# GADD34 induces p21 expression and cellular senescence

KAHORI MINAMI[1,3], HIROKAZU INOUE[2], TAKAO TERASHITA[3], TAKAHIRO KAWAKAMI[1], RYOSUKE WATANABE[1], MASATAKA HANEDA[4], KEN-ICHI ISOBE[4], HIDETOSHI OKABE[1] and TOKUHIRO CHANO[1,5]

Departments of [1]Clinical Laboratory Medicine and [2]Microbiology, Shiga University of Medical Science, Shiga 520-2192; [3]Department of Applied Biological Chemistry, Faculty of Agriculture, Kin-ki University, Nara 631-8505; [4]Department of Immunology, Nagoya University School of Medicine, Nagoya 466-8550; [5]PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan

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**Abstract.** We previously identified GADD34 (growth arrest and DNA damage protein 34) by screening for genes involved in oncogenic-transformation and/or cellular senescence in Ras-transformed rat F2408 fibroblasts (7EJ-Ras), which exhibit anchorage-independent growth and do not senesce. In the current study, we found that transduction of 7EJ-Ras cells with a retroviral vector expressing GADD34 suppressed their proliferation. Furthermore, we observed that fibroblasts derived from GADD34-knockout mice (GADD34-KO MEFs) did not undergo senescence. Whereas the expression of p21 was decreased in GADD34 KO MEFs, its expression was rescued in these cells by ectopic expression of GADD34 by retroviral transduction. These findings suggest that GADD34 contributes to the regulation of p21 expression, and that it suppresses cellular proliferation through the induction of cellular senescence.

## Introduction

Expression of oncogenic Ras in normal fibroblasts promotes cellular senescence. This phenomenon is termed premature senescence, a repressive state that counteracts proliferative stimulation due to mitogenic signaling, and prevents the induction of malignant transformation. 7EJ-Ras is a rat fibroblastic cell line expressing activated H-ras. Although these cells are transformed by H-ras, they are not susceptible to premature senescence and are immortalized. The cells also grow in soft agar, and as such we can make use of these cells to search for genes associated with oncogenic transformation, including those involved in the ability to avoid senescence.

*Correspondence to*: Dr Tokuhiro Chano, Department of Clinical Laboratory Medicine, Shiga University of Medical Science, Shiga 520-2192, Japan
E-mail: chano@belle.shiga-med.ac.jp

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We employed a previously described cDNA library screening strategy using 7EJ-Ras cells to identify genes linked to oncogenic transformation and/or premature senescence following transformation with activated Ras (1-3). Using the screen, we found that *GADD34* could suppress anchorage-independent growth of 7EJ-Ras cells.

GADD34 belongs to a family of proteins that are up-regulated by growth arrest and DNA damage (GADD). GADD34, like GADD45 and GADD153, was originally discovered as a UV-inducible transcript in Chinese hamster ovary cells (4). GADD34 regulates translation during conditions of cellular stress, such as heat shock (5), virus infection (6), nutrient deprivation (7), and exposure of cells to agents that cause misfolding of proteins in the ER (8). Overexpression of GADD34 in several cell lines leads to apoptosis or cell cycle arrest (9), suggesting that it is a tumor suppressor gene.

We previously reported that GADD34 enhances the stability of p53 by phosphorylating the latter at serine 15 (10), and that expression of GADD34 enhanced the expression of the mRNA encoding p21 (11). In this study, we show that embryonic fibroblasts isolated from GADD34-knockout mice (KO MEFs) do not exhibit the premature senescence that characterizes normal fibroblasts. Furthermore, we show that GADD34 enhances p21 expression, induces cellular senescence and inhibits tumor growth.

## Materials and methods

*Cell culture*. 7EJ-Ras is a rat fibroblastic cell line transformed by activated H-ras. 7EJ-Ras mouse embryonic fibroblasts (WT MEFs) and GADD34 KO MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). We constructed a recombinant retrovirus expressing *GADD34* (pCX bsr/GADD34). Cells were selected in the presence of blasticidin and clones were isolated and expanded. 7EJ-Ras cells and GADD34 KO MEFs were transduced with pCXbsr/GADD34 according to a previously described method (12).

*Protein extraction and Western blot analysis*. GADD34 expression was confirmed by Western blot analysis. Cells were ruptured in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM

NaCl, 5 mM EDTA, 1% NP-40, 1 mM $Na_3VO_4$, and containing a mixture of phosphatase and protease inhibitors). Cell extracts were kept on ice for 15 min and centrifuged at 20,000 x g for 10 min (4˚C), and the supernatant was boiled in SDS sample buffer and stored at -80˚C. Protein extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore), and exposed to the indicated antibodies. Anti-GADD34 (H-193) was obtained from Santa Cruz Biotechnology. Anti-α-tubulin (DM 1A) was from Sigma.

*Soft agar assay*. Cells ($1x10^4$) were mixed with Sea-plaque-agarose at a final concentration of 0.4% in DMEM, and overlaid onto a 0.8% agarose in 35-mm plates. The plates were incubated for 10 days. Colonies were counted and photographed using an inverted microscope.

*Senescence assay*. Cells were cultured using the 3T3 method, which involved the passage and culture of the cells every 3 days, to compare the rate of proliferation of KO and WT MEFs. For senescence-associated ß-galactosidase (SA-ß-gal) activity, a senescence detection kit (Bio Vision) was used according to the manufacturer's instructions.

*RT-PCR and quantitative real-time PCR*. Total-RNA was isolated from cells using TRIzol (Gibco-BRL) and first-strand cDNA was synthesized using Superscript III reverse transcriptase and oligo-d(T)12-18 primer (Invitrogen). The gene primer sequences were as follows respectively: murine *Gadd34*: forward, 5'-CTGCAAGGGGCTGATAAGAG and reverse, 5'-AGGGGTCAGCCTTGTTTTCT; *Rb1*: forward, 5'-CACG TGTAAATTCTGCTGCAA and reverse, 5'-CCTGGT GGAGGCATACTGTAA; *cyclin D3*: forward, 5'-TAG GCGCCTGCTCTATGTCT and reverse, 5'-ATCTGT GGGAGTGCTGGTCT; *p21*: forward 5'-GTCCAATCC TGGTGATGTCC and reverse, 5'-CAGGGCAGAGGA AGTACTGG; *Gapdh*: forward, 5'-CATGACAACTTTGGC ATTGTG and reverse, 5'-GTTGAAGTCGCAGGAGAC AAC; human GADD34: forward, 5'-GAATCAAGCCA CGGAGGATA and reverse, 5'-CAGGGAGGACACTC AGCTTC. The PCR protocols comprised 32 (Gadd34 and GADD34) and 28 (Rb1, *cyclin D3*, *p21* and *Gapdh*) cycles of 30-sec denaturation at 95˚C, 30-sec annealing at 55˚C and 30-sec extension at 72˚C. For real-time PCR, iQSYBR-Green Supermix (Bio-Rad Laboratories) was used according to the manufacturer's instructions. Mouse *p21* primer sequences for real-time PCR were as follows: forward 5'-CCCTCTATT TTGGAGGGTTAATCT and reverse, 5'-GTACCCTGC ATATACATTCCCTTC.

## Results

*GADD34 suppresses anchorage-dependent growth of rat fibroblast cells transformed by v-H-ras*. To further evaluate the putative tumor suppressor activity of GADD34, we transduced F2408 rat fibroblasts, which are transformed by activated H-ras (7EJ-Ras), with a retroviral vector expressing GADD34. Western blot analysis confirmed expression of exogenous GADD34 in the transduced cells (Fig. 1a). We first compared

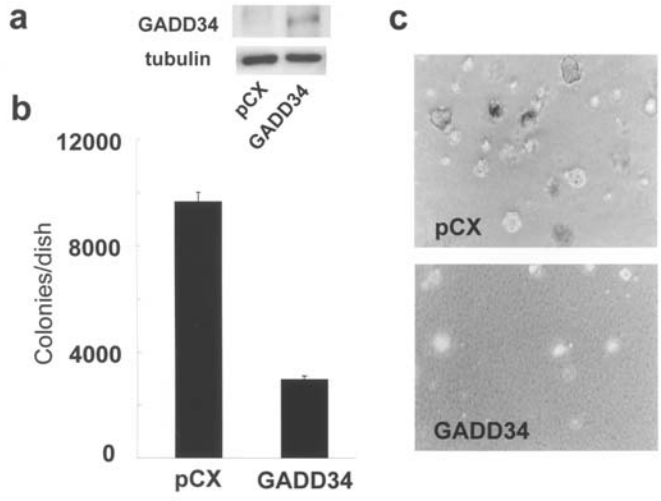


Figure 1. GADD34 suppresses anchorage-independent growth in 7EJ-Ras cells. (a) Expression of GADD34 after retroviral gene transfer was evaluated by Western blot analysis of cellular lysates. GADD34, cells transduced with the retroviral vector expressing GADD34; pCX, cells transduced with the empty control vector. (b) Colony formation in soft agar. Number of colonies formed by 7EJ-Ras cells transduced with the GADD34 expression vector (GADD34) or the control vector (pCX). (c) Photographs of colonies in soft agar after incubation for 10 days.

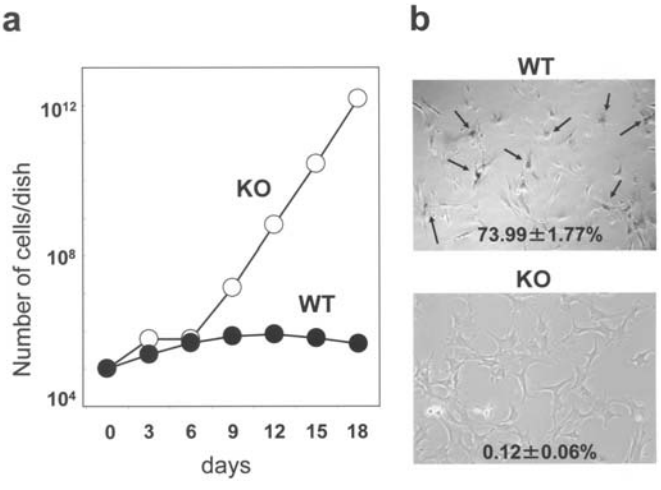


Figure 2. Evaluation of cellular senescence in GADD34 WT and KO MEFs. (a) Growth curves of GADD34 WT and KO MEFs. Cells were counted every 3 days. KO MEFs continued to proliferate, whereas WT MEFs underwent growth arrest on day 9. (b) Assay of senescence-associated ß-galactosidase (SA-ß-gal) activity in WT and KO MEFs.

anchorage-independent growth and the induction of premature senescence in GADD34 vector (pCX/GADD34)-transduced and control-vector (pCX)-transduced cells. 7EJ-Ras fibroblasts expressing GADD34 formed significantly fewer colonies in soft agar than did cells transduced with the empty control vector (Fig. 1b and c). However, expression of GADD34 did not lead to premature senescence. These results suggest that expression of GADD34 suppresses oncogenic transformation by inhibiting anchorage-independent growth.

*GADD34-knockout MEFs do not senesce*. We did not observe the formation of colonies in soft agar by either wild-type

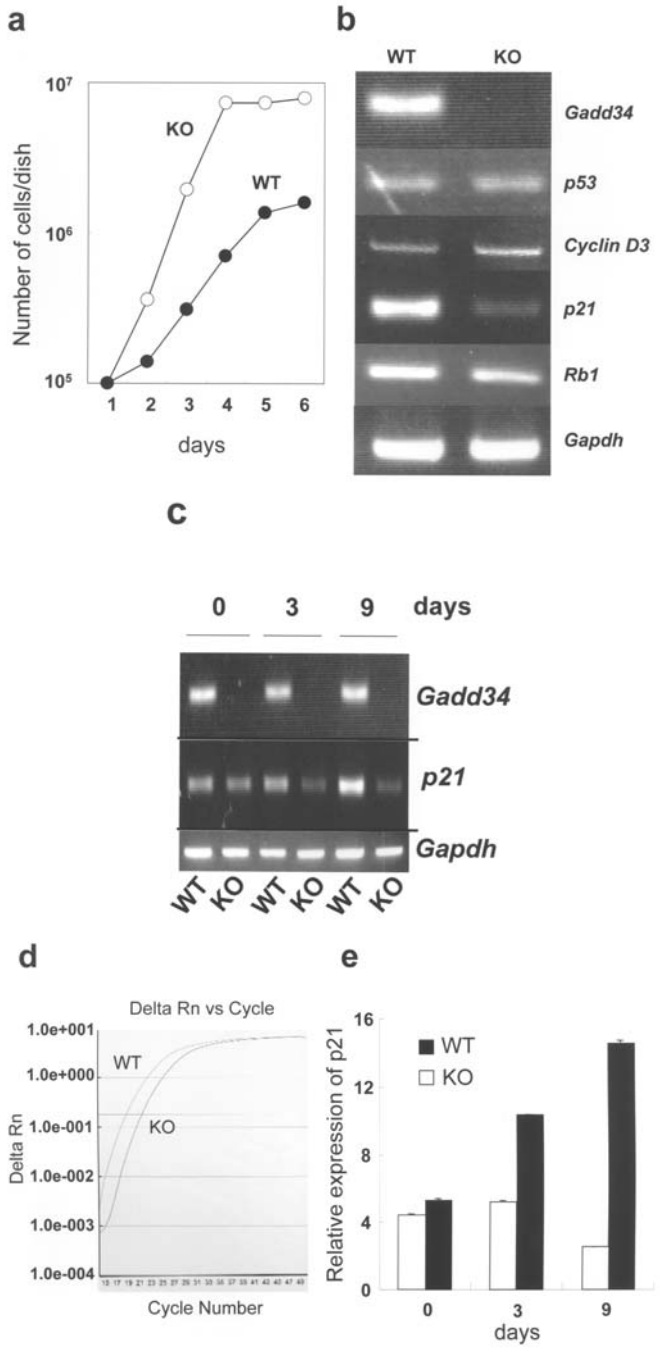


Figure 3. Growth curves and p21 expression in GADD34 WT and KO MEFs. (a) Daily monitoring of the proliferation of GADD34 WT and KO MEFs. (b) Expression of cell cycle regulators in WT and KO MEFs. (c) Evaluation of the expression of GADD34 and p21 by semi-quantitative RT-PCR in WT MEFs and KO MEFs at 0, 3 and 9 days after initiation of culture. (d) Evaluation of the level of p21 mRNA by quantitative real-time PCR in WT and KO MEFs. (e) The relative expression of p21 mRNA in RNA isolated from WT and KO MEFs.

(WT) or GADD34-knockout (KO) MEFs (data not shown). However, we observed that the KO MEFs did not senesce and instead that they continued to proliferate in culture. To evaluate whether GADD34 is involved in the regulation of cellular senescence, we made use of the 3T3 method to compare the rate of proliferation of KO and WT MEFs. We found that WT MEFs underwent growth arrest at day 9 of culture, whereas KO MEFs continued to proliferate (Fig. 2a). In addition, 73% of the WT MEFs exhibited activity of senescence-associated ß-galactosidase (SA-ß-gal), indicating that most of the cells were senescencing. In contrast, KO MEFs were virtually negative for SA-ß-gal activity (Fig. 2b). These findings indicate that GADD34 may play a role in the regulation of cellular senescence, as evidenced by the fact that KO MEFs avoided cellular senescence and continued to proliferate.

*Up-regulation of p21 is disturbed in GADD34-KO MEFs.* We also observed that the rate of proliferation of KO MEFs was up to one order of magnitude greater than that of the WT MEFs (Fig. 3a). We then used semi-quantitative RT-PCR to compare the expression of various regulators of the cell cycle in WT and KO MEFs (Fig. 3b). We found that the expression of p21 was significantly lower in KO than it was in WT MEFs, whereas the expression of other cell-cycle regulators such as p53, cyclin D3, and Rb1 was not significantly different between KO and WT MEFs. To determine whether increased expression of p21 correlated with GADD34 expression, we compared the expression over time of GADD34 and p21 between WT and KO MEFs. As shown in Fig. 3c, the expression of p21 mRNA progressively increased in WT MEFs, whereas it progressively decreased during the same period in KO MEFs. Furthermore, to measure the level of p21 mRNA more precisely, we performed quantitative real-time PCR on samples of RNA isolated from WT and KO MEFs (Fig. 3d). We observed that the level of p21 mRNA in KO MEFs was approximately 50% of the level detected in WT MEFs on day 3. The level of p21 in KO MEFs decreased by approximately 50% from day 3 to day 9, whereas it increased by approximately 33% in WT MEFs over the same period (Fig. 3e). These results strongly suggest that GADD34 is involved in up-regulation of p21 expression.

*Re-introduction of GADD34 into KO MEFs suppresses growth and increases p21 expression.* To investigate further the effect of GADD34 on p21 expression, we transduced the KO MEFs with the control and GADD34-expressing retroviral vector (12). We detected the expression of GADD34 protein in KO MEFs transduced with the GADD34 vector (Fig. 4a). Re-introduction of the GADD34 gene resulted in both suppression of growth of the KO MEFs (Fig. 4b) and recovery of the expression of p21 compared to control-vector transduced cells (Fig. 4c). Analysis of p21 expression by quantitative real-time PCR confirmed that transduction with the GADD34-expressing vector maintained the expression of p21, whereas p21 expression decreased in KO MEFs transduced with the control vector (pCX) (Fig. 4d and e). These findings suggested that GADD34 is involved in the maintenance and up-regulation of p21 transcription, and that its expression suppresses cell growth.

## Discussion

Hayflick and colleagues were the first to describe cellular senescence as the finite replicative life span of primary mammalian fibroblasts (13). Under ordinary culture conditions, cellular senescence is an irreversible blockade of cell cycle

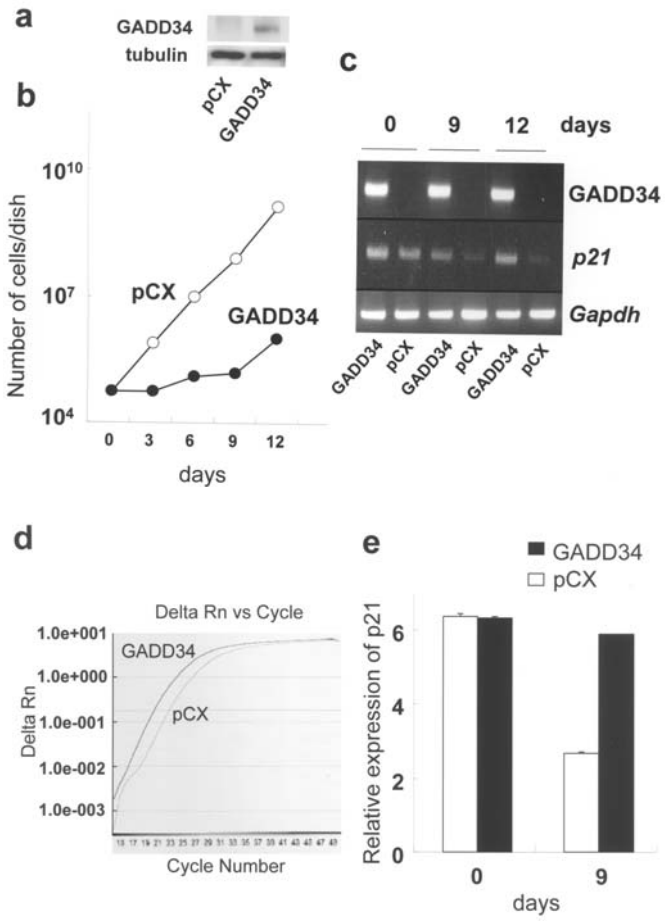


Figure 4. Growth rate and p21 expression in KO MEFs transduced with a control (pCX) or GADD34-expressing (GADD34) vector. (a) Western blot analysis of the expression of GADD34 in lysates prepared from the transduced cells. (b) Numbers of transduced KO MEF cells counted every three days after initiation of culture. (c) RT-PCR analysis of the expression of both GADD34 and p21 in the transduced KO MEFs. (d) Real-time PCR analysis of the expression of p21 in the transduced cells. (e) Relative expression of p21 mRNA in KO MEFs transduced with the GADD34-expressing or control (pCX) vectors.

progression that depends solely on the accumulated number of cell divisions. Escape from cellular senescence is a prerequisite for oncogenic transformation. Over the ensuing decades, many kinds of oncogenic stimuli were found to induce cellular senescence. This effect has been termed premature senescence. Many primary rodent cells undergo premature senescence following expression of various oncogenes, such as Ras or Myc. These findings suggest that the onset of cellular senescence does not simply reflect the accumulation of cell divisions, but can be prematurely activated in response to oncogenic stimuli (14).

Premature senescence is thought to be an intrinsic anti-tumorigenic barrier that can irreversibly arrest the growth of cells that have suffered carcinogenic damage. Tumor suppressors and oncogenes participate in the regulation of senescence-inducing pathways (15). Early tumorigenic cells can also undergo cellular senescence through the overexpression of several tumor suppressor proteins, such as the CDK inhibitors p16, p21 or p27. p21 and p16 can also induce cellular senescence. p21 expression is increased in response to various stimuli, including expression of oncogenic Ras, exposure of cells to DNA-damaging drugs, ionizing radiation, or agents affecting DNA replication, mitosis or cellular differentiation. p21 induction is essential for the onset of cell cycle arrest following induction of senescence in response to DNA or cell damage, and the induction of its expression is mediated mainly by p53 (16).

In this study, we found that GADD34 expression can inhibit the anchorage-independent growth of cells expressing oncogenic Ras. In addition, we observed that GADD34 KO MEFs were able to avoid senescence and continued to proliferate under ordinary culture conditions, whereas WT MEFs underwent senescence after a defined number of passages. In support of this, we did not observe SA-ß-gal activity in KO MEFs, whereas a majority of WT MEFs exhibited this activity. These findings strongly suggest that GADD34 suppresses tumor growth and that it is involved in the induction of cellular senescence. We next evaluated the expression of various cell-cycle regulators in the WT and KO MEFs. We found that the expression of p21 was reduced in KO MEFs, relative to its level of expression in WT MEFs. Furthermore, p21 expression was restored by transduction of KO MEFs with a retroviral vector expressing GADD34. These results strongly suggest that GADD34 is involved in the regulation of both the expression of p21 and the suppression of cell growth.

We have previously reported that GADD34 stabilizes the p53 protein by inducing the phosphorylation of the latter at serine 15 (10), leading to enhanced expression of p21 (11). The induction of the expression of p21 in response to activation of p53 is an important pathway leading to cellular senescence. In the present study, we found that GADD34 deficiency led to a reduction in the expression of p21 and allowed cells to avoid senescence. Furthermore, restoration of GADD34 expression by retroviral transduction into the deficient cells rescued p21 expression. Taken together with previous results, this study suggests that GADD34 mediates p53 activation and subsequent expression of p21, and that GADD34 may be an important player in the regulation of cellular senescence. Further clarification of the mechanism by which GADD34 contributes to the regulation of cellular senescence is necessary, and may eventually lead to new approaches to the prevention of diseases such as cancer.

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