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## Chronic Exposure to Arsenic Causes Increased Cell Survival, DNA Damage, and Increased Expression of Mitochondrial Transcription Factor A (*mtTFA*) in Human Prostate Epithelial Cells

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Abstract

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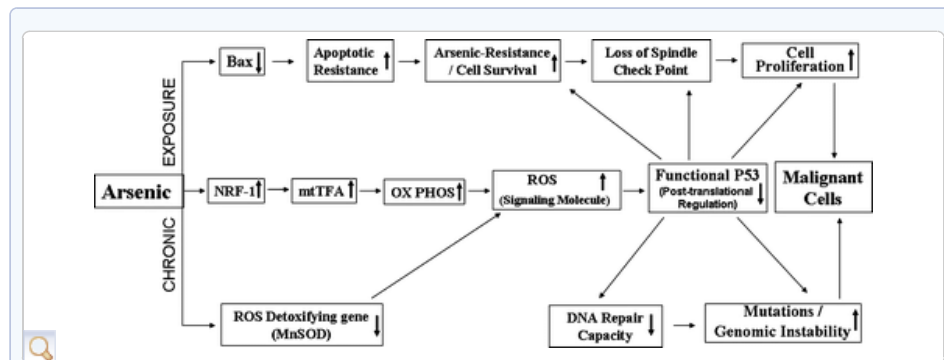
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## Abstract



Arsenic is a known carcinogen, and its exposure is associated with cancers in multiple target organs including the prostate. Whether arsenic causes cancer by increased cell proliferation or cell survival is not clear. Additionally, mitochondria have been shown to play important roles in arsenic-induced DNA damage and carcinogenesis. However, the mechanism of mitochondrial involvement in arsenic-induced cancer is not clear. Therefore, the objectives of this study were to investigate the effect of arsenic on cell proliferation/survival and genotoxicity, and to determine the effect of arsenic on the expression of mitochondrial transcription factor A (*mtTFA*) in human prostate epithelial cells, RWPE-1. Results of this study revealed that chronic exposure to arsenic causes increased cell survival. Arsenic also induced nuclear DNA damage and mutations in mitochondrial DNA. Expressions of DNA repair genes *ERCC6*, *XPC*, *OGG1*, and reactive oxygen species (ROS) scavenger *MnSOD* was also altered in arsenic-exposed cells. Arsenic concentration-dependent increased expression of *mtTFA* and its regulator *NRF-1* was observed in arsenic-exposed cells, suggesting that arsenic


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regulates mitochondrial activity through an *NRF-1*-dependent pathway. In summary, this study suggests that chronic exposure to arsenic causes DNA damage and increased cell survival that may ultimately result in neoplastic transformation of human prostate epithelial cells. Additionally, this study also provides evidence that arsenic controls mitochondrial function by regulating *mtTFA* expression.

## 1 Introduction

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Epidemiological and experimental studies have shown a significant association between chronic exposure to inorganic arsenic and prostate cancer. (1-4) However, the mechanism of arsenic-induced prostate cancer is not well understood. Mitochondria are a major source of oxygen-derived free radicals, also collectively known as reactive oxygen species (ROS). Increased production of ROS and the resultant damage to both mtDNA and nuclear DNA have long been thought to play a key role in carcinogenesis. (5) Mitochondrial aberrations have been identified in cancer of the prostate, bladder, breast, colon, head and neck, kidney, liver, lung, stomach, and in the hematologic malignancies, leukemia and lymphoma. (5) Published reports suggest that oxidants play important roles in mediating the biological effects of arsenic. Arsenic generates ROS during its metabolism in cells (6) and induces oxyradicals. (7) Involvement of mitochondria, a major source of intracellular ROS, in arsenic-induced DNA damage and carcinogenesis has been reported. (8, 9) In addition, genotoxicity of arsenic through mitochondrial damage has also been suggested. (10) However, the mechanistic basis for the involvement of mitochondria in mediating genotoxic and carcinogenic effects of arsenic is not clear.

Mitochondrial transcription factor A (*mtTFA*) is a nuclear encoded gene. Its transcription is regulated by another redox-sensitive transcription factor known as nuclear respiratory factor-1 (*NRF-1*). (11) *mtTFA* binds to the D-loop regulatory region of mitochondrial DNA and controls transcription and replication of the mitochondrial genome. (12, 13) Previous studies have shown that maintenance of mtDNA copy number and gene expression by *mtTFA* is essential for the proper functioning of mitochondria and also for cell growth. (12, 14) A recent study has shown that increased expression of *mtTFA* is associated with the progression of endometrial adenocarcinoma and poor prognosis for endometrial cancer patients. (15) These studies suggest that aberrant expression of *mtTFA* caused by endogenous or exogenous factors may lead to the abnormal function of mitochondria that may ultimately result in genotoxicity and carcinogenesis.

There are several reports on mitochondrial involvement in arsenic-induced genotoxicity and carcinogenesis; however, the effect of arsenic on the *mtTFA* and its transcriptional regulator *NRF-1* is not known. Therefore, the objectives of this study were (a) to determine whether the arsenic-induced genotoxic effect involves mitochondrial transcription factor A (*mtTFA*) and its upstream regulator and redox sensitive transcription factor *NRF-1* and (b) to evaluate whether arsenic exposure causes a mitogenic effect or increases cell survival in prostate epithelial cells.

## 2 Materials and Methods

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### 2.1 Cell Culture and Arsenic (Sodium Arsenite, NaAsO<sub>2</sub>) Treatment

Immortalized normal prostate epithelial cells, RWPE-1 (ATCC), were treated with either acute (72 h) or chronic (90 days) sodium arsenite (Sigma) in keratinocyte serum free medium supplemented with 50 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, and 1% antibiotic. For the acute treatment, RWPE-1 cells were plated (200,000 cells per well) in six-well tissue culture plates and allowed to attach for 24 h. The growth medium was replaced with growth factor-free medium, and the cells were then treated with sodium arsenite at concentrations of 1 pg/mL, 100 pg/mL, 1 ng/mL, 100 ng/mL, and 1 µg/mL or with the vehicle only (sterile deionized water) for 72 h. Similarly, for chronic (90 days) exposure to arsenic, the cells were grown in keratinocyte serum free medium supplemented with 50 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, and 1% antibiotic and sodium arsenite at

concentrations of 1 pg/mL, 100 pg/mL, 1 ng/mL, and 100 ng/mL, respectively. Every 6 days, cells grown to near confluence were subcultured in fresh culture medium containing arsenic at the respective concentration. Following this procedure, cells were grown and maintained in arsenic-containing growth medium for 90 days. Parallel cultures grown in arsenic-free medium (treated with the deionized water vehicle) were used as passage-matched controls.

## 2.2 Cell Proliferation Assay

Cell count analysis was performed to determine the effect of both acute and chronic exposure to arsenic on the proliferation of RWPE-1 cells. Cells were given an acute (72 h) treatment of arsenic as described above. After 72 h, the culture medium was removed, and cells were detached by adding 500  $\mu$ L of 1 $\times$  trypsin-EDTA to each well. After harvesting the cells by centrifugation, cell pellets were suspended in 1 mL of 1 $\times$  PBS and counted with a hemacytometer. Similarly, the RWPE-1 cells with chronic (90 days) exposure to arsenic and passage-matched untreated control cells were plated (200,000 cells per well) in six-well tissue culture plates and allowed to attach and grow. After 72 h of seeding, the culture medium was removed, and cells were detached by trypsin digestion using 1 $\times$  trypsin-EDTA as described above. Cells were then collected and suspended in 1 mL of 1 $\times$  PBS, and cell count analysis was performed with a hemacytometer or cell counter. The cell count experiments were repeated three times, and each trial was performed in triplicate.

## 2.3 Quantitative Real-Time PCR

Gene expression was measured by real-time quantitative reverse transcription PCR using the One-Step real-time quantitative reverse transcription PCR kit with SYBR green (BioRad, Hercules, CA). Single-step real-time quantitative reverse transcription PCR amplifications starting with total RNA (200 ng) were performed in 96-well optical reaction plates using an iCycler (BioRad) programmed for reverse transcription (50  $^{\circ}$ C for 15 min, denaturation and reverse transcriptase enzyme inactivation at 95  $^{\circ}$ C for 5 min, followed by 40 cycles of 10 s denaturation at 95  $^{\circ}$ C, and 30 s primer annealing and strand extension at 60  $^{\circ}$ C). The specificity of PCR products was verified by melting curve analysis between 55 and 95  $^{\circ}$ C at 0.5  $^{\circ}$ C temperature increments, and the size-specificity of the PCR product was further confirmed on an agarose gel. A single peak in the melt curve analysis and the expected size of the PCR product on an agarose gel confirmed the specificity of the primers. Threshold cycle number (Ct value) was analyzed using iCycler IQ optical system software (BioRad, version 3.0a). The Ct values of the genes of interest were normalized to the Ct value of *GAPDH* from the same sample, and the fold-change in the expression of each gene was calculated by using the delta  $-\Delta$  Ct method.<sup>(16)</sup> Amplification reactions for each sample were performed in triplicate. A nontemplate control was included in each experiment. The primer sequences used for the real-time quantitative reverse transcription PCR experiments are provided in Table 1.

**Table 1. List of Forward and Reverse Primer Sequences Used for Gene Expression Analysis by Quantitative Real-Time PCR and for Multiplex PCR Amplification of Mitochondrial DNA Regions**

gene	forward primer (5' to 3')	reverse primer (5' to 3')	PCR product size (bp)
Primers for Gene Expression Analysis by Real-Time PCR			
GAPDH	GGTGGTCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT	116
CyclinD1	AACTACCTGGACCGCTTCCT	CCACTTGAGCTTGTTACCA	204
PCNA	TGCGGCCGGGTTCCAGGAGTCA	CAGGCAGCGGGAAGGAGGAAAGT	348
P53	GGCCCACTTACCCTACTAA	GTGGTTTCAAGCCAGATGT	156
Bcl2	GGATGCCTTTGTGGAAGTGT	AGCCTGCAGCTTTGTTTCAT	236
Bax	TTTGCTTCAGGGTTTCATCC	CAGTTGAAGTTGCCGTCAGA	246
MnSOD	GCTGACGGCTGCATCTGTT	CCTGATTTGGACAAGCAGCAA	101
mtTFA	TATCAAGATGCTTATAGGGC	CACTCCTCAGCACCATATTTTCG	430
NRF-1	GTCCAGATCCCTGTGAGCAT	GGTGACTGCGCTGTCTGATA	117
ERCC6	TTGAGCTGCAGGGTTTGGGTG	TGCATCCTCCTCCAGACTGGC	383
XPC	CCAGAGCAGCGCAAGACAAGA	AAGCGGGCTGGGATGATGGAC	215
OGG1	GACAAGAGCCAGGCTAGCAG	CTCTTGAAGTGGGAGTCCA	126

## Primers for Multiplex PCR of Mitochondrial DNA

tRNA <sup>lys</sup> /ATPase	CTACCCCTCTAGAGCCAC	TGTCGTGCAGGTAGAGGCTT	922
tRNA <sup>leu</sup>	AGGACAAGAGAAATAAGGCC	AGTAGAATGATGGCTAGGGTGAC	629
ND4	CCGGCGCAGTCATTCTCA	GGAGTATAGGGCTGTGACTA	293

## 2.4 Comet Assay

The comet assay was performed under alkaline conditions as previously reported.<sup>(17)</sup> Briefly, cells were detached by 1× trypsin-EDTA treatment for 5 min and collected by centrifugation. Cell pellets were washed twice with 1× PBS and then suspended in 1× PBS. Fifty microliters of cell suspension (5000 cells) was mixed with 500 μL of 1% (w/v) low melting point (LMP) agarose dissolved in 1× PBS. Cell suspension in agarose was then quickly pipetted into each of 3 wells of a comet slide (Trevigen, Gaithersburg, MD) and allowed to set at 4 °C for 10 min in the dark. The slides were then immersed in prechilled lysis solution (2.5 M NaCl, 100 mM sodium-EDTA, and 10 mM Tris at pH 10) for 60 min at 4 °C to remove cellular proteins. Following lysis, the slides were placed in a horizontal gel electrophoresis unit and then electrophoresed (1 V/cm tank length) in freshly prepared alkaline electrophoresis buffer (300 mM NaOH and 1 mM EDTA at pH 13) for 45 min at room temperature. Following electrophoresis, slides were immersed in neutralization buffer (0.4 M Tris-HCl, pH 7.5) and then gently washed three times for 5 min at 4 °C to remove alkalis. Finally, slides were fixed in 70% ethanol for 5 min and stored in the dark to dry completely. Just before image analysis, gels on each slide were stained with 50 μL of diluted SYBR green (1:10,000 dilution in 10 mM Tris at pH 8.0, and 1 mM-EDTA buffer) in the dark for 5 min at 4 °C. A coverslip was placed over the moist gel, and the slides were examined under an epifluorescence microscope (Nikon) at 460 nm. Images were captured using a digital camera and saved as TIFF/JPEG files. All steps were performed under reduced light to minimize DNA damage from ambient ultraviolet radiation.

DNA damage was analyzed by visual scoring of comet tail morphology (intact nuclei with no tails indicated no DNA damage vs nuclear DNA with tails indicating DNA damage), and comet tail length. The percentage of comet tail DNA was chosen as the parameter for the measurement of DNA damage because it has been shown to have minimum variation between experimental trials and has also been recommended for DNA damage analysis in the comet assay.<sup>(18-20)</sup>

## 2.5 Western Blot Analysis

Proteins from total cell lysate were separated by electrophoresis using a 10% SDS-PAGE gel, followed by protein transfer onto nitrocellulose membranes. The membranes were then blocked overnight at 4 °C with 5% nonfat dried milk in 1× PBS containing 0.05% Tween 20. Blots were then reacted with diluted primary antibody for 1 h at room temperature. On the basis of the reaction efficiency of the primary antibodies, dilutions of 1:200 GAPDH (Santa Cruz, Cat # sc-25778), 1:100 NRF-1 (Santa Cruz, Cat # sc-33771), 1:100 mtTFA (Santa Cruz, Cat # sc-19050), 1:400 PCNA (Santa Cruz, Cat # sc-56), 1:400 XPC (Santa Cruz, Cat # sc-30156), and 1:100 P53 (Santa Cruz, Cat# sc-126) were used. After 3 washes with washing buffer (1× PBS containing 0.05% Tween 20), membranes were incubated in the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:1000 for 1 h at room temperature. Membranes were again given 3 washes with washing buffer, and the specific protein bands were then visualized using an enhanced chemiluminescence system (Amersham, NJ).

## 2.6 Mitochondrial DNA Amplification by Multiplex PCR and DNA Sequencing

### 2.6.1 Multiplex PCR

Three mutation hot spots (tRNA<sup>lys</sup>/ATPase, tRNA<sup>leu</sup> (UUR), and ND<sub>4</sub> regions) in the mitochondrial genome were amplified with the three pairs of primers listed in Table 1. Each PCR mixture (100 μL) contained 1× PCR buffer (10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.01% (w/v) gelatin), 0.2 mM of each dNTPs, 0.5 μM of each primer, 1 U of *Taq* DNA polymerase, and 100 ng of total DNA. The reaction mixture was denatured at 94 °C for 3 min, followed by 25 cycles of denaturation (45 s) at 94 °C, 1 min of primer-annealing at 55 °C, and 2 min of extension at 72 °C. The PCR was completed by a final extension cycle at 72 °C for 7 min. After amplification, 50 μL of the PCR mixture was analyzed on a 1.5% agarose gel. The bands were

then cut from the gel, and the mtDNA fragments were gel eluted. The mtDNA fragments were then precipitated, washed, and dissolved in 10 mM Tris-1 mM EDTA at pH 7.5.

### 2.6.2 Sequencing of PCR Products

To remove the unincorporated nucleotides, multiplex PCR amplified products were purified using a PCR purification kit (Qiagen). Sequencing of the purified PCR products was performed on an Applied Biosystems 3130 genetic analyzer using the Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystem, Foster City, CA).

### 2.7 Statistical Analysis

*t*-Test (two-tailed, paired samples for means, and hypothesized difference of 0) was performed on the data. An ANOVA was performed to determine if the source of variation in the data was between or within treatment groups. Alpha was set at 0.05 as the significance criterion for all statistical tests; data with  $p \leq 0.05$  were considered as significantly different.

## 3 Results

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### 3.1 Arsenic-Induced Cell Proliferation

#### 3.1.1 Effect of Acute (72 h) Exposure to Arsenic on Cell Proliferation

To determine the effect of acute exposure to arsenic on cell proliferation, cell count analysis was performed on RWPE-1 cells treated with various concentrations (1 pg/mL, 100 pg/mL, 1 ng/mL, 100 ng/mL, and 1  $\mu$ g/mL) of sodium arsenite for 72 h. Data from cell count analysis revealed a small but significant increase ( $p = 0.008$ ) in the growth of 100 pg/mL treated cells and a significant decrease ( $p = 0.009$ ) in the growth of 1  $\mu$ g/mL treated cells (Figure 1, left panel). Acute exposure to 1 pg/mL, 1 ng/mL, and 100 ng/mL arsenic had no significant ( $p > .05$ ) effect on the proliferation of RWPE-1 cells. This suggests that acute exposure to a relatively low concentration (1 pg/mL) of arsenic has no mitogenic or toxic effects on RWPE-1 cells, whereas the higher concentration (1  $\mu$ g/mL) is toxic to these cells.

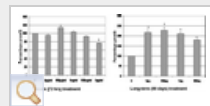


Figure 1. Effect of acute (left panel) and chronic (right panel) exposure to arsenic on cell proliferation in RWPE-1 human prostate epithelial cells. RWPE-1 cells were treated with various concentrations of arsenic for either short or long durations as described in [Materials and Methods](#). Cell count analyses were performed, and the data shown are the means  $\pm$  SEM of two independent experiments each with triplicate flasks. An \* indicates a significant ( $p < 0.05$ ) difference between control and arsenic-exposed cells.

#### 3.1.2 Effect of Chronic (90 Days) Exposure to Arsenic on Cell Proliferation

The effect of chronic exposure to arsenic on cell proliferation was also determined by cell count analysis of RWPE-1 cells. Cells were grown in growth media containing various concentrations (1 pg/mL, 100 pg/mL, 1 ng/mL, and 100 ng/mL) of arsenic and maintained at a semiconfluent level as described in the [Materials and Methods](#) section. A significant and arsenic concentration-dependent increase in cell proliferation was observed for lower concentrations (1 pg/mL,  $p = 0.031$ , and 100 pg/mL,  $p = 0.028$ ) of arsenic used in this study (Figure 1, right panel). Chronic exposure to a relatively high concentration (100 ng/mL) of arsenic also induced significant ( $p = 0.012$ ) growth; however, this concentration was not as effective as the lower concentrations in inducing cell proliferation. This indicates that chronic exposure to low levels of arsenic results in increased growth of RWPE-1 cells; these cells become resistant to the cytotoxic effects of high arsenic concentrations after chronic exposure.

### 3.2 Arsenic-Induced Changes in the Cell Cycle

Flow cytometry analysis was performed to evaluate the effect of chronic exposure to arsenic on the cell cycle of prostate epithelial cells. Percentage of cells in G0/G1, S, and G2/M stages from arsenic-treated groups and the untreated group are given in Figure 2. Arsenic-treated groups had a lower percentage of cells in G0/G1 stages, as compared to the untreated control group. However, these differences were not statistically significant ( $p > 0.05$ ). The decrease in percentage of cells in S phase from the 100 pg/mL arsenic-treated group compared to that of the controls was statistically significant ( $p = 0.031$ ), whereas no significant ( $p = 0.110$ )

difference in the percentage of cells in S phase from 100 ng/mL and the untreated control group was observed. In addition, a higher percentage of cells in G2/M stages from arsenic-exposed groups as compared to the unexposed control groups was observed. The increase in the percentage of cells in G2/M stages from the 100 pg/mL arsenic-exposed group was not significant, whereas the increase in the 100 ng/mL arsenic-exposed group was statistically significant ( $p = 0.020$ ) (Figure 2).

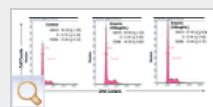


Figure 2. Effect of arsenic exposure on the cell cycle of RWPE-1 cells as determined by flow cytometry. Arsenic-exposed as well as the passage-matched unexposed control cells were collected and processed as described in [Materials and Methods](#) for flow cytometric analysis. Histograms represent FACS data showing cell population in G0/G1, S, and G2/M stages of the cell cycle.

### 3.3 Arsenic-Induced Changes in Gene Expression

To understand the molecular mechanism for increased cell growth and survival as a result of chronic exposure to arsenic, the expression of some genes associated with the cell cycle, cell survival, and DNA repair as well as the transcription factors regulating mitochondrial gene expression were measured at the transcript level using real-time quantitative reverse transcription PCR. Western blot analyses of selected proteins were also performed to confirm the arsenic-induced changes at the protein level. Gene expression analysis was performed on RWPE-1 cells with chronic exposure to 100 pg/mL and 100 ng/mL arsenic. Details of the arsenic-induced changes observed in the expression of each category of genes are described under the following subheadings.

#### 3.3.1 Cell Proliferation and Survival-Related Genes

To understand the molecular basis for arsenic-induced growth of RWPE-1 cells, mRNA expression of cell proliferation (*CyclinD1*, *PCNA*), cell cycle control (*P53*), pro-apoptotic (*Bax*), and antiapoptotic (*Bcl2*) marker genes were analyzed by real-time quantitative reverse transcription PCR. There was no significant ( $p > 0.05$ ) change in the expression of *CyclinD1* and *P53* mRNAs between arsenic-treated cells compared to that in passage-matched untreated control cells. A concentration-dependent and significant increase in expression of *PCNA* mRNA (2.4-fold at 100 pg/mL,  $p = 0.001$ , and 5.35-fold at 100 ng/mL,  $p = 0.004$ ) was observed in arsenic-exposed cells (Figure 3). *Bcl2* mRNA expression was increased 1.4-fold and 2.1-fold in 100 pg/mL and 100 ng/mL arsenic-treated cells, respectively. An arsenic concentration-dependent and significant decrease in the expression of *Bax* mRNA with a maximum decrease of 32-fold ( $p = 0.005$ ) at the lower concentration (100 pg/mL) and a minimum decrease of 8.1-fold ( $p = 0.004$ ) at the higher concentration (100 ng/mL) was observed (Figure 3).

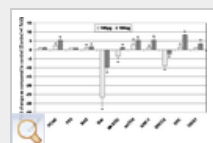


Figure 3. Real-time quantitative reverse transcription PCR analysis of gene expression in arsenic-treated cells. RWPE-1 cells were exposed for 90 days to arsenic as described in [Materials and Methods](#). The expression of genes at the transcription level was measured by real-time quantitative reverse transcription PCR. Threshold cycle number (Ct value) for each gene obtained by real-time quantitative reverse transcription PCR was normalized to the Ct value of *GAPDH* from the same sample, and the fold-change in expression for each gene was obtained using the delta-delta Ct method. The graph shows the means of triplicate values. An \* indicates a significant ( $p < 0.05$ ) difference between control and arsenic-exposed cells.

#### 3.3.2 DNA Repair Genes

The effect of arsenic on the mRNA expression of representative genes for DNA nucleotide excision repair (*ERCC6* and *XPC*) and base excision repair (*OGG1*) was also determined. A statistically significant decreased expression of *ERCC6* mRNA in arsenic-treated cells with a maximum decrease of 9.2-fold ( $p < 0.001$ ) at the lower concentration (100 pg/mL) and a minimum decrease of 2.6-fold ( $p = 0.003$ ) at the higher concentration (100 ng/mL) was observed. The expression of *XPC* and *OGG1* transcripts, however, was increased in arsenic-treated cells in a concentration-dependent manner (Figure 3). The increased expression of *XPC* mRNA was statistically significant in both 100 pg/mL ( $p = 0.042$ ) and 100 ng/mL ( $p < 0.001$ )

arsenic-exposed cells. The increased expression of *OGG1* mRNA in 100 pg/mL arsenic-exposed cells was not significant ( $p = 0.113$ ), whereas 100 ng/mL arsenic-exposed cells had a significant ( $p = 0.003$ ) increase in *OGG1* transcript expression.

### 3.3.3 Transcription Factors mtTFA and NRF-1

In order to determine the involvement of mitochondria in mediating arsenic effects, the mRNA expression of *mtTFA* and the redox sensitive transcription factor *NRF-1* that regulates *mtTFA* were also measured. A concentration-dependent increase in the expression of *mtTFA* and its regulator *NRF-1* gene transcripts was observed in arsenic-treated cells compared to that of passage-matched untreated control cells. Expression of *mtTFA* transcripts was significantly increased 4.0-fold ( $p = 0.054$ ) in 100 pg/mL and 6-fold ( $p = 0.006$ ) in 100 ng/mL arsenic-treated cells. Similarly, the expression of *NRF-1* transcripts was also significantly increased 1.3-fold ( $p = 0.028$ ) in 100 pg/mL and 6-fold ( $p = 0.014$ ) in 100 ng/mL arsenic-treated cells (Figure 3).

### 3.3.4 Confirmation of Gene Expression Changes by Western Blot Analysis

Arsenic-induced changes in expression of selected genes were further confirmed at the protein level using Western blot analysis (Figure 4, left panel). Quantification of protein band intensities were analyzed by Image J software (Figure 4, right panel). An increase in the level of NRF-1 and mtTFA proteins in cells exposed to relatively higher concentrations of arsenic (100 pg/mL, 1 ng/mL, and 100 ng/mL) and a decrease in the level of these two proteins in cells exposed to relatively lower concentration (1 pg/mL) of arsenic as compared to that of passage-matched controls was observed. Statistical analysis of the protein band intensities data revealed significant increased expression of mtTFA proteins in 100 pg/mL ( $p = 0.025$ ) and 100 ng/mL ( $p = 0.003$ ) arsenic exposed cells, whereas the decrease in the mtTFA protein level in 1 pg/mL and 1 ng/mL arsenic-treated cells was not significant. The change in NRF-1 proteins in 100 ng/mL was also significant ( $p = 0.011$ ), whereas changes in NRF-1 protein in 1 pg/mL, 100 pg/mL, and 1 ng/mL arsenic-treated cells were not significant. A concentration-dependent and statistically significant ( $p < 0.05$ ) increase in the expression of cell proliferation marker protein PCNA and DNA repair protein XPC was observed in arsenic-treated cells compared to that of passage-matched controls (Figure 4). As observed in the case of mtTFA and NRF-1 proteins, the expression of P53 protein was also significantly decreased ( $p = 0.047$ ) in 1 pg/mL arsenic-treated cells, whereas it was significantly increased in 100 ng/mL ( $p = 0.041$ ) arsenic-treated cells. Expression of P53 protein in 100 pg/mL and 1 ng/mL arsenic-treated cells was also decreased; however, these decreases were not statistically significant ( $p > 0.05$ ). Therefore, results of the Western blot analysis confirmed the changes observed at the transcript level in the expression of *NRF-1*, *mtTFA*, *PCNA*, and *XPC* genes in 100 pg/mL and 100 ng/mL arsenic-treated cells.

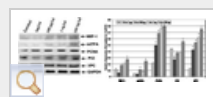


Figure 4. Representative Western blots (left panel) and the relative band intensity histograms (right panel) showing the effect of arsenic on the expression of selected genes at the protein level. Arsenic (As)-exposed and passage-matched unexposed control (C) RWPE-1 cells were grown for 90

days as described in [Materials and Methods](#). Expression of the genes at the protein level was measured in whole cell lysates using Western blot analysis. The intensity of each protein band was quantified using Image J software as described in [Materials and Methods](#). Histograms represent the signal intensity of protein bands in arbitrary units after normalization with the signal intensity of GAPDH internal control for each sample. The graph shows the means of duplicate values. An \* indicates a significant ( $p < 0.05$ ) difference between control and arsenic-exposed cells.

## 3.4 Nuclear and Mitochondrial DNA Damage by Arsenic

### 3.4.1 Nuclear DNA Damage Analysis by the Comet Assay

Single cell gel electrophoresis (commonly known as the comet assay) was performed to evaluate the effect of chronic exposure to arsenic on nuclear DNA damage. The results of the comet assay revealed the presence of nuclear DNA forming tail-like structures in 100 pg/mL arsenic-treated cells (Figure 5, left panel), whereas the nuclear DNA of untreated passage-matched control cells was intact with no tailed DNA (Figure 5, right panel). The presence of tails in arsenic-treated cells indicates that DNA is damaged and that broken DNA emerged from

the nucleus and formed tail-like structures following alkaline single cell gel electrophoresis.

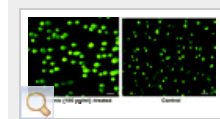


Figure 5. Representative photographs of the comet assay from arsenic-exposed cells (left panel) and untreated control cells (right panel). Arsenic-exposed cells and passage-matched untreated control cells were grown for 90 days as described in [Materials and Methods](#). DNA damage in individual cells was measured by single cell gel electrophoresis (comet assay).

Magnification scale bar (—) = 10 $\times$ .

### 3.4.2 Mitochondrial DNA Mutation Detection by Multiplex PCR and Sequencing

Multiplex PCR was used to amplify mitochondrial DNA regions of *tRNA<sup>lys</sup>/ATPase*, *tRNA<sup>leu</sup> (UUR)*, and *ND<sub>4</sub>* followed by sequencing of the PCR products to detect mutations. The multiplex PCR of mitochondrial DNA did not reveal any change in the size of PCR products, indicating no major deletions or insertions in the sequence of mitochondrial DNA in arsenic-treated cells. In order to detect point mutations, if any, PCR products were purified (to get rid of unincorporated nucleotides) and then subjected to DNA sequencing. Sequence analysis revealed an insertion of a "G" base in the sequence of mitochondrial *ATPase* gene from 100  $\mu$ g/mL arsenic-treated cells (Figure 6).

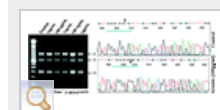


Figure 6. Representative photograph of multiplex PCR amplification products (left panel) and DNA sequence chromatogram showing mutations in the mtATPase region (right panel) in RWPE-1 cells with chronic (90 days) exposure to arsenic. Mitochondrial DNA regions of *tRNA<sup>leu</sup> (UUR)*, *tRNA<sup>lys</sup>/ATPase*, and *ND<sub>4</sub>* were amplified by multiplex PCR with total DNA

isolated from arsenic-exposed and passage-matched untreated RWPE-1 cells. Mitochondrial DNA specific sequences were used as primers as given in Table 1. M: DNA marker. The PCR products were purified and sequenced as described in [Materials and Methods](#). The mutated base in DNA from arsenic-exposed cells and its corresponding normal bases in DNA from passage-matched untreated RWPE-1 cells are indicated by arrows.

## 4 Discussion

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The most important and novel finding of this study was that chronic exposure to arsenic results in increased cell survival by decreasing the expression of pro-apoptotic genes. Additionally, the findings of this study also suggest that arsenic is mutagenic to mitochondrial DNA and can potentially induce nuclear DNA damage by activating mitochondrial ROS through increased expression of *mtTFA*.

Results of gene expression analysis revealed no increase in levels of cell cycle gene *Cyclin D1* in cells with chronic exposure to arsenic. This was further confirmed by data from flow cytometry analysis indicating no increase in the percentage of cells in S-phase following arsenic exposure. Despite results at the molecular level showing no induction of S-phase cells and *CyclinD1* expression, cell count data revealed increased growth of cells chronically exposed to arsenic. Overall cell growth in arsenic-treated cells can be explained by the mechanism of increased cell survival. This is further supported by our data on decreased expression of the pro-apoptotic gene *Bax* and an increase in the percentage of arsenic-treated cells at G2/M stage compared to untreated control cells. Decreased expression of *Bax* is supportive of the hypothesis that arsenic exposure results in increased cell survival. Cell cycle analysis revealed an increase in the G2/M percentage in arsenic-exposed cells compared to that in unexposed control cells. Therefore, the cell growth and cell cycle data indicate that arsenic is able to delay the progression from G2 or M phase; however, it is not able to induce apoptosis. These findings are consistent with previous reports of arsenic-induced G2 phase delay<sup>(21-23)</sup> and prevention of mitotic arrest-associated apoptosis in arsenic-exposed cells.<sup>(23)</sup> The cell cycle analysis and decreased expression of the pro-apoptotic gene *Bax* further indicate that the observed increase in the growth of cells with chronic exposure to arsenic is due to increased cell survival and adaptation capacity.

Loss of spindle checkpoints could be another potential cause for increased proliferation of cells

chronically exposed to arsenic. Arsenic-induced mitotic arrest and apoptosis has been shown to be dependent on functional spindle checkpoints. (24) This is further supported by the absence of mitotic arrest as a result of the loss of mitotic spindle checkpoints in arsenic-resistant cells. (24) Although binding of arsenic to both  $\alpha$ - and  $\beta$ -tubulins has been observed, (25) its biological consequences are still debatable. (26, 27) On the basis of the lack of mitotic arrest due to aberrant spindle checkpoints (24) in arsenic-resistant cells, it is likely that the increased cell proliferation of RWPE-1 cells observed in this study could be due to aberrant spindle checkpoints. Previous studies have shown that failure of spindle checkpoints or their aberrant activities can initiate an untimely anaphase that may cause increased cell proliferation and predispose cells to genomic instability and malignant transformation. (28) Therefore, chronic exposure to arsenic may have caused aberrant spindle checkpoints in arsenic-exposed RWPE-1 cells resulting in aneuploidy and genomic instability and consequently the selective cell proliferation advantage of these cells over the normal (unexposed) cells. Further study is needed to understand the effects of chronic exposure to arsenic on mitotic spindle checkpoints and their subsequent impact on the proliferation of RWPE-1 cells.

Arsenic has been shown to increase the production of intracellular ROS, (8, 29) and up-regulate the redox sensitive transcription factors *AP-1*, *CREBP*, and *NF-kappaB* in human epidermal keratinocytes and breast cancer cells. (30, 31) The role of nuclear factor erythroid-derived factor 2-related factor-1 (*Nrf-1*), a transcription factor, has also been recently reported to increase the cell survival capacity by regulating antioxidant genes and protecting cells from arsenic cytotoxicity. (32) Arsenic exposure also causes the activation of *Gadd45a* through the production of hydrogen peroxide. (33) These studies suggest that arsenic exposure results in increased ROS that act as signaling molecules to regulate several cellular functions by regulating gene expression and function. Mitochondria are a major source for intracellular ROS. *NRF-1* is a redox-responsive transcription factor and has been shown to regulate the mitochondrial function either directly by regulating the nuclear encoded cytochrome *c* oxidase (*COX*) subunits (34) or indirectly by activating *mtTFA*. (35, 36) Previous reports suggest the involvement of mitochondria and mtROS in mediating the effects of arsenic. (8-10) However, the mechanistic basis for the regulation of mitochondrial function and mtROS by arsenic is not clear. *mtTFA* controls mitochondrial gene expression and thereby mitochondrial function including the generation of mtROS. In this study, gene expression analysis revealed an increased expression of *mtTFA* and its regulator *NRF-1* in arsenic-exposed cells. Because we observed increased expression of *mtTFA* by arsenic, one of our important findings is that arsenic regulates the mitochondrial function and thereby mtROS production by increasing the expression of *mtTFA* and its activator *NRF-1*.

Previous research has shown that chronic exposure to arsenic induces genetic and epigenetic changes. (37) In this study, the comet assay revealed that arsenic can induce nuclear DNA damage. Mitochondrial DNA multiplex PCR and sequence analysis also revealed that arsenic is a mutagen and can induce point mutations in mitochondrial DNA. Genotoxic effects of carcinogens that induce DNA breaks, if not repaired, may contribute to the propagation of mutations and consequently malignant transformation. Various types of DNA damage have been found to be associated with cancer development, and therefore, mutations resulting from DNA damage are considered as a hallmark of cancer. (38) Arsenic has been shown to induce malignant transformation in RWPE-1 cells, (39) but the arsenic-induced DNA damage in these cells is not known. Therefore, to our knowledge this is the first report on arsenic-induced DNA damage in human prostate epithelial cells. The mechanism for the DNA damage observed in this study remains to be determined. Previous research has shown that arsenic biomethylation is required for oxidative DNA damage. (40) RWPE-1 cells have been shown to be deficient in arsenic biomethylation, and no increase in oxidative DNA damage was reported in these cells following arsenic exposure for 30 weeks. (40) On this basis, the role of arsenic-induced ROS in oxidative DNA damage is very unlikely and needs further investigation. It is possible that arsenic-induced ROS (as a signaling molecule) aberrantly regulate the expression and function of DNA repair genes and thereby hinder the repair of spontaneous DNA damage originating from endogenous sources. This possibility is further supported by the observation of decreased expression of NER gene *ERCC 6* in this study and the BER gene *APE1* in arsenic-exposed aging

mice.(41)

In this study, no change in *P53* transcripts was observed in arsenic-exposed cells. However, Western blot analysis revealed decreased *P53* protein in cells treated with lower concentrations of arsenic and increased *P53* protein in cells treated with a higher concentration (100 ng/mL) of arsenic. The arsenic-induced changes in *P53* expression at the protein level but not at the transcript level suggest that arsenic regulates *P53* at the post-translational level, potentially through arsenic-induced ROS as a signaling molecule. This argument is further supported by a recent report of arsenic-induced post-translational modification of *P53* poly (ADP-ribosyl) ation, and consequently its inactivation in human skin cells chronically exposed to arsenic.(42) Among the DNA repair genes analyzed in this study, increased expression of *XPC* and *OGG1* was also observed in arsenic-exposed cells. A similar result of increased expression of *XPC* protein by arsenic exposure has also been reported recently.(43) Despite the increased expression of DNA repair genes *OGG1* and *XPC* in arsenic-exposed cells (should be able to repair DNA damage), the persistence of DNA damage in arsenic-exposed cells was observed in this study. It is not clear how arsenic-induced DNA damage persists even though the level of DNA repair protein *XPC* is increased in arsenic-exposed cells. *XPC* protein requires several other proteins, such as TFIIH complex and *XPE*, for DNA damage recognition and repair.(44) Therefore, the dysfunctional *XPC* complex potentially due to either the lack of *XPC*-interacting protein(s) or nonfunctional-interacting protein (s) in the *XPC* complex could be a potential explanation for the persistence of DNA damage even when *XPC* protein level is increased in arsenic-exposed cells. Previous research suggests that functional *P53* is required for efficient global nucleotide excision repair by *XPC*.(45) Additionally, the epigenetic events of histone modifications, some of which are dependent on *P53*, and chromatin remodeling are needed to allow the entrance of NER factors including the *XPC* to the site of DNA lesion.(46) Therefore, the increased level of *XPC* protein by itself is not enough to repair DNA damage, and aberrations in any of these genetic and epigenetic events that are required for *XPC* mediated DNA repair could result in the loss of DNA repair capacity and accumulation of DNA damage. The reduced expression of NER gene *ERCC6* expression could be contributing further to the accumulation and persistence of DNA damage and mutations.

Increased DNA damage should result in the increased expression of the *P53* gene and increased apoptotic pathway, ultimately reducing the chance of tumorigenesis. Contrary to this usual mechanism, a decrease in the pro-apoptotic gene *Bax* and a decrease in *P53*, a mediator of apoptosis, were observed in cells exposed to lower concentrations of arsenic. This suggests that arsenic inhibits the apoptosis of DNA-damaged cells by increasing cell survival through decreasing the genes that mediate apoptosis, such as *Bax* and *P53*. The resistance to apoptosis of DNA-damaged cells may ultimately result in the accumulation of mutations and consequently the neoplastic transformation of cells. Therefore, results of this study showing increased survival of arsenic-exposed cells through the decreased expression or function of *P53* and *mtTFA*-mediated increased mitochondrial activity could be a potential new mechanistic basis for arsenic-induced prostate cancer. The findings of this study and previous reports on the loss of mitotic spindle checkpoints in arsenic-resistant cells are summarized in a proposed model that can explain the malignant transformation of cells with chronic exposure to arsenic (Figure 7). This is the first report of changes in marker genes *NRF-1* and *mtTFA* (for mitochondrial function) and *ERCC 6* (for DNA repair) in arsenic-exposed cells.



Figure 7. Proposed model for malignant transformation of arsenic-exposed cells. The findings of this study and previous reports can be rationalized in this model for arsenic-induced carcinogenesis. Arrow signs (↑ and ↓) in the boxes indicate increase and decrease, respectively, in the expression/function of the gene or in the biological response mentioned in the text boxes of this model.

Changes in the remaining marker genes analyzed in this study are consistent with previous reports on gene expression changes in cells with chronic exposure to arsenic. For example, the decreased expression of *Bax* and *P53* observed in this study is consistent with previous reports

of decreased expression of *Bax* in arsenic-exposed RWPE-1 cells(47) and rat liver TRL1215 cells.(48) The decreased expression of *P53* observed in this study at lower doses of arsenic is also consistent with previous reports of loss of functional *P53* and its role in arsenic-induced aneuploidy in human fibroblast cells,(49) reduced expression of wild type *P53* in human small airway epithelial cells (SAECs) transformed by chronic exposure to arsenic,(50) and inhibition of *P53* functional activation by inhibition of its phosphorylation and nuclear localization in an arsenic-treated mice model(51) as well as in mouse epidermal cells.(52) Similarly, the observation of increased expression of *PCNA* is consistent with previous reports of increased expression of *PCNA* in rat liver epithelial cells TRL 1215 transformed by arsenic exposure(53) and arsenic-induced liver tumors in male mice(54) as well as in arsenic-exposed mouse testicular leydig cells.(55) The increased expression of *OGG1* observed in this study is also consistent with previous reports of increased expression of *OGG1* transcripts in arsenic-exposed human populations.(56) The novel findings of this study, such as the involvement of *NRF-1* and *mtTFA* in mediating arsenic effects, arsenic-induced DNA damage in RWPE-1 cells that lack arsenic-biomethylation capacity (required for oxidative DNA damage), cells acquiring resistance to arsenic-induced toxicity, and increased cell growth without the involvement of *CyclinD1* and S-phase increases after chronic arsenic exposure, provide the basis for further study to understand the target organ and cell type-dependent toxicity and carcinogenesis from arsenic exposure.

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