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Arsenic-induced apoptosis in the p53-proficient and p53-deficient cells through differential modulation of NFκB pathway

Lei Yin^{1,2}, Xiaozhong Yu^{1,*}

¹Department of Environmental Health Science, College of Public Health, University of Georgia, 150 Green Street, Athens, GA 30602, USA. Fax: 706.542.7472.

²ReproTox Biotech LLC, 111 Riverbend Drive, Athens, Georgia

Abstract

Arsenic is a well-known environmental carcinogen and an effective chemotherapeutic agent. The underlying mechanism of this dual-effect, however, is not fully understood. In this study, we applied mouse p53^{+/+} and p53^{-/-} cells to examine the NFκB pathway and proinflammatory cytokines after arsenic treatment. Arsenic reduced cell viability and increased more apoptosis in the p53^{-/-} cells as compared to p53^{+/+} cells, which was correlated with activation of SAPK/JNK, p38 MAPK, and AKT pathways. A transcriptional regulatory network analysis revealed that arsenic activated transcription regulatory elements E2F, Egr1, Trp53, Stat6, Bcl6, Creb2 and ATF4 in the p53^{+/+} cells, while in the p53^{-/-} cells, arsenic treatment altered transcription factors NFκB, Pparg, Creb2, ATF4, and Egr1. We observed dynamic changes in phosphorylated NFκB p65 (p-NFκB p65) and phosphorylated IKKαβ (p-IKKαβ) in both genotypes from 4h to 24h after treatment, significant decreases of p-NFκB p65 and p-IKKαβ in the p53^{-/-} cells, whereas increases of p-NFκB p65 and p-IKKαβ were observed in the p53^{+/+} cells. Our study confirmed the differential modulation of NFκB pathway by arsenic in the p53^{+/+} or p53^{-/-} cells and this observation of the differential mechanism of cell death between the p53^{+/+} and p53^{-/-} cells might be linked to the unique ability of arsenic to act as both a carcinogen and a chemotherapeutic agent.

Keywords

arsenic; p53; NFκB; gene expression profiling; apoptosis and transcription factors

INTRODUCTION

Arsenic is a well-known environmental toxicant and carcinogen that has been associated with numerous human health impacts, including diabetes mellitus, dermatosis, cardiovascular disorders, and cancer (Tapio and Grosche, 2006). Arsenic has been used as a drug for over 2000 years in both traditional Chinese Medicine and the western world (Forkner and Scott, 1931). Within the last decade, arsenic has been used as a chemotherapeutic agent for acute promyelocytic leukemia (APL) and is approved by the Food and Drug Administration (FDA) as a chemo-therapeutic agent for the treatment of

*To whom correspondence should be addressed: Department of Environmental Health, University of Georgia Xiaozhong Yu, yuxz@uga.edu.

Acute Promyelocytic Leukemia (APL) (Antman, 2001; Chen et al., 1997; FDA, 2000; Miller et al., 2002). Arsenic has been shown to induce apoptosis in a relatively broad spectrum of tumors such as lung, ovarian, gastric, neuroblastoma, and breast cancer (Bode and Dong, 2002). Currently, there are over 100 clinical trials underway to extend its therapeutic application (<http://www.clinicaltrials.gov>). These clinical trials include treatments for multiple myeloma, advanced solid tumors, malignant glioma, advanced or metastatic breast cancer, liver, or non-small cell lung cancer with single arsenic or combining treatment with ascorbic acid or other anti-cancer drugs. The underlying mechanism of this dual-effect on carcinogenesis and chemotherapy, however, is not fully understood.

Tumor suppressor gene p53 is crucial in maintaining genome integrity through the induction of cell cycle arrest or apoptosis with irreparable damage (Ashcroft et al., 2000; Vogelstein et al., 2000). P53 inactivation is one of the most frequent genetic events during carcinogenesis, and p53 mutations have been linked to more than 50% of all human cancers (Olivier et al., 2002). The deletion of p53 significantly attenuated cell cycle alteration and apoptosis in the treatment with metals such as cadmium and methyl mercury, as expected from the role of p53 in regulating cell cycle and apoptosis (Gribble et al., 2005a; Sidhu et al., 2005; Yu et al., 2008b; Yu et al., 2011). However, the arsenic treatment caused the toxicological changes in both genotypes, with more sensitivity in the p53^{-/-} mouse embryonic fibroblasts cells (MEFs) (Yu et al., 2008b). This study suggests that arsenic induces apoptotic process in the p53^{-/-} MEF cells might be through the down-regulation of NFκB in a p53-independent pathway (Yu et al., 2008b). NFκB is another fundamental transcription factor that plays an important role in cell death/survival and cytokine production (Egan and Toruner, 2006; Li and Verma, 2002; Perkins and Gilmore, 2006). The loss of p53 activity and the activation of NFκB in tumors are associated with faster tumor progression and resistance to cancer treatment (Cheney et al., 2008; Komarova et al., 2005; Tanaka et al., 2006; Yan et al., 2008). In the present study, we continued to apply mouse embryonal fibroblasts (MEFs) isolated from the p53^{+/+} and p53^{-/-} mouse to examine the NFκB pathway by comparing transcriptional factors and proinflammatory cytokines in these cells with or without arsenic treatment. Arsenic induced more morphological changes and reduced more cell viability in the p53^{-/-} cells than that in the p53^{+/+} cells. We found arsenic-induced more apoptosis in the p53 deficient cells, which was correlated with activation of SAPK/JNK, p38 MAPK, and AKT pathways. A transcriptional regulatory network analysis revealed that arsenic treatment resulted in activation of transcription regulatory elements E2F, Egr1, Trp53, Stat6, Bcl6, Creb2 and ATF4 in the p53^{+/+} cells, while in the p53^{-/-} cells, arsenic treatment altered transcription factors such as NFκB1 and Pparg in addition to Creb2, ATF4, and Egr1. These results were further verified by comparing the protein levels of phosphorylated NFκB p65 (p-NFκB p65), IKKαβ as well as pro-inflammatory cytokines secretion profile. We observed dynamic changes in p-NFκB p65 and p-IKKαβ in both genotypes from 4h to 24h after Arsenic treatment, significant decreases of p-NFκB p65 and p-IKKαβ in the p53^{-/-} cells, whereas increases of p-NFκB p65 and p-IKKαβ were observed in the p53^{+/+} cells. Our study confirmed the differential modulation of NFκB pathway by arsenic in the p53^{+/+} or p53^{-/-} cells and this observation of the differential mechanism of cell death between the p53^{+/+} and p53^{-/-} cells might be linked to the unique ability of arsenic to act as both a carcinogen and a chemotherapeutic agent. Our finding of the unique response to arsenic in

p53 genotype cells provides a potential new direction for exploring the role of p53 in carcinogenesis as well as p53-independent apoptosis in cancer treatment with arsenic.

Material and Methods

Reagents and chemicals

Dulbecco's modified Eagle's medium (DMEM): F12 media, penicillin-streptomycin, L-glutamine, trypsin-EDTA, balanced salt solutions, and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA). Nu-Serum was obtained from Becton Dickinson (Bedford, MA). Tissue culture-treated dishes and flasks were obtained from Corning (Corning, NY). Neutral red (NR), MG132, lactacystin, protease, and phosphatase inhibitor cocktails were obtained from Sigma (St Louis, MO). Sodium arsenite was obtained from Sigma (St Louis, MO). Cell lysis buffer and all phospho-specific antibodies were obtained from Cell Signaling (Beverly, MA). All other antibodies used in this study were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The enhanced chemiluminescence-based Western blotting detection reagent was obtained from BioRad (Hercules, CA). Proteasome inhibitors, MG132 and lactacystin (Lact, Calbiochem, La Jolla, CA) and protein synthesis inhibitor, anisomycin (Axxora, San Diego, CA) were prepared in DMSO at 5 mM (MG132 and lactacystin) and 50 mM (anisomycin).

Cell culture

Mouse embryonic fibroblasts (MEFs) were isolated from heterozygous mice for the wild-type and null p53 allele of a C57BL/6 $\times$ 129/Sv background following a modified method from previously described protocols (Yu et al., 2008b; Yu et al., 2010). Briefly, embryos were separated from the uteri of pregnant females on day 14 of gestation. The torso and limbs were dissected to a single fibroblast suspension. DNA obtained from the tail was used for PCR genotyping. The single cell suspensions were inoculated in 100 mm dishes in DMEM F-12 with 10% v/v Nu-Serum. The ease of isolation and the ability to derive these cells from mice harboring various genetic alterations has made the MEFs an ideal model system for studying aspects of cell growth control and functional genetics (Vengellur et al., 2003). In past studies, this culture system has been successfully applied to investigate the molecular mechanism of metal-induced cell cycle arrest and apoptosis, as well as the role of p53 (Gribble et al., 2005a; Yu et al., 2008b).

Arsenic treatments, morphology, and cytotoxicity

MEFs, at passage 6, were grown to 85% confluency in 10% Nu-serum DMEM/F-12 media and then switched to lower serum (containing 5% Nu-serum) DMEM F-12 culture medium for 24h. MEFs were treated with sodium arsenic at concentrations of 0–20 μ M for 24 h. Western blot analysis using anti-cleaved caspase-3 antibody (Cell Signaling, Beverly, MA) (Yu et al., 2005). All cultures were viewed with an Olympus (CK40) inverted microscope equipped with phase-contrast optics (Olympus, Japan) at intervals during culture to assess their general appearance. Resultant images were captured and digitized using a Coolsnap Camera (Roper Scientific, Inc., Duluth, GA). Cell viability was determined using the neutral

red (NR) uptake assay (Yu, Robinson et al. 2008). Briefly, the cells were cultured in a 96-well plate and were treated with different concentrations of Arsenic. The media with treatment was removed, and fresh media containing 50 µg/ml NR was added to the dish. After more 3h incubation at 37°C, 5% CO₂, the cells were washed with PBS and NR was eluted with 1% acetic acid/50% ethanol solution. The absorbance value of the resulted NR solution was measured at 540 nm using a Synergy HT Reader (Gen5 VT, BioTek). The cell viability was calculated as the ratio of the treatment versus control after subtracting the background control. The data represented the average ± standard deviation of six replicates and were expressed as a fold change of the treated versus untreated control.

Apoptosis associated endpoints were determined in cell extracts by western analysis using an anti-caspase-3 antibody, which detects the cleaved and uncleaved form of caspase-3, or by measuring functional caspase 3/7 activities using caspase 3/7-specific fluorogenic substrate DEVD-AMC (Axxora, San Diego, CA). 10 µg of cell extract was added in duplicate to a 96-well plate format. Reaction buffer containing the fluorogenic substrate was added to initiate the reaction and was incubated at 37 °C for 2 h. The enzyme-catalyzed release of 7-amino-4-methyl coumarin (AMC) was measured by a fluorescence microplate reader at excitation 360 nm and emission 460 nm. Fluorescent units were converted to *p* mol of AMC released per µg of protein and incubation time (2 h) using a standard curve generated with known serial dilutions of AMC. We then converted the absolute activities due to metal treatments relative to untreated controls by expressing the former as a percentage of the control,

Western blot Analysis

At the stated time points, the cells were washed with ice-cold Phosphate-buffered saline (PBS) twice and lysed by 0.5 ml of cell lysis buffer (Cell Signaling, Beverly, MA), containing additional inhibitors of phosphatase and protease cocktail (Sigma, St Louis, MO). Cells were harvested by scraping in cell lysis buffer and then placed on ice. All extracts were homogenized by sonication and then centrifuged to remove insoluble material. The resulting supernatant was gently collected, and total protein was determined using the protein assay kit (Bio-Rad, Hercules, CA). Western blot analysis for the selected proteins was performed as the previously described (Yu et al., 2005). Briefly, the equal amount of protein was separated on the SDS-PAGE gel and then transferred to polyvinylidene difluoride nylon membranes (PVDF, Millipore/Sigma) for immunoblot analyses. Membranes were rinsed briefly in Tris-buffered saline, pH 7.6 (TBS), blocked with 5% nonfat dried milk in TBS with 0.1% Tween-20 (T-TBS) for 60 min. Membranes were then incubated overnight with primary antibody at 4C° and then incubated with secondary antibody for 1.5h at room temperature. Following each antibody incubation, the membrane was washed four times for 5min with T-TBS. The primary antibodies included phospho-SAPK/JNK (Thr183/Tyr185, #9255, Cell Signaling, Inc), phospho-p38 MAPK (Thr180/Tyr182, D3F9, #4511, Cell Signaling, Inc), Phospho-Akt (Ser473, D9E #4060, Cell Signaling, Inc), cleaved caspase-3 (#9961, Cell Signaling, Inc), and NFκB Pathway antibodies including phospho-IKKα/β (Ser176/180), NFκB p65 (C22B4) Rabbit mAb # 4764, phospho-NFκB p65 (Ser536) (93H1) Rabbit mAb # 3033, (Cell Signaling, Inc). β-actin (Santa Cruz Biotechnology, CA) was used as an internal control to ensure equal loading. After hybridization with secondary

antibodies conjugated to horseradish peroxidase, the immunocomplex was detected with the enhanced chemiluminescence (ECL) detection reagent (BioRad, Hercules, CA) and exposed to X-ray films. Quantification of band intensities was achieved using the NIH Image J (1.30 V, NIH, USA) and the results were expressed as the percentage of the corresponded control after normalization to β -actin.

Immunofluorescence staining for NF κ B p65

Cells were fixed in ice-cold 50% ethanol for 5 min. The samples were incubated with anti-NF κ B p65 antibody (C22B4, Cell Signaling Technology) overnight for 24 h at 4°C, washed, and incubated with anti-rabbit IgG Alexa Fluor 488 antibody (1/200 dilution) (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Then, the nuclei were counterstained with Hoechst 33342 in mounting medium and the fluorescence images were obtained using a Olympus IX71 fluorescence imaging system.

Microarray hybridization and transcription factor analysis

The cells were treated with arsenic (5 μ M) for 24h, then total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA), and quality was assayed on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). The procedure for oligonucleotide microarray hybridization was reported previously (Yu et al., 2008b). Briefly, hybridization of cRNA was carried out for 18 h on an orbital shaker set at 300 rpm and 37 °C. After removing the hybridization chamber, arrays were washed with 0.75 $\times$ TNT for 1 h at 46 °C. Incubation for 30 min with AlexaFluor 647-streptavidin (Molecular Probes, Inc., Eugene, OR) was followed by four 5 min washes in 1 $\times$ TNT and two vigorous rinses in 0.05% Tween-20. Slides were dried and arrays were scanned on an Axon GenePix 4000 Scanner (Axon Instruments, Union City, CA) set to a wavelength of 635 nm. CodeLink array data was first run through accompanying software from Amersham Biosciences. This raw data was imported to BRB ArrayTools v3.0 and a log base 2 transformation was applied and normalized each array by using the median intensity over entire arrays (Wright and Simon, 2003). Microarray analysis was conducted as previously reported (Yu et al., 2006; Yu et al., 2008b). Genes significantly changed in the p53 $-/-$ cells versus p53 $+/+$ as well as genes changed after treatment with arsenic in both p53 $+/+$ and p53 $-/-$ cells ($p < 0.01$ and fold change > 1.5) were output and transcription factor analyses were performed to identify biologically enriched transcription regulatory elements (TREs) found in the regulatory regions of these significantly changed genes in the BRB-ArrayTools. Quantitative analysis of gene expression in the antioxidant pathway, Gsta2, and cell cycle regulation of Ccnb1, Cdc25c, Uchl1 and Ube2c was confirmed using quantitative Real-time PCR (qRT-PCR) (Yu et al., 2008b). A significant correlation between the gene expression results in the microarray analyses and the qRT-PCR analysis for these genes was observed ($r = 0.94$, $P < 0.001$). All genes queried in this analysis algorithm have been cataloged to the transcription factor responsive categories based upon experimentally verified transcription factor responsiveness. Transcription factor-binding information in the Transcriptional Regulatory Element Database (TRED) was used to eliminate targets without any experimental verification (Zhao et al., 2005).

Statistical analysis

Cell viability is expressed as a fold change of the treated versus untreated control after subtraction with the blank controls. Densitometric analysis of protein band from the western blot analysis was performed using the Image J 1.49 (NIH). Differences between the treatments and the genotypes were examined for statistical significance using two-way analysis of variance (ANOVA). A p-value of ≤ 0.05 denoted the presence of a statistically significant difference (ANOVA ≤ 0.05) among the genotypes or doses. We also compared the individual treatment with the control by Tukey-Kramer multiple comparisons and a p-value of ≤ 0.05 denoted the presence of a statistically significant difference between the individual treatments with the control. Statistical analysis was conducted using the JMP program (SAS Inc).

RESULTS

Arsenic induced more morphological changes and reduced more cell viability in the p53^{-/-} cells than that in the p53^{+/+} cells

No significant morphological changes were observed at concentration $\leq 5 \mu\text{M}$ in either genotype. Significant morphological changes were observed in p53^{-/-} cell at $7.5 \mu\text{M}$, including irregular cell shape and condensation. Significant disruptions of morphology such as roundup, and detachment were observed in both genotypes at high concentrations ($\geq 20 \mu\text{M}$, data not included). The effect of arsenic on cytotoxicity was examined in both p53^{+/+} and p53^{-/-} MEFs. Neutral red (NR) uptake assay was used to quantitatively estimate the number of viable cells after treatments. As shown in Figure 1, arsenic treatment reduced NR uptake in a dose-dependent manner. Significant decreases in NR dye uptake were observed at 7.5 and $10 \mu\text{M}$ in the p53^{-/-} cells, while significant decreases were observed at $10 \mu\text{M}$ in the p53^{+/+} cells (Figure. 1). However, there were significant differences of cell viability between the p53^{+/+} and p53^{-/-} cells (ANOVA, $p < 0.05$), and significant reductions of cell viability were observed at the concentration of 7.5 and $10 \mu\text{M}$ in the p53^{-/-} cells as compared to that in the p53^{+/+} cells.

Arsenic-induced more apoptosis in the p53 deficient cells is correlated with activation of SAPK/JNK, p38 MAPK, and AKT pathways

Caspase 3 is one of the key executioners of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly ADPribose polymerase (PARP). Activation of caspase 3 requires proteolytic processing of its inactive zymogen into activated p17 and p19 fragments. Cleaved Caspase 3 (Asp175) antibody detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175. As shown in Figure 2, the representative western blot detection of cleaved caspase 3 (Figure 2) demonstrated a significant increase of cleaved caspase-3 in both cell types at the highest dose, but there was a more significant response in the p53^{-/-} cells as compared to the p53^{+/+} cells (A and B). Dose-dependent increases in the cleaved caspase 3 (Figure 2) were observed following arsenic treatment in

both genotypes (C). These responses were more significant in the p53^{-/-} cells at concentrations of 10 and 20 μ M as compared to the p53^{+/+} cell at equivalent concentrations. We used lactacystin, MG132 and anisomycin as positive stress controls. Although there was an increasing trend in the cleaved caspase 3 in the treatment with MG132 at 0.5 μ M, there was no statistical significance between two genotypes at the dose tested. Treatment with anisomycin at 10 μ M led to a significant increase of cleaved caspase 3 and higher up-regulation in the p53^{+/+} cells. We further measured the caspase 3/7 activity and found arsenic treatment induced a dose-dependent increase of caspase 3/7 activity treated in both genotypes, with significant more increase in the p53^{-/-} cells. These data confirmed our previous finding that arsenic induced significantly higher apoptosis in the p53^{-/-} cells as compared to the p53^{+/+} cells.

Signal transduction pathways controlled by cascade kinases regulate critical cellular functions such as cell growth, differentiation, and apoptosis, including major kinase cascades in the activation of mitogen-activated protein kinases (MAPK): SAPK/JNK, p38 and AKT (Kyriakis and Avruch, 1996). Utilizing a combination of western blot analysis and phospho-specific antibodies, we examined whether the activation of cleaved caspase 3 by arsenic in the p53^{-/-} cells are associated with the increases in the activation of cellular stress signaling SAPK/JNK and p38 MAPK. We measured the phosphorylation of SAPK/JNK, p38 MAPK and AKT expression levels in both genotypes of cells treated with arsenic. As shown in Figure 3, arsenic treatment induced dose-dependent activation of SAPK/JNK in both p53^{+/+} and p53^{-/-} cells. Arsenic induced more activation of p-SAPK/JNK in the p53^{-/-} cells as compared to p53^{+/+} cells. This suggests that the stress responses induced by arsenic is independent of p53. Significant activation of SAPK/JNK was observed in the treatment with anisomycin at 10 μ M in both genotypes but not in the control cells treated with MG132 and lactacystin (Figure 3). Arsenic induced a dose-dependent increase of p-p38 in p53^{-/-} cells, but not in the p53^{+/+} cells (Figure 4). No significant up-regulation of p-38 was observed in the treatments with MG132 and lactacystin (Figure 4D). However, a significant increase in p-p38 was found in anisomycin-treated MEFs (Figure 4A and 4D). Arsenic exposure induced phosphorylation of AKT (p-AKT) at concentrations of less than 10 μ M in both genotypes, but it significantly decreased p-AKT at concentrations of 20 μ M only in the p53^{-/-} cells (Figure 5). Significant activation of p-AKT was observed in the treatment with anisomycin at 10 μ M in both genotypes, but not in MG132 and lactacystin at the doses tested (Figure 5).

Differential activities of transcriptional factors in the p53^{+/+} and p53^{-/-} cells and alterations by arsenic

As previously reported, deletion of the p53 (p53^{-/-} MEFs) resulted in dramatic changes of gene expressions as compared to the p53^{+/+} cells (Yu et al., 2008b). A transcriptional regulatory network analysis was performed to identify biologically enriched transcription regulatory elements (TREs) found in the regulatory regions of these significantly changed genes (Figure 6). As compared to the p53^{+/+} cell, the p53^{-/-} cells have a significantly altered transcription factor (Figure 6A). Alteration in the Trp53 transcription factor was observed due to the deletion of p53 gene in the p53^{-/-} cell, validating the use of

transcriptional regulatory network analysis. Enriched changes of genes in the NF κ B1, Rela, Hoxc8, Ppara, Ppard, Pparg, Smad1, Atf4, and Bcl6 transcription factors were observed in the p53 $^{-/-}$ cell, suggesting the deletion of p53 altered the balance of these signalings and lead to the up-regulated activity such as NF κ B1 and CREB2. For the microarray-based gene expression analysis, treatments were restricted to one time point (24 h) and a single concentration (5 μ M). The concentration selected was based on minimal impacts on morphology, cell viability and caspase 3/7 activity after 24 h treatment. Arsenic treatment in the p53 $^{+/+}$ wildtype cells for 24 h resulted in the cluster of differential expressed genes enriched for E2F, Erg1, Hoxc8, Stat6, Trp53, Pou5f1, Bcl6, Creb2 and Atf4 transcription factors (Figure 6B). In contrast, in the p53 $^{-/-}$ MEF, arsenic treatment for 24 h induced cluster of differential expressed genes enriched for Fos, Pparg, Creb2, Atf4, Nfkb1, Creb1, Jun, Erg1, Aft1 and Smad transcription factors (Figure 6C). Treatment of arsenic significantly reduced the expression levels of genes regulated by transcription factors c-Rel and NF κ B1 (Figure 6D).

Arsenic altered protein levels of the NF κ B signaling pathway

In order to confirm the results from the transcriptional regulatory network analysis, we further measured the temporal changes of protein levels within NF κ B signaling pathways (Figure 7). Arsenic treatment after 4, 8 and 24 h did not significantly change the total NF κ B p65 in both p53 $^{+/+}$ and p53 $^{-/-}$ cells (Figure 7). Dynamic changes of protein expression of p-NF κ B p65 were observed in both p53 $^{+/+}$ and p53 $^{-/-}$ cells in a dose- time-dependent manner ($p < 0.05$, Two-way ANOVA, time and treatments in each genotype, Figure 7B). In the p53 $^{+/+}$ cells, there is no significant change of p-NF κ B p65 in any treatments at 4 h; however, significant inductions were observed at 5 μ M and 7.5 μ M for 8 h and 24 h in the p53 $^{+/+}$ cells. A significant increase of p-NF κ B p65 at the concentration of 7.5 μ M of arsenic was found at 24 h as compared to the control. In the p53 $^{-/-}$ cells, a significant increase of p-NF κ B p65 was observed at concentrations of 5 and 7.5 μ M of arsenic at 4 h, and higher levels of induction of p-NF κ B p65 were observed at concentrations of 5 and 7.5 μ M of arsenic at 8h. At 24 h time-point, significant decreases of p-NF κ B p65 were observed at treatments of arsenic at 5 and 7.5 μ M. Arsenic reduced the level of p-IKK $\alpha\beta$ in the p53 $^{-/-}$ cells at concentrations of 7.5 μ M at 8 and 24h. On the other hand, arsenic treatment did not affect the expression levels of p-IKK $\alpha\beta$ in the p53 $^{+/+}$ cells at indicated time points (Figure 7A and 7B). As shown in Figure 7C, there was a significant difference of NF κ B p65 expression in the p53 $^{+/+}$ and p53 $^{-/-}$ cells. The levels of both cytoplasm and nuclear NF κ B p65 (white arrows) were much higher in the p53 $^{-/-}$ cells than that in the p53 $^{+/+}$ cells. Consistent with the result from the western blot analysis, arsenic treatment at 7.5 μ M for 24 h induced the translocation of NF κ B p65 from the cytoplasm to the nucleus in the p53 $^{+/+}$ (black arrows). However, noticeable reduced-levels of NF κ B p65 in cytoplasm and nucleus were observed in the p53 $^{+/+}$ after arsenic treatment for 24h.

Arsenic induced differential proinflammatory cytokines and chemokines in the p53^{+/+} and p53^{-/-} cells

We further tested whether the loss of p53 affects the cytokines and chemokine levels. We examined the secreted cytokines in the supernatants of both p53^{+/+} and p53^{-/-} MEFs using cytokines protein array (Figure 8A). The results revealed high expression of almost all cytokines /chemokines in the p53^{-/-} cell tested in the array, except MIP-1 γ as compared to the p53^{+/+} cells. Furthermore, we examined the secretion levels of cytokine/chemokines in both p53 genotypes after arsenic treatment for 24h. As shown in Figure 8B, the cytokine secretion patterns were changed depending on the p53 status. Treatment with arsenic in the p53^{-/-} cell significantly reduced the levels of cytokines and restored the levels of cytokines as comparable to the p53^{+/+} cells.

Discussion

Arsenic is a well-known environmental toxicant and carcinogen, but also approved chemotherapy for leukemia (Gao et al., 1986; Ho and Lai, 2004). Based on unique signaling pathways revealed in the arsenic-induced apoptosis in the p53 deficient cells (Yu et al., 2008b), we tested the hypothesis that arsenic treatment modulates NF κ B signaling pathways differently in the p53 proficient and deficient cells. We demonstrated that arsenic induced more apoptosis and reduced production of inflammatory cytokines through an NF κ B-mediated mechanism in the p53^{-/-} cells, but not in the p53^{+/+} cells.

As consistent with our previous study (Yu et al., 2008b), significant morphological changes, and dose-dependent decreases in cell viability were observed in both p53^{-/-} and p53^{+/+} cells after arsenic treatment. Most interestingly, the p53^{-/-} cells exhibited higher sensitivity to arsenic exposure, as indicated by morphological alterations and cytotoxicity as well as evidence of apoptosis and stress responses (caspase-3, p-p38, and p-SAPK/JNK at 10 μ M). In contrast, neither apoptosis nor stress responses were observed up to this concentration in the p53^{+/+} cells. Our current finding, as well as previous reports, suggests that arsenic might activate alternative pathways in the p53^{-/-} cells, which is suppressed in the p53 proficient cells. This is also a unique response as compared to the positive control MG132, lactacystin and anisomycin, as well as other metals such as methylmercury and cadmium, showing the p53^{+/+} cells were more sensitive to the treatment (Gribble et al., 2005b; Yu et al., 2008a; Yu et al., 2010; Yu et al., 2011).

Arsenic-induced cytotoxicity or apoptosis is commonly associated with increased generation of reactive oxygen species (ROS), depletion of cellular antioxidant system, such as GSH, inhibition of DNA repair and DNA methylation, and decreased mitochondrial membrane potential accompanied by cytochrome c release and caspase activation (Miller et al., 2002). P53 protein is a key component in the regulation of cytotoxicity and apoptosis, and the alterations of p53 induced by various exogenous agents may lead to aberrant cellular effects (Ashcroft et al., 2000; Vogelstein et al., 2000). Many studies report that wild type of p53 is crucial for the induction of apoptosis after DNA damage, such as cisplatin and methylmercury (Bragado et al., 2007; Gribble et al., 2005a); however, the thymocytes apoptosis by irradiation was almost completely blocked in p53-deficient mice (Clarke et al.,

1993). Arsenic is a unique metal, and reported to cause DNA damage, cell cycle perturbations, and apoptosis through the p53 dependent and independent pathways (Huang and Lee, 1998; Liu et al., 2003; McCabe et al., 2000; Park et al., 2000; Yih and Lee, 2000). In the presence of functional p53, exposure to 5 μ M arsenic has been found to induce DNA strand breaks and increase p53 phosphorylation paralleled with increases in p53 target genes p21 and MDM-2 (Shen et al., 2000; Yih and Lee, 1999; Yih and Lee, 2000; Yih et al., 2002). In the p53 deficient cells, arsenic treatment also disrupted mitosis and induced apoptotic cell death (Huang and Lee, 1998; Liu et al., 2003; McCabe et al., 2000; Salazar et al., 1997). Huang reported that arsenic induced apoptosis similarly in the p53^{+/+} and p53^{-/-} cells (Huang et al., 1999), which was different from our study as well as other studies (Huang and Lee, 1998; Liu et al., 2003; McCabe et al., 2000; Salazar et al., 1997). The difference might result from the high concentrations used in Huang's study from 12.5 μ M to 200 μ M of arsenic (Huang et al., 1999). We did not observe any difference between the genotypes of p53 if the concentration of arsenic used over 20 μ M. These concentrations might be too high and un-relevant to the clinical use of arsenic in the cancer treatment, and the plasma arsenic concentrations are reported to be from 0.5 μ M to 7.30 μ M (Burnett et al., 2015; Chen et al., 2001b; Firkin et al., 2015).

NF κ B is a family of transcription factors that plays an essential regulatory role in inflammation, the immune response, cell proliferation, and apoptosis (Cheney et al., 2008; Komarova et al., 2005; Li and Verma, 2002; Tanaka et al., 2006). Dysregulations of NF κ B-associated pathway are associated with various types of human cancers or neoplastic transformation (Egan and Toruner, 2006; Li and Verma, 2002; Perkins and Gilmore, 2006). Deletion of p53 in MEF cells leads cells to grow faster than the p53^{+/+} cells and the activations of NF κ B pathways (Dijsselbloem et al., 2007; Komarova et al., 2005; Lee et al., 2008). Our preliminary study strongly suggests that arsenic induces apoptotic process in the p53^{-/-} MEF cells through down-regulation of NF κ B, a p53-independent pathway (Yu et al., 2008b). Arsenic significantly affects signal transduction molecules that are involved in mediating cellular proliferation or apoptosis, including MAPKs, p53, activator protein-1 (AP-1) and NF κ B (Huang et al., 2005; Kitchin, 2001; Qian et al., 2003). Our current examination of arsenic treatment consistently induced dose-dependent activations of SAPK/JNK and p38 in both p53^{+/+} and p53^{-/-} cells, with significant more activation of SAPK/JNK in p53^{-/-} cells than that in the p53^{+/+} (Figure 3), suggesting SAPK/JNK pathway might be involved in the increased cell death in p53^{-/-} cells. Dynamic changes of protein expression of p-NF κ B p65 were observed in both p53^{+/+} and p53^{-/-} cells (Figure 7). In the p53^{+/+} cells, arsenic treatment significantly up-regulated p-NF κ B p65 at concentration of 5 μ M at 8 h and 7.5 μ M at 24 h. In the p53^{-/-} cells, arsenic significant increased p-NF κ B p65 at concentrations of 5 and 7.5 μ M at 4 h time-point, and significant decrease of p-NF κ B p65 at 5 and 7.5 μ M at 24 h. In the p53^{-/-} cells, significant decreases of p-IKK $\alpha\beta$ (Figure 7) were observed at the concentration 5 and 7.5 μ M of arsenic. These results were consistent with the results from the gene expression profiling analysis, showing arsenic uniquely modulate NF κ B as well as other transcriptional factors in the p53^{-/-} cells (Yu et al., 2008b). Our finding suggests that several uncharacterized or unstudied transcriptional factors may play a role in arsenic modulation of NF κ B. Further examination

of these transcriptional factors may represent novel mechanisms involved in arsenic in the p53 deficient cancers.

p53 and NF κ B are two major transcription factors important in controlling cell survival or death (Egan and Toruner, 2006; Li and Verma, 2002; Perkins and Gilmore, 2006). Dynamic crosstalk has been demonstrated between the p53 and NF κ B pathways (Tergaonkar and Perkins, 2007). NF κ B and p53 can work cooperatively, either through the co-regulation of specific target genes or through the pro-apoptotic activity of NF κ B in response to certain inducers (Jeong et al., 2005; Tergaonkar and Perkins, 2007). Huang et al. reported that I κ B kinase α (IKK α) could determine whether a cell proliferates or undergoes apoptosis through the regulation of the CBP-mediated crosstalk between NF κ B and p53 (Huang et al., 2007). Either activation or inactivation of NF κ B by arsenic has been reported and the contradictory results were observed due to the dose or different cell type tested (Bode and Dong, 2002; Chen et al., 2001a; Chen and Shi, 2002). Low and non-cytotoxic concentrations of arsenic (1–10 μ M) usually activate NF κ B, while high concentrations of arsenic (>10 μ M) generally inhibit this transcription factor (Bode and Dong, 2002; Chen et al., 2001a; Chen and Shi, 2002). Barchowsky *et al.* (Barchowsky et al., 1996) reported that lower concentrations of arsenic activated NF κ B through increasing intracellular oxidant levels in vascular endothelial cells. Later studies indicate that the induction of NF κ B by arsenic is cell-type dependent (Hamilton et al., 1998). Arsenic is able to induce NF κ B in MDA epithelial-like cells, whereas arsenic is unable to induce NF κ B in H4IIE rat hepatoma cells. Mechanistic studies by Jaspers *et al.* (Jaspers et al., 1999) indicated a possible non-classic signaling pathway involved in arsenic-induced NF κ B in airway epithelial cells. At higher concentrations (>50 μ M), arsenic appears to exhibit an inhibitory effect on NF κ B by interfering with the DNA binding of NF κ B (Jaspers et al., 1999; Shumilla et al., 1998). Our current finding provided a new insight into the role of arsenic on NF κ B signaling in p53 proficient cells and p53 deficient cells.

Combining with our previously published results based on the gene expression (Yu et al., 2008b), we propose a integrate mechanism of arsenic induced cell death or apoptosis either dependent or independent on the p53 pathway. Arsenic treatment induces cell cycle arrest, activation of stress signaling (p-p38 and p-SAPK/JNK) and apoptosis in both p53^{+/+} and p53^{-/-} cells, with more significant activation of stress signaling and induction of apoptosis in p53^{-/-} cells. The induction of cell cycle arrest and apoptosis in p53^{+/+} and p53^{-/-} is through the activation of different cellular signaling pathways. In the presence of functional p53, p53 is activated by several post-transcriptional modifications in response to DNA damage, which in turn activates its downstream targets, such as p21, Gadd45, cyclin B, and cause cell cycle arrest and Noxa, Puma, and Perp, leading to apoptosis. In contrast to effects of arsenic mediated apoptosis via p53 pathway, arsenic triggers prominent apoptosis in the p53^{-/-} cells through the p53 independent pathway. Treatment with arsenic in the p53^{-/-} cells uniquely induces alterations in the immune responses through the inhibition of NF κ B pathway, which leads to activation of MAPK stress signaling, alteration of p53-independent cell cycle regulatory genes and pro-apoptotic machinery. In summary, our finding reveals the unique responses by the treatment of arsenic in the p53^{+/+} and p53^{-/-} cells, which provides a potential new direction for exploring the role of p53 in carcinogenesis as well as p53-independent apoptosis in cancer treatment with arsenic.

Supplementary Material

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Figure 1. Arsenic induced a dose-dependent cytotoxicity in p53+/+ and p53-/- cells.

The p53 wildtype (p53+/+) and knockout (p53-/-) MEF cells were exposed to various concentrations of Arsenic for 24 h. Cell viability was assessed utilizing the Neutral Red (NR) uptake assay, which indirectly reflects the uptake function of the cells. Cells were seeded to the 96-well plates, treated with different compounds at four doses with 5 replicates. Cells treated with vehicle (0.05% DMSO) were used as the background group with cell viability set as 100%. After 24 h, the medium was replaced with a medium containing NR dye (50 μg/ml). Following 3h incubation, the supernatants were removed, the cells were washed with PBS twice, and NR was eluted with 100 μl of a 0.5% acetic acid/50% ethanol solution. The absorbance values were measured at 540 nm with a Synergy HT microplate reader (BioTek, VT). Cell viability was expressed as a fold change of the mean of treated condition versus controls after subtracting the background control. Statistical analysis of cell viability was conducted using two-way analysis of variance (ANOVA) followed by Dunnett's method to the control, with a significance level of $p < 0.05$ (*). Data were presented as mean $\pm$ SD, $n = 5$. The experiments were repeated three times.

Figure 2. Arsenic induced activation of cleaved caspase 3 (A-D) and increased the activity of caspase 3/7 (E) in p53^{+/+} and p53^{-/-} cells.

The mouse embryonic fibroblasts cells were exposed to various concentrations of arsenic (μM), and proteasomal inhibitors, MG 132 (0.5 μM) and Lactacystin (Lact, 1 μM) or Anisomycin (Anis, 10 μM) for 24h. Cell extracts were prepared and subjected to western blot analysis to determine the cleaved caspase 3 using specific caspase 3 antibody (A and B) as stated under Materials and Methods. Quantifications of western blot bands of cleaved caspase 3 after treatments (C and D) were conducted using NIH Image J (V1.51j 4) and the results were expressed as the percentage of the corresponded control after normalization to β-actin. Functional caspase 3/7 activities were measured using caspase 3/7-specific fluorogenic substrate DEVD-AMC and fluorescent units were converted to *p* mol of AMC released per μg of protein using a standard curve generated with known serial dilutions of AMC. The results were expressed as the percentage of the untreated control (E). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. * indicates significance at *p* = 0.05 of the individual treatment versus the control. The experiments were repeated three times, and the representative western blot images are displayed.

Figure 3. Arsenic induced activation of phosphorylation of SAPK/JNK in p53^{+/+} (A) and p53^{-/-} (B).

Cells were exposed to various concentrations of arsenic (μM), and proteasomal inhibitors MG 132 (0.5 μM) and Lactacystin (Lact, 1 μM) or Anisomycin (Anis, 10 μM) for 24h. Cell extracts were prepared and subjected to western blot analysis of phospho-specific antibodies against p-SAPK/JNK (A and B). Quantifications of western blot bands after treatments (C and D) were conducted in NIH Image J and the results were expressed as the percentage of the corresponded control after normalization to β -actin. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons as compared with the control for each dose. * indicates significance at $p < 0.05$ of the individual treatment versus control. The experiments were repeated three times, and the representative western blot images are displayed.

Figure 4. Arsenic induced activation of phosphorylation of p38 MAPK in p53+/+ (A) and p53-/- (B).

Cells were exposed to various concentrations of arsenic (μM), and proteasomal inhibitors MG 132 (0.5 μM) and Lactacystin (Lact, 1 μM) or Anisomycin (Anis, 10 μM) for 24h. Cell extracts were prepared and subjected to western blot analysis of phospho-specific antibodies against phospho-p38 MAPK (A and B). Quantifications of western blot bands were conducted in NIH Image J (C and D) and expressed as the percentage of the corresponded control after normalization to β -actin. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons as compared with the control for each dose. * indicates significance at $p < 0.05$ of the individual treatment versus control. The experiments were repeated three times, and the representative western blot images are displayed.

Figure 5. Arsenic induced activation of phosphorylation of AKT in p53^{+/+} (A) and p53^{-/-} (B) cells.

The mouse embryonic fibroblasts cells were exposed to various concentrations of arsenic (μM), proteasome inhibitors MG 132 (0.5 μM) and Lactacystin (Lact, 1 μM) or Anisomycin (Anis, 10 μM) for 24h. Cell extracts were prepared and subjected to western blot analysis to determine the phosphorylation status using phospho-specific antibody AKT. Quantifications of western blot bands of phosphor-AKT after exposure to arsenic (C) and proteasomal inhibitors and anisomycin (D) were conducted using NIH Image J and expressed as the percentage of the corresponded control after normalization to β -actin. Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. * indicates significance at $p < 0.05$. The experiments were repeated three times, and the representative western blot images are displayed.

Arsenic induced differential activation of transcriptional regulatory network

Figure 6. Arsenic induced differential activations of transcriptional regulatory networks in the p53^{+/+} and p53^{-/-} cells.
MEF cells, p53^{+/+} and p53^{-/-}, were exposed to arsenic (5 μ M) for 24h. A microarray-based gene expression analysis was conducted. Genes significantly changed in the p53^{-/-} cells versus p53^{+/+} as well as genes changed after arsenic treatment in both p53^{+/+} and p53^{-/-}

cells ($p < 0.01$ and fold change > 1.5) were output, and transcription factor analyses were performed to identify biologically enriched transcription regulatory elements (TREs) in the regulatory regions of these changed genes using the BRB-ArrayTools. Transcription factor-binding information in the Transcriptional Regulatory Element Database (TRED) was used to eliminate targets without any experimental verification (Zhao et al., 2005). Transcription factor enriched in the p53^{-/-} versus p53^{+/+} cells (A), transcription factor enriched by arsenic treatment in the p53^{+/+} cells (B) and the p53^{-/-} cells (C), as well as altered gene-networks under the regulation of NF κ B (D) were shown.

Figure 7. Arsenic altered NF κ B signaling pathway proteins in the p53 $^{+/+}$ and p53 $^{-/-}$ cells.

Cells were exposed to various concentrations of arsenic for 4, 8 and 24 h. Cell extracts were prepared and subjected to western blot analysis of total NF κ B p65, phospho-NF κ B p65 and IKK α / β as well as β -actin (A). Quantifications of western blot bands were conducted in NIH Image, and the results of NF κ B p65, p-NF κ B p65 and p-IKK α / β (B) were expressed as the percentage of the corresponded control after normalization to the internal control level of β -actin. Representative immunostaining of NF κ B p65 in the p53 $^{+/+}$ and p53 $^{-/-}$ cells treated with arsenic (7.5 μ M) or control were shown in C. Black arrows show the translocation of NF κ B p65 from the cytoplasm to nucleus after arsenic treatment for 24h (7.5 μ M) in the p53 $^{+/+}$ cells. White arrows show the staining of NF κ B p65 in both cytoplasm and nucleus in the p53 $^{-/-}$ control cells. Statistical significance was determined by ANOVA followed by Tukey-Kramer multiple comparisons as compared with the control for each dose. * indicates significance at $p < 0.05$ of the individual treatment versus control. The experiments were repeated three times, and the representative western blot images are displayed.

Figure 8. Arsenic induced differential secretion of proinflammatory cytokines and chemokines in the p53^{+/+} and p53^{-/-} cells.

MEF cells, p53^{+/+} and p53^{-/-}, were exposed to arsenic (5 μ M) for 24 h, and the conditioned media were hybridized to RayBio® Mouse Cytokine Antibody Array 3. Densitometric quantification of dots for each cytokine was achieved using the NIH ImageJ and expressed as mean $\pm$ standard deviation (SD), n = 3. The ratio of each cytokine between p53^{-/-} and p53^{+/+} was calculated. The results were averaged from three independent experiments. **A** shows the comparison of secreted cytokines between p53^{+/+} and p53^{-/-} cells. **B** shows the comparison of secreted cytokines between the p53^{+/+} and p53^{-/-} cells treated with arsenic.