

Suppression of Gadd45 Alleviates the G2/M Blockage and the Enhanced Phosphorylation of p53 and p38 in Zinc Supplemented Normal Human Bronchial Epithelial Cells

RITA S. M. SHIH,* STEPHEN H. K. WONG,* NORBERTA W. SCHOENE,† AND KAI Y. LEI*¹

*Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742; and †Diet, Genomics, and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

An adequate zinc status is essential for optimal cellular functions and growth. Yet, excessive zinc supplementation can be cytotoxic and can impair cell growth. Gadd45 plays a vital role as cellular stress sensor in the modulation of cell signal transduction in response to stress. The present study was designed to determine the influence of zinc status on Gadd45 expression and cell cycle progression in zinc deficient and supplemented normal human bronchial epithelial (NHBE) cells, and to decipher the molecular mechanism(s) exerted by the suppression of Gadd45 expression on cell growth and cell cycle progression in this cell type. Cells were cultured for one passage in different concentration of zinc: <0.4 μ M (ZD) as severe zinc deficient; 4 μ M as normal zinc level in culture medium; 16 μ M (ZA) as normal human plasma zinc level; and 32 μ M (ZS) as the high end of plasma zinc attainable by oral supplementation. Inhibition of cell growth, upregulation of Gadd45 mRNA and protein expression, and blockage of G2/M cell cycle progression were observed in ZS cells. In contrast, little or no changes in these parameters were seen in ZD cells. The siRNA-mediated knocking down of Gadd45 was found to relieve G2/M blockage in ZS cells, which indicated that the blockage was Gadd45 dependent. Moreover, the enhanced phosphorylation of p38 and p53 (ser15) in ZS cells was normalized after suppression of Gadd45 by siRNA, implicating that the enhanced phosphorylation of these proteins was Gadd45 dependent. Thus, we demonstrated for the first time

that an elevated zinc status modulated signal transduction to produce a delay at G2/M during cell cycle progression in NHBE cells. *Exp Biol Med* 233:317–327, 2008

Key words: Gadd45; p38; p53; siRNA; G2/M; zinc supplementation

Introduction

Bronchial epithelial cells are the physical barrier that separates airway connective tissue and smooth muscle from the airway luminal contents. In the absence of intact epithelial barrier, the airway lumen may contain harmful substances, which could initiate inflammatory reactions in the submucosa (1–3). Exposure to high concentration of zinc in the air may cause significant health risk (4). Zinc toxicity can cause acute respiratory tract inflammation together with bronchial hyper-responsiveness. Studies have shown that workers in mining industries had increased polymorpho-nuclear leukocytes and incidence of pulmonary inflammation (5). In view of the prevalence and clinical significance of zinc deficiency in human populations, as well as extensive use of zinc supplementation in animal production and to a lesser extent in human populations, we have initiated studies designed to examine the influence of zinc status on the expression of stress inducible gene, the growth arrest and DNA damage-induced gene (Gadd) Gadd45, in Normal Human Bronchial Epithelial (NHBE) cells. NHBE cells have been selected for this study because they are more representative of cell population during lung tissue transformation and are considered to be progenitor cells for human bronchial cancer.

Gadd45 was originally identified as the mRNA transcript that was rapidly induced in response to UV radiation (6). Gadd45 is an ubiquitously expressed 21kD acidic protein in response to genotoxic agent, and is involved in

This work was supported by US Department of Agriculture National Research Initiative Grant 2002-35200-12241 (K.Y. Lei).

¹ To whom correspondence should be addressed at Department of Nutrition and Food Science, 0121 Skinner Building, College Park, MD 20742. E-mail: dlei@umd.edu

Received August 15, 2007.
Accepted October 28, 2007.

DOI: 10.3181/0708-RM-220
1535-3702/08/2333-0317\$15.00
Copyright © 2008 by the Society for Experimental Biology and Medicine

many biological processes related to the maintenance of genomic stability and apoptosis. Gadd45^{-/-} knockout mice were more susceptible to DNA-damage induced tumors when subjected to carcinogens (7, 8, 9). Over expression of Gadd45 in tumor cell lines was found to inhibit cell growth (10). Moreover, Gadd45 induction through tet-off inducible system suppressed cell growth (11). Initially, over expression of Gadd45 by microinjecting Gadd45 expression vector was found to induce G2/M cell cycle arrest (12). The importance of Gadd45 in G2/M regulation was further supported by findings of the inability of cells from Gadd45 knockout mice to arrest at the G2/M phase after exposure to UV radiation. In cells under hyperosmolarity stress, inhibition of cell cycle progression was accompanied by Gadd45 induction, which was partially dependent on p38 kinase activity (13). Various studies demonstrated a functional association between stress-activated mitogen-activated protein kinase pathway and Gadd45 in response to environmental stresses (14). Gadd45 induction also depends on p38 activity in other cell types exposed to oxidative stress (15), flavonoids (16), or peripheral benzodiazepine receptor-specific ligands (17). Conversely, p38 activity depends on MEKK4 activation mediated by Gadd45 protein in response to an array of stimuli (13, 18–20). Tumor suppressor gene p53 plays an important role in the maintenance of genomic fidelity by controlling cell cycle checkpoints and apoptotic process following cell exposure to genotoxic stress. The dependence of Gadd45 induction on normal cellular p53 function is well established (21). In response to DNA damage, Gadd45 was found to contribute to the stability of p53.

The present study was designed to determine the influence of zinc status on Gadd45 expression and cell cycle progression in NHBE cells, and to decipher the molecular mechanism(s) exerted by the suppression of Gadd45 expression on cell growth and cell cycle progression in this normal human cell type. Findings from this study demonstrated that zinc supplementation of normal human bronchial cells produced adverse effects by increased expression of Gadd45 resulting in cycle blockage at G2/M and an increase in phosphorylation of tumor suppressor genes p53 and p38.

Materials and Methods

Cell Culture. NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were plated at 3,500 cells/cm² in tissue culture dishes containing bronchial epithelial cell growth medium (BEGM), supplemented with 0.5 µg/ml epinephrine, 10 µg/ml transferrin, 5 µg/ml insulin, 0.1 ng/ml retinoic acid, 52 µg/ml bovine pituitary extract, 0.5 µg/ml hydrocortisone, 0.5 pg/ml human recombinant epidermal growth factor, and 6.5 ng/ml triiodothyronine (as growth supplements) without antibiotics, and cultured at 37°C in a 5% CO₂ incubator. Endotoxin-free medium was used (<0.005 endotoxin units/

ml). The medium was changed at day 1 and subsequently every 48 h. The cells were grown to 80% confluence for 6 days, and subcultured using trypsin-EDTA at a ratio of 1:8 at passage 3 for experimental zinc treatment.

A zinc-free BEGM baseline medium, in which Cambrex omitted the addition of ZnSO₄, was used as the zinc-depleted medium. This medium consisted of Bronchial Epithelial Basal Medium (BEBM) supplemented with growth components, and contained residue amounts of zinc (<0.4 µM), as detected by flame atomic absorption spectrophotometry. The zinc-free basal medium of <0.4 µM was used as the zinc-depleted medium (ZD). For the other four treatment groups, zinc was added to the media in the form of ZnSO₄ so that the only difference between these media was the zinc concentration. For the zinc-normal medium (ZN) contained 4 µM of ZnSO₄, the zinc-adequate medium (ZA) contained 16 µM of ZnSO₄, and the zinc-supplemented medium (ZS) contained 32 µM ZnSO₄. The ZN medium was used as a comparison to standard culture media and was used as the control group for experiments. The ZA treatment was used as a representative of human plasma zinc levels, and the ZS group was used to represent the high end of plasma zinc levels attainable by oral supplementation in humans (22). After NHBE cells were subcultured into one of the four corresponding groups, the cells were cultured overnight in ZN media before changing to their respective medium. Cells were then cultured in ZD, ZN, ZA and ZS media for 6 days. The cell number was determined by using a hemocytometer, and cell viability was assessed by trypan blue dye exclusion. Cell morphology was evaluated by using a phase-contrast microscope (Olympus, Tokyo, Japan).

Cellular Zinc and DNA Determination. After reaching 80% confluence, cells were harvested by treatment with trypsin-EDTA for 5 min in 37°C incubator. Both cells and media were collected by scraping from tissue culture dishes. And cell suspensions were then centrifuged at 500 × g for 5 min at 4°C, and cell pellets were washed three times with phosphate-buffered saline (PBS). Cells were resuspended into 1.5ml PBS and sonicated for two 30 sec intervals. An aliquot of the sonicated cell suspension was immediately aspirated into the flame atomic absorption spectrophotometer for the measurement of cellular zinc content by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). Zinc standard solutions (Fisher, Pittsburgh, PA) ranging from 0.05 ppm to 1.0 ppm were used to generate a linear standard curve. The zinc content of the cells was determined based on these zinc reference solutions. In addition, the certified zinc solutions were compared to bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. From the same sample, a small aliquot of the sonicated cell suspension was used to measure cellular DNA content using diphenylamine (23). Data were expressed as cellular zinc per microgram of DNA because of

the linear relationship between cellular DNA and cell number we previously established (23).

RNase Protection Assay. Total RNA was isolated from NHBE cells using the RNeasy Lysis Kit (Qiagen, Valencia, CA), according to manufacturer's instruction and the integrity of the RNA was verified by electrophoresis and quantified by spectrophotometry. The mRNA abundance of human Gadd45 gene was measured by the non-radioactive RNase Protection Assay (Pharmingen, San Diego, CA). The human GAPDH probe was also included in the multi-probe and was used as housekeeping gene for normalization. Labeled riboprobes were synthesized using the Non-Radioactive In Vitro Transcription kit with T7 RNA polymerase (Pharmingen, San Diego, CA), and Biotin-dUTP (Roche, Alameda, CA).

RNase Protection Assay (RPA) was performed using the Pharmingen RPA kit. Each sample contained 10 µg of total RNA from NHBE cells, and 2 µg of the multi-riboprobe. The RNA and labeled probes were co-precipitated with ammonium acetate and ethanol, and resuspended in hybridization buffer at 56°C for 16 h. The RNase digestion was performed at 30°C for 45 mins, followed by inactivation with proteinase K cocktail, and subsequent precipitation. Protected fragments were separated by Tris-Urea Polyacrylamide Gel Electrophoresis (PAGE) using pre-casted gel from BioRad (Hercules, CA). Control samples were processed without RNase digestion and only full-length probes were applied. No protected bands appeared in controls, in which yeast RNA replaced NHBE RNA indicating that digestion was complete. The PAGE gel resolved protected probes were transferred to nylon membrane and subjected to UV crosslinking. The biotin-labeled protected cDNA transferred to the membrane were detected by chemiluminescent signal and visualized by X-ray film exposure.

Cell Cycle Analysis. DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca²⁺, Mg²⁺-free), and fixed in 70% cold ethanol. Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg per ml propidium iodide, 100 U per ml RNase in PBS), and incubated at room temperature for 1 h. Staining was quantitated with a FACSCalibur cytometer (Becton Dickinson, San Jose, CA). Flow cytometric data files were acquired for 10,000 cellular events with the CELLQuestPro software program (Becton Dickinson, San Jose, CA). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). This same software can also be used to estimate the percentage of DNA fragments found in the sub G0/G1 region to give an estimate of apoptosis (24). The calibration standard LinearFlow green and the DNA QC Particle kit, for verification of instrument

performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

Nuclear and Cytoplasmic Extract Preparation. The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation according to the manufacturer's instructions, which are based on the method of Smirnova et al. (25). Nuclear and cytoplasmic extracts were then stored in aliquots at -80°C. Protein concentrations were determined by using the BCA Protein Assay Reagent kit (Pierce). Contaminations of nuclear extracts by cytoplasmic proteins or contamination of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of Hsp90 or Oct-1, respectively, were routinely found to be less than 5% in our lab.

Western Blot Analysis. Nuclear and cytoplasmic protein concentrations were determined by using the BCA kit (Pierce). Forty µg of protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Germany) by using a mini-transfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10mM phosphate buffer pH7.3, 137mM NaCl, 2.7mM KCl, and 0.1% Tween 20) for 1 h at room temperature, prior to incubation with 1 µg/ml of primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4 °C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz, Santa Cruz, CA) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Anti-Gadd45 (H165), anti-phospho-p53 (Ser15), anti-phospho-p38 (D-8), anti-actin (I19) and anti-histone H1 (Fl 219) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

siRNA Transfection. RNA interference of Gadd45 was performed by using a 23 bp (including 2-deoxynucleotide overhang) siRNA duplex with the DNA sequence AAAGTCGCTACATGGATCAAT. A control siRNA that specifically targeted the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG was used as a non-silencing control. siRNA duplexes were synthesized from Qiagen (Valencia, CA). For cell transfection with siRNA, NHBE cells cultured on culture dishes at 60% confluency, and siRNA (600pmol) were introduced into the cells using RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Briefly, cells were cultured under different zinc status in cell culture dishes, in the following treatments: ZD-Luciferase, ZN-Luciferase, ZS-Luciferase, ZD-Gadd45, ZN-Gadd45, and ZS-Gadd45. Aliquots of 600 pmol of siRNA-Gadd45 or siRNA-Luciferase were diluted with Opti-MEM medium (Invitrogen, Carlsbad, CA), and another

Table 1. The Influence of Zinc Status on Cell Growth and Cellular Zinc Level in NHBE Cells

	Zinc concentration in NHBE medium			
	Zinc deficient (ZD)	Zinc normal (ZN)	Zinc adequate (ZA)	Zinc supplemented (ZS)
	0.4 μ M	4 μ M	16 μ M	32 μ M
DNA content (% of ZN) ¹	83 $\pm$ 1 ^{3 b}	100 $\pm$ 4 ^c	79 $\pm$ 3 ^b	67 $\pm$ 4 ^a
Cellular zinc level (zinc/DNA, ng/ μ g) ²	1.6 $\pm$ 0.2 ^a	4.3 $\pm$ 0.5 ^b	9.1 $\pm$ 1.7 ^c	17.1 $\pm$ 1.1 ^d

¹ DNA content per plate in normal human bronchial epithelial (NHBE) cells after cultured in respective zinc media for one passage. DNA content was determined by diphenylamine method.

² Cellular zinc level in NHBE cells treated with different concentrations of zinc (ZD, ZN, ZA and ZS) for one passage. Cellular zinc was measured by flame atomic absorption spectrophotometry.

³ Values are means $\pm$ SEM from three experiments. Similar results were obtained from experiments with cells from another subject.

solution containing lipofectamine transfection reagent was also diluted with Opti-MEM medium. These two mixtures were combined together at room temperature with gentle vortex, and incubated for 20 min, and followed by addition of 10 ml corresponding medium for the treatment groups. The mixture was then overlaid onto the 60% confluent NHBE cells. After 48 hours, cells were harvested for cell cycle and western blot analyses.

Statistical Analysis and Data Presentation. Each experiment was repeated at least three times. Data were expressed as mean $\pm$ SEM. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

Data were presented in table or figure format and mostly expressed as percent of ZN for ease of comparison between treatments. Most importantly, this approach will allow the figures with essential gel data to be appreciated next to the corresponding bar graphs.

Results

Zinc Supplementation Markedly Reduced Cell Growth in Normal Human Bronchial Cells. In ZD, ZA and ZS cells, cell growth as measured by DNA content per plate, was found to be reduced to 83%, 79%, and 67%, respectively, of the ZN cells (Table 1). The culture of cells in zinc-depleted medium resulted in about 50% reduction of cellular zinc as compared to control ZN cells (Table 1). Moreover, cellular zinc level in ZA and ZS cells was about 200% and 400% of that of ZN control cells, respectively. Thus, cell growth was reduced both by the low-zinc and high-zinc status, particularly in ZS cells. Furthermore, a dose-dependent elevation in cellular zinc content was observed as the zinc concentration in the media was increased.

Gadd45 mRNA Abundance Was Up-Regulated in ZS NHBE Cells. *Gadd45* mRNA abundance was up-regulated in ZA and ZS NHBE cells to 149 $\pm$ 11% and 185 $\pm$ 5%, respectively, of ZN control cells (100 $\pm$ 1%) (Fig. 1A). Although the mRNA abundance in ZD cells appeared to be lower than ZN cells, the difference was not

significant. Thus, in ZA and ZS cells, the marked elevations in *Gadd45* mRNA levels were associated with increases in cellular zinc levels and reductions in cell growth.

Gadd45 Protein Level Was Markedly Elevated in ZS NHBE Cells. In ZS cells, western blot analysis indicated an almost 5-fold increase in cytoplasmic (Fig. 1B) and a 4-fold increase in nuclear (Fig. 1C) *Gadd45* proteins when compared with the ZN cells. The cytoplasmic and nuclear *Gadd45* protein levels were increased 1.4-fold and 1.6-fold, respectively, in ZD cells; as well as 2.2-fold and 1.3-fold, respectively, in ZA cells, as compared to ZN control cells. These findings indicated that the magnitude of increases in *Gadd45* protein among the treatment groups were higher than the increases in mRNA levels, especially for the ZS group. Thus, there may be additional posttranscriptional regulation involved in the enhancement of the protein stability of *Gadd45*. Because the depression of cell growth induced by an upregulation of *Gadd45* protein is well established, the marked elevation in *Gadd45* protein in ZS cells may contribute to the observed reduction in cell growth.

Zinc Supplementation Delayed G2/M Cell Cycle Progression in NHBE Cells. To uncover the mechanism responsible for the marked cell growth reduction in ZS cells, we next examined the cell cycle progression by flow cytometry. A marked delay in G2/M cell cycle progression was observed in ZS NHBE cells (24.39 $\pm$ 0.53%) when compared to ZN cells (13.15 $\pm$ 0.12%) (Fig. 2). The delay was smaller in magnitude in ZA cells, with 15.59 $\pm$ 0.20% of cells in G2/M. In contrast, no change was observed in ZD cells, with 12.98 $\pm$ 0.60% cells in G2/M, when compared to ZN cells.

siRNA Mediated Gene Silencing Knocked Down Gadd45 Protein Expression. The approach of siRNA-mediated gene silencing of *Gadd45* was used to establish whether the knock down of the marked elevation of *Gadd45* in ZS cells would normalize the delayed in G2/M cell cycle progression. After *Gadd45* siRNA transfection, the *Gadd45* protein levels were knocked down to similar levels in all *Gadd45* siRNA transfected zinc treatments, ZD-Gad (56 $\pm$ 14%), ZN-Gad (44 $\pm$ 10%) and ZS-Gad (53 $\pm$ 8%), as compared to ZN-C controls (100 $\pm$ 9%) (Fig. 3).

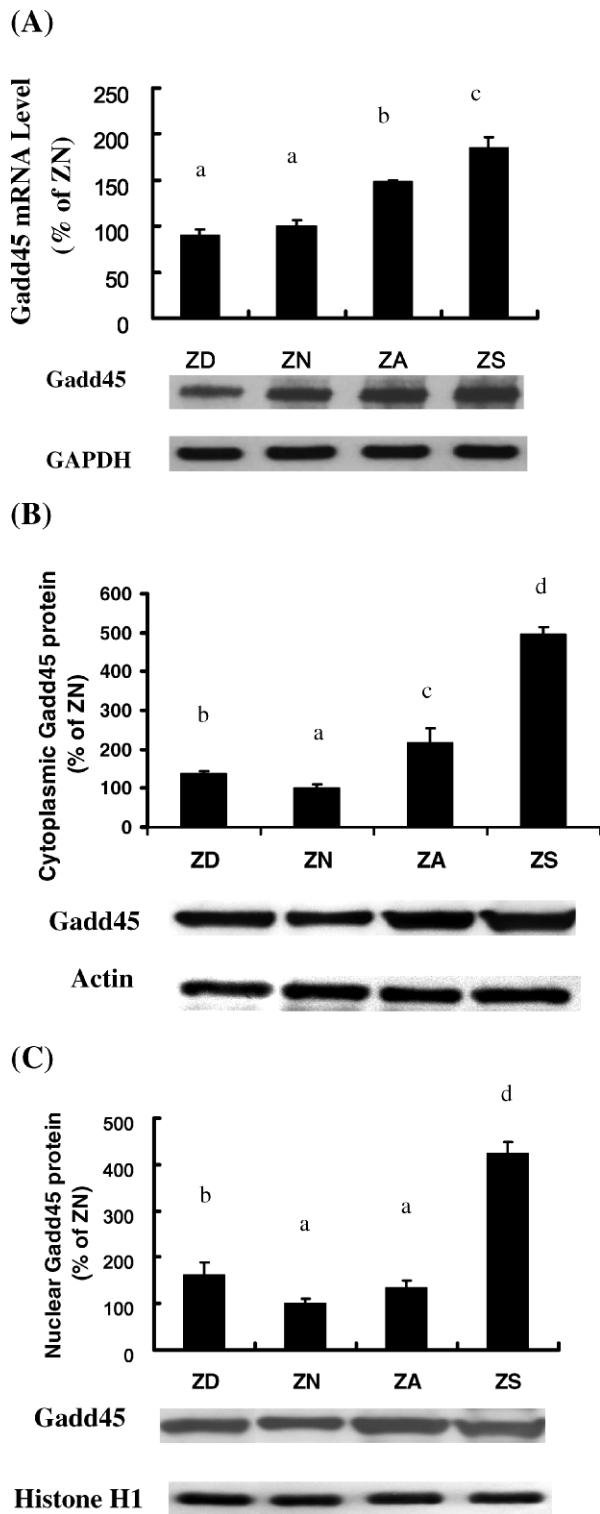


Figure 1. Gadd45 mRNA abundance (A) as well as Gadd45 protein level in the cytoplasm (B) and nucleus (C) were up-regulated in ZS NHBE cells. Cells were cultured in ZD ($<0.4 \mu\text{M}$ zinc), ZN ($4.0 \mu\text{M}$ zinc), ZA ($16.0 \mu\text{M}$ zinc), and ZS ($32.0 \mu\text{M}$ zinc) media. Cells were cultured for 1 passage in BEGM with zinc added as a supplement to the ZD medium. (A) Gadd45 mRNA level was measured by RNase Protection Assay (RPA). RNase protection products were separated on a polyacrylamide gel and quantitated by laser densitometry. GAPDH was used as an internal reference, and values are expressed as a percentage of ZN controls. (B) Cytoplasmic and

siRNA Mediated Gadd45 Knocked Down Abrogated the Blockage in G2/M Cell Cycle Progression and Triggered Apoptosis in ZS NHBE Cells. The blockage in G2/M cell cycle progression was partially abrogated in ZS-Gad group. Similarly, ZD-Gad and ZN-Gad showed the same amount of cells in G2/M after siRNA transfection (Fig. 4). The extent of DNA fragments found in the sub G0/G1 region was used as an estimate of apoptosis. Even with the knock down of Gadd45, the amount of DNA in the region was found to be modest and essentially the same for all treatment groups.

siRNA Mediated Suppression of Gadd45 Depressed the Enhanced Phosphorylation of p53 at Position ser 15 and Phosphorylation of p38 in ZS NHBE Cells. Phosphorylated p53 (ser 15) (Fig. 5A) and phosphorylated p38 (Fig. 5B) protein levels in ZS cells were induced to around 2-fold and 2.5-fold, respectively, higher than ZN cells. Interestingly, after the suppression of Gadd45 mediated by siRNA, these two induced phosphorylated proteins were decreased to around the same level of ZN control cells in all siRNA (ZD-Gad, ZN-Gad, ZS-Gad) treatment groups. These findings suggest that the up regulation of these phosphorylated proteins was Gadd45 dependent.

Discussion

In this report, we provide evidence that the G2/M blockage found in physiological range of zinc supplementation in NHBE cells is associated with an up regulation of Gadd45 expression. The administration of small interference RNA targeting Gadd45 abrogated the G2/M blockage in zinc supplemented NHBE cells. The silencing of Gadd45 also suppressed the phosphorylated-p53 (ser 15) and phosphorylated-p38 levels, which indicated that Gadd45 might have contributed to the enhanced phosphorylation of p53 and p38 in response to the stress induced by zinc supplementation in NHBE cells.

Zinc Supplementation Inhibits Normal Human Bronchial Cells Proliferation. In zinc supplemented NHBE cells, cellular zinc was fourfold higher than that of ZN control group (Table 1), and cell growth as measured by cell number was decreased to 60% of ZN (Table 1). Our study is the first to demonstrate that zinc supplementation, within the physiological range, is capable of reducing the

(C) nuclear protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and incubated with anti-Gadd45 antibody. Cytoplasmic and nuclear samples were probed with antibodies against Actin and Histone H1, respectively, for normalization. Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Densitometric data are expressed as a percentage of ZN controls. Representative samples from each treatment group are shown below the bar graph. Values are means $\pm$ SEM from three experiments. Different letters indicate significantly different means, $P < 0.05$. Treatments with the same letters indicate no significant difference. Similar results were obtained from experiments with cells from another subject.

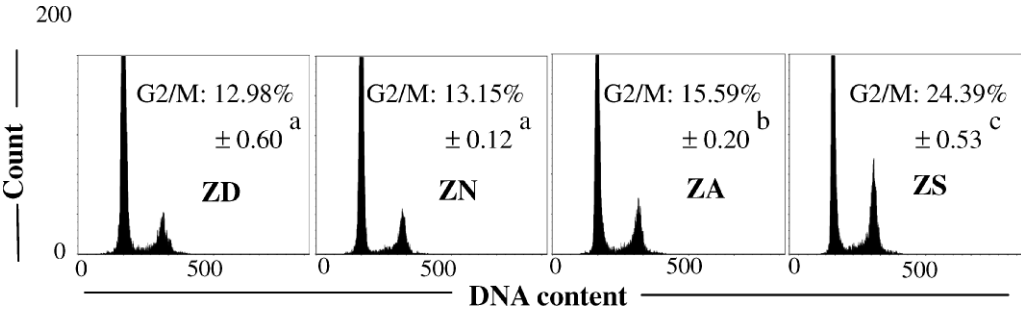


Figure 2. Zinc supplementation blocked G2/M progression in NHBE cells. Cell cycle analysis of NHBE cells was assayed by flow cytometry using a FACScalibur cytometer. Cells were cultured in ZD, ZN, ZA, and ZS media for one passage. Washed cells were fixed in ethanol and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed by the CELLQuest program. Cell cycle distribution percentages of stained nuclei were calculated using Modfit LT software. The calibration standard LinearFlow green and the DNA QC particle kit were used for verification of instrument performance. Histograms are representative of three independent experiments. The proportions of cells in G0/G1, S phase and the G2/M ratios are indicated for each treatment group. Similar results were obtained from experiments with cells from another subject.

growth of NHBE in primary culture. Thus, high physiologic level of zinc also imposes adverse effect in normal cells. Controversy towards the efficacy of zinc supplementation in the prevention of cancer growth has been reported. Several studies have indicated that high cellular zinc levels inhibit cancer cell growth (26–28). Moreover, studies also reported

that treatment with high level of zinc not only inhibited proliferation of cancer cells but also induced G2/M arrest (29, 30). However, an epidemiological study has reported an increase in risk for cancer development with usage of high level of zinc supplement. (31). Even though zinc supplementation can inhibit cancer cell proliferation, the cytotoxic

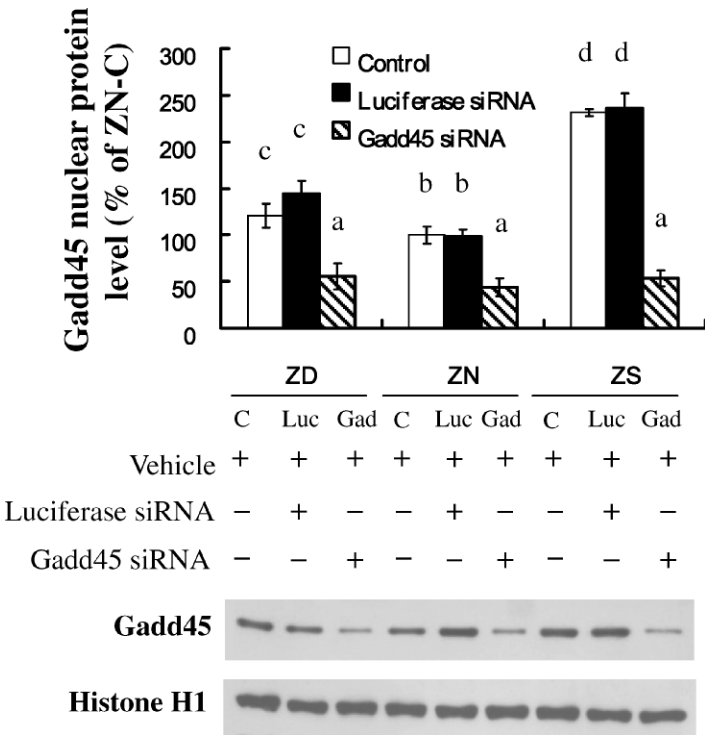


Figure 3. siRNA mediated gene silencing knocked down Gadd45 protein expression. Nuclear Gadd45 protein levels are presented as percentage of ZN-C (control in ZN group). NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups (ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc). Control groups (ZD-C, ZN-C and ZS-C) were transfected with lipofectamine only (vehicle). Nuclear Gadd45 protein levels were knocked down by siRNA targeting Gadd45 in ZD-Gad, ZN-Gad and ZS-Gad treatment groups. Nuclear protein extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and probed with anti-Gadd45 antibody. Nuclear samples were probed with antibody Histone H1 for normalization. Autoradiography was visualized by using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means $\pm$ SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$. Similar results were obtained from experiments with cells from another subject.

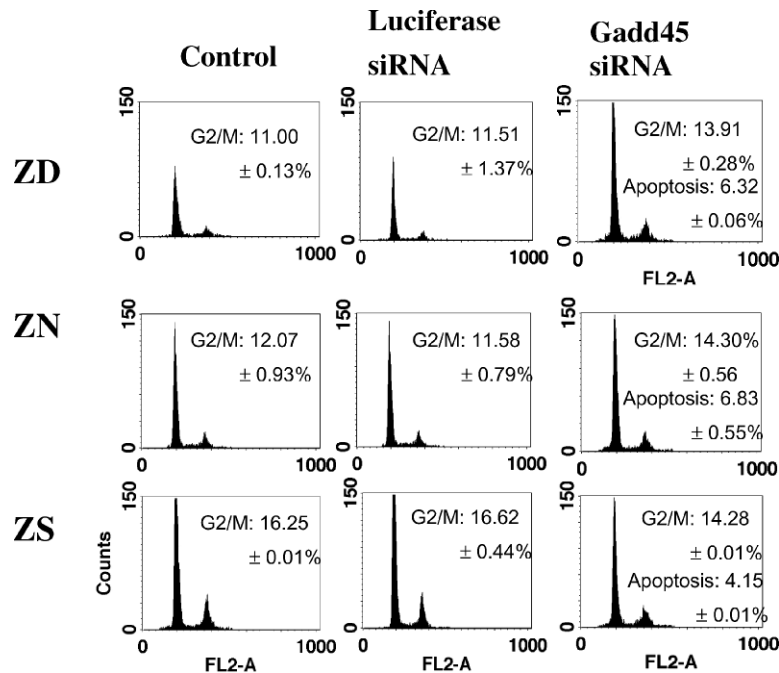


Figure 4. siRNA mediated Gadd45 knocked down relieved the delay in G2/M cell cycle progression in ZS NHBE cells. Cell cycle analysis was performed after siRNA transfection. NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups including ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc. Cells were harvested and washed, followed by fixation in ethanol, and stained with propidium iodide for DNA content. Flow cytometric data files were collected and analyzed by the CELLQuest program. Cell cycle distribution and apoptosis percentages were calculated using Modfit LT software.

effect of zinc (90 and 900 $\mu\text{mol/L}$) resulting in the induction of DNA fragmentation has been observed in human primary liver cells (32).

Upregulation of Gadd45 Is Associated with Cell Growth Suppression in Zinc Supplemented NHBE Cells. Our results demonstrate that cell growth suppression was accompanied by marked induction of Gadd45 expression, which suggests that Gadd45 may be the main regulator in cell growth inhibition in zinc supplemented NHBE cells. This hypothesis is supported by numerous findings regarding the function of Gadd45 reported by other research groups. Several reports have established that over-expression of Gadd45, by transient transfection and tet-off inducible system, substantially inhibited cell growth in multiple tumor lines (33, 11). In addition, Gadd45 has been established to be downstream of p53-dependent pathway in cells harboring functional p53 or null-p53. To determine whether Gadd45 is the single regulator of p53-dependent pathway in response to cellular stress, an experiment is in progress, and p21^{WAF/CIP1} is the candidate gene to be studied. Although Gadd45 reveals a similar growth suppressive property as p21^{WAF/CIP1}, it does not utilize Rb-related pathways and may act differently to exert its growth-suppressive function (34).

G2/M Blockage Was Found in ZS NHBE Cells. In our study, cell cycle analysis by flow cytometry demonstrated that there was G2/M blockage in ZS cells (Fig. 2). In ZS cells, G2/M blockage was accompanied by three to fivefold induction of nuclear and cytoplasmic

Gadd45 proteins levels, respectively (Fig. 1B and 1C), and by twofold increase in mRNA abundance (Fig. 1A) as compared to ZN control cells. These results suggest that zinc supplementation may cause cellular stress, which induces Gadd45 expression. With the increase in Gadd45 expression in ZS cells, G2/M blockage occurs to delay the transition and hinder cell growth. The underlying mechanism by which Gadd45 blocks the G2/M cell cycle progression needs to be further investigated. This probably is due to the binding of Gadd45 to Cdk1, which decreases Cdk1 kinase activity, and hinders the cell progression from G2 to M (11). High dosage of zinc has been reported to induce DNA fragmentation in human cells, implying that DNA damage may occur in high zinc supplementation (30). The molecular mechanism by which stress signals arrest cell cycle progression at the G2/M checkpoint is still not fully established. The cell cycle control system is based on the cyclin-dependent protein kinases (Cdk) and the cyclins. Cyclin B1 that undergoes a cycle of synthesis and degradation with each division cycle, is capable of binding to Cdk1. This dimer forms the M phase-promoting factor (MPF) during G2 and is required for entry into mitosis. Gadd45 has been shown to bind Cdk1 and inhibit its activity or alter the subcellular level of Cyclin B1 in human cells when exposed to stresses like ionizing radiation or ultra violet radiation (11, 35, 36). Gadd45 induces G2/M arrest through p53-dependent and independent pathways by decreasing Cdk1 kinase activity and changing subcellular Cyclin B1 levels in many cell types when exposed to

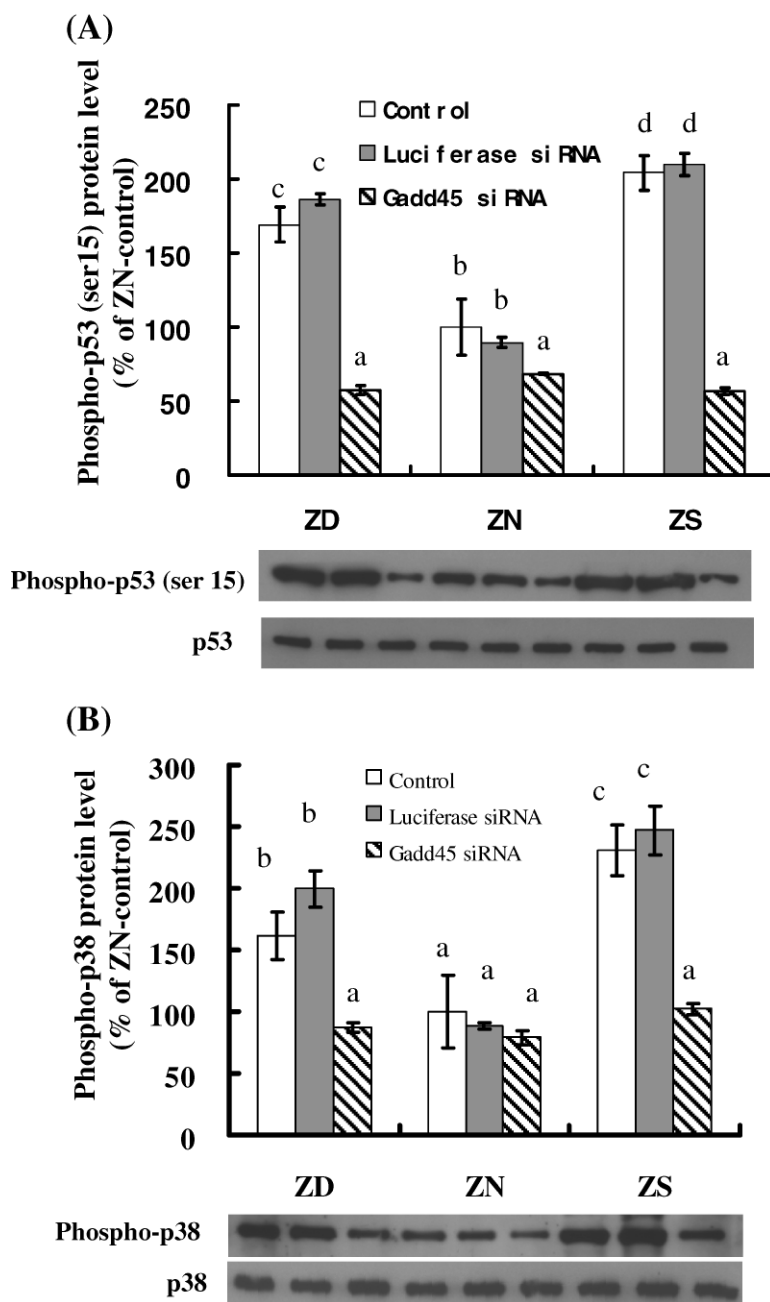


Figure 5. The enhanced phospho-p53 (ser15) protein level (A) were depressed and phosphorylation of p38 (B) was normalized in ZS and ZD after knock down of Gadd45 by siRNA. NHBE cells were transiently transfected with either siRNA targeting Gadd45 (Gad) or Luciferase (Luc) in the treatment groups (ZD-Gad, ZN-Gad, ZS-Gad, ZD-Luc, ZN-Luc and ZS-Luc). Control groups (ZD-C, ZN-C and ZS-C) were transfected with lipofectamine only (vehicle). After 48 hours, cells were lysed and analyzed. Total cell extracts were separated on 10% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes, and probed with anti-phospho-p53 (ser15) antibody (A) and with anti-phospho-p38 antibody (B). Autoradiography was visualized using enhanced chemiluminescence and quantitated by densitometry. Values are expressed as a percentage of ZN controls. Representative samples, from each treatment group, are shown below the bar graph. Values are means $\pm$ SEM from 3 experiments. Different letters indicate significantly different means, $P < 0.05$.

stresses (37, 41). The effect of Gadd45 on the G2/M transition may be due to its ability to dissociate complexes of Cyclin B1 and Cdk1 as well as to inhibit the activity of Cdk1/Cyclin B1 in vitro (35, 36). In the present study, no G1 arrest was observed in ZS cells. This is consistent with data from another study, which reported that Gadd45 did not efficiently inhibit Cdk2/Cyclin E, nor caused G1 arrest

when microinjected (35). However, the observed G2/M blockage may require p53 if Gadd45 acts directly on Cdk1/Cyclin B1. One explanation is that Gadd45 may cooperate with other downstream targets of p53, such as p21, whose constitutive expression is lost upon deletion of p53 (42).

The Upregulation of Gadd45 Accounts for the G2/M Blockage in ZS NHBE Cells. To show the

involvement of Gadd45 in G2/M cell cycle regulation in zinc supplemented NHBE cells, we initiated the small interfering RNA targeting Gadd45 experiment to knock down Gadd45. The success of knocking down Gadd45 was confirmed by the marked reduction in Gadd45 protein level (Fig. 3). Gadd45 protein was knocked down to 40–50% of ZN control group (ZN-C). Consistent with reports from other research groups (43), which studied the effect of knocking out Gadd45, cell cycle analysis of NHBE cells by flow cytometry indicated a decrease in G2/M blockage in ZS cells after targeting Gadd45 siRNA (ZS-Gad) than in ZS control groups (ZS-C & ZS-Luc; Fig. 4). Thus, the up-regulated Gadd45 expression may partially accounts for the G2/M blockage in zinc-supplemented cells. After the knocked down of Gadd45, a small but similar extent of apoptosis was observed among all zinc treatment groups in NHBE cells. This is because the suppression of Gadd45 can no longer protect cells from apoptosis under the cytotoxic status induced by zinc supplementation. The reason for the abrogated G2/M blockage not normalizing to the level of ZN (ZN-C) cells may be due to another signaling pathway independent of Gadd45 that regulates G2/M transition, which may include p38. This project provides evidence that GADD45 plays a direct role in the G2/M cell cycle checkpoint in zinc supplemented NHBE cells. In addition, the findings are consistent with those from other studies which reported the correlations of Gadd45 upregulation with G2/M arrest in human fibroblasts (37, 39, 43).

Possible Pathways Regulate Gadd45 in G2/M Blockage in ZS NHBE Cells. Gadd45 has been shown to be a direct target gene of p53 tumor suppressor gene, and to play a vital role in cell cycle progression. Gadd45 can be regulated either by p53-dependent or –independent pathway in response to stress. In this study, the p53 mRNA and protein expressions exhibited similar increases in ZS NHBE cells (data not shown). Moreover, the silencing of Gadd45 suppressed the phosphorylation of p53 at position serine 15, which is required for activation of p53, suggesting that Gadd45 can behave as an upstream activator of the p53 phosphorylation process through other mechanism. Our findings on the relationship of Gadd45 and p53 indicated that the upregulation of Gadd45 enhanced the phosphorylation of p53. Similarly, other studies have shown that the upregulation of Gadd45 is capable of enhancing p53 protein level through a positive feedback loop by binding to MTK1 and activates p38 indirectly (44, 45). Moreover, studies have established that Gadd45 can bind with PCNA (46, 48) and p21^{WAF/CIP1} (49), which are downstream regulators of p53. Furthermore, chromatin immunoprecipitation studies have shown that p53 preferentially binds to the responsive element in the promoters of p21 and GADD45 but not to promoters of other target genes that recruited p53 following DNA damage (50). Therefore, the observed enhanced Gadd45 expression possibly may act through a p53-dependent pathway in the G2/M blockage in the ZS NHBE cells.

Another possible regulatory pathway responsible for the upregulation of Gadd45 in ZS NHBE cells is the interaction of Gadd45 protein with the activated p38 MAPK directly or indirectly. Indirectly, Gadd45 can bind to the N-terminal of MTK1 and disrupt the N-C interaction within MTK1 itself. This Gadd45-binding induces MTK1 N-C dissociation, dimerization, and autophosphorylation and leads to activation of MAPK, including p38 (51). There is one p38 interacting domain in the region of amino acids 71 to 96 in Gadd45. And this region is required to activate p38 in the presence of H-ras oncogene (20). In our study, we demonstrated that silencing Gadd45 suppressed p38 phosphorylation, suggesting that the activation of p38 is mediated through Gadd45 protein. The Gadd45 protein may directly bind to p38 upstream regulator, such as MTK1 and trigger the phosphorylation of p38 in ZS NHBE cells. The activated p38 then binds to downstream regulator, p53, and activates its function, and consequently arrests the NHBE cells in G2/M. Apart from interacting with Gadd45 in stress induced G2/M arrest, p38 also acts independently of p53 pathway by shuttling phosphatase Cdc25B from nucleus to cytoplasm and recruited 14-3-3 to bind to Cdc25B (52). Cdc25B is required to activate Cdk1 by removing two phosphate groups and the activated Cdk1 is required for entry into mitosis.

In summary, the novel findings presented above demonstrated that zinc supplementation, in a physiologically relevant system, impaired cell growth, markedly elevated Gadd45 expression, inhibited G2/M progression, and enhanced phosphorylation of p53 (ser 15) and p38 in NHBE cells. The data also contributed to establishing a possible mechanism responsible for the observed cell cycle arrest in normal human cells. A better understanding of the mechanisms involved in the protection of normal cells from zinc cytotoxicity is essential for the identification of potential targets, such as Gadd45, p53 and p38. Nevertheless, the applicability of these in vitro mechanistic data to in vivo conditions, such as industrial pulmonary exposure and extensive use of dietary zinc supplementation, would require detailed in vivo studies to establish cellular and subcellular zinc concentrations as well as to examine the parameters studied here.

The assistance provided by Ms. Noella A. Bryden in the analyses of trace minerals in Dr. Richard A. Anderson's Lab at ARS/USDA is gratefully acknowledged.

1. Folkerts G, Nijkamp FP. Airway epithelium: more than just a barrier. *Trends Pharmacol Sci* 19:334–341, 1998.
2. Thompson PJ. Unique role of allergens and the epithelium in asthma. *Clin Exp Allergy* 28:110–116, 1998.
3. Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF. The bronchial epithelium as a key regulator of airway inflammation and remodeling in asthma. *Clin Exp Allergy* 29:90–95, 1999.
4. Merchant J, Webby R. Metal fume fever: a case report and literature review. *Emerg Med Fremantle* 13:373–375, 2001.

5. Blanc P, Wong H, Bernstein MS, Boushey HA. An experimental human model of metal fume fever. *Ann Intern Med* 114:930–936, 1991.
6. Fornace AJ Jr, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ. Mammalian genes coordinately regulated by growth arrest signals and DNA damaging agents. *Mol Cell Biol* 9: 4196–4203, 1989.
7. Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX, Fornace AJ Jr. Genomic instability in Gadd45a-deficient mice. *Nat Genet* 23:176–184, 1999.
8. Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, Fornace AJ Jr. Dimethylbenzanthracene carcinogenesis in Gadd45a-null mice is associated with decreased DNA repair and increased mutation frequency. *Cancer Res* 61:2487–2491, 2001.
9. Hildesheim J, Bulavin DV, Anver MR, Alvord WG, Hollander MC, Vardanian L, Fornace AJ Jr. Gadd45a protects against UV irradiation-induced skin tumors, and promotes apoptosis and stress signaling via MAPK and p53. *Cancer Res* 62:7305–7315, 2002.
10. Zhan Q, Lord KA, Alamo I Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ Jr. The gadd and MyD Genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol Cell Biol* 14:2361–2371, 1994.
11. Jin S, Tong T, Fan W, Fan F, Antinore MJ, Zhu X, Mazzacurati L, Li X, Petrik KL, Rajasekaran B, Wu M, Zhan Q. GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. *Oncogene* 21: 8696–8704, 2002.
12. Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ Jr, Harris CC. GADD45 induction of a G2/M cell cycle checkpoint. *Proc Natl Acad Sci U S A* 96:3706–3711, 1999.
13. Kultz D, Madhany S, Burg MB. Hyperosmolarity causes growth arrest of murine kidney cells induction of Gadd45 and Gadd153 by osmosensing via protein kinase 2. *J Biol Chem* 273:13645–13651, 1998.
14. Takekawa M, Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* 95:521–530, 1998.
15. Oh-Hashi K, Maruyama W, Isobe K. Peroxynitrite induces GADD34, 45, and 153 via p38 MAPK in human neuroblastoma SH-SY5Y cells. *Free Radical Biol Med* 30:213–221, 2001.
16. O'Prey J, Brown J, Fleming J, Harrison PR. Effects of dietary flavonoids on major signal transduction pathways in human epithelial cells. *Biochem Pharmacol* 66:2075–2088, 2003.
17. Sutter AP, Maaser K, Barthel B, Scherubl H. Ligands of the peripheral benzodiazepine receptor induce apoptosis and cell cycle arrest in oesophageal cancer cells: involvement of the p38MAPK signaling pathway. *Br J Cancer* 89:564–572, 2003.
18. Chi HB, Lu BF, Takekawa M, Davis RJ, Flavell RA. GADD45 β /GADD45 γ and MEKK4 comprise a genetic pathway mediating STAT4-independent IFN γ production in T cells. *EMBO J* 23:1576–1586, 2004.
19. Lu BF, Yu H, Chow CW, Li BY, Zheng WP, Davis RJ, Flavell RA. GADD45 γ mediates the activation of the p38 and JNK MAP kinase pathways and cytokine production in effector TH1 cells. *Immunity* 14: 583–590, 2001.
20. Bulavin DV, Kovalsky O, Hollander MC, Fornace AJ. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of Gadd45a. *Mol Cell Biol* 23: 3859–3871, 2003.
21. Carrier F, Georgel PT, Pourquier P, Blake M, Kontny HU, Antinore MJ, Gariboldi M, Myers TG, Weinstein JN, Pommier Y, Fornace AJ Jr. Gadd45, a p53-responsive stress protein, modifies DNA accessibility on damaged chromatin. *Mol Cell Biol* 19:1673–1685, 1999.
22. Hambidge KM, Casey CE, Krebs NF. Zinc. In: Mertz W, Ed. *Trace Elements in Human and Animal Nutrition* (5th ed.). Academic Press, pp1–137, 1986.
23. Williams DL, Newman TC, Shelness GS, Gordon DA. Measurement of apolipoprotein mRNA by DNA-excess solution hybridization with single-stranded probes. *Methods Enzymol.* 128:671–689, 1986.
24. Riccardi C, Nicolette I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 1:14581–1461, 2006.
25. Smirnova IV. Zinc and cadmium can promote rapid nuclear translocation of metal responsive element-binding transcription factor-1. *J Biol Chem* 275:9377–9384, 2000.
26. Bataineh ZM, Bani-Hani IH, Al-Alami JR. Zinc in normal and pathological human prostate gland. *Saudi Med J* 3:218–220, 2002.
27. Feng P, Liang JY, Li TL, Guan ZX, Zou J, Franklin R, Costello LC. Zinc induces mitochondria apoptosis in prostate cells. *Mol Urol* 4: 31–36, 2000.
28. Uzzo RG, Leavis P, Hatch W, Gabai VL, Dulin N, Zvartau N, Kolenko VM. Zinc inhibits nuclear factor-kappaB activation and sensitizes prostate cancer cells to cytotoxic agents. *Clin Cancer Res* 8:3579–3583, 2002.
29. Jaiswal AS, Narayan S. Zinc stabilizes adenomatous polyposis coli (APC) protein levels and induces cell cycle arrest in colon cancer cells. *J Cell Biochem* 93:345–57, 2004.
30. Liang JY, Liu YY, Zou J, Franklin RB, Costello LC, Feng P. Inhibitory effect of zinc on prostatic carcinoma cell growth. *Prostate* 40:200–207, 1999.
31. Leitzmann MF, Stampfer MJ, Wu K, Colditz GA, Willett WC, Giovannucci EL. Zinc supplement use and risk of prostate cancer. *J Natl Cancer Inst* 95:1004–1007, 2003.
32. Paramanantham R, Bay BH, Sit KH. Flow cytometric evaluation of the DNA profile and cell cycle of zinc supplemented human Chang liver cells. *Acta Paediatr Jpn* 38:334–338, 1996.
33. Zhan KA, Lord I, Alamo I Jr, Hollander MC, Carrier F, Ron D, Kohn KW, Hoffman B, Liebermann DA, Fornace AJ Jr. The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol Cell Biol* 14:2361–2371, 1994.
34. Zhao HC, Jin SQ, Antinore MJ, Lung FDT, Fan FY, Blank P, Roller P, Fornace AJ, Zhan QM. The control region Gadd45 is required for its interaction with p21/waf1. *Exp Cell Res* 258:92–100, 2000.
35. Zhan Q, Antinore MJ, Wang XW, Carrier F, Smith ML, Harris CC, Fornace AJ Jr. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. *Oncogene* 18: 2892–2900, 1999.
36. Jin S, Antinore MJ, Lung FD, Dong X, Zhao H, Fan F, Colchagie AB, Blanck P, Roller PP, Fornace AJ Jr, Zhan Q. The GADD45 inhibition of Cdc2 kinase correlates with GADD45-mediated growth suppression. *J Biol Chem* 275:16602–16608, 2000.
37. Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ Jr, Harris CC. GADD45 induction of a G2/M cell cycle checkpoint. *Proc Natl Acad Sci U S A* 96:3706–3711, 1999.
38. Mullan PB, Quinn JE, Gilmore PM, McWilliams S, Andrews H, Gervin C, McCabe N, McKenna S, White P, Song YH, Maheswaran S, Liu E, Haber DA, Johnston PG, Harkin DP. BRCA1 and GADD45 mediated G2/M cell cycle arrest in response to antimicrotubule agents. *Oncogene* 20:6123–6131, 2001.
39. Maeda T, Hanna AN, Sim AB, Chua PP, Chong MT, Tron VA. GADD45 regulates G2/M arrest, DNA repair, and cell death in keratinocytes following ultraviolet exposure. *J Invest Dermatol* 119:22–26, 2002.
40. Jiang F, Li P, Fornace AJ Jr, Nicosia SV, Bai W. G2/M arrest by 1,25-dihydroxyvitamin D3 in ovarian cancer cells mediated through the

- induction of GADD45 via an exonic enhancer. *J Biol Chem* 278: 48030–48040, 2003.
41. Mak SK, Kultz D. Gadd45 proteins induce G2/M arrest and modulate apoptosis in kidney cells exposed to hyperosmotic stress. *J Biol Chem* 279:39075–39084, 2004.
 42. Tang HY, Zhao K, Pizzolato JF, Fonarev M, Langer JC, Manfredi JJ. Constitutive expression of the cyclin-dependent kinase inhibitor p21 is regulated by the tumor suppressor protein p53. *J Biol Chem* 273: 29156–29163, 1998.
 43. Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM, Fornace AJ Jr. Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. *Oncogen* 13:2255–2263, 1996.
 44. Jin SQ, Mazzacurati L, Zhu XC, Tong T, Song YM, Shao SJ, Petrik KL, Rajasekaran B, Wu M, Zhan QM. Gadd45a contributes to p53 stabilization in response to DNA damage. *Oncogene* 22:8536–8540, 2003.
 45. Takekawa M, Saito H. A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* 95:521–530, 1998.
 46. Ohtani-Fujita N, Minami S, Mimaki S, Dao S, Sakai T. p53-independent activation of the Gadd45 promoter by Delta12-prostaglandin J2. *Biochem Biophys Res Commun* 251:648–652, 1998.
 47. Kearsey JM, Coates PJ, Prescott AR, Warbrick E, Hall PA. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21Cip1. *Oncogene* 11: 1675–1683, 1995.
 48. Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM, Fornace AJ Jr. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science* 266:1376–1380, 1994.
 49. Vairapandi M, Balliet AG, Fornace AJ Jr, Hoffman B, Liebermann DA. The differentiation primary response gene MyD118, related to GADD45, encodes for a nuclear protein which interacts with PCNA and p21WAF1/CIP1. *Oncogene* 12:2579–2594, 1996.
 50. Jackson JG, Pereira-Smith OM. p53 is preferentially recruited to the promoters of growth arrest genes p21 and GADD45 during replicative senescence of normal human fibroblasts. *Cancer Res* 66:8356–8360, 2006.
 51. Miyake Z, Takekawa M, Ge Q, Saito H. Activation of MTK1/MEKK4 by GADD45 through induced N-C dissociation and dimerization-mediated trans-autophosphorylation of the MTK1 kinase domain. *Mol Cell Biol* Jan 22 [Epub ahead of print], 2007.
 52. Bulavin DV, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E, Fornace AJ Jr. Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* 411:102–107, 2001.