Stress regulated transcription factor ATF4 promotes neoplastic transformation by suppressing expression of the INK4a/ARF cell senescence factors

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Abstract

Many cancers overexpress ATF4, a stress-induced transcription factor that promotes cell survival under hypoxic conditions and other stresses of the tumor microenvironment, but the potential contributions of ATF4 to oncogenesis itself have been little explored. Here we report that ATF4 promotes oncogene-induced neoplastic transformation by suppressing the expression of cellular senescence-associated genes. Strikingly, primary embryo fibroblasts from ATF4-deficient mice were resistant to transformation by co-expression of H-rasV12 and SV40 large T antigen. In wild-type cells these oncogenes induced expression of the murine Atf4 gene along with the cyclin-dependent kinase inhibitor Cdkn2a, which encodes the cell senescence-associated proteins p16INK4 and p19ARF. Elevated levels of ATF4 were sufficient to suppress expression of these proteins and drive oncogenic transformation. Conversely, genetic ablation of ATF4 led to constitutive expression of p16INK4a and p19ARF, triggering cellular senescence. Our findings define a central function for ATF4 in promoting oncogenic transformation by suppressing a central pathway of cellular senescence.
Introduction

cAMP-responsive element-binding proteins (CREBs) and activating transcription factors (ATFs) are basic region leucine zipper protein, which act as transcriptional activators or repressors. Activating transcription factor-4 (ATF4), a member of CREB/ATF family, is ubiquitously expressed throughout the body and is induced in response to various stress signals including anoxia, hypoxia, endoplasmic reticulum stress, amino acid deprivation, and oxidative stress (1). The stress-induced expression of ATF4 causes adaptive responses in cells through regulating the expression of target genes involved in amino acid synthesis, differentiation, metastasis, angiogenesis, and drug resistance (1). Excessive expression of ATF4 is often observed in malignant tumors in both human and rodents (2). The highly expressed ATF4 is thought to facilitate tumor progression, because transcription of genes involved in tumor cell proliferation is modulated by ATF4 (3). However, the role of ATF4 in the malignant transformation of normal cells remains to be elucidated.

In this study, we found that embryonic fibroblasts prepared from Atf4-null (Atf4-/-) mice showed resistance to oncogenic transformation induced by concomitant expression of proto-oncogenic RAS (H-rasV12) and simian virus 40 large T-antigen (SV40LT). On the basis of these findings, we further characterized the role of ATF4 during the malignant transformation. Our present results suggest that ATF4 acts as a suppressor of cellular senescence thereby promoting oncogenic transformation.

Materials and methods

Animals and cells. Heterozygous Atf4-null (Atf4+/-) mice and male balb/c Nu/Nu mice were cared for in accordance with the guidelines established by the Animal Care and Use
Committee of Kyushu University. Mouse embryonic fibroblasts (MEFs) were prepared by standard techniques from littermate embryos of wild-type or Atf4−/− mice, and cells were maintained in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 10% FBS, 20 μM β-mercaptoethanol, and 1x nonessential amino acid mix. For oncogenic transformation, MEFs were infected with 1x10⁶ cfu/ml retroviral vectors expressing H-rasV¹² and SV40LT (4). Anchorage-independent growth of oncogene-infected cells was evaluated by soft-agar colony formation assay. Nu/Nu mice were inoculated with cells (2.5X10⁶ cells/mouse) infected with oncogenes. Tumor size was measured as described previously (5).

**Cell cycle analysis.** Single cell suspension was prepared from tumor masses. Cells were incubated with 0.05mg/ml propidium iodide for specific DNA staining. The samples were analyzed on the EPICS Elite flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). The total number of cells analyzed from each sample was 10000.

**Microarray gene expression analysis.** The quality of extracted RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The cRNA was amplified and labeled using a Low Input Quick Amp Labeling Kit. Labeled cRNA was hybridized to a 44K Agilent 60-mer oligomicroarray (Whole Mouse Genome Microarray Kit Ver.2.0). To identify up or down-regulated genes, we calculated Z-scores and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe. We set criteria for up-regulated genes: Z-score ≥ 3.0 and ratio ≥ 2-fold; and for down-regulated genes: Z-score ≤ -3.0 and ratio ≤ 0.5. All microarray data were submitted
to the Gene Expression Omnibus at the National Center for Biotechnology Information (accession number GSE31405). The functional analysis of the differentially expressed genes was done by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database on DAVID system (6).

**Quantitative RT-PCR analysis.** The complementary DNA (cDNA) was synthesized by reverse-transcribing using ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) (7). Diluted cDNA samples were analyzed by real-time PCR using THUNDERBIRD™ SYBR qPCR Mix (Toyobo) and the 7500 Real-time PCR system (Applied Biosystems). Sequences for PCR primers are described in **Supplementary Table S1**.

**Luciferase reporter assay.** The surround regions of C/EBP-ATF response element (CARE) in the mouse *p16Ink4a* and *p19ARF* promoter were amplified using DNA extracted from wild-type MEFs. The PCR products were ligated into a pGL4 Basic vector (*p16Ink4a*-Luc and *p19ARF*-Luc) as shown in **Supplementary Fig. S1**. The sequences of the CARE in luciferase vectors were mutated using a QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA). Expression vectors for mouse ATF4 and C/EBPα were constructed as described previously (5). MEFs were transfected with 100 ng per well of reporter vectors and 500 ng per well of expression vectors using Lipofectamine LTX reagent (Invitrogen). phRL-TK vector (Promega) at 0.5 ng per well of was also cotransfected as an internal control reporter.

**Chromatin immunoprecipitation assays.** MEFs were treated with 8% paraformaldehyde to cross-link the chromatin. Those samples were incubated with
antibodies against ATF4, C/EBPα, and rabbit-IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Chromatin/antibody complexes were extracted using a protein G agarose kit (Roche, Germany). Isolated DNA was subjected to PCR using the following primer pair: 5’-GATTTCATACTGGGCCTGGT-3’ and 5’-GCTGGCCTCTCTCTGGAC-3’.

**Western blotting.** Nuclear or cytoplasmic proteins prepared from tumor masses or MEFs were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were reacted with antibodies against ATF4, p16INK4a, p19ARF, Retinoblastoma protein (Rb), p53, β-ACTIN (Santa Cruz Biotechnology), or phosphorylated-Rb (Phospho.-Rb; Invitrogen, Carlsbad, CA). The immunocomplexes were reacted with horseradish peroxidase conjugated secondary antibodies.

**Histochemical analysis.** Frozen tumor mass sections were prepared on day 14 after inoculation of mice with cells infected with oncogenes. Senescence-associated β-galactosidase (SA β-gal) positive cells were detected using a senescence detection Kit (BioVision, Palo Alto, CA). TUNEL staining was performed using the *in situ* Apoptosis Detection Kit (Takara, Osaka, Japan). The percentage of SA β-gal-positive and TUNEL-positive cells was determined by counting the numbers of positive cells among at least 10,000 cells from each sample.

**Statistical analysis.** The statistical significance of differences among groups was analyzed by analysis of variance (ANOVA) and post-hoc Bonferroni comparisons. A 5% probability was considered significant.
Results and Discussion

To characterize the role of ATF4 in oncogenic transformation, we prepared mouse MEFs from wild-type and Atf4-/- mice, and infected the cells concomitantly with retrovirus vectors expressing the H-ras^{V12} and the SV40 LT (4). These oncogenes were equally expressed in wild-type and Atf4-/- cells (Fig. S2A). Concomitant introduction of H-ras^{V12} and SV40 LT significantly enhanced the growth of both wild-type and Atf4-/- cells, but the growth rate of Atf4-/- cells was modest as compared to that of wild-type cells (Fig. S2B). Introduction of oncogenes also failed to induce anchorage-independent growth of Atf4-/- cells as shown by the formation of colonies on soft agar plate (P<0.01, Fig. 1A), suggesting that the loss of ATF4 resulted in the phenotype resisting oncogene-induced malignant transformation. This notion was also supported by the fact that ectopic expression of ATF4 significantly enhanced oncogene-induced anchorage-independent growth of Atf4-/- cells (P < 0.05, Fig. 1B). Furthermore, overexpression of ATF4 in wild-type cells also accelerated oncogene-induced formation of colonies on soft agar plate (P < 0.05, Fig. S2C). ATF4 appears to play as an essential role in oncogene-induced malignant transformation.

Next, we tested this hypothesis by using an implant model in mice. Equal numbers of wild-type or Atf4-/- cells infected with oncogene (H-ras^{V12} and SV40 LT) were inoculated into the flanks of balb/c nu/nu mice. Although mice inoculated with wild-type cells infected with oncogenes showed a significant growth of tumor masses (Fig. 1C), mice inoculated with Atf4-/- cells showed no palpable tumor masses until 9 days after inoculation. On day 14 after inoculation of cells, the average of tumor weight in mice inoculated with the Atf4-/- cells was 9.28 ± 4.13 mg, representing an 94.82 % reduction.
from that in mice inoculated with the wild-type cells (179.1 ± 11.97 mg) (P<0.01, Fig. 1C inside panel).

Comparison of flow cytometry (FACS) histograms from the wild-type and the Atf4-/- tumors also revealed a significant difference in the cell cycle distribution between the genotypes. As compared to wild-type tumor cells, Atf4-/- cells were highly accumulated in the G0/G1 phase, whereas the population of the G2/M phase of Atf4-/- cells was considerably smaller than that of wild-type cells (Fig. 1D). These results indicate that the loss of ATF4 attenuates the tumorigenicity of mouse fibroblasts and the G0/G1 phase arrest of the cell cycle underlies the resistance of Atf4-/- cells to oncogenic transformation.

To identify genes that enable Atf4-/- cells to resist oncogenic transformation, we performed microarray analysis using RNA isolated from oncogene-introduced wild-type or Atf4-/- cells. A large number of genes would be induced or repressed by oncogenic stimuli. We thus focused on genes whose responsiveness to oncogenic stimuli was altered in Atf4-/- cells. On day 7 after concomitant introduction of H-rasV12 and SV40LT, 326 genes in Atf4-/- cells were differentially expressed as compared to wild-type cells. Among these genes, 216 genes in Atf4-/- cells were up-regulated by oncogenic stimuli, whereas 110 genes were down-regulated in oncogene-infected Atf4-/- cells (Supplementary Table S2). The functional analysis of the genes, done using KEGG database (6), showed that 12 biological pathways were enriched in a statistically significant manner (P<0.05; Table S3). Among these pathways, we further focused on the cell cycle pathway because Atf4-/- cells introduced with oncogenes resulted in G0/G1 phase arrest (Fig. 1D). In this pathway, only Cdkn2a, a canonical inducer of cellular
senescence, was highly expressed in Atf4/- cells, despite a decrease in the expression of other cell cycle regulatory genes (Table 1). These molecular findings prompted us to explore the possibility that ATF4 suppresses the transcription of cellular senescence-associated genes.

Recent studies have demonstrated that tumor suppressor gene Cdkn2a acts as a canonical inducer of cellular senescence (8,9). Cdkn2a generates several different transcript variants using different first exons and alternate polyadenylation sites (Fig. 2A) (10,11). The p16Ink4a variants encode structurally related protein isoforms that inhibit CDK4 kinase (9). The CDK4 inhibitor prevents phosphorylation of Rb by disrupting the activity of CDK4-Cyclin D complex, thereby causing G1 phase arrest. The remaining transcript includes an alternate first exon located 20 kb upstream from the remainder of the p16Ink4a gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to that of p16INK4a protein (10,11). p19ARF (also known as p14ARF in human) stabilizes p53 by sequestering MDM2, a protein responsible for degrading p53 (12). In spite of the structural and functional differences, p16INK4a and p19ARF play an important role in the cell cycle’s G1/S progression: the induction of these proteins is critical for causing cellular senescence (8,9). Introduction of H-rasV12 and SV40LT into wild-type cells significantly and consistently increased the levels of Atf4 mRNA and its protein throughout the experimental period (Fig. 2B and C). Introduction of the oncogene also induced the expression of p16INK4a and p19ARF, but the induction was transient; mRNA levels of cellular senescence-associated genes reached a peak level from 1 to 2 days after oncogene introduction and decreased to basal level within 4 days (Fig. 2B and C). In contrast,
introduction of H-ras\textsuperscript{V12} and SV40LT into \textit{Atf4}/- cells caused a significant and consistent increase in the expression of p16INK4a and p19ARF at both mRNA and protein levels (P<0.05; \textbf{Fig. 2B and C}). The increase in the expression of p16INK4a and p19ARF in oncogene-introduced \textit{Atf4}/- cells was suppressed by ectopic expression of ATF4 (\textbf{Fig. 2D}), suggesting that excess ATF4 suppresses the expression of cellular senescence-associated genes induced by oncogenic stimuli.

ATF4 positively or negatively regulate the transcription of its target genes by forming homo- or hetero-dimer with Jun, AP-1, and CCAAT/enhancer binding proteins (C/EBPs) (13,14). The dimerization partners appear to determine the diverse actions of ATF4. The heterodimerization of ATF4 with C/EBP\textalpha represses transcription on CARE (15). A consensus sequence of CARE was found in the upstream region of mouse \textit{p16Ink4a} gene (\textbf{Fig. 2A}). The sequence inspection of the upstream region of mouse \textit{p19ARF} gene also revealed a CARE that matches 8 bp of the 9 bp consensus sequence TGATGXAAX (where X = A, T, or C). The CARE sequences were located at similar position on the human CDKN2A locus. To explore whether ATF4 acts as a transcriptional repressor of cellular senescence-associated genes, we constructed two luciferase reporter vectors containing the CARE derived from the upstream region of mouse \textit{p16Ink4a} and \textit{p19ARF}. Neither ATF4 nor C/EBP\textalpha had a considerable effect on the luciferase activity of \textit{p16Ink4a}-Luc, but co-transfection of \textit{p16Ink4a}-Luc with both ATF4 and C/EBP\textalpha resulted in a 90% reduction of the luciferase activity (\textbf{Fig. 2E}). A similar transrepression was also found when cells were co-transfection of \textit{p19ARF}-Luc with both ATF4 and C/EBP\textalpha (\textbf{Fig. 2E}). The transrepression effects of ATF4-C/EBP\textalpha on the \textit{p16Ink4a}-Luc and \textit{p19ARF}-Luc were dependent on the CAREs, because mutation of...
the sequences attenuated the suppressive action of ATF4-C/EBPα to the basal level (Fig. 2E).

The results from chromatin immunoprecipitation analysis also revealed that the amounts of ATF4 binding to the Cdkn2a locus CARE located on the upstream of p16INK4a in wild-type cells consistently increased from day 3 after introduction of H-rasV12 and SV40LT (Fig. 2F). On the other hand, no obvious binding of ATF4 to the Cdkn2a locus CARE was observed in oncogene-introduced Atf4-/− cells. The correlation between the binding activity of ATF4 to CARE and its transcriptional regulation of the p16INK4a and p19ARF suggests that ATF4 functions as a transcriptional repressor of p16INK4a/ARF during oncogenic transformation.

During oncogenic transformation, cancer cells acquire genetic mutations that override the normal cell cycle mechanism, resulting in abnormal proliferation. Failure of cells to override the mechanism often causes reversible cell cycle arrest, apoptotic cell death, or cellular senescence (16). In contrast to reversible cell cycle arrest, cellular senescence is defined by irreversible loss of proliferative potential, acquisition of characteristic morphology, and expression of specific biomarkers, such as SA β-gal (8,17). On day 14, after inoculation of mice with oncogene-introduced Atf4-/− cells, a significant increase in the protein levels of p16INK4a and p19ARF were observed in tumor masses formed by Atf4-/− cells (P<0.05, respectively; Fig. 3A). Induction of p16INK4a and p19ARF ultimately causes cellular senescence by modulating the activity of Rb and p53 (10-12). In fact, phosphorylation of Rb was significantly suppressed in tumor masses formed by Atf4-/− cells (P<0.05; Fig. 3B), whereas a significant accumulation of p53 was detected in Atf4-/− tumors (P<0.05; Fig. 3B). Similar decrease in the levels of phosphorylated Rb and accumulation of p53 protein was also detected...
when cultured *Atf4/-* cells were infected with H-ras^{V12} and SV40LT (P<0.05, respectively; **Fig. S3**). These results suggest that ATF4 acts to suppress cellular senescence during oncogenic transformation. This notion was also supported by the fact that the number of SA β-gal positive-cells in tumor masses formed by *Atf4/-* cells was significantly higher than that in tumor masses formed by wild-type cells (P<0.05; **Fig. 3C**). Furthermore, the number of TUNEL-positive cells, as an index of apoptotic cell death, also increased in tumor masses formed by *Atf4/-* cells (P<0.05; **Fig. 3D**). Since mice inoculated with *Atf4/-* cells introduced with oncogenes showed a modest tumorigenicity (**Fig. 1C**), the induction of cellular senescence and apoptotic cell death seemed to be associated with modest tumor formation of *Atf4/-* cells.

In this study, we used retrovirus vectors expressing SV40LT as a transforming agent, together with H-ras^{V12}. As SV40LT inactivates p53 and prevents the phosphorylation of Rb by protein-protein interaction (18,19), the SV40LT-transduced cells are thought to be insensitive to growth arrest by p16INK4a and p19ARF. Inactivation of the growth-suppressive properties of p53 has shown to be essential for immortalization of MEFs by SV40LT (18). Concomitant introduction of H-ras^{V12} and SV40LT induced the expression of p53 in *Atf4/-* cells, so that the amount of p53 proteins in oncogene-introduced *Atf4/-* cells was much greater than that in wild-type cells (**Fig. S3**). The most amount of p53 protein in oncogene-introduced wild-type cells was precipitated together with SV40LT (**Fig. S4**). However, a large amount of p53 protein in *Atf4/-* cells was unable to be precipitated together with SV40LT, suggesting that extensive expression of p53 in oncogene-introduced *Atf4/-* cells overrides the binding capacity of SV40LT, and allows induction of cellular senescence.
The present findings suggest the mechanism by which ATF4 promotes oncogenic transformation by suppressing the expression of cellular senescence-associated proteins (Fig. 3E). ATF4, p16INK4a, and p19ARF are expressed in response to oncogenic stimuli, but in turn, an overabundance of ATF4 suppresses the expression of these cellular senescence-associated proteins through binding to the CARE in the Cdkn2a locus. Decreases in the levels of cellular senescence-associated proteins restore stabilization of p53 by p19ARF and prevention of Rb hyperphosphorylation by p16INK4a, thus transforming cells so as to increase proliferation and oncogenic potentials. In fact, downregulation of Cdkn2a by siRNA enhanced oncogene-induced anchorage-independent growth of Atf4-/- cells (Fig. S5). ATF4 is highly expressed in malignant tumors not only in rodents but also in human (2). During oncogenic transformation of human cells, ATF4 may also play a role in overriding the normal cell cycle mechanism, resulting in abnormal proliferation.
Footnotes

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References


Figure legends

Figure 1. Resistance of \textit{Atf4}-/- cells to oncogene-induced tumorigenesis. (A) Attenuated anchorage-independent growth of \textit{Atf4}-/- cells with concomitant introduction of H-ras\textsuperscript{V12} and SV40LT (1x10\textsuperscript{6} cfu/ml each). The cells infected with oncogenes were subjected to a soft agar colony formation assay. Colonies were stained with crystal violet. (B) Enhancement of oncogene-induced anchorage-independent growth of \textit{Atf4}-/- cells by ectopic expression of ATF4. (C) The growth of tumors in mice inoculated with wild-type or \textit{Atf4}-/- cells infected with oncogenes. The inside panel shows the weight of tumor masses on day 14 after inoculation. (D) The analysis of cell cycle distribution of cells prepared from tumor masses formed by wild-type or \textit{Atf4}-/- cells. All values are shown as means \pm S.E. (n=3-9). **P <0.01, *P < 0.05 compared between the two groups. ##P <0.01, #P < 0.05 compared with the wild-type group at the corresponding day.

Figure 2. Transcriptional regulation of cellular senescence-associated genes by ATF4. (A) Location of CARE in the mouse Cdkn2a locus. The Cdkn2a gene has a unique first exon, exon 1\textalpha{} and 1\textbeta{}. The sequence inspection of Cdkn2a locus reveals two CAREs that matche 8 bp and 9 bp, respectively, of the 9 bp consensus sequence TGATGXAAX (where X = A, T, or C). (B and C) Temporal profiles of protein (B) and mRNA (C) levels of ATF4, p16INK4a, and p19ARF in wild-type or \textit{Atf4}-/- cells infected with oncogenes (H-ras\textsuperscript{V12} and SV40LT). (D) Suppressive action of ATF4 on oncogene-induced expression of p16INK4a and p19ARF in \textit{Atf4}-/- cells. (E) ATF4-C/EBP\alpha{}-mediated repression of p16INK4a and p19ARF transcription. Reporters contained either native (\textit{p16Ink4a-Luc} and \textit{p19ARF-Luc}) or mutated CARE
(Δp16Ink4a-Luc and Δp19ARF-Luc). The presence or absence of reporter (50 ng) and expression plasmids (100 ng of ATF4 and C/EBPα) is indicated by (+) or (-). Activity of each reporter construct is relative to response in empty vectors (pcDNA3.1). Values are mean ± S.E. (n = 6). (F) Temporal binding profiles of ATF4 and C/EBPα to the CARE in the Cdkn2a locus. Allows indicate amplified area. All values are shown as means ± S.E. (n = 3-9). **P <0.01, compared between the two groups. #P < 0.05 compared with wild-type group at the corresponding day.

Figure 3. Induction of cellular senescence in tumor masses formed by Atf4-/- cells. (A) The protein abundance of p16INK4a, and p19ARF in tumor masses on day 14 after inoculation of mice with wild-type or Atf4-/- cells infected with oncogenes (H-rasV12 and SV40LT). (B) The abundance of phosphorylated Rb (Phospho. Rb) and p53 in tumor masses on day 14 after inoculation of mice with wild-type or Atf4-/- cells infected with oncogenes. **P <0.01, *P < 0.05 compared between the two groups. (C and D) The SA β-gal (C) and TUNEL (D) staining cells in tumor masses on day 14 after inoculation of mice with with wild-type or Atf4-/- cells infected with oncogenes. (E) Schematic mechanism of promotional action of ATF4 to oncogenic transformation by suppressing cellular senescence. All values are shown as means ± S.E. (n =3-9).
Table 1 Differentially expressed cell cycle regulatory genes between oncogene-introduced wild-type and *Atf4*-/- cells.

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