI-1 is expressed in endothelial cells of small vessels in human colon adenocarcinomas and in proliferative vessels in high-grade gliomas and metastatic tumors[39,40] suggest that PAI-1 also plays a role in angiogenesis in human cancers. Overexpression of *MMP-2* and *PAI-1* has a poor prognosis in lung cancer tissues, especially in lung adenocarcinomas.[41–43] Therefore, absence of *ana* might also be a prognostic marker of lung adenocarcinomas.

In conclusion, we hypothesize that Ana functions as a tumor suppressor, and in addition negatively regulates the angiogenesis, invasion, and metastasis of cancer. Further studies on the correlation between *ana* and other cancer-related genes would help elucidate the mechanism of lung adenocarcinoma development.

Acknowledgments

We thank K. Miyazono (Department of Molecular Pathology, University of Tokyo, Japan) for providing the plasmid p800LUC. We also thank Drs John D. Minna (University of Texas South-western Medical Center, Dallas, TX, USA), Y. Hayata (Tokyo Medical University, Tokyo, Japan), T. Terasaki (Kanagawa Institute of Technology, Kanagawa, Japan), S. Hirohashi (National Cancer Center, Tokyo, Japan), and M. Takada (National Hospital Organization Kinki-chuo Chest Medical Center, Osaka, Japan) for providing us with several lung cancer cell lines. This work was supported in parts by Grants-in-Aid for priority area of cancer research from the MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, the research fund from the Uehara Memorial Foundation, and the Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms) from the MEXT of Japan.

References

1 Yokota J, Kohno T. Molecular footprints of human lung cancer progression. *Cancer Sci* 2004; **95**: 197–204.

2 Yoshida Y, Matsuda S, Ikematsu N *et al*. ANA, a novel member of Tob/BTG1 family, is expressed in the ventricular zone of the developing central nervous system. *Oncogene* 1998; **16**: 2687–93.

3 Ikematsu N, Yoshida Y, Kawamura-Tsuzuku J *et al*. Tob2, a novel antiproliferative Tob/BTG1 family member, associates with a component of the CCR4 transcriptional regulatory complex capable of binding cyclin-dependent kinases. *Oncogene* 1999; **18**: 7432–41.

4 Guardavaccaro D, Corrente G, Covone F *et al*. Arrest of G (1) –S progression by the p53-inducible gene PC3 is Rb dependent and relies on the inhibition of cyclin D1 transcription. *Mol Cell Biol* 2000; **20**: 1797–815.

doi: 10.1111/j.1349-7006.2008.01030.x

5 Maekawa M, Nishida E, Tanoue T. Identification of the anti-proliferative protein Tob as a MAPK substrate. *J Biol Chem* 2002; **277**: 37783–7.
6 Suzuki T, Kawamura-Tsuzuku J, Ajima R, Nakamura T, Yoshida Y, Yamamoto T. Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. *Genes Dev* 2002; **16**: 1356–70.
7 Yoshida Y, Nakamura T, Komoda M *et al*. Mice lacking a transcriptional corepressor Tob are predisposed to cancer. *Genes Dev* 2003; **17**: 1201–6.
8 Iwanaga K, Sueoka N, Sato A *et al*. Alteration of expression or phosphorylation status of Tob, a novel tumor suppressor gene product, is an early event in lung cancer. *Cancer Lett* 2003; **202**: 71–9.
9 Ito Y, Suzuki T, Yoshida H *et al*. Phosphorylation and inactivation of Tob contributes to the progression of papillary carcinoma of the thyroid. *Cancer Lett* 2005; **220**: 237–42.
10 Kawakubo H, Carey JL, Brachtel E *et al*. Expression of the NF-κB-responsive gene BTG2 is aberrantly regulated in breast cancer. *Oncogene* 2004; **23**: 8310–9.
11 Struckmann K, Schraml P, Simon R *et al*. Impaired expression of the cell cycle regulator BTG2 is common in clear cell renal cell carcinoma. *Cancer Res* 2004; **64**: 1632–8.
12 Horai R, Asano M, Sudo K *et al*. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. *J Exp Med* 1998; **187**: 1463–75.
13 Kitamura T, Koshino Y, Shibata F *et al*. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003; **31**: 1007–14.
14 Kohno T, Kawanishi M, Matsuda S *et al*. Homozygous deletion and frequent allelic loss of the 21q11.1-q21.1 region including the ANA gene in human lung carcinoma. *Genes Chromosomes Cancer* 1998; **21**: 236–43.
15 Nagayama K, Kohno T, Sato M, Arai Y, Minna JD, Yokota J. Homozygous deletion scanning of the lung cancer genome at a 100-kb resolution. *Genes Chromosomes Cancer* 2007; **46**: 1000–10.
16 Ten Have-Opbroek AA, Benfield JR, van Krieken JH, Dijkman JH. The alveolar type II cell is a pluripotential stem cell in the genesis of human adenocarcinomas and squamous cell carcinomas. *Histol Histopathol* 1997; **12**: 319–36.
17 Otto WR. Lung epithelial stem cells. *J Pathol* 2002; **197**: 527–35.
18 Edwards DR, Murphy G, Reynolds JJ *et al*. The Transforming growth factor-β modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J* 1987; **6**: 1899–904.
19 Reiss M. TGF-β and cancer. *Microbes Infect* 1999; **1**: 1327–47.
20 Kawakubo H, Brachtel E, Hayashida T *et al*. Loss of B-cell translocation gene-2 in estrogen receptor-positive breast carcinoma is associated with tumor grade and overexpression of cyclin d1 protein. *Cancer Res* 2006; **66**: 7075–82.
21 Aoki T, Du Mori TX, Nisihira T, Matsubara T, Nakamura Y. Allelotype study of esophageal carcinoma. *Genes Chromosomes Cancer* 1994; **10**: 177–82.
22 Sakata K, Tamura G, Nishizuka S *et al*. Commonly deleted regions on the long arm of chromosome 21 in differentiated adenocarcinoma of the stomach. *Genes Chromosomes Cancer* 1997; **18**: 318–21.
23 Ohgaki K, Iida A, Kasumi F *et al*. Mapping of a new target region of allelic loss to a 6-cM interval at 21q21 in primary breast cancers. *Genes Chromosomes Cancer* 1998; **23**: 244–7.
24 Yamamoto N, Uzawa K, Yakushiji T, Shibahara T, Noma H, Tanzawa H. ANA lysis of the ANA gene as a candidate for the chromosome 21q oral cancer susceptibility locus. *Br J Cancer* 2001; **84**: 754–9.
25 Melamed J, Kernizan S, Walden PD. Expression of B-cell translocation gene 2 protein in normal human tissues. *Tissue Cell* 2002; **34**: 28–32.
26 Ajima R, Akiyama T, Usui M *et al*. Osteoporotic bone formation in mice lacking *tob2*; involvement of Tob2 in RANK ligand expression and osteoclasts differentiation. *FEBS Lett* 2008; **582**: 1313–18.
27 Risch A, Plass C. Lung cancer epigenetics and genetics. *Int J Cancer* 2008; **123**: 1–7.
28 Matsumoto S, Iwakawa R, Takahashi K *et al*. Prevalence and specificity of LKB1 genetic alterations in lung cancers. *Oncogene* 2007; **26**: 5911–8.
29 Harada N, Maniwa Y, Yoshimura M *et al*. E1B-deleted adenovirus replicates in p53-deficient lung cancer cells due to the absence of apoptosis. *Oncol Rep* 2005; **14**: 1555–63.
30 Ou YH, Chung PH, Hsu FF, Sun TP, Chang WY, Shieh SY. The candidate tumor suppressor BTG3 is a transcriptional target of p53 that inhibits E2F1. *EMBO J* 2007; **26**: 3968–80.
31 Prevot D, Morel A, Voeltzel T *et al*. Relationships of the antiproliferative proteins BTG1 and BTG2 with CAF1, the human homolog of a component of the yeast CCR4 transcriptional complex: involvement in estrogen receptor alpha signaling pathway. *J Biol Chem* 2001; **276**: 9640–8.
32 Swiatkowska M, Pawlowska Z, Szemraj J, Drzewoski J, Watala C, Cierniewski CS. Cerivastatin, a HMG-CoA reductase inhibitor, reduces plasminogen activator inhibitor-1 (PAI-1) expression in endothelial cells by down-regulation of cellular signaling and the inhibition of *PAI-1* promoter activity. *Jpn J Pharmacol* 2002; **90**: 337–44.
33 Mauxion F, Faux C, Séraphin B. The BTG2 protein is a general activator of mRNA deadenylation. *EMBO J* 2008; **27**: 1039–48.
34 Yoshida Y, Yoshida EH, Nakamura T, Yamamoto T. Association of ANA, a member of the antiproliferative Tob family proteins, with a Caf1 component of the CCR4 transcriptional regulatory complex. *Jpn J Cancer Res* 2001; **92**: 592–6.
35 Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis. *Int J Cancer* 1997; **72**: 1–22.
36 Itoh T, Tanioka M, Yoshida H *et al*. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res* 1998; **58**: 1048–51.
37 Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. *Semin Cancer Biol* 2000; **10**: 415–33.
38 Devy L, Blacher S, Grignet-Debrus C *et al*. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J* 2002; **16**: 147–54.
39 Pyke C, Kristensen P, Ralfkiaer E, Eriksen J, Dano K. The plasminogen activation system in human colon cancer: messenger RNA for the inhibitor *PAI-1* is located in endothelial cells in the tumor stroma. *Cancer Res* 1991; **51**: 4067–71.
40 Kono S, Rao JS, Bruner JM, Sawaya R. Immunohistochemical localization of plasminogen activator inhibitor type 1 in human brain tumors. *J Neuropathol Exp Neurol* 1994; **53**: 256–62.
41 Pederson H, Brunner N, Francis D *et al*. Prognostic impact of urokinase, urokinase receptor, and type 1 plasminogen activator inhibitor in squamous and large cell lung cancer tissue. *Cancer Res* 1994; **54**: 4671–5.
42 Hoikkala S, Paakko P, Soini Y, Makitaro R, Kinnula V, Turpeenniemi-Hujanen T. Tissue *MMP-2*/TIMP-2-complex are better prognostic factors than serum *MMP-2*, MMP-9 or TIMP-1 in Stage I–III lung carcinoma. *Cancer Lett* 2006; **236**: 125–32.
43 Pappot H, Pedersoen AN, Brunner N, Christensen IJ. The complex between urokinase (uPA) and its type-1 inhibitor (PAI-1) in pulmonary adenocarcinoma: relation to prognosis. *Lung Cancer* 2006; **51**: 193–200.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *ana* expression in lung cancer specimens. *ana* mRNA expression level was evaluated for clinical samples of 10 squamous-cell carcinomas (Sq, green) and two large-cell carcinomas (LCC, purple) by real-time reverse transcription–polymerase chain reaction. *ana* expression in the cancerous tissue was divided by that in corresponding noncancerous tissue to obtain the expression level.

Fig. S2. *ana* expression in lung cancer cell lines. The level of *ana* mRNA expression in normal lung tissue purchased from Clontech was set to 1. *ana* expression level was normalized by *GAPDH*. SCC (red), small-cell carcinoma cell lines; Ad (yellow), adenocarcinoma cell lines; Sq (green), squamous-cell carcinoma cell lines; LCC (purple), large-cell carcinoma cell lines. NCI-H596 (blue graph) and HCC366 cells are adenosquamous carcinoma cell lines. The cells written in red lacked *ana* expression.

Fig. S3. Developmental expression of *ana* in lung. X-gal staining of lung from $ana^{+/-}$ mice at various developmental stages. *ana* expression in different developmental stages was monitored by X-gal staining. Black arrows indicate bronchial epithelial cells. Original magnification: ×200.

Fig. S4. Ana does not affect cell proliferation. Proliferation in A549, NCI-H322, NCI-H1299, and HCC366 cells transfected with the retrovirus carrying *ana* cDNA (A) or with control retrovirus (V). Error bars represent ± SE. (a) Soft agar colony formation assay was performed. (b) Cells were cultured under low serum conditions. Triplicate samples were counted at the indicated times. (c) Western blot analysis of protein extracts from control cells and Ana-overexpressing cells to check Ana protein expression.

Fig. S5. *tob2* expression in lung. Expression of *tob2* was monitored by X-gal staining of the lung from 3-month-old *tob2* hetero-deficient mice. X-gal positive cells were stained blue. Sections were counterstained with Kernechtrot stain solution (red). Original magnification: ×200.

Fig. S6. Ana represses transcriptional activity on *PAI-1* promoter. A549 cells (3×10^5 cells per well in 12-well tissue culture plates) were cotransfected with FuGENE6 Transfection Reagent (Roche Diagnostics) and the following plasmids: p800LUC containing *PAI-1* promoter fragment corresponding to positions from +71 to –800 (80 ng/well), Ana expression vector (A), or empty vector (V) (500 ng/well). After 24 h, cell extracts were analyzed for luciferase activity with the Dual-Luciferase Reporter System (Promega). Transfection efficiency was standardized with an internal control plasmid, pRL-β-actin (0.5 ng/well).

Table S1. Primer or probe sets for real-time reverse transcription–polymerase chain reaction (RT-PCR)

Table S2. Various tumors in $ana^{-/-}$ and wild-type mice

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

doi: 10.1111/j.1349-7006.2008.01030.x