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Impact of Prenatal Arsenate Exposure on Gene Expression in a Pure Population of Migratory Cranial Neural Crest Cells

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Abstract

Prenatal exposure to arsenic, a naturally occurring toxic element, causes neural tube defects (NTDs) and, in animal models, orofacial anomalies. Since aberrant development or migration of cranial neural crest cells (CNCCs) can also cause similar anomalies within developing embryos, we examined the effects of *in utero* exposure to sodium arsenate on gene expression patterns in pure populations of CNCCs, isolated by fluorescence activated cell sorting (FACS), from *Cre/LoxP* reporter mice. Changes in gene expression were analyzed using Affymetrix GeneChip® microarrays and expression of selected genes was verified by TaqMan quantitative real-time PCR. We report, for the first time, arsenate-induced alterations in the expression of a number of novel candidate genes and canonical cascades that may contribute to the pathogenesis of orofacial defects. Ingenuity Pathway and NIH-DAVID analyses revealed cellular response pathways, biological themes, and potential upstream regulators, that may underlie altered fetal programming of arsenate exposed CNCCs.

Keywords

Arsenate; Craniofacial development; Embryo; Gene expression profiling; Microarray; Neural crest cells

1. INTRODUCTION

The naturally occurring heavy metal arsenic is a ubiquitous environmental contaminant with demonstrated adult toxicity in humans and teratogenicity in both laboratory animals and humans [1–6]. The predominant developmental effects of *in utero* arsenic exposure in laboratory animals are neural tube defects (NTDs), craniofacial malformations including exencephaly, renal agenesis and minor skeletal anomalies [1, 2, 7–9]. Within the past decade, particular interest has been focused on the potential teratogenicity of arsenic in

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humans. Epidemiological studies have suggested associations between clusters of adverse pregnancy outcomes and occupational/environmental arsenic exposures [10–12]. Arsenic ingestion, through drinking contaminated water during pregnancy, was reported to increase the incidence of spontaneous abortion, still births and reduced birth weight [10, 13]. Maternal arsenic exposure was also reported to result in chromosomal aberrations in fetal cells [14–16], and developmental anomalies in offspring [4–6, 17–20].

Mouse models, susceptible to arsenic-induced congenital malformations, represent useful paradigms to examine mechanistic linkages between exposure and abnormal development. Indeed, an earlier study from our laboratory documented an array of developmental anomalies including NTDs and orofacial malformations in murine embryos/fetuses exposed *in utero* to pentavalent arsenate [21].

Cranial neural crest cells (CNCCs) represent a population of pluripotent embryonic cells that play a critical role in craniofacial ontogeny [22, 23], and whose migration from the cranial neural folds has been suggested to be a prerequisite for proper folding/elevation of the neural folds [23, 24]. We have thus employed a mouse model where neural crest cells (NCCs), and their derivatives, are indelibly (genetically) marked with enhanced green fluorescent protein (EGFP) [22, 25] in order to investigate the impact of maternal sodium arsenate exposure on post-migratory murine CNCCs. Findings from the current study facilitated identification of *novel* putative cellular and molecular targets of arsenic's teratogenicity, as well as understanding of the effects of *prenatal* arsenate exposure on *cranial neural crest (NC)* proliferation, survival and migration.

2. MATERIALS AND METHODS

2.1 Generation of a two-component (*Wnt1-Cre/Z/EG*) murine animal model and animal dosing:

Generation of *Wnt1-Cre/Z/EG* transgenic mice, animal husbandry and developmental staging of embryos were conducted as described previously [22]. In brief, the *Wnt1* proto-oncogene encodes an intercellular signaling molecule that is initially expressed throughout the presumptive midbrain during development of the central nervous system [26]. NCCs are derived from such *Wnt1*-expressing precursor cells. Expression of Cre recombinase from the *Wnt1-Cre* transgene mirrors that of *Wnt1* [27]. Crossing the *Wnt1-Cre* recombinase transgenic mouse line with the *Z/EG* transgenic mouse reporter strain [28], in which enhanced green fluorescent protein (EGFP) is expressed by a cell only in the presence of Cre recombinase, results in offspring in which the *Wnt1-Cre* transgene activates EGFP expression in the NCC of the developing embryo.

Wnt1-Cre mice were generously provided by Dr. Andrew McMahon (Harvard University, Cambridge, MA) and *Z/EG* mice (*Tg [ACTB-Bgeo/GFP] 21Lbe*) were obtained from Jackson Laboratory (Bar Harbor, ME). All studies were reviewed and approved, before their conduct, by the University of Louisville Institutional Animal Care and Use Committee (IACUC). Pregnant *Wnt1-Cre/Z/EG* dams were dosed *in utero* via intraperitoneal injection with 20 mg/kg pentavalent sodium arsenate (Na_3AsO_4) on gestation day (GD) 7.5 and 8.5. In murine development, this represents a temporal window when the neural tube is going

through a cascade of developmental stages/processes that lead to its closure on GD 9.5 – 10.0. Control dams were dosed similarly with saline. Dams were euthanized on GD 9.5 by carbon dioxide asphyxiation/cervical dislocation, and embryos isolated by cesarean section and immediately placed in ice-cold phosphate-buffered saline (PBS). Genotyping of *Wnt1-Cre/Z/EG* transgenic embryos was performed as reported earlier [22].

2.2 Visualization of EGFP-labeled CNCCs:

EGFP-labeled CNCCs were visualized in intact embryos and photographed as described previously [22].

2.3 Fluorescence-activated cell sorting (FACS):

Gestation day 9.5 embryos were dissected in ice-cold calcium-magnesium-free PBS (CMF-PBS) (0.2 g KCl, 0.2 g KH₂PO₄, 8.0 g NaCl, 2.16 g Na₂HPO₄·7H₂O, pH 7.2 per liter) and heads removed above the second branchial arch. Following removal of the midbrain and hindbrain, the remaining tissue, comprising the frontonasal region and the first branchial arch (Figure 1), was incubated with 1.5 mL (0.5%) trypsin at 37°C for 10 minutes followed by centrifugation and resuspension in tissue culture medium with 5% fetal bovine serum (FBS). Cell sorting was performed using a Mo-Flo DakoCytomation (Fort Collins, CO) as described [25]. Each sample consisted of pooled cells from multiple embryos from the same dam and independent cell samples were generated from pooled embryos from three independent dams.

2.4 Total RNA isolation and production of antisense RNA:

Cells (approximately 7500) isolated by FACS were collected and processed using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) according to the manufacturer's protocol. Briefly, 11 µL extraction buffer was placed into a microcentrifuge tube containing the sorted cells and incubated for 30 minutes at 42°C. After incubation, the cell lysate was loaded onto a spin column, washed, and total cellular RNA was eluted in 10 µL elution buffer. The eluant (total RNA) was used as template to produce antisense RNA (aRNA) using the RiboAmp HS kit (Arcturus) following manufacturer's instructions. Approximately 400 ng of Poly(dI) carrier was added to each total RNA sample before amplification. RNA amplification, *in vitro* transcription of the double-stranded cDNA, as well as generation and fragmentation of biotin-labeled cRNA, were performed as described earlier [22]. Twenty micrograms of fragmented, biotin-labeled cRNAs corresponding to either sodium arsenate- or saline (vehicle)-exposed EGFP-labeled, FACS-isolated CNCCs were hybridized to individual GeneChips from an identical lot of Murine Genome 430A GeneChip® arrays (Affymetrix) for 16 hr. GeneChip® arrays were washed and stained using antibody-mediated signal amplification according to the Affymetrix Fluidics Station's standard Eukaryotic GE Wash 2/ protocol. Three independent biological samples were prepared for each sample type, and each of the three biological replicates was hybridized to a single GeneChip®.

2.5 Reduction, statistical analysis, and biological interpretation of microarray data:

Scanning of GeneChip® arrays, subsequent image processing using Affymetrix GCOS v1.2 software, Robust Multi-chip Average, with GC-content background correction (GC-RMA)

pre-processing, and ‘per gene normalization’ with GeneSpring v7.2 (Silicon Genetics, Inc., Redwood City, CA) were performed as described earlier [29]. One-way ANOVA (parametric test, assuming equal variances) was employed using the “Benjamini and Hochberg false discovery rate” as the multiple testing correction ($p = 0.05$; probes with p values of less than 0.05 were considered to be expressed significantly above background) to define a set of statistically significant, differentially expressed genes. One-way ANOVA was applied to define genes that are significantly altered in arsenate-treated CNCCs vs. saline (control)-treated CNCCs. This restriction tested each of the approximately 45,000 transcripts (corresponding to about 34,000 genes and ESTs) on the microarray and generated a list of genes with statistically significant expression values. Finally, a “filter” on the fold change (probes with fold differences >1.5 were considered significant) was applied to the list of aforementioned genes, and lists of genes were generated based on the treatment conditions (e.g. saline-treated sample #1 vs. arsenate-treated sample #1, or saline-treated sample #2 vs. arsenate-treated sample #2 and so on). The gene lists include those genes/ESTs whose expression was either >1.5 -fold up- or down-regulated in a statistically significant manner ($p < 0.05$) as a function of the treatment condition. GeneSpring GX software (version 11.0, Agilent Technologies, Palo Alto, CA), was used for hierarchical clustering analysis to create a condition tree representing all of the differentially expressed genes based on their expression profiles. NIH-DAVID software (DAVID v 6.7; <http://david.abcc.ncifcrf.gov>; [30]) was employed for functional annotations of affected genes, as well as to discern various biological/canonical pathways impacted in murine CNCCs by prenatal arsenate exposure. This was accomplished by converting the probe IDs of all the differentially expressed gene transcripts and ESTs to unique DAVID IDs and matching those to the KEGG (Kyoto Encyclopedia of Genes and Genomes; <http://www.genome.jp/kegg/pathway.html>), Biocarta (https://cgap.nci.nih.gov/Pathways/BioCarta_Pathways) and Panther pathway databases (<http://www.pantherdb.org/pathway/>).

In addition, computational gene interaction predictions were made utilizing Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) to establish an overview of diverse cellular processes and biological pathways within CNCCs impacted by *in utero* arsenate exposure. Several hundred differentially expressed genes detected from the microarray analysis, were used to execute core analysis and to construct interactive gene association maps for predicting the teratogenic effects of arsenate on a range of cellular and molecular events within post-migratory CNCCs.

2.6 TaqMan™ quantitative real-time PCR:

cDNA synthesis from amplified RNA (aRNA), quantitative real-time PCR (qRTPCR) (TaqMan®) analysis and processing of raw data, were performed as previously reported [29]. Briefly, the expression of a selected panel of 20 genes was assessed by qRTPCR. The data was reduced by the Ct method [31] using *Gapdh* as an internal control. The data are expressed as fold change in arsenate-treated cells, relative to that in saline-treated control samples. The data are reported as average fold-change of 3 independent experiments $\pm$ S.E.M. p values were determined by one-way ANOVA. Predesigned forward and reverse primers and their corresponding fluorescent probes for each of the genes that were examined

were purchased from Thermo Fisher/Life Technologies (Carlsbad, CA, USA). The assay ID numbers of these TaqMan probe-primers are listed in Supplementary Table 1.

3. RESULTS

3.1 Phenotypes generated by *in utero* arsenate exposure:

In utero treatment of pregnant dams with 20 mg/kg pentavalent sodium arsenate (Na_3AsO_4) on GD-7.5 and -8.5 resulted in 25% of the embryos/fetuses demonstrating neural tube and orofacial defects, including exencephaly (GD-10.5), facial clefts (GD-10.5), and midfacial hypoplasia (GD-17.5) when compared to the saline-treated controls, as reported earlier [21].

3.2 Characterization of EGFP-labeled cranial neural crest cells:

Generation of a compound transgenic mouse model in which constitutive expression of EGFP was induced in cells with an activated *Wnt1* promoter has been previously reported by our laboratory [22]. Under these conditions, EGFP was expressed only in the NCCs. Figure 1 demonstrates the location of post-migratory CNCCs in a GD 9.5 embryo. Fluorescence labeling of CNCCs permitted the FACS-based isolation of sufficient numbers of such cells for adequate RNA extraction and amplification for hybridization to Affymetrix GeneChip® microarrays.

3.3 Differential gene expression between maternal arsenate- and saline (control)-exposed cranial neural crest cells:

Genes and ESTs differentially expressed between *in utero* arsenate- and saline exposed CNCCs are listed in Supplementary Table 2. Only those genes exhibiting a change in expression equal to or greater than 1.5 fold are listed. *In utero* exposure to 20 mg/kg pentavalent sodium arsenate resulted in 303 genes exhibiting at least a 1.5 fold *increase* in expression in CNCCs, and 275 genes exhibited at least a 1.5 fold *decrease* in expression, compared to their relative level of expression in saline treated control tissue. GeneSpring GX software (version 11.0) was used to generate a clustered image map (heat map) comprising all 578 differentially expressed genes demonstrating consistent differences between the two treatments (Figure 2A). An additional clustered image map of the top thirty genes displaying greatest differential expression between prenatal saline- and arsenate-exposed CNCCs is presented in Figure 2B. Differentially regulated genes and ESTs were also clustered into various functional categories (Supplementary Table 3) following Gene Ontology analysis with the GeneSpring GX (V. 11.0) and the Ingenuity Pathway Analysis (IPA) programs. Heat maps of these clusters are shown in Supplementary Figure 1 A–J. Examples of affected functional categories include: apoptosis, stress response (including oxidative stress), ribosomal functions, DNA damage response/repair, mitochondria/electron transport chain (ETC)/oxidative phosphorylation, cell migration, cell adhesion, growth and differentiation, signaling mediators as well as proliferation and cell cycle (Supplementary Table 3). Results indicate that an array of genes encoding proteins involved in these functional categories were upregulated in CNCCs following maternal arsenate exposure, when compared to their saline-exposed counterparts (Supplementary Table 3A,B,D–J). In contrast, most of the genes encoding ribosomal proteins were downregulated in CNCCs as a result of prenatal arsenate-exposure (Supplementary Table 3C).

3.4 Bioinformatic analysis of biological functions of genes differentially expressed within cranial neural crest cells exposed *in utero* to arsenate or saline (control):

Ingenuity Pathway Analysis (IPA) was utilized to examine the biological functions of differentially expressed genes in CNCCs. IPA uses a Fisher exact test (EASE score) to rank biological themes. Cell death and survival, cell cycle, cellular movement, and cellular growth and proliferation, were some of the key developmental processes that emerged as statistically significant, and potential teratogenic targets of *in utero* arsenate exposure within CNCCs (Supplementary Figure 2). Differentially expressed genes representing each of these biological processes, that were significantly altered, are listed in Supplementary Table 4. In *in utero* arsenate-exposed CNCCs: (1) 25 genes associated with varied cell signaling processes were significantly downregulated and of these, 14 genes belong to the protein kinase cascade (Supplementary Table 5- 'Cell signaling'); (2) 6 genes involved in small GTPase mediated signal transduction (Supplementary Table 5 - 'Small GTPase-mediated signaling'), and three involved in sequestration/release of Ca^{2+} displayed significantly decreased expression (Supplementary Table 5- 'Cell signaling'); (3) 14 genes whose altered expression had been documented to be linked to neonatal and/or perinatal survival/death, were all significantly repressed (Supplementary Table 5 - "Neonatal Survival-Death" & "Perinatal Survival-Death").

3.5 Identification of relevant biological networks and canonical pathways involving genes differentially expressed within cranial neural crest cells following maternal arsenate exposure:

Investigation of functional relationships between arsenate-induced differentially expressed genes using IPA program, revealed various cellular response pathways, and biological themes, underlying altered fetal programming of CNCCs. Five major interactive networks involving the differentially expressed genes were discovered. It is important to note that up- and down-regulated (i.e. differentially expressed) genes present in these networks are represented in shades of red and green, respectively, and the extent of up- or down-regulation of these differentially expressed genes is proportional to the intensity of the red or green color. One such network comprising 20 differentially expressed genes underscored cell death/apoptosis as a key biological event affected within CNCCs following *in utero* arsenate exposure (Supplementary Figure 3). Three other networks containing 39, 20 and 26 differentially expressed genes highlighting crucial physiological processes such as cell migration and adhesion (Supplementary Figure 4), cell proliferation and differentiation (Supplementary Figure 5), and embryogenesis (Supplementary Figure 6), respectively, were also found to be potentially affected within CNCCs as a result of maternal arsenate exposure. Yet another network associating 50 differentially expressed genes with various signaling pathways that are likely to be impacted within CNCCs by prenatal arsenate exposure, is presented in (Supplementary Figure 7).

IPA also identified a number of critical canonical pathways operative within post-migratory CNCCs which were likely to be adversely impacted by arsenate exposure (Supplementary Table 6). Several of these signaling cascades have significant enrichment p-values, indicating that it is highly implausible that these pathways were detected by chance. Examples of some of the high scoring (p-value < 0.05) canonical pathways are: pyridoxal 5'-

phosphate salvage pathway, NADH repair, myo-inositol biosynthesis & degradation, IL8 signaling, mitochondrial dysfunction, calcium transport, Cdc42 signaling, mTOR signaling, and Cxcr4 signaling. In addition, maternal arsenate exposure was likely to have potential unfavorable influences on the following canonical cascades in CNCCs - as anywhere from 4 to 9 genes (some being common to several of these pathways) encoding proteins associated with these pathways were significantly up- and/or down-regulated (Supplementary Table 7): Integrin signaling (9 genes up); RhoGDI signaling (8 genes up); Regulation of actin-based motility by Rho (5 genes up); Rho family of GTPases (9 genes up); Cxcr4 signaling (7 genes up); Paxillin signaling (5 genes up- and 1 gene down); Remodeling of epithelial adherens junctions (4 genes up); Protein ubiquitination pathway (10 genes up); PAK signaling (4 genes up); Ephrin receptor signaling (6 genes up); Calcium signaling (5 genes up); mTOR signaling (4 and 5 genes up and down, respectively); EIF2 signaling (5 genes down); and Wnt- β -catenin signaling (5 genes down).

Bioinformatic analyses with the IPA program also revealed, several “upstream regulators” (based on the differential expression of their respective downstream target genes in arsenate-exposed CNCCs), that are predicted to be activated (e.g. miR-124-3p, miR-16-5p and Retinoic acid) or silenced (e.g. miR-1/miR-206 family of miRNAs) in CNCCs following prenatal arsenate exposure. Downstream gene targets of these regulators are presented in Supplementary Tables 8a–d.

To further verify our bioinformatic analyses, NIH-DAVID database (DAVID v 6.7; <http://david.abcc.ncifcrf.gov>) was utilized to identify various biological and metabolic pathways over-represented in genes that are either up- or downregulated in post-migratory CNCCs following *in utero* arsenate exposure (Table 1). This list was obtained by matching the list of differentially expressed genes against the reference pathways in the KEGG, Biocarta and Panther pathway databases within the DAVID v 6.7 program. It is evident from Table 1 that for the upregulated genes, the highest ranking pathways are related to cell adhesion, DNA replication, chemokine signaling, etc., in contrast to the downregulated genes, where the top-ranking pathways are represented by various signal transduction pathways.

3.6 TaqMan™ quantitative real-time PCR verification of microarray results:

Expression of the NC marker *Crabp1* (cellular retinoic acid binding protein 1) and a negative control gene, *En1* (Engrailed 1), *not* known to be expressed by CNCCs, were used to validate (by TaqMan™ qRT-PCR) the purity of the purified cell populations and the specificity of the FACS procedure. While *Crabp1* was expressed specifically in the FACS-captured cells, *En1* was not (results not shown). An earlier study from our lab demonstrated that *Crabp1* expression is strictly restricted to the CNCCs [25]. Twenty genes (ten upregulated and ten down-regulated) were randomly selected from our gene list and their expression patterns determined by qRT-PCR using diluted cRNA as template. The overall expression profiles of 19 out of the 20 mRNAs tested were found to be in agreement with the results of GeneChip® microarray (Table 2).

4. DISCUSSION

Heavy metals, such as arsenic, can bind to structural proteins, enzymes, and nucleic acids, and interfere with their functioning. Since normal embryogenesis is reliant on precise and finely orchestrated morphological and molecular events, it is reasonable to posit that exposure to arsenic can adversely effect embryonic development. Arsenic may be particularly toxic to the growing fetus [32], in part because it can cross the placenta and accumulate in the fetus [33, 34]. Indeed, arsenic is a known teratogen causing adverse developmental outcomes [1, 35, 36]. Specifically, previous studies have documented an increased incidence of craniofacial defects in mice prenatally exposed to pentavalent sodium arsenate [1, 21, 37–39]. A very recent study documented positive associations between maternal arsenic exposure and cleft lip and cleft palate formation in humans [40].

The embryonic first and second branchial arches represent tissue primordia that develop into elements of the orofacial region, pharynx, and the outer and middle ear [41]. The mesenchyme of the branchial arches is composed of paraxial mesodermal cells and post-migratory NCCs. By GD 9.5 the first murine branchial arch displays a distinctive swelling caused, in part, by the migration of CNCCs into it, and proliferative activity of these post-migratory NCCs (Fig. 1). Temporospatial orchestration of NCC migration, differentiation, proliferation, and apoptosis is indispensable for normal development of craniofacial structures [41, 42]. Thus, one of the mechanisms of arsenic's teratogenicity could be its impact on various cellular functions of CNCCs. While several studies have demonstrated changes in gene expression in diverse cell and tissue types following exposure to trivalent or pentavalent arsenic in humans and laboratory animals [7, 8, 12, 43–45], no study has to date examined such potential alterations in a pure population of NCCs exposed to arsenicals.

The present study has, for the first time, identified genes within embryonic CNCCs whose expression are significantly altered as a result of *in utero* exposure of dams (at GD-7.5 and -8.5) to 20 mg/kg sodium arsenate. For assessing the effects of arsenate in mice, i.p. injections ranging from 30–40 mg/kg body wt have been typically used [1,38]. These doses are in excess of any human exposures but take into account that lethal doses for experimental animals are typically higher than those estimated for humans [42]. Studies at 40 mg/kg body wt. take advantage of the high penetrance of NTDs (100%) without incurring high lethality [7,8]. For the present study, an i.p.dosage of 20 mg/kg body wt. was chosen because at this dosage, a higher frequency of liveborns and lower resorption rates are observed while still manifesting the salient features of arsenic toxicity [21,38]. More importantly, this dosage allows the isolation of a sufficient number of viable embryos for harvesting EGFP-labeled CNCCs *via* FACS.

Apoptosis:

The present study revealed significant differential expression of a panoply of genes involved in cell survival and/or apoptosis within CNCCs as a consequence of maternal arsenate exposure. One example, Trp63 (transformation related protein 63), represents a gene involved in cell cycle regulation and which is also essential for developmental neuronal death by acting as an obligate pro-apoptotic partner for p53 [46]. This gene is significantly upregulated (>8.0-fold; Supplementary Table 3A) in response to arsenate exposure. Studies

have also documented that p63 promotes *survival* of embryonic neural precursor cells and neurons, antagonizing p53 [47, 48]. Our observation is consistent with those of Pallocca et al. [12] who found that the p53 signaling pathway is among the most affected pathways in CNCCs exposed to arsenate (As_2O_3). In addition to upregulation of *Trp63*, other pro-apoptotic genes, such as *Irf6*, *Tex261*, *Mitch1*, *Col18a1* and *Ambra1* (Supplementary Table 3A), were also upregulated. Simultaneously, several anti-apoptotic genes (*Bcl-2*, *Mcl1*, *Bmf* and *Thoc1*) were seen to be *downregulated* (Supplementary Table 3A). These observations support the conclusion that prenatal arsenate exposure enhances cellular death within CNCCs, primarily via apoptosis, and reinforces similar findings in embryonic maxillary mesenchymal cells exposed *in vitro* to sodium arsenate [21]. Lastly, two genes, *Csrnp1* and *Gja1* (also known as *Cx43*), involved in regulation of apoptosis, [49, 50], were significantly downregulated in arsenate-exposed CNCCs (Supplementary Table 3A). Mice with a mutated *Cx43* gene display oculodentodigital dysplasia (ODDD), as observed in humans, including diverse craniofacial anomalies [49], whereas, mice harboring a mutated *Csrnp1* have palatal and facial dysmorphologies [50]. These findings signify that several of the aforementioned arsenate target genes are not only important for regulation of apoptosis but are also key to normal orofacial ontogenesis. Arsenate targeting of genes involved in regulating apoptosis and/or pathways governing those genes is further supported by our gene association map (Supplementary Fig. 3) which highlights apoptosis/cell death within *in utero* arsenate-exposed CNCCs. Moreover, bioinformatic analysis with the IPA program revealed that cell death was the most statistically significant biological function impacted by maternal arsenate exposure in CNCCs, and that an array of genes (14) reported to be associated with both neo- and perinatal survival was significantly repressed in CNCCs exposed prenatally to arsenate (Supplementary Table 5 - “Neonatal Survival-Death” & “Perinatal Survival-Death”).

Stress response:

Prenatal arsenate exposure significantly altered the expression of a number of genes related to stress response in post-migratory CNCCs (Supplementary Table 3B). Interestingly, most of the genes (14 out of 18) in this category were significantly upregulated, indicating that arsenate exposure confers considerable stress upon the developing embryo. It is interesting to note that defense mechanisms may be operative to possibly counter the stress response. Support for this speculation comes from the finding that *Asna1*, encoding an arsenic-translocating ATPase and the catalytic component of an oxyanion pump responsible for resistance to arsenicals [51], and *Alas 2* (Aminolevulinic acid synthase 2), known to be stimulated by hypoxia [52], are both significantly upregulated in arsenate-exposed CNCCs. Increased expression of heme oxygenase 1 (Hmox1), a hallmark for arsenic-induced stress, and several heat shock proteins (*e.g.*, Hsp-60, -70 and -90) has been reported to be significantly elevated in mouse liver treated with inorganic arsenicals [53, 54]. This is in agreement with our results which demonstrated that arsenate exposure increased expression of Hmox2, a stress-induced heme oxygenase isoform, as well as genes encoding a number of stress-activated Hsp70-related proteins. Significant upregulation (~4-fold) of *Stub1*, a stress-activated, Hsp70-binding ubiquitin ligase, by arsenate in CNCCs raises the compelling possibility of inhibition of BMP and TGF β signaling (through ubiquitin-dependent degradation of Smad proteins) – two canonical pathways indispensable for normal craniofacial development [55]. Such inhibition of TGF β superfamily signaling by arsenate

could account for various arsenic-induced dysmorphologies observed in CNCC-derived craniofacial structures [21]. In addition, a gene encoding Map4k4 a mitogen-activated protein kinase (mapk) expressed in response to stress, was significantly upregulated in CNCCs following prenatal arsenate exposure. Map4k4 functions as an upstream activator of various stress-activated kinases/maps (such as, JNK 1 and 2, ERK1/2, and p38 SAP kinase) [56]. Finally, arsenate differentially altered expression of genes encoding members of three stress-activated pathways - Nrf2-mediated oxidative stress response, endoplasmic reticulum (ER) stress pathway, and acute phase response signaling, in CNCCs (Supplementary Table 6). The Nrf2 pathway, responsible for transactivation of detoxifying and antioxidant enzymes in response to oxidative stress, is modulated in various human cell types exposed to arsenic [57]. Arsenic-induced alteration of the ER stress pathway - involved in degrading accumulated misfolded proteins (following harmful cellular insults) - has also been reported [58].

Reactive oxygen species (ROS):

Arsenic is known to generate, in a dose-dependent manner [59, 60], various reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide-derived hydroxyl ion ($*OH$), and peroxy ($ROO*$) radicals [61–63]. Our demonstration of massive upregulation (64-fold; Supplementary Table 2) of the gene encoding hemoglobin alpha 1 (*Hba-a1*), a member of the fetal hemoglobin family, and a well-known free radical scavenger, lends credence to potential augmentation of ROS production in arsenate-exposed CNCCs [64]. Results from the present study also demonstrate robust upregulation of *Igfbp2* (Insulin-like growth factor binding protein 2; ~8.4-fold; Supplementary Table 2), whose overexpression has been reported to increase ROS production [65]. We further document significant upregulation of genes encoding Mpo (myeloperoxidase) and Prdx6 (peroxiredoxin 6) (Supplementary Table 3B), induced in response to oxidative stress and ROS, respectively [66]. Collectively, these data document adverse effects of arsenate on the antioxidant defenses of CNCCs, potentially leading to deleterious outcomes within developing orofacial tissues.

Ribosomes:

In the present study, almost all ribosomal components and ribosome-binding proteins were significantly downregulated in NCCs as a result of maternal arsenate exposure (Supplementary Table 3C). Examples of such genes include those encoding ribosomal proteins (Rpl23, Rpl37a, Rps11, Rps13, and Rps21) and components of mitochondrial ribosomes (Mrpl27 and Mrpl42). These results are in excellent corroboration with the findings of earlier studies demonstrating repression of several genes involved in RNA processing and protein synthesis, and deregulation of the ribosomal pathway, following arsenate exposure [54, 67]. Bioinformatic (NIH-DAVID) analysis also highlighted “ribosome” as one of the top ranking terms enriched within the genes downregulated in arsenate-exposed CNCCs (Table 1). In addition, Dnajc1, a ribosome-binding protein involved in coordination of ribosomes, regulation of protein translation and protein folding, was significantly repressed by arsenate in CNCCs (Supplementary Table 3C). Finally, IPA revealed significant upregulation of genes encoding 12 members of the Protein Ubiquitination pathway, underlining increased degradation of proteins in arsenate-exposed

CNCCs (Supplementary Table 6). These findings underscore the possibility that synthesis of *de novo* cellular and mitochondrial proteins as well as overall stability of cellular proteins are markedly compromised in CNCCs following prenatal arsenate exposure, conditions detrimental to normal embryonic development.

DNA damage and DNA repair:

In utero exposure to sodium arsenate activated a number of genes associated with DNA damage and DNA repair in CNCCs (Supplementary Table 3D). Examples of such genes include those encoding Sfpq, Fancg, Xab2, Ttc5, Rnf8, and Rpain, among others. Genes encoding two death domain kinases, Ripk1 and Ripk4, essential for the activation of NF- κ B following DNA damage [68], were also upregulated. Activation of NF- κ B is a key cellular response leading to alteration of cell proliferation and/or apoptosis [68, 69]. This finding raises the possibility of arsenate induction of NF- κ B within CNCCs (impacting their proliferation and survival). These results are in agreement with prior studies documenting chromosomal aberrations and DNA damage in arsenic-exposed fetal cells [14–16]. In addition, arsenate exposure significantly repressed the expression of genes encoding DNA polymerase beta (Polb) and Rint1 in CNCCs. Polb catalyzes base excision repair required for DNA maintenance, replication, and recombination [70], whereas, Rint1 participates in DNA repair, cell cycle control and survival, following DNA damage [71]. Altogether, these observations suggest that arsenate-induced DNA damage/chromosomal aberrations in CNCCs affect their proliferation and survival.

Mitochondrial function:

Arsenate is a phosphate analog [72] and can competitively replace phosphate in ATP, thereby uncoupling mitochondrial oxidative phosphorylation [73]. It has been demonstrated that mitochondrial ATP synthase (Complex V) which phosphorylates ADP to ATP can utilize arsenate as a substrate, converting it to arsenite [74]. The uncoupling mechanism is attributed to the highly unstable arsenate–phosphate ester that is rapidly hydrolyzed leading to a deficit in ATP production and conservation [72, 75]. Moreover, perturbation of the mitochondrial ETC increases ROS levels that can also inhibit aerobic respiration, alter membrane potential and reduce ATP levels [76, 77]. Not surprisingly, mitochondrial components, including enzymes involved in ETC/oxidative phosphorylation represent a significantly affected category of genes whose expression is altered in CNCCs in response to maternal arsenate exposure (Supplementary Table 3E). Pathway and Gene ontology analyses with Ingenuity and NIH-DAVID convincingly substantiate this premise further (Table 1 and Supplementary Table 6). Indeed, KEGG analysis reveals that oxidative phosphorylation is the second most downregulated process (Table 1) with many of the affected genes encoding components of the ETC. These include genes encoding subunits of Complex I (NADH: ubiquinone oxidoreductase) – Ndufa5, Ndufa8, Ndufa12, Ndufc1, Ndufv1; Complex III (cytochrome bc1 complex) – Uqcrc1; Complex IV (cytochrome c oxidase) – Cox7c, Cox8c, Cox15; and, complex V (ATP synthase) – ATP5a1. These findings are in broad agreement with a recent study that observed decreases in the activities of various mitochondrial complexes (I, II and IV) in microglial cells exposed to arsenate [76]. Altered expression of these genes support the premise that mitochondria are one of the major targets of arsenate's toxicity in CNCCs and that key mitochondrial processes (including ETC/oxidative

phosphorylation) are adversely impacted. Pathway and Gene ontology analyses with Ingenuity and NIH-DAVID convincingly substantiate this premise (Table 1 and Supplementary Table 6). Altogether, these results underscore the likelihood that prenatal arsenate exposure can trigger altered energy metabolism within CNCCs resulting in damage to the developing embryo. Mitochondrial dysfunction may also augment CNCC apoptosis. This notion is well-supported by studies demonstrating apoptosis of embryonic maxillary mesenchymal cells due to mitochondrial membrane perturbation resulting from arsenate exposure [21] and decreased cell viability, increased apoptosis, oxidative stress damage and mitochondrial dysfunction in neurons exposed to arsenic [78].

Cell migration and cell adhesion:

Cells of the NC lineage possess the potential of long-range migration at various stages of development. In the present study, expression of numerous genes known to be involved in regulating cell migration were differentially altered in post migratory CNCCs following *in utero* arsenate exposure (Supplementary Table 3F; Supplementary Table 6; Supplementary Figure 4). This allows for the suggestion that not all CNCCs harvested for analysis in the present studies were *post*-migratory. Maternal arsenate exposure was previously postulated to adversely impact NCC migration [7]. Our data indicate that genes and pathways known to govern cellular migration were affected, and hence the “migratory potential” of the CNCCs were most likely altered, following arsenate exposure. This reinforces prior published evidence that one of the plausible mechanisms of prenatal arsenic exposure-induced craniofacial anomalies could be via modulation of CNCC migration.

Further support for the premise that exposure to arsenate adversely impacts CNCC migration comes from the altered expression of an array of genes involved in governing cellular adhesion in CNCCs — a process critical for cell migration [79] (Supplementary Table 3G). Among these genes, *Irf6*, involved in palatal adhesion and fusion competence [80] and *Cdh1*, key to embryonic stem cell migration [81], were both upregulated. Since overexpression of cadherins inhibits emigration of NCCs via increasing cell-cell adhesion [82, 83], our data suggest that arsenate may be altering the migratory capabilities of CNCCs by modulating their adhesive potential. This notion is supported by the interactive gene network which highlights cellular adhesion as a key physiological process targeted by arsenate within CNCCs (Supplementary Figure 4). KEGG pathway analysis also revealed cell adhesion as one of the most significantly affected pathways within arsenate-exposed CNCCs (Table 1). Moreover, IPA revealed a number of pathways directing cellular adhesion and migration (such as, RhoGDI-, Paxillin-, Integrin-, and Epithelial Adherens Junction signaling, among others) that are highly likely to be affected in arsenate-exposed CNCCs, compromising their motility and adhesion (Supplementary Table 6).

Growth factors, signal transducers and cell proliferation:

Toxic/teratogenic effects of arsenic on cell proliferation/cell cycle [8, 84], growth and differentiation [85, 86] and a panoply of signal transduction pathways [63], have been well-documented. In the present study, maternal arsenate exposure also triggered differential regulation of an array of genes encoding numerous growth and differentiation factors, signaling mediators as well as proteins involved in governing cell proliferation, within

CNCCs (Supplementary Table 3H–J, respectively). Earlier studies reported that p63 and IRF6, functioning within a regulatory loop, orchestrate cellular proliferation and differentiation during normal orofacial development, and that mutations in *p63* and *IRF6* have been linked to increased risk for cleft lip and/or palate [87, 88]. Notably, in the current study, arsenate-induced significant upregulation of *Irf6* and *Trp63* (Supplementary Table 3H) could underlie abnormal CNCC proliferation and differentiation, resulting in the arsenate-induced orofacial anomalies previously reported [21]. Further support for the possibility that arsenic teratogenicity is mediated via targeting genes governing cellular proliferation, growth and differentiation, and signal transduction comes from the following: (1) IPA analysis (of genes differentially expressed in CNCCs subsequent to maternal arsenate exposure) revealed two interactive gene association maps, one highlighting cellular proliferation and differentiation and the other accentuating a multitude of signaling pathways (Supplementary Figures 5 and 7, respectively), and (2) analyses with the KEGG, Panther and Biocarta pathway databases (using the NIH-DAVID program) and IPA, also revealed an array of canonical cascades (such as, the FGF-, p38MAPK-, Wnt/ β -catenin-, PDGF-, JAK/STAT-, EGF receptor-, VEGF- and mTOR signaling pathways) operative within NCCs, that were potentially impacted by *in utero* arsenate exposure (Table 1 and Supplementary Table 6). These signaling pathways are known to direct diverse physiological processes including cellular proliferation and differentiation [63, 89]. Intriguingly, in the present study, maternal arsenate exposure altered expression of genes encoding 9 members of the mTOR signaling pathway within CNCCs (Supplementary Table 6). Although previously unreported in an embryonic cell system, arsenate exposure-induced modulation of mTOR signaling and resultant altered cellular proliferation and apoptosis have been reported in various adult cell types [90, 91]. Another novel finding from the present study is the potential impact of maternal arsenate exposure on RhoGDI signaling. Eight genes that have been documented to regulate cellular proliferation, migration and apoptosis [92], and are members of the RhoGDI cascade, were differentially expressed in CNCCs (Supplementary Table 7). Yet another unique finding is the emergence of two closely-related pathways: Pyridoxal 5'-phosphate Salvage Pathway, and the Salvage Pathways of Pyrimidine Ribonucleotides, as the two most enriched canonical cascades in arsenate-exposed CNCCs, several gene members of which displayed significant differential expression (Supplementary Table 6). Abnormal pyridoxal 5'-phosphate (PLP; Vitamin B6) metabolism alone or in tandem with anomalous folate metabolism has been linked to craniofacial dysmorphologies [93, 94].

Regulation of gene expression:

Bioinformatic analysis (IPA) predicted several upstream regulators of gene expression that are potentially activated or inhibited in CNCCs following prenatal arsenate exposure (Supplementary Table 8). Of these, two microRNAs (miR-124-3p and miR-16-5p), small non-coding RNAs that function in mRNA silencing, were potentially “activated” by arsenate, as 15 and 14 of their respective gene-targets were significantly downregulated in CNCCs following prenatal arsenate exposure. In contrast, the miR-1/miR-206 family of miRNAs were potentially “repressed” by arsenate as 14 of their gene targets displayed significantly enhanced expression in arsenate-exposed CNCCs. Key roles and differential expression of miR-124, miR-16 and miR-206 during neural tube and orofacial development

have been well-documented [95–99]. In addition, miR-124 and miR-206 were characterized as differentially expressed miRNAs in sodium arsenite-induced NTDs in chick embryos [100]. IPA also predicted retinoic acid (RA) as one of the potentially “activated” upstream regulators of gene expression within CNCCs following maternal arsenate exposure. In the present study, arsenate exposure resulted in significant upregulation of 31 out of 41 RA target genes in CNCCs offering credence to this prediction (Supplementary Table 8). Since RA-induced NTDs and craniofacial anomalies are thought to be mediated via effects on CNCCs [101–103], *these findings underscore the potential for unique miRNA/RA-mediated teratogenic mechanisms within CNCCs.*

Collectively, these data document potential adverse impact(s) of prenatal arsenate exposure on various growth factor-mediated signaling pathways (FGF-, p38MAPK-, Wnt/ β -catenin-, Ras, PDGF-, JAK/STAT-, EGF receptor-, VEGF- and RA-), as well as, epigenetic regulators (miR-16, -124 and -206), directing crucial cellular processes central to normal craniofacial morphogenesis. Such insult(s) can potentially result in abnormal cellular proliferation, differentiation, and survival, as well as DNA damage, generation of ROS, altered energy metabolism/mitochondrial dysfunction and decreased protein synthesis while increasing degradation of key proteins, within CNCCs.

5. CONCLUSIONS:

This is the first study where a highly purified population of CNCCs was exposed *in utero* to the teratogen, sodium arsenate, and a gene expression signature created using high-density DNA microarrays. Utilization of bioinformatic tools, such as NIH-DAVID and Ingenuity pathway analysis, enabled identification of biological themes that are enriched in CNCCs as a consequence of prenatal arsenate exposure. *These biological themes may hold the key towards understanding the precise mechanisms by which arsenic, in particular, and other teratogens, in general, may compromise the developing orofacial region including the upper lip and secondary palate.*

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Isolated pure population of mouse cranial neural crest cells (CNCCs).
- Studied the effects of *in utero* exposure to sodium arsenate on CNCCs.
- Reported arsenate-induced alterations in the expression of novel candidate genes.
- Identified novel signaling pathways potentially impacted by arsenate exposure.
- Identified novel genes & canonical cascades that may contribute to arsenate-induced orofacial defects.

Fig. 1. EGFP-labeled neural crest cells in *Wnt1-Cre/Z/EG* transgenic mouse embryos. Photomicrograph of a GD-9.5, two-component *Wnt1-Cre/Z/EG* transgenic embryo, under darkfield (left) and epifluorescence (right) optics. The region, demarcated by the yellow line, was excised from the sodium arsenate- and saline-exposed embryos for fluorescence-activated cell sorting (FACS). FN: frontonasal region; MB: midbrain; HB: hindbrain.

Fig. 2.

Clustered image maps (heat maps) illustrating all genes (A) and the top thirty genes (B) differentially expressed between prenatal saline- and arsenate-exposed cranial neural crest cells (CNCCs). Each row of the clustered image map represents a gene, and each column represents one array for treatment type examined [saline (S) or arsenate (A)]. The color saturation represents the level of gene expression. The three columns marked 'S' represent clusters of genes from the three independent sets of CNCCs prenatally-exposed to saline (control), that exhibit increased (red) and decreased (blue) expression. The three columns marked 'A' represent clusters of the same genes from the three independent sets of CNCCs prenatally-exposed to arsenate. Clustered are those genes that were either up regulated (red) or down regulated (blue) in arsenate-exposed CNCCs when compared to their relative expression in saline exposed CNCCs. GeneSpring GX software (v 11.0, Agilent Technologies) was used for hierarchical clustering analysis to create a condition tree representing all of the differentially expressed genes based on their expression profiles. Genes demonstrating a 1.5-fold or greater difference ($p < 0.05$) in expression are included in the map.

Table 1:

Pathways Altered in Cranial Neural Crest Cells following *In Utero* Exposure to Sodium Arsenate.

Upregulated Genes			
Category	Term	P-Value ²	Number of genes ³
KEGG	Cell adhesion molecules (CAMs)	1.27E-02	23
	DNA replication	1.91E-02	14
	Pyrimidine metabolism	2.01E-02	6
	Chemokine signaling pathway	2.90E-02	10
	Focal adhesion	4.28E-02	4
	Ubiquitin mediated proteolysis	4.76E-02	7
PANTHER	Inflammation mediated by chemokine and cytokine signaling pathway	1.84E-02	10
	Parkinson disease	3.84E-02	9
	Axon guidance mediated by semaphorins	4.72E-02	3
	Dopamine receptor mediated signaling pathway	4.76E-02	4
	Metabotropic glutamate receptor group II pathway	4.89E-02	4
	Muscarinic acetylcholine receptor 2 and 4 signaling pathway	4.92E-02	4
Downregulated Genes			
Category	Term	P-Value	
KEGG	Oxidative phosphorylation	1.44E-02	11
	Ribosome	1.69E-02	9
	Alzheimer's disease	5.14E-02	4
BIOCARTA	MAPKinase Signaling Pathway	1.54E-02	10
PANTHER	Ras Pathway	3.33E-02	6
	PDGF signaling pathway	3.64E-02	7
	JAK/STAT signaling pathway	3.72E-02	3
	EGF receptor signaling pathway	4.51E-02	6
	VEGF signaling pathway	5.00E-02	5

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Highest ranked pathways impacted by *in utero* arsenate exposure in the cranial neural crest cells were delineated by utilizing the NIH-DAVID program and by matching the statistically significant up- and downregulated pathways to the KEGG (Kyoto Encyclopedia of Genes and Genomes), Biocarta and Panther pathway databases.

P value indicates that the overrepresentation of a particular pathway is a statistically significant event and that the probability of that pathway appearing on the list is *not* a random event.

Number of genes within each pathway.

Table 2.

TaqMan™ Quantitative Real-Time PCR^{1/} Verification of Differentially Expressed Genes in Cranial Neural Crest Cells (CNCCs) Exposed *in utero* to Sodium Arsenate or Saline (vehicle control).

Gene ²	Entrez Gene	Affymetrix Probe ID	Mean Fold Change ± SEM Arsenate vs. Saline (Microarray Data)	Mean Fold Change ^{3,4,5} ± SEM Arsenate vs. Saline (Individual TaqMan Assay)	Concordance ⁶
Upregulated Genes					
Arfip1	54208	1451131_at	3.34 ± 0.11	15.30 ± 1.25	+/+
Asna1	56495	1418292_at	12.00 ± 1.09	20.25 ± 1.23	+/+
Cdh1	12550	1448261_at	12.50 ± 1.30	19.83 ± 1.02	+/+
Cdk9	107951	1417269_at	1.90 ± 0.07	8.31 ± 0.53	+/+
Foxp1	108655	1455242_at	3.59 ± 0.15	2.71 ± 0.92	+/+
Hmox2	15369	1416399_a_at	2.00 ± 0.06	3.87 ± 0.19	+/+
Id2	15902	1453596_at	3.09 ± 0.14	4.92 ± 0.72	+/+
Irf6	54139	1418301_at	14.93 ± 1.48	16.37 ± 1.08	+/+
Mef2a	17258	1452347_at	3.66 ± 0.17	11.87 ± 1.26	+/+
Nr2f2	11819	1416159_at	2.40 ± 0.11	4.11 ± 1.00	+/+
Downregulated Genes					
Apc2	23805	1435199_at	-2.47 ± 0.12	-3.26 ± 0.69	+/+
Bcl2	12043	1440770_at	-9.80 ± 0.67	-8.53 ± 1.22	+/+
Cdgap (Arhgap31)	12549	1455164_at	-4.24 ± 0.10	-3.45 ± 0.68	+/+
Foxc1	17300	1419486_at	-3.62 ± 0.16	-13.36 ± 1.29	+/+
Gja1	14609	1438945_x_at	-1.65 ± 0.04	-2.47 ± 0.33	+/+
Meis2	17536	1440091_at	-6.23 ± 0.39	-7.03 ± 0.96	+/+
Nischarin	64652	1430151_at	-3.77 ± 0.25	-1.08 ± 0.03	+/-
Pias1	56469	1446448_at	-5.17 ± 0.45	-4.72 ± 0.97	+/+
Rgmb	68799	1442811_at	-4.34 ± 0.15	-2.60 ± 0.60	+/+
Stat3	20848	1459961_a_at	-3.50 ± 0.23	-2.30 ± 0.33	+/+

^{1/}The differential expression of twenty genes in cranial neural crest cells following *in utero* exposure to saline (vehicle control)- and sodium arsenate, was compared using Affymetrix GeneChip® arrays and TaqMan™ quantitative real-time PCR as detailed in Experimental Procedures.

^{2/}Target genes were selected randomly.

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³ cDNA samples were prepared from saline (vehicle control)-, and sodium arsenate-exposed CNCCs and subjected to TaqMan® quantitative real-time PCR (qRT-PCR) for each target gene. Analyses were performed in triplicate using data from three independent experiments.

⁴ Ct values represent the number of cycles during the exponential phase of amplification necessary to reach a predetermined threshold level of PCR product as measured by fluorescence. The more template present at the start of a reaction, the fewer the cycles required to synthesize enough fluorescent product to be recorded as statistically above background. All data were normalized to the amplification signal from the housekeeping gene, *Gapdh*. The Ct values represent these normalized signals, $Ct = Ct_{\text{sample}} - Ct_{\text{Gapdh}}$. Data presented represent mean $\pm$ standard deviation over three replicates.

Negative methodological control reactions, which lacked reverse transcriptase, did not amplify any detectable product.

⁵ Fold-change (FC) values were determined according to the relationship: $FC = 2^{-\Delta Ct}$, where ΔCt is the difference in Ct values between arsenate-exposed CNCC samples vs. vehicle (saline)-exposed CNCC samples [31]. Statistical analysis comparing the two exposures (vehicle and arsenate) was done with one-way ANOVA of the Ct values and adjustment for multiple comparisons using Dunnett's method. 95% confidence intervals for the FC were calculated by taking the appropriate transformation of the 95% confidence limits for the estimated difference in Ct values. A negative fold-change value indicates down regulation of gene expression relative to control samples and a positive fold-change value indicates up regulation. Only those genes that demonstrated a statistically significant (adjusted $p < 0.05$) increase or decrease in expression for the arsenate vs. vehicle (saline) expression comparison, were included in this table.

⁶ Full concordance in the pattern or level of gene expression obtained using the Affymetrix GeneChip® arrays and the TaqMan™ qRT-PCR is represented as "+/+";