**Activating Transcription Factor 3, a Stress-inducible Gene, Suppresses Ras-stimulated Tumorigenesis**

Received for publication, August 23, 2005, and in revised form, December 21, 2005. Published, JBC Papers in Press, February 9, 2006, DOI 10.1074/jbc.M509278200

Dan Lu, Curt D. Wolfgang1, and Tsonwin Hai2

From the Ohio State Biochemistry Program, Department of Molecular and Cellular Biochemistry and Center for Molecular Neurobiology, Ohio State University, Columbus, Ohio 43210

**ATF3** is a stress-inducible gene that encodes a member of the ATF/CREB family of transcription factors. Current literature indicates that **ATF3** affects cell death and cell cycle progression. However, controversies exist, because it has been demonstrated to be a negative or positive regulator of these processes. We sought to study the roles of **ATF3** in both cell death and cell cycle regulation in the same cell type using mouse fibroblasts. We show that **ATF3** promotes apoptosis and cell cycle arrest. Fibroblasts deficient in **ATF3** ([**ATF3**/−/−]) were partially protected from UV-induced apoptosis, and fibroblasts ectopically expressing **ATF3** under the tet-off system exhibited features characteristic of apoptosis upon **ATF3** induction. Furthermore, **ATF3**+/− fibroblasts transitioned from G1 to S phase more efficiently than the **ATF3**+/+ fibroblasts, suggesting a growth arrest role of **ATF3**. Consistent with the growth arrest and pro-apoptotic roles of **ATF3**, **ATF3**+/− fibroblasts upon Ras transformation exhibited higher growth rate, produced more colonies in soft agar, and formed larger tumor upon xenograft injection than the **ATF3**+/+ counterparts. **ATF3**/−/− cells, either with or without Ras transformation, had increased RB phosphorylation and higher levels of various cyclins. Significantly, **ATF3** bound to the cyclin D1 promoter as shown by chromatin immunoprecipitation (ChIP) assay and repressed its transcription by a transcription assay. Taken together, our results indicate that **ATF3** promotes cell death and cell arrest, and suppresses Ras-mediated tumorigenesis. Potential explanations for the controversy about the roles of **ATF3** in cell cycle and cell death are discussed.

During cancer development, the cells encounter many stress signals, including genotoxic damages, inappropriate activation of oncogenes, telomere erosion, hypoxia, and nutrient deprivation in the tumor microenvironment (for review, see Ref. 1). All along, the cells have built-in mechanisms to restrain or eliminate themselves (for review, see Ref. 2). A prominent example is p53, which upon stress induction either arrests or kills cells (for reviews, see Refs. 3 and 4). Another example is oncogene-induced killing: oncogenic stress, such as inappropriate activation of the E2F1 and c-Myc oncogenes, triggers apoptosis (for reviews, see Refs. 1 and 5). Therefore, the successful cancer cells are those that manage to foil the hardwired stress response to eliminate themselves during the process of transformation from normal cells to cancerous cells. Thus, to understand cancer development, it is important to study the stress response genes that may play an important role in this self-eliminating safeguard process.

Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors. Overwhelming evidence indicates that **ATF3** is a stress-inducible gene: its mRNA level is low or not detectable in most cells, but is greatly induced by a variety of stress signals, including genotoxic agents such as ultraviolet light (UV), benz[a]pyrene diol epoxide (BPDE), ionizing radiation, and methyl methane-sulfonate (for reviews, see Refs. 6 and 7). In addition, **ATF3** is induced by ischemia (8, 9) and hypoxia, conditions encountered by cancer cells in the tumor microenvironment. Emerging evidence suggests that **ATF3** may play a role in cancer development. It has been reported to affect cell death and cell cycle progression, two processes that regulate the growth of cancer cells. However, controversies remain for its roles in these processes. For cell death, **ATF3** has been reported to be either pro-apoptotic or anti-apoptotic. Ectopic expression of **ATF3** induced apoptosis in ovarian cells (11) and enhanced the ability of etoposide or camptothecin to induce apoptosis in HeLa cells (12), suggesting a pro-apoptotic role of **ATF3**. Consistently, primary islets derived from **ATF3** knock-out (ATF3−/−) mice were partially protected from cytokine- and nitric oxide-induced apoptosis (13). Furthermore, antisense **ATF3** reduced stress-induced apoptosis in endothelial cells (14). Therefore, both gain-of-function (ectopic expression) and loss-of-function (knock-out and antisense) approaches support a pro-apoptotic role of **ATF3**. However, several reports suggest that **ATF3** is anti-apoptotic. Adenovirus-mediated expression of **ATF3** reduced nerve growth factor (NGF) withdrawal-induced apoptosis in superior cervical ganglion neurons in *vitro* (15), suppressed kainic acid-induced death in hippocampal neurons in *vivo* (16), and inhibited adriamycin-induced apoptosis in primary cardiomyocytes in *vitro* (17). Thus far, the anti-apoptotic role of **ATF3** has not been demonstrated by the loss-of-function approach. For cell cycle regulation, some reports suggest that **ATF3** promotes cell proliferation. Ectopic expression of **ATF3** by transient transfection moderately induced DNA synthesis in hepatic tumor cells (18). Furthermore, retrovirus-mediated stable expression of **ATF3** promoted the proliferation of chick embryo fibroblasts under low serum concentrations (19). In contrast, however, ectopic expression of **ATF3** suppressed cell cycle progression in HeLa cells (20). Therefore, similar to the situ-

---

1. This work was supported by Grant DK59605 (to T. H.) from the National Institutes of Health and Grant 7-05-RA-52 (to T. H.) from the American Diabetes Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

2. Current address: Vanda Pharmaceuticals, Inc., 9620 Medical Center Dr., Suite 201, Rockville, MD 20850.

3. K. Ameri, C. Culmsee, M. Raida, D. M. Katschinski, R. H. Wenger, T. Hai, E. Wagner, and A. L. Harris, submitted manuscript.
ATF3 as a Tumor Suppressor

One potential explanation for the above conflicting results is the diverse cell types used in the studies, ranging from primary islets or neurons to hepatic tumor cells. Other explanations include the varying levels and durations of ATF3 expression, and the differences in the approaches used in the studies; some used the gain-of-function approach whereas others used the loss-of-function approach. We sought to study the roles of ATF3 in cell death and cell cycle regulation in the same cell type using fibroblasts. In addition, we tested the hypothesis that ATF3 plays a role in cancer development using the Ras-stimulated transformation of fibroblasts as a model, a well established and widely accepted model for studying tumorigenesis. In this report, we show that ATF3 promoted apoptosis and cell cycle arrest. Its action on cell cycle arrest correlated with reduced phosphorylation of Rb and reduced protein levels of various cyclins in ATF3+/- cells compared with that in intact cells. Furthermore, we show that ATF3 suppressed Ras-stimulated tumorigenesis, at least in part, by inhibiting cell proliferation and promoting cell death.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and the JNK-I Inhibitor—ATF3 knock-out mice in the C57BL/6 background were described previously (13). Primary mouse embryonic fibroblasts (MEFs) were isolated from day 13.5 wild-type C57BL/6 or ATF3 knock-out embryos and immortalized by the 3T3 protocol: passaged at the density of 9 × 10^5 cells per 6-cm plate every 3 days (21). MEFs were maintained in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM nonessential amino acid, and 55 μM β-mercaptoethanol. Phoenix ecotropic virus packaging cells were maintained in DMEM supplemented with 10% FBS. pRetro-off-HA-ATF3 was generated by inserting hemagglutinin (HA)-tagged human ATF3 open reading frame into the NotI site of pRetro-off vector (Clontech), pRetro-off-HA-ATF4 by inserting the HA-ATF4 open reading frame into the BamHI site of the vector, and pRetro-off-HA-ATF3 (1–100) by inserting the DNA fragment encoding HA-tagged ATF3 amino acids 1–100 into the NotI site. pBabe-puro, pBabe-hygro, and pBabe-puro-H-Ras(V12) were kindly provided by Dr. Gustavo Leone (Ohio State University). pBabe-hygro-ATF3 was generated by inserting the open reading frame of human ATF3 into the EcoRI and SalI sites of pBabe-hygro. JNK-I, a cell-permeable peptide that inhibits the activation of the JNK pathway (22), was from the Cleveland Clinic Foundation.

Retrovirus Production and Infection—The Phoenix ecotropic virus packaging cells were transfected with pBabe constructs using the calcium phosphate method; the medium containing the viruses was collected 48 h after transfection and aliquots kept at −80 °C until use. MEFs were infected with high titer retroviruses in the presence of 4 μg/ml polybrene and selected by adding the appropriate antibiotics (puromycin 2.5 μg/ml or hygromycin 250 μg/ml) at 48 h after infection. In general, more than 50% of the cells survived selection, and by day 4 most of the non-transduced cells had died off. Transformation was judged successful if the cells displayed morphological changes characteristic of Ras transformation (highly refractile with thin and long projections). The resulting pools of transfected cells on day 7 were used in subsequent experiments. All results presented were derived from at least three repeated experiments using independently transfected cells and were reproducible using two batches of immortalized cells derived from different litters of mice.

Generation of Tet-off Stable Cells and Apoptosis Assays—Stable cell lines were established by transfecting the wild-type fibroblasts with various pRetro-off constructs followed by puromycin selection. Individual colonies were selected, expanded, and maintained in the presence of tetracycline and puromycin. Each stable cell line was grown in the absence of tetracycline for the indicated times to induce the expression of the transgenes before assays. For trypan blue stain, both the floating and attached cells were collected and stained, and the blue cells scored as dead. For Annexin V stain, the cells were grown on Superfrost Plus slides (VWR Scientific); the unfixed cells were stained with 5% Annexin V-FITC (BD Pharmingen) and 5 μg/μl propidium iodide (P1) (Sigma), and visualized using a Bio-Rad MRC 1024 confocal microscope. For DAPI stain, the cells were fixed with 4% paraformaldehyde and stained with 1 μM DAPI. For TUNEL assay, the cells were fixed, permeabilized, and incubated with hydrogen peroxide before incubated with biotin-dCTP and terminal transferase (Invitrogen). The signals were detected by the ABC complex followed by the DAB substrate solution (Vector). For DNA laddering, both the floating and attached cells were collected and the genomic DNAs extracted for analysis on a 2% agarose gel.

Serum Stimulation and BrdU Labeling—Cells at about 50% confluency were serum-starved with 0.1% FBS for 24 h and restimulated with 10% FBS for indicated times before harvesting for immunoblot analysis (below), transcription assay (below), or BrdU labeling (Roche Applied Science) according to the manufacturer’s instructions.

UV Treatment and Viability Assay—2 × 10^5 cells were seeded on 6-cm plates and treated with UV at the dose indicated in the Fig. 1 legend. At various times after UV treatment, cells were either harvested for immunoblot analysis (below) or assayed for viability using crystal violet stain quantified by A595 reading (24). The A595 reading at 48 and 72 h after UV treatment was standardized against the A595 of the respective cells upon seeding (at 4 h after seeding when the cells became attached) to reduce the artifacts caused by seeding variations. The standardized A595 reading of the wild-type cells at each time point was arbitrarily defined as 1 to obtain the relative cell viability of the knock-out cells.

Chromatin Immunoprecipitation (ChIP) Assay—Cells were incubated with 1% formaldehyde at room temperature for 10 min to cross-link proteins and DNAs, followed by sonication to shear the DNAs to an average size of 500–1000 bp. Immunoprecipitation was carried out using 2 μg of ATF3 antibody (Santa Cruz Biotechnology) or IgG. After reversal of cross-linking, the DNA fragments were purified by phenol extraction and ethanol precipitation, followed by PCR analysis using primers flanking the CRE/ATF site on the cyclin D1 promoter: 5′-CGAGCGATTTGCATATCTACC-3′ (upstream) and 5′-GTAGTCCGTGTGTGACGTTACTG-3′ (downstream).

Transcriptional Assay of Endogenous Cyclin D1 Gene—Nuclear RNAs were isolated from the nuclei of serum-starved and restimulated cells using the TRIzol method (Invitrogen) and treated with DNase I to remove the contaminating genomic DNAs. The cyclin D1 pre-mRNAs were assayed by reverse transcription coupled with polymerase chain reaction (RT-PCR) as detailed previously (8) using a primer set targeted to the exon 2 and intron 2 of the corresponding gene: 5′-TTGACTGCCCAGAAGTTGCTG-3′ (upstream) and 5′-ACAGAGGTAGATGGTTTG-3′ (downstream). A control RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included using the following primers: 5′-CCCGATCTCCGGAAAGCTTGTATCATCAG-G3′ (upstream) and 5′-GGCTCGAGGCAGTGAAGCCGACCG-3′ (downstream). Reactions without reverse transcriptase were included to confirm that the signals were not derived from the genomic DNAs.
Cell Growth at Low Concentrations of Serum and Colony Formation in Soft Agar—For cell growth analysis, 1 x 10⁴ cells were seeded into each well of a 6-well plate and grown in the presence of 0.1–2% FBS for 1–7 days as indicated in the Fig. 5 legend. Cells were stained by crystal violet and the A595 readings measured. For anchorage-independent growth, 5 x 10⁶ cells were resuspended in 4 ml of growth medium containing 0.3% agarose and plated on 6-cm plates containing a solidified bottom layer made of 0.6% agarose in medium. After the 0.3% agarose solidified, 3 ml of growth medium were added to the plates and replaced every 3 days. 21 days after plating, colonies were stained with methylthiazolyldiphenyl-tetrazolium (MTT) and imaged at x10 magnification. Each experiment was performed with duplicate plates.

Xenograft Tumor Formation Assays—2 x 10⁶ cells were resuspended in 100 μl of sterile phosphate-buffered saline and injected subcutaneously (s.c.) into the flank or intravenously (i.v.) into the tail vein of 8–10 week-old athymic NCr male mice (Taconic). For s.c. injection, ATF3+/+ and ATF3−/− cells were injected into the right and left flanks of the same mouse to eliminate the differences due to the host. Tumor size was determined at 3-day intervals by measuring the length (L) and width (W) of the tumor using a pair of calipers, and the tumor volume calculated as (L x W²)/2 (23). At 21 days after injection, mice were weighed to obtain their body weights and euthanized. Subcutaneous tumors and the lungs were excised and weighed, and the ratio of lung to total body weight was calculated. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Ohio State University.

Immunoblot and Immunohistochemistry Analysis—Equal amounts of whole cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblot using polyvinylidene fluoride membrane (Immobilon-P; Millipore) and various primary antibodies: ATF3, Cdk2, Cdk4, cyclin A, cyclin E, Rb, Erk (Santa Cruz Biotechnology), p-Rb, cleaved caspase 3, cleaved PARP (Cell Signaling), actin (Sigma), and cyclin D1 (Calbiochem). Bound primary antibodies were detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) and Lumi-Light Western blotting substrate (Roche Applied Science). Paraffin-embedded xenograft tumor sections were analyzed for phosphohistone H3 and cleaved caspase 3 by immunohistochemistry as described before (8) using the antibody against phosphohistone H3 (Upstate Biotechnologies) or cleaved caspase 3 (Cell Signaling). To quantify the phosphohistone H3-positive cells, the positive cells within the entire tumor sections were counted. The tumor area was measured by the Meta Vue program under ×40 magnification, and the positive cells per mm² were calculated.

Statistical Analysis—All numerical values are mean ± S.E. Comparison between two groups was made by two-sample Student’s t test, and comparison among three groups by one-way analysis of variance (ANOVA). p < 0.05 was considered statistically significant.

RESULTS

ATF3 Is Pro-apoptotic in Immortalized Fibroblasts—We isolated mouse embryonic fibroblasts (MEFs) from wild-type (ATF3+/+) and ATF3 knock-out (ATF3−/−) mice (13), and immortalized them as detailed under “Experimental Procedures.” To test whether ATF3 deficiency affects the cells in their response to stress-induced apoptosis, we used the UV-induced apoptosis as a paradigm. Fig. 1A and B show that UV induced ATF3 in the wild-type fibroblasts but not the knock-out cells. The induction was detected 2 h after treatment, consistent with previous reports that ATF3 is induced by stress signals within 2 h in most stress paradigms (for a review, see Ref. 6). Significantly, ATF3−/− cells were partially protected from UV-induced apoptosis as evidenced by increased cell viability at 48 and 72 h after treatment (Fig. 1C), decreased activation of caspase 3, and decreased cleavage of poly(ADP-ribose) polymerase (PARP) (Fig. 1D). Fig. 1C shows the averages from three experiments and Fig. 1D is a representative result. Taken together, these results suggest a pro-apoptotic role of ATF3. To confirm this conclusion by a complementary approach, we attempted to express ATF3 ectopically in the wild-type fibroblasts. Although stable cells constitutively expressing ATF3 were reported by others in HeLa cells (12), HT1080 cells (24), and chicken embryo fibroblasts (19), we were unable to establish such stable lines using the mouse fibroblasts. Therefore, we used the tet-off system to express ATF3 in an inducible manner. We analyzed three clones and compared them to three control cell lines generated in parallel: (a) ATF3−/− line expressing a mutant ATF3 that lacks the leucine zipper domain and does not bind to DNA (25), (b) ATF4 line expressing another member of the ATF/CREB family of transcription factors, (c) a vector control line. As shown in Fig. 2A, expression of ATF3 led to reduced cell viability as assayed by trypan blue exclusion test; however, expression of ATF3−/− or ATF4 did not. We then analyzed one ATF3 clone (clone 38) and compared it to the vector control cells. As shown in Fig. 2B–E, expression of ATF3 led to features characteristic of apoptosis: 1) membrane inversion by Annexin V stain (panel B), 2) pyknotic nuclei by DAPI stain (panel C), DNA fragmentation by TUNEL assay (panel D), and DNA laddering (panel E). Therefore, both loss-of-function and gain-of-function approaches demonstrated that ATF3 is pro-apoptotic in mouse fibroblasts.

ATF3 Inhibits Serum Stimulation-induced Cell Cycle Progression—To determine whether ATF3 plays a role in cell cycle progression, we serum-starved the ATF3+/+ and ATF3−/− cells for 3 days and simulated them with 10% serum. As shown in Fig. 3A, serum stimulation transiently induced ATF3 expression in the wild-type cells. BrdU labeling indicated that ATF3−/− cells progressed from G1 to S phase more efficiently than the ATF3+/+ cells. At 16 h after serum stimulation, about 60% (62 ± 4%) of the ATF3−/− cells underwent DNA replication, but only about 45% (44 ± 2%) of the ATF3+/+ cells did (Fig. 3B, p < 0.05). The difference between the wild-type and knock-out cells remained at 20 h after serum stimulation (Fig. 3B, p < 0.05). At 9 and 12 h after

FIGURE 1. ATF3−/− fibroblasts were partially protected from UV-induced apoptosis. A and B, ATF3+/+ (A) or ATF3−/− and ATF3−/− cells (B) were treated with 45 J/m² of UV, harvested at the indicated times after treatment, and analyzed by immunoblot using the antibody against ATF3 or Erk (control). C, ATF3+/+ and ATF3−/− cells were treated with 80 J/m² of UV, stained by crystal violet at the indicated times after treatment, and the relative cell viability was calculated as detailed under “Experimental Procedures.” *, p < 0.05 (ATF3−/− versus ATF3+/+). D, ATF3+/+ and ATF3−/− cells were treated with 80 J/m² of UV, harvested at the indicated times after treatment, and analyzed by immunoblot using antibodies against activated caspase 3, cleaved PARP, and actin.

ATF3 as a Tumor Suppressor

JOURNAL OF BIOLOGICAL CHEMISTRY 10475

APRIL 14, 2006 VOLUME 281 NUMBER 15

CELL BIOLOGY
serum stimulation, some cells had already progressed into S phase in both wild-type and knock-out cells, but there was no difference between them. Fig. 3B shows the average of three experiments and Fig. 3C shows a representative image of BrdU-stained cells at different time points.

**ATF3 as a Tumor Suppressor**

ATF3 Inhibits Ras-stimulated Cell Growth at Low Serum Concentrations and in Soft Agar—Because cell cycle arrest and cell death are important brakes for cancer cell progression, the above results suggested that *ATF3* may function as a tumor suppressor. To test this hypothesis, we used the Ras-stimulated transformation of immortalized fibroblasts, a well established and widely accepted paradigm for studying transformation. We transduced the immortalized wild-type (*ATF3*/*H11001*/H11001) and knock-out (*ATF3*/*H11003*/H11003) fibroblasts with a retroviral vector expressing oncogenic H-Ras (RasV12) or an empty vector, and selected the transduced cells with appropriate antibiotics. As shown in Fig. 4A, *ATF3*
expression was induced in the wild-type cells upon Ras(V12) transduction. This is an important result, because ATF3, as an inducible gene, is not expressed (or expressed at a very low level) in untreated wild-type cells. If ATF3 is not induced by oncogenic Ras in the wild-type cells, the deficiency of ATF3 in the knock-out cells may not have any detectable consequences in this paradigm. Oncogenic Ras activates many signaling pathways, including the JNK pathway (for review, see Ref. 26). Because JNK pathway is involved in the induction of ATF3 by various signals (13, 27), we examined whether the JNK pathway is involved in the induction of ATF3 by Ras. Fig. 4B shows that treatment of the Ras-transformed cells with JNK-I, a cell-permeable inhibitor of the JNK pathway (22), reduced the expression of ATF3, indicating that the induction of ATF3 by H-Ras(V12) is mediated, at least in part, by the JNK pathway.

For the convenience of discussion, we will refer to the Ras-transformed wild-type cells as Ras/ATF3+/+ and the Ras-transformed knock-out cells as Ras/ATF3−/− cells. All results presented below involving the Ras-transformed cells were derived from at least three independent experiments. For each experiment, pools of retrovirus-transduced cells (detailed under "Experimental Procedures") were used for the assays. To rule out the possibility that the differences between Ras/ATF3+/+ and Ras/ATF3−/− cells were caused by the immortalization process rather than the ATF3 deficiency in the knock-out cells, we generated a second batch of immortalized ATF3+/+ and ATF3−/− MEFs and transformed them with H-Ras(V12). Similar results were obtained from the second batch of immortalized cells (data not shown).

To examine whether ATF3 deficiency affects Ras-stimulated transformation, we examined the growth of Ras/ATF3+/+ and Ras/ATF3−/− cells at low concentrations of serum. Time course analysis showed that the cell number of Ras/ATF3−/− cells increased faster than that of Ras/ATF3+/+ cells; the difference was detectable starting at day 3 after seeding and continued to day 7 when the plates became confluent and the experiments terminated (Fig. 5A, *p < 0.05, **p < 0.01). Dose analysis indicated that the increase of cell number over a 4-day period (from day 2 to day 6) was statistically different (p < 0.05) between Ras/ATF3+/+ and Ras/ATF3−/− cells at 1% of serum (Fig. 5B). To test whether the difference was caused by the deficiency of ATF3 in the knock-out cells, we complemented the Ras/ATF3−/− cells with ATF3 by retroviral transduction. As shown by both time course and dose analyses, ATF3 add-back reduced the cell number to a level comparable to that of the
Ras/ATF3+/− cells (Fig. 5, A and B). At 0.1% serum, none of the cells grew much, and there were no differences among the three groups of cells. Because a decrease in cell number could be caused by a decrease in cell proliferation, an increase in cell death, or both, we examined the cells by BrdU labeling. As shown in Fig. 5C, Ras/ATF3+/− cells proliferated better than Ras/ATF3−/− cells at 1% of serum: about 60% (58.1 ± 0.9%) of the cycling Ras/ATF3−/− cells were in the S phase, but only about 40% (43.3 ± 0.4%) of the cycling Ras/ATF3+/− cells were (p < 0.01). ATF3 add-back reduced the proliferation of the Ras/ATF3−/− cells. We did not observe obvious cell death in either Ras/ATF3+/+ or Ras/ATF3−/− cells under this condition (data not shown). Thus, ATF3 deficiency promoted the proliferation of Ras-transformed cells under low concentrations of serum. We also compared Ras/ATF3+/+ and Ras/ATF3−/− cells for Rb phosphorylation and found that ATF3 deficiency promoted colony formation. Fig. 5D shows a photograph of the colonies in soft agar (a representative of four experiments). Control cells transduced by vector only did not result in any colonies (data not shown).

ATF3 Modulates the Expression of Various Cell Cycle Components—Because hyperphosphorylation of retinoblastoma (Rb) protein is a key marker for G1 to S transition (for reviews, see Refs. 28 and 29), we examined the phosphorylation of endogenous Rb in wild-type and knock-out cells, either with or without Ras transformation. As shown in Fig. 6 (left panel), Rb phosphorylation is induced upon serum stimulation in both ATFS+/+ and ATFS−/− cells. However, the level of phosphorylation was higher in the ATFS−/− cells than that in the ATFS+/+ cells. Similarly, the level of Rb phosphorylation was higher in the Ras/ATFS−/− cells than that in the Ras/ATFS+/+ cells when the cells were grown at 1% serum (Fig. 6, right panel), a condition under which the Ras/ATFS+/+ and Ras/ATFS−/− cells had significant difference in their proliferation (as shown in Fig. 5C). Because phosphorylation of Rb requires the activity of cyclin/Cdk complexes (for reviews, see Refs. 28 and 29), we examined various cell cycle regulators and found that the steady-state protein levels of cyclin A, cyclin D1, and cyclin E were higher in the ATFS−/− cells than that in the ATFS+/+ cells, but the level of Cdk4 was about the same (Fig. 6, left panel). Although the level of Cdk2 was also higher in the ATFS−/− cells, the difference was subtle. We also examined the wild-type and knock-out cells after oncogenic Ras transformation and found that the levels of cyclin A, cyclin D1, and cyclin E were slightly higher in the Ras/ATFS−/− cells than that in the Ras/ATFS+/+ cells (right panel). Taken together, our results indicate that ATF3 inhibits G1 to S transition in mouse fibroblasts, at least in part, by directly or indirectly affecting the steady-state protein levels of various cyclin molecules.

The cyclin D1 promoter contains a CRE/ATF site (5′-TAACGTCACA-3′), a potential binding site for ATF3. Because ATF3 is a transcriptional repressor, the lower protein levels of cyclin D1 in the ATFS+/+ cells prompted us to examine whether ATF3 represses the transcription of the cyclin D1 promoter. An established method to detect endogenous gene transcription is to measure the primary transcripts (pre-mRNAs) (30–32). We thus isolated the nuclear RNAs from serum-stimulated Ras/ATFS+/+ and ATFS−/− cells and examined their cyclin D1 pre-mRNA levels by RT-PCR using an upstream primer targeted at exon 2 and a downstream primer targeted at intron 2 of the cyclin D1 gene. If ATF3 represses the transcription of the cyclin D1 gene, its pre-mRNA level should be lower in the ATFS+/+ cells than that in ATFS−/− cells. Fig. 7A shows the expected results. The lack of RT-PCR signals in the absence of the reverse transcriptase (RT−) confirmed that the signals were not derived from the genomic DNAs. We next examined the binding of ATF3 to the cyclin D1 promoter by ChIP assay. As shown in Fig. 7B, ATF3 bound to the cyclin D1 promoter in vivo. Taken together, our results support the notion that cyclin D1 is a direct target gene of ATF3 and ATF3 represses its expression.

ATF3 Suppresses Ras-stimulated Tumorigenesis in Vivo—To examine whether ATF3 can inhibit tumor formation in vivo, we injected (s.c.) the Ras/ATFS+/+ and Ras/ATFS−/− cells in nude mice. To avoid potential differences because of the host, we injected these cells separately to the right and the left flanks of the same mouse. The experimenter injecting the cells was unaware of the genotypes. As shown in Fig. 8A, Ras/ATFS−/− cells formed larger tumors than Ras/ATFS+/+ cells at 21 days after injection (p < 0.05). Representative pictures of the tumors both macroscopically and microscopically after hematoxylin and eosin (H&E) stain are shown in panel B. Time course measurement of the tumor size at 3-day intervals indicated that the Ras/ATFS−/− tumors...
DISCUSSION

ATF3 in Cell Death: Pro- or Anti-apoptotic?—In this report, we show that ATF3 is pro-apoptotic in mouse fibroblasts by a gain-of-function approach using the tet-off system to express ATF3 and by a loss-of-function approach using ATF3 knock-out cells. We note that the fibroblasts cell lines expressing ATF3 under the tet-off system were not stable. After several passages (fewer than 5), induction of ATF3 resulted in cell cycle arrest (data not shown), instead of apoptosis. Although the cells were maintained and propagated in the presence of tetracycline to turn off the expression of ATF3, we speculate that ATF3 was expressed at a low leaky level, resulting in the selection of cells with genetic alterations that led to phenotypic changes upon ATF3 induction. In addition to its pro-apoptotic function in fibroblasts described in this report, ATF3 was demonstrated to be pro-apoptotic in several other cell types: ovarian epithelial cells (11), HeLa cells (12), primary islets (13), and primary endothelial cells (14). The pro-apoptotic function of ATF3 is consistent with our previous reports that ATF3 was deleterious to various tissues upon transgenic expression in mice: (a) mice expressing ATF3 in the heart had conduction abnormalities and contractile dysfunction (35); (b) mice expressing ATF3 in the liver had liver dysfunction (36); (c) mice expressing ATF3 in the pancreas had islet dysfunction and defects in glucose homeostasis (8, 13).

However, as described in the introduction some reports showed an anti-apoptotic role of ATF3 (15–17). One explanation for this apparent discrepancy is that the function of ATF3 is context-dependent. That cellular context affects the function of a given gene is a recurring theme in biology; examples include NFκB, p53 and TGFβ (37–41). For ATF3, we speculate that at least two aspects of the cellular contexts, cell type and the status of malignancy, affect its function. Thus far, all reports derived from acute neuronal injury models have been consistent: ATF3 expression is protective. Ectopic expression of ATF3 prevented NGF withdrawal-induced apoptosis (15), prevented kainic acid-induced death (16), enhanced neuronal protrusion (42), and promoted neuronal regeneration.5 Therefore, ATF3 appears to be protective in neuronal cells during acute injuries. Another aspect of cellular context that appears to affect the role of ATF3 in apoptosis is the malignancy of the cells. We found that ATF3 promoted apoptosis in a non-malignant epithelial cell line, but failed to do so in a highly malignant line derived from this cell.6 Clearly, much remains to be determined regarding the cellular contexts that affect the function of ATF3. Our proposed explanations (cell type and the status of malignancy) do not resolve two discrepancies in the literature. First, ATF3 was demonstrated to be both anti-apoptotic (43) and pro-apoptotic (14) in TNFα-induced cell death in primary endothelial cells. Because the cell type (human umbilical vein
grew faster and had a shorter lag time before obvious size increase (Fig. 8C). We also injected the tumor cells in the nude mice intravenously (i.v.) via the tail vein and assayed the ability of these cells to establish tumors in the lung. As shown in Fig. 8D, Ras/ATF3−/− cells again formed larger tumors than Ras/ATF3+/− cells at 21 days after injection (p < 0.01). Representative pictures of the lung tumors both macroscopically and microscopically are shown in panel E.

As described above, ATF3 had pro-apoptotic and growth arrest function in immortalized fibroblasts (Figs. 1–3, 5, 6). To determine whether these functions contributed, at least in part, to the smaller size of the Ras/ATF3+/− tumors than the Ras/ATF3−/− tumors, we assayed the tumors by immunohistochemistry for proliferation using antibody against phosphohistone H3, a mitotic marker (33, 34), and for apoptosis using antibody against activated caspase 3, an apoptotic marker. As shown in Fig. 9A, solid tumors (s.c. injection) derived from the Ras/ATF3−/− cells had a higher mitotic index than that from the Ras/ATF3+/− cells (51 ± 1/mm² versus 34 ± 2/mm²; p < 0.05). Lung tumors showed the same trend (Fig. 9B, 67 ± 6/mm² versus 43 ± 3/mm²; p < 0.05). Representative pictures are shown on the right of each panel. Activated caspase 3 stain indicated that Ras/ATF3−/− tumors contained fewer apoptotic cells than the Ras/ATF3+/− tumors (Fig. 9C). Taken together, our results indicate that ATF3 promoted apoptosis and cell cycle arrest in immortalized mouse fibroblasts that were either transformed (Figs. 5, 6, and 9) or untransformed by oncogenic Ras (Figs. 1–3 and 6).
ATF3 as a Tumor Suppressor

FIGURE 9. Ras/ATF3−/− tumors had higher mitotic index and lower apoptosis than Ras/ATF3+/− tumors. A and B, subcutaneous (A) or lung (B) tumors derived from Ras/ATF3−/− and Ras/ATF3+/− cells were analyzed for phosphohistone H3, a mitotic marker, by immunohistochemistry. Phosphohistone H3-positive cells per mm² were determined as detailed under "Experimental Procedures"; the mean ± S.E. from three mice is shown. *, p < 0.05 and **, p < 0.01 (Ras/ATF3−/− versus Ras/ATF3+/−). Representative images are shown on the right. C, indicated subcutaneous tumors were analyzed for apoptosis by immunohistochemistry using antibody against activated caspase 3. Representative images are shown.

endothelial cells) and stress paradigm (TNFα) were the same, it is not clear why opposite results were obtained. Second, the roles of ATF3 in cardiac cells remain to be resolved. Adenovirus-mediated expression of ATF3 prevented primary cardiomyocytes from adriamycin-induced apoptosis (17), but transgenic mice expressing ATF3 in the heart had cardiac dysfunctions (35). One possibility is that the process of isolation or adenoviral infection affected the cells, resulting in different responses of cardiac cells to ATF3 expression in vitro versus in vivo. Another possibility is the duration of ATF3 expression: the in vitro experiments entailed transient expression of ATF3, whereas the in vivo transgenic mouse model entailed constitutive expression of ATF3.

ATF3 in Cell Cycle Regulation: Cell Cycle Arrest or Progression?—Our results support a cell cycle arrest role of ATF3: ATF3−/− cells, whether transformed by oncogenic Ras or not, proliferated better than the corresponding ATF3+/− cells in vitro (Figs. 3, 5, and 6) and in vivo as xenograft tumors (Fig. 9). All these results were derived from a loss-of-function approach. Two observations using the gain-of-function approach support a growth arrest role of ATF3. First, Fan et al. (20) demonstrated that ectopic expression of ATF3 suppressed cell cycle progression in HeLa cells. Second, the tet-off ATF3 cells described in this report, after several passages, exhibited phenotypic change from apoptosis to G₁ arrest upon ATF3 induction (as discussed above). Thus, both gain- and loss-of-function approaches support a growth arrest function of ATF3. However, this conclusion is in conflict with that from several other reports. Allan et al. (18) reported that ATF3 promoted G₁ to S transition, albeit moderately. One potential explanation for the discrepancy is the cell type difference: Allan et al. used hepatic tumor cells rather than the fibroblasts reported here and the adenocarcinoma cells of the cervix by Fan et al (20). Our results are also in contrast to the report that ATF3 can partially transform chick embryo fibroblasts at least in part by promoting proliferation in low serum concentrations (19). Chick embryo fibroblasts are known to behave differently from mouse embryo fibroblasts, because they can be transformed by a single oncogene (Ref. 44 and references therein) in contrast to the need for cooperating oncogenes to transform rodent fibroblasts (45). Therefore, the differences between our report and that of Perez et al. (19) may be caused by the species difference. During the preparation of this report, Tamura et al. (46) reported that ATF3 is a target gene of c-Myc and promotes cell cycle progression. One explanation for this discrepancy is that their studies were carried out in rat fibroblasts whereas ours were in mouse fibroblasts. An example of previously known discrepancies between mouse and rat fibroblasts is c-Myc deficiency, which completely blocks the proliferation of mouse fibroblasts (47, 48) but not that of rat fibroblasts (49). Therefore, similar to the situation in apoptosis, the function of ATF3 in cell cycle regulation appears to be context-dependent and much remains to be determined.

ATF3 in Cancer Development—In this report, we demonstrate for the first time that ATF3 could function as a tumor suppressor in Ras-mediated tumorigenesis in vitro and in vivo. We note that solid tumors derived from the Ras/ATF3−/− cells grew fast with a rapid increase in size starting at day 9 after injection. However, tumors from Ras/ATF3+/− cells grew slowly with a long lag time, and did not start to increase in size obviously until 2 weeks after injection (Fig. 8). Interestingly, the expression of ATF3 in the Ras/ATF3+/− tumors at 21 days after injection was not detectable (data not shown), an observation consistent with the notions that ATF3 can function as a tumor suppressor and that the cells expressing ATF3 have selection disadvantages. Several reports in the literature also support the tumor suppressor role of ATF3. First, ATF3 is expressed in the colorectal tumor cells at a lower level than that in the adjacent non-tumor cells (50) and has an anti-invasive activity in the colorectal cancer cells (51). Second, the expression of ATF3 is induced by anti-tumor agents such as curcumin (52), progesterone (11), and cyclooxygenase inhibitor (50). Third, ATF3 is a downstream target of the JNK stress signaling pathway in several stress paradigms (13, 27, 53). Interestingly, oncogenic Ras has been demonstrated to activate the JNK pathway (26, 54) and inhibition of JNK reduced oncogenic Ras-induced ATF3 expression (Fig. 4B). Recently, JNK was demonstrated to suppress Ras-mediated transformation. Using immortalized MEFs derived from knockout mice deficient in both JNK1 and JNK2, Kennedy et al. (55) demonstrated that the knock-out cells upon
ATF3 as a Tumor Suppressor

Ras transformation form larger tumors than the wild-type cells. Therefore, it is reasonable that ATF3, a downstream target of INK, also suppresses Ras-mediated transformation. However, it is not clear how critical ATF3 is in INK-mediated tumor suppression.

In contrast to its tumor suppressor function, however, ATF3 has also been demonstrated to promote cancer development. The expression of ATF3 correlates with increased metastasis in melanoma and breast cancer cells (56, 57), and antisense knockdown of ATF3 reduced the ability of HT29 colon cancer cells to invade through Matrigel in vitro (58). These, in combination with the reports that ATF3 can promote cell cycle progression and inhibit apoptosis (discussed above), suggest that ATF3 may also be an oncogene. Thus, we speculate that ATF3 plays a dichotomous role in cancer development by functioning as a tumor suppressor or an oncogene, presumably in a context-dependent manner.

This speculation is consistent with the induction of ATF3 by TGFβ (59), an agent that is well known to play a dichotomous role in cancer development by functioning as a tumor suppressor or an oncogene (60–63). Thus far, the mechanisms by which ATF3 functions are not well understood. It has been demonstrated to stabilize p53 (64) but also to antagonize p53 transcriptional activity (24), again indicating that ATF3 can have opposite functions. Clearly, further analyses are required to solve the paradox of ATF3. Because Ras is one of the most widely mutated proto-oncogenes in human tumors (10, 65, 66), this report using Ras transformation provides a useful model for future investigation of the mechanisms by which ATF3 affects cancer development.

Acknowledgment—We thank Dr. G. Leone for pBabe-hygro, pBabe-puro, and pBabe-puro-H-Ras(V12).

REFERENCES

1. Evan, G. I., and Vousden, K. H. (2001) Nature 411, 342–348
2. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
3. Ko, L. J., and Prives, C. (1996) Genes Dev. 10, 1054–1072
4. White, E. (1996) Genes Dev. 10, 1–15
5. Sherr, C. J. (1998) Genes Dev. 12, 2984–2991
6. Hai, T., Wolfgang, C. D., Marsee, D. K., Allen, A. E., and Sivaprasad, U. (1999) J. Biol. Chem. 274, 29507–29514
7. Lin, K. I., DiDonato, J. A., Flanagan, L., Tenniswood, M. P., Guha, C., Lisanti, M. P., Pestell, R. G., and Scherer, P. W. (2000) Mol. Biol. Cell 11, 233–241
8. Mochida, T., Udagawa, S., and Tsuuo, T. (2001) Oncogene 20, 1774–1787
9. Mochida, T., Udagawa, S., and Tsuuo, T. (2001) Cancer Surv. 36, 351–358
10. Sherr, C. J., and McCormick, F. (2002) Curr. Opin. Cell Biol. 14, 187–188
11. Stommel, J. W., Trunzer, K., Shokat, K. M., Marukawa, M., Saka, S., Nakanishi, A., and Johnson, B. T. (2000) J. Biol. Chem. 275, 44817–44826
12. Yan, C., Wang, H., and Boyd, D. D. (2002) J. Biol. Chem. 277, 10804–10812
13. Yan, C., Wang, H., and Boyd, D. D. (2002) J. Biol. Chem. 277, 10829–10839
14. Ueda, H., and Kanda, H. (1998) Oncogene 17, 1395–1413
16. Francis, J. S., Dragunow, M., and During, M. J. (2004) J. Biol. Chem. 279, 1774–1787
17. Nakagomi, S., Suzuki, Y., Namioka, K., Kiyura, S., and Kiyama, H. (2003) J. Neuropathol. 62, 518–578
18. Francis, J. S., Dragunow, M., and During, M. J. (2004) Brain Res. Mol. Brain Res. 124, 199–203
19. Nobori, K., Ito, H., Tamamori-Adachi, M., Adachi, S., Ono, Y., Kawaguchi, J., Kitajima, S., Marumo, F., and Isobe, I. (2002) J. Mol. Cell. Cardiol. 34, 1387–1397
20. Allan, A. L., Albanese, C., Pestell, R. G., and LaMarre, J. (2001) J. Biol. Chem. 276, 27272–27280
21. Perez, S., Vial, E., van Dam, H., and Castellazzi, M. (2001) Oncogene 20, 1135–1141
22. Tanaka, A., Numano, F., and Kitajima, S. (2002) J. Cell. Physiol. 188, 352–358
23. Lin, K. I., DiDonato, J. A., Flanagan, L., Tenniswood, M. P., Guha, C., Lisanti, M. P., Pestell, R. G., and Scherer, P. W. (2000) Oncogene 22, 6480–6483
24. Iyengar, P., Combs, T. P., Shah, S. J., Gouon-Evans, V., Pollard, J. W., Albanese, C., Iyer, P. R., and Scherer, P. W. (2000) Oncogene 19, 57–70
25. Sherr, C. J. (1996) Science 274, 1672–1677
26. Sherr, C. J., and McCormick, F. (2002) Cancer Cell 2, 103–112
27. Chen, H., Pan, Y. X., Dudenhausen, E. E., and Kilberg, M. S. (2004) J. Biol. Chem. 279, 50829–50839
28. Gerald, D., Berra, E., Frapart, Y. M., Chan, D. A., Giaccia, A. J., Mansuy, D., Pouyssegur, J., Yaniv, M., and Mechta-Grigoriou, F. (2004) Cell 118, 781–794
29. Lipson, K. E., and Baserga, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 9774–9777