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Arsenic- and Cadmium-Induced Toxicogenomic Response in Mouse Embryos Undergoing Neurulation

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Abstract

Arsenic (As) and cadmium (Cd) are well-characterized teratogens in animal models inducing embryotoxicity and neural tube defects (NTDs) when exposed during neurulation. Toxicological research is needed to resolve the specific biological processes and associated molecular pathways underlying metal-induced toxicity during this timeframe in gestational development. In this study, we investigated the dose-dependent effects of As and Cd on gene expression in C57BL/6J mouse embryos exposed *in utero* during neurulation (GD8) to identify significantly altered genes and corresponding biological processes associated with embryotoxicity. We quantitatively examined the toxicogenomic dose-response relationship at the gene level. Our results suggest As and Cd induce dose-dependent gene expression alterations representing shared (cell cycle, response to UV, glutathione metabolism, RNA processing) and unique (alcohol/sugar metabolism) biological processes, which serve as robust indicators of metal-induced developmental toxicity and indicate underlying embryotoxic effects. Our observations also correlate well with previously identified impacts of As and Cd on specific genes associated with metal-induced toxicity (*Cdkn1a*, *Mt1*). In summary, we have identified in a quantitative manner As and Cd induced dose-dependent effects on gene expression in mouse embryos during a peak window of sensitivity to embryotoxicity and NTDs in the sensitive C57BL/6J strain.

Keywords

Arsenic; Cadmium; Metabolism; Cell Cycle; Toxicogenomics; NTD

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Introduction

Derived from natural and anthropogenic sources, widespread environmental exposure to arsenic (As) and cadmium (Cd) remains of public health concern due to their potential to cause adverse effects in the human population. In animal models, As and Cd are well-characterized teratogens inducing embryotoxicity, including growth effects, mortality and a range of congenital malformations (Ferm and Carpenter, 1967; Ferm, 1977; Salvatori *et al.*, 2004). Exposures to either As or Cd during neurulation result in neural tube defect (NTD) formation, dependent on several factors including, dose, timing of exposure, strain, exposure route, ionic state (As) and nutritional status (Nakashima *et al.*, 1987; Hovland *et al.*, 1999; Machado *et al.*, 1999). In the C57BL/6J mouse strain and related substrains, a general dose response relationship has been established between As, Cd and embryotoxicity using intraperitoneal (primarily) or subcutaneous dosing during this window in gestational development. Maternal exposure to 5mg/kg As (As³⁺, ip, GD8, C57BL/6J) induces modest increases in malformations but not NTDs, while 10mg/kg associates with increased resorptions and NTD (exencephaly, 17%) formation (Machado *et al.*, 1999). For Cd (C57BL/6J, GD8 or GD9, ip, acute) doses ranging from 0.5–1 mg/kg are relatively non-toxic while doses ≥ 2 mg/kg induce developmental malformations (Hovland *et al.*, 1999; Lutz and Beck, 2000; Lee *et al.*, 2006; Robinson *et al.*, 2009b). For example, maternal exposure to 4mg/kg Cd (ip, GD8.0) induces exencephaly in 33% of C57BL/6J fetuses (Hovland *et al.*, 1999; Robinson *et al.*, 2009b) As and Cd pass across the placenta and distribute to the embryo dependent on dose, time of observation and route of administration (Dencker, 1975; Christley and Webster, 1983; Lindgren *et al.*, 1984; Hanlon and Ferm, 1986; Hood *et al.*, 1988), and they have been identified in the neuroepithelium of the neural tube as well as other tissues including the limb buds and gut (Christley and Webster, 1983; Lindgren *et al.*, 1984).

Embryonic uptake of As and/or Cd results in changes at the morphological, cellular and molecular level in a time dependent manner. Cd studies (GD8.0, Cd 4mg/kg) suggest peak cellular morphological changes (increased pyknotic nuclei) to occur 10–12h correlated with subsequent alterations in neural tube development (Webster and Messerle, 1980). Under these conditions, As and Cd exposures can affect DNA-damage, cell cycle arrest, oxidative stress and cell death pathways (Pulido and Parrish, 2003; Beyersmann and Hartwig, 2008), yet the means by which these metals interfere with signaling pathways and subsequent morphogenesis remains unresolved. Investigations suggest additional effects such as glucose impairment and disruption in ion (e.g. Zn) or folic acid regulation also may be potential mechanisms of metal-induced embryotoxicity (Włodarczyk *et al.*, 2001; Fernandez *et al.*, 2004; Fernandez *et al.*, 2007; Hill *et al.*, 2009; Robinson *et al.*, 2009b; Robinson *et al.*, 2010b).

Toxicogenomic approaches provide researchers with the ability to simultaneously examine multiple biological processes or pathways across dose and time and, thus, these approaches hold promise to make cross comparisons between variables of interest (e.g. teratogens, time, dose, strain). Dose- and time-response assessments are needed to define mechanisms of action for both of these metals in order to develop sensitive indicators of toxicity as well as characterize shared and unique mechanisms of embryotoxicity. Emerging bioinformatic tools, such as GO-Quant, allow assessment of toxicogenomic data in the context of risk assessment, enabling dose- and time-response calculations within subsets of genes associated with specific functional families (i.e. Gene Ontologies) (Yu *et al.*, 2006). While debate still remains over the application of toxicogenomic data in the risk assessment arena (Freeman, 2004), examination of toxicogenomic data using tools, like GO-Quant, can assist in determining robust and sensitive responses in relation to environmental teratogens and developmental toxicity.

Building upon previous studies using a similar experimental design in terms of exposure and gestational timing (acute exposure on GD8.0, ip, C57BL/6J strain), we examined dose-dependent effects of As and Cd on gene expression in association with increased embryotoxicity in C57BL/6J mouse embryos undergoing neurulation. In these preliminary assessments we identified overlapping and non-overlapping metal-induced gene expression alterations which associate with embryotoxicity and NTD development *in vivo*.

Methods

Animals and Metal Exposure

Following procedures detailed in our previous studies (Robinson *et al.*, 2009b), colonies of C57BL/6J (Jackson Labs, Bar Harbor, MA) were maintained at the University of Washington, Department of Environmental and Occupational Health Sciences, housed in filter covered transparent plastic cages with water and food available ad libitum. Mice were bred in climate-controlled rooms under an alternating 12h light/dark cycle. Timed matings were generated by placing single male mice into cages containing two females in the late afternoon. C57BL/6J females used for mating were aged between 3–5 months and weighed between 17–24g. Copulatory plugs were identified in the early morning (8:00am $\pm$ 1h) the following day and designated as gestational day (GD) 0. Pregnant mice were administered single doses via intraperitoneal injection (ip) on GD 8.0 at 8:00am ($\pm$ 1h), with either sodium arsenite (2.5, 5, or 10mg/kg BW, Alfa Aesar, Ward Hill, MA), or cadmium chloride (0.5, 2 or 4mg/kg/BW, Alfa Aesar, Ward Hill, MA) dissolved in deionized water, or vehicle control (water, 10ul/g). Exposure groups were prearranged before mating. The administration time (GD8) and methodology (intraperitoneal injection) were selected due to previous dose-response studies assessing developmental toxicity in C57BL/6J mouse embryos with the primary endpoint of interest being the formation of NTDs (exencephaly) (Hovland *et al.*, 1999; Machado *et al.*, 1999; Robinson *et al.*, 2009b). GD8 represents the optimal day of exposure regarding the aims of this study due to factors including high NTD sensitivity, a relatively low resorption rate (less sensitive than earlier timepoints in neurulation (<GD8)) and the ability to acquire enough embryonic material for gene expression analyses.

Pregnant C57BL/6J females were euthanized on 0, 8 and 12h post injection (GD8.0). Contemporaneous controls were performed for the three developmental times investigated (GD8 + 0, 8, and 12 h) for a sample of the collection dates. However, it was not possible to have contemporaneous controls for all combinations of developmental times on all collection dates due to failed pregnancies and time constraints regarding the extraction of the embryos. Whole embryos were isolated from the uterus. All extraembryonic membranes, including the yolk sac, were removed. Embryos were quickly examined for intended developmental stage of interest (GD8, 0h; Thelie stage (TS) ~12, GD8.3, 8h; TS ~12.5 and GD8.5, 12h; TS ~13). Litters observed to be the incorrect stage (>0.5 TS) due to suspected earlier mating were not used for this study. Embryos were washed in cold CMF-PBS, placed in liquid nitrogen, and stored at -80°C . Complete pooled litters were kept separate.

RNA Isolation

Embryos were placed in 500ul of RTL Cell Lysis buffer (Qiagen, Valencia, CA) and lysed with a 30G needle to homogenize the tissue. RNA was purified using the RNeasy kit (Qiagen, Valencia, CA). Quality and quantity was assessed using Nanodrop ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and the “6000” assay on the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The quality of RNA samples were assessed based on absorbance values ranging from 1.9–2.1 (A260/A240), distinct peaks of 18S and 28S rRNA subunits and profiles showing minimal degradation. As displayed in Supplemental Table 1, exposed and non-exposed litters were collected and used for gene

expression analysis at the following timepoints: 0h (GD8.0) control (n=3); 8h (GD8.0 +8h), control, 2.5, 5, 10mg/kg sodium arsenite and 0.5, 2, 4 mg/kg cadmium chloride (n=4, except 0.5mg/kg Cd, n=3); 12h (GD8.0 +12h) control (n=5), 10mg/kg sodium arsenite and 4mg/kg cadmium chloride (n=4).

Oligonucleotide Microarrays

Microarray hybridizations were completed using the oligonucleotide-based Affymetrix platform (Mouse 430 2.0), with 1 µg of total RNA from each litter. Prior labeling was completed with the GeneChip One-Cycle Target Labeling kit (Affymetrix, Inc., Santa Clara, CA) following the manufacturer's protocol. The amount and quality of the cRNA was assessed using a Nanodrop ND-100 spectrophotometer and an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). cRNA was fragmented and hybridized to the arrays (Affymetrix, Inc., Santa Clara, CA) for 17h, prior to washing and scanning.

Data Processing

Intensity values were extracted from scanned images using GeneChip Operating Software (Affymetrix, Inc., Santa Clara, CA) for all 45101 probes. Raw intensities were normalized using GCRMA and transformed by log base 2 (BRBarraytools, NCI) (Simon *et al.*, 2007).

Identification of Significantly Altered Genes Due to Metal Exposure

Figure 1 represents the statistical approach used in this study to explore dose- and time-dependent effects of As and Cd exposure on gene expression and corresponding GO biological processes. To identify As and Cd dose-dependent alterations, we employed two discrete linear models to assess genes that were altered by dose (As or Cd) or changing across time. Model 1 assessed the effect of As on gene expression ($B_{\text{Dose_Arsenic}}$) and the effect of time (B_{Time}). Model 2 assessed the effect of Cd on gene expression ($B_{\text{Dose_Cadmium}}$) and the effect of time (B_{Time}). Two separate models were created due to initial statistical assessments suggesting differential distributions of significant effects between As and Cd. Both dose and time effects were examined in our model due to their relevance and significance.

$$\text{Model 1: } \log_2[\text{Exp}_n]_{\text{C57BL/6J}} = B_{\text{Dose_Arsenic}} x_1 + B_{\text{Time}} x_2$$

$$\text{Model 2: } \log_2[\text{Exp}_n]_{\text{C57BL/6J}} = B_{\text{Dose_Cadmium}} x_1 + B_{\text{Time}} x_2$$

$$[B_{\text{Dose_Arsenic}} x_1] \text{ Coding: "0", control; "1", 2.5mg/kg, "2", 5mg/kg, "3", 10mg/kg As}$$

$$[B_{\text{Dose_Cadmium}} x_1] \text{ Coding: "0", control; "1", 0.5mg/kg, "2", 2mg/kg, "3", 4mg/kg Cd}$$

$$[B_{\text{Time}} x_2] \text{ Coding: "0", 0h, "1", 8h, "2", 12h}$$

We determined significant As or Cd effects using a cutoff value of significance ($p < 0.0001$) for the primary dose effect ($B_{\text{Dose_Arsenic}}$ or $B_{\text{Dose_Cadmium}}$) within Model 1 and 2 to assess

significant metal effects across dose over time (Simon *et al.*, 2007). Adjusted false discovery rates were below 1% for both gene subsets for As or Cd (Benjamini and Hochberg, 1995). We established our cutoff values to identify roughly 2–4% of the probes to be differentially expressed across dose and time. Approximately 10–20% of all significantly altered genes were represented by more than one probe. Due to the relatively small proportion of genes represented by more than one probe, we herein use the term “genes” instead of probes to enhance ease of readership. Redundant probes were assessed separately and corresponding fold change (FC) values were not averaged. Post-hoc analyses were conducted to examine the dose relationship between each dose group and its comparative concurrent control using a Student t-test ($p < 0.05$) for all significantly altered genes (As or Cd, $p < 0.0001$). To assess similarities in gene expression changes across dose and time for both metals, we conducted principal components analysis (PCA) (Genemaths XT, Applied Maths, Sint-Martens-Latem, Belgium) and hierarchical clustering analysis (Eisen *et al.*, 1998). PCA plots were constructed using Ward linkage and Euclidean distance to assess similarities between each exposure group (average). PCA was conducted in a supervised manner (all genes impacted by As and/or Cd, $p < 0.0001$) and across all 45,101 probes. Hierarchical clustering plots were performed by calculating ratios ($\log_2$), between gene intensities on each single array versus the average intensity for each gene across all included exposure groups. Using average linkage and Euclidean dissimilarity methods, gene relationships were clustered to show commonalities and differences between single arrays.

Enrichment Analysis of GO Biological Processes

We conducted Gene Ontology (GO) analysis (Doniger *et al.*, 2003) to identify enriched GO gene categories (classified by biological process, molecular function, and cellular component) within genes identified to be significantly altered by As or Cd exposure ($p < 0.0001$). Since our aims in this preliminary study were to explore overlapping and non-overlapping impacts of metals on biological processes and relative molecular pathways, GO analyses within this manuscript focused primarily on GO categories classified by biological process which includes broad biological processes (e.g. developmental process, metabolic process) and more defined categories representing molecular pathways of interest (e.g. Wnt-signaling pathway). Separate analyses were conducted for each metal. Significantly enriched GO categories were based on a set criteria of permutation value ($p < 0.05$), Z-score (> 2) and genes changed within each specific GO gene category (> 2). A ranking value (GO-Enrichment Score) was calculated using $-\log(\text{permutation } p\text{-value}) * Z$ score to compare enrichment of GO biological processes between As and Cd.

Quantitative Analysis of Enriched GO Biological Processes

To quantitatively evaluate changes within gene-expression linked GO gene categories, we used GO-Quant (Yu *et al.*, 2006) to calculate the absolute average FC associated within each GO subset. Enriched GO gene categories were analyzed across dose and time.

Cluster Gene Analysis to Examine Dose-Response Relationship at the Gene and GO Level

K-means cluster gene analysis was conducted to identify similar expression patterns amongst significantly altered genes (TIGR MEV, average linkage, default parameters) (Saeed *et al.*, 2003). Using genes identified to be significantly altered by As or Cd ($p < 0.0001$), we began our analyses by clustering gene expression patterns across dose and time into 10 dose-response relationships to visualize similar unique relationships. Clusters were reduced if we determined similar directionality in dose-response patterns between clusters. Enrichment analysis of GO biological processes was conducted using both MappFinder (same criteria as above) and DAVID (≥ 3 genes, $p < 0.05$) (Dennis *et al.*, 2003). For analyses completed using DAVID GO biological processes are separated by “levels” within the GO hierarchical system (1–5 levels, 1 represents most broad, 5 represents high

specificity), with biological level 4 representing categories with moderate-high specificity within the GO hierarchy tree. GO analysis was conducted across all levels, however, only GO categories identified within DAVID's classification "biological level 4" were displayed to reduce redundancy of similar GO terms. We used the combination of MappFinder and DAVID to improve GO assessments due to differences in GO databases and the lack of linkage using MAPPFinder in particular clusters. Using the GO-Quant methodology to quantitatively assess gene responses within each cluster, we calculated the average FC associated within genes of each cluster across dose and time.

Enrichment Analysis of Transcription Factor Binding Sites within Overlapping Metal-Induced Alterations

Similar to above, K-means cluster gene analysis was conducted to identify similar expression patterns within genes significantly altered by both As and Cd ($p < 0.0001$) across dose and time (TIGR MEV). Overrepresented transcription factor binding sites (TFBS) within genes identified to be significantly altered by both As and Cd were identified using oPOSSUM (default criteria) (Ho Sui *et al.*, 2005). Approximately 70% of these genes were linked with the TFBS database. All TFBS with a $Z > 2.5$ represented by more than 5 genes were selected as enriched. In addition, TFBS analysis was conducted within each cluster (I-III) to identify if overrepresented TFBS were enriched within each cluster. Associations with selected GO biological processes were determined using MappFinder and DAVID.

Results

As and Cd Induce Gene Expression Alterations in a Dose-Dependent Manner

As shown in Figure 2A, we identified 1960 and 775 genes to be significantly altered by As and Cd, respectively, across dose and time, with 116 of these genes identified to be significantly altered by both As and Cd (ANOVA, $p < 0.0001$). Using PCA we observed a dose-dependent relationship in the distance between exposure groups of As and Cd (8h) in comparison with the 8h control within genes altered by As and/or Cd (Figure 2B). Furthermore, we identified high dose groups of As (10mg/kg) and Cd (4mg/kg) to display similar extreme differences from control (8 or 12h), irrespective of time. Within genes identified to be significantly altered by As or Cd, we identified a monotonic dose-dependent relationship in the total number of genes altered by metal exposure, specifically at 8h (Figure 2C and 2D). With As (Figure 2C), we identified a relatively small increase (6%) in the amount of genes altered at 8hr between 2.5 and 5mg/kg compared to the dramatic 84% increase in the amount of genes altered at 8hr between 5 and 10mg/kg. At 8 and 12h, we observed a similar response in the amount of genes altered and the percentage of up- and downregulated genes with 10mg/kg As. With Cd at 8hr (Figure 2D), we observed a 27% increase in the amount of genes significantly altered between dose groups 0.5 and 2mg/kg and 2 and 4mg/kg. With 4mg/kg Cd, we observed a 12% increase in the amount of probes altered at 12h compared to 8h. At 8h, with 0.5 and 2mg/kg Cd, a higher proportion of genes were identified to be upregulated than downregulated. In contrast, with 4mg/kg Cd, more genes were identified to be downregulated (8h and 12h). Comparing both concentrations, which were chosen since they have previously induced NTDs (high dose), we observed differential proportions of up/down regulated genes, whereas with 10mg/kg As, we observed 54 and 55% of altered genes to be upregulated (8h and 12h) and with 4mg/kg Cd, we observed only 14 and 18% of altered genes to be upregulated.

As and Cd Impact Gene-Expression Linked GO Biological Processes

Conducting enrichment analysis of GO biological processes within genes found to be significantly altered by either As or Cd, we identified 172 biological processes to be overrepresented, including 25 biological processes identified to be enriched with both As

and Cd (Figure 3). Both As and Cd (Quadrant I) significantly impacted genes enriched for GO biological processes related to general metabolism (e.g. cellular metabolic process), transcriptional and translational activities (e.g. RNA processing, translation), mitochondrial organization, and cell cycle and environmental stress response-related categories (e.g. cell cycle regulation, glutathione metabolic process, response to UV) (GO-Enrichment Score >2.6). In addition, specific biological processes were uniquely enriched with As (Quadrant II) and Cd (Quadrant IV), including alcohol-, sugar- and sterol-metabolism as well as biopolymer modification, identified to be enriched by only As and additional environmental stress-related biological processes (e.g. apoptotic mitochondrial changes with As or response to DNA damage stimulus with Cd).

As and Cd Impacts Enriched GO Biological Processes in a Dose and Time-Dependent Manner

In Figure 4, we quantitatively describe the effect of As (A) or Cd (B) on selected enriched GO biological processes across dose and time, using the absolute average FC (log 2 scale) of all significantly altered genes ($p < 0.0001$) within each GO biological process. For both As and Cd, we identified the GO biological process “response to UV” to display the greatest FC response at 12h (As, 1.3, Cd, 1.5, log 2 scale). At 8h, this category was also one of the most robust, in terms of the degree of FC, showing peak effects with 10mg/kg As and 2mg/kg Cd. Other related GO biological processes previously linked with environmental stress (glutathione metabolic process, regulation of cell cycle) showed less robust, but similar dose-response relationships for both As and Cd. Enriched GO biological processes, such as translation or RNA processing, showed smaller changes in comparison with environmental stress-related biological processes as well as the average of all gene FC values with As or Cd (Figure 4A, B). Specifically, with As exposure (Figure 4A), uniquely enriched processes related to sterol and alcohol/glucose metabolism (i.e. glycogen metabolic process, alcohol metabolic process, glucose metabolic process, monosaccharide metabolic process, carbohydrate metabolic process) displayed monotonic dose dependent changes. For example, across doses the absolute average FC for the 22 significantly altered genes ($p < 0.0001$) related to glucose metabolic process was 0.08, 0.12, 0.73 (8h) and 0.90 (12h) (log 2 scale) with doses 2.5, 5, 10mg/kg (8h) and 10mg/kg (12h) As, respectively. These GO biological processes were similarly affected or, in some cases, more affected as categories reflective of environmental stress pathways (response to UV, apoptotic mitochondrial changes, regulation of cell cycle, glutathione metabolic process, cell cycle) for As. Due to the number of enriched GO biological processes impacted by As and Cd, only selected GO categories are presented across dose and time.

Enriched GO Biological Processes Altered by As or Cd Dependent on Dose-Response Relationship

To further investigate the dose-relationship between metal exposure, gene expression alterations and associated GO categories, we conducted K-means clustering within genes significantly altered by As or Cd ($p < 0.0001$) (Figure 5 and 6). We determined 2 common dose-relationships with As exposure in C57BL/6J embryos (Cluster I and II, Figure 5A). In general, Cluster I represented 1112 genes upregulated with 10mg/kg at both 8h and 12h, while Cluster II represented 848 genes downregulated with 10mg/kg at 8h and 12h. (Figure 5A). Only minimal changes were identified with 2.5 and 5mg/kg within these two clusters on average (Figure 5C). Common GO biological processes were identified in genes within Cluster I and II, including genes involved in protein metabolism/transport (red), cellular localization/organization (purple), DNA metabolism (grey), biopolymer modification (mocha), and cell cycle regulation (light blue) (Figure 5B). Unique categories in Cluster I represented genes involved in alcohol, carbohydrate, and sugar metabolism, stress response (e.g. glutathione metabolic process, response to oxidative stress), programmed cell death,

intracellular signaling, cellular homeostasis, and heart development. Biological processes related to RNA metabolism, sterol metabolism, DNA repair were uniquely identified in Cluster II.

We identified 4 unique dose-response relationships within significant gene expression alterations associated with Cd exposure across dose and time (Cluster I-IV, Figure 6). Cluster I represented 211 genes upregulated with 0.5 and 2mg/kg Cd (8h p.i.), and downregulated with 4mg/kg at 8h and 12h (Figure 6A). The 147 genes within Cluster II showed minimal changes with 0.5mg/kg Cd and downregulation with 2 and 4mg/kg. Greater changes were observed with 2mg/kg (−0.5 FC, log 2 scale) compared to 4mg/kg (−0.2 FC, log 2 scale) (Figure 6C). In parallel, but directionally opposite to observations in Cluster II, genes within Cluster IV (180 genes) showed upregulation, again with peak changes occurring in the 2mg/kg group (Figure 6A, C). Genes within Cluster III showed minimal changes with lower doses (0.5, 2mg/kg); however, with 4mg/kg, this group of genes (137 total) showed, on average, reduced expression by ~50% at 8h and 12h. Strong similarities between 8h and 12h for 4mg/kg Cd were observed in terms of magnitude and directionality for Clusters I-IV (Figure 6C). Common enriched GO biological processes were identified within more than one cluster, including RNA metabolism (I-III, green), transcription (I, II, green) and protein metabolism/translation (II, III, red) (Figure 6B). Unique categories included biopolymer modification, negative regulation of programmed cell death, cell development, negative regulation of neurogenesis (Cluster I); mitochondrial organization, regulation of cell cycle (Cluster II); chromosomal organization, DNA metabolism, mitosis (Cluster III) and cytoskeleton organization, intracellular signaling cascade, glutathione metabolism, negative regulation of progression through the cell cycle, and response to stress (Cluster IV).

Overlapping Gene Expression Responses of Arsenic and Cadmium

Using K-means clustering analysis, we identified 3 unique relationships across dose and time within the 116 genes identified to be significantly altered by both As and Cd (Figure 7). Cluster I represented 49 genes that were upregulated with As (5, 10mg/kg) and Cd (2 and 4mg/kg) (Figure 7A–C). These genes were linked with GO biological processes such as: cell cycle process (Cdk1na, Mapre1, Re811, Sash1), stress response (Ercc5, Cdk1na, Pmaip1, Ubf3b), cellular homeostasis (Gsr, Mt1, Txndd3), tube morphogenesis (G6pdx, Fzd6) and transport (Slc19a2, 26100029101Rik, Npc2, Stau2, Txndc13, Fzd6). Genes within Cluster III displayed a relationship opposite to Cluster I; in general, genes were downregulated with As (5, 10mg/kg) and Cd (2 and 4mg/kg) (Figure 7B, C) and included genes (55 total) linked with cell cycle process (Hus1, Cdk9) and RNA processing (672048F09Rik, Gtpb3, Tyw3, Tsen2, Dlm1) (Figure 7A). The 12 genes represented in Cluster II displayed similarities in response with Cluster I, upregulated response with 2.5, 5, 10mg/kg As and 0.5 and 2mg/kg Cd, however, showed striking dissimilarities with 4mg/kg Cd (downregulated versus upregulated) at both 8h and 12h.

Enrichment of Transcription Factor Binding Sites within As and Cd Commonly Impacted Genes

Within the 116 genes identified to be significantly altered by both As and Cd., we identified enrichment of 7 TFBS, including, Foxa2 (35 genes), Nfkb1 (13), Nk2-5 (54), Nr2f1 (21), Pax5 (10), Sox9 (46) and Stat1 (11) (Figure 7D). Upon further analysis, overrepresentation of specific TFBS were identified to be cluster dependent. For example, Foxa2, Nk2-5 and Stat1 were found to be overrepresented in only Cluster I.

Discussion

In this study, using a toxicogenomic approach, we investigated the dose-response relationship associated with either As or Cd in exposed embryos during the neurulation period of development. We examined overlapping and non-overlapping GO biological processes impacted by each metal across dose using a combination of data-driven methodologies, data mining and bioinformatic resources. We discuss the effects of As and Cd on gene expression response across dose and time in context with the dose-response relationship between metals and developmental toxicity and NTD formation as summarized in Supplemental Table 2.

Dose-Dependent Gene Expression Alterations in Relation to the Dose-Response Relationship of Metals and Developmental Toxicity

A collection of developmental toxicity studies investigating the effects of As and Cd on neurulation in mouse have established a dose response relationship between As, Cd and embryotoxicity, with doses of As (~5mg/kg) and Cd (~1mg/kg) producing minimal embryotoxicity and doses of As (10mg/kg) and Cd (4mg/kg) inducing NTDs, other structural defects, growth alterations and increased mortality (Hovland *et al.*, 1999; Machado *et al.*, 1999; Lutz and Beck, 2000; Lee *et al.*, 2006; Robinson *et al.*, 2009b; Robinson *et al.*, 2010b). Using a similar experimental design in terms of dose, administration of exposure, strain and gestational timing as the aforementioned studies, in this study, we observed a monotonic dose response relationship, with increased alterations in gene expression (at 8h) coinciding with increased dose (Figure 2). Our study suggests that at this timepoint increased alterations in gene expression is a measure of metal-induced response corresponding with increased developmental toxicity that precedes morphological effects determined later in gestation.

Arsenic and Cadmium Induce Environmental Stress Response-Related Impacts in Association with Developmental Toxicity

In general toxicological studies, four common predominant mechanisms have been proposed regarding metal toxicity: 1) alterations in cell proliferation pathways, 2) inhibition of major DNA repair systems, 3) interference with cellular redox regulation/generation of oxidative stress, and 4) induction of apoptosis or cell death (Pulido and Parrish, 2003; Beyersmann and Hartwig, 2008). In this first attempt toward understanding the toxicogenomic dose-response relationship between metals (As and Cd) and developmental toxicity during the neurulation window of sensitivity, we provide evidence that these pathways are disrupted in the developing embryo during neural tube development. This disruption appears to be dose-dependent at the gene and GO biological process level in relation to developmental toxicity (Figure 2–7). In addition, these results are supported by initial studies assessing mechanisms of metal-induced developmental toxicity during neurulation as discussed below (Włodarczyk *et al.*, 1996; Włodarczyk *et al.*, 1996; Fernandez *et al.*, 2003; Kultima *et al.*, 2006; Paniagua-Castro *et al.*, 2007; Robinson *et al.*, 2009a; Robinson *et al.*, 2009b; Robinson *et al.*, 2010a; Robinson *et al.*, 2010b).

In this study, enriched GO categories representative of biological processes controlling cell cycle regulation (i.e. cell cycle, cell cycle process, regulation of cell cycle) were observed with both As and Cd (Figure 3). Metals impacted cell cycle-related genes in a dose-dependent manner (Figure 4) and bi-directionally (Figure 5, Clusters I, II and Figure 6, I, IV). Specific cell cycle genes, such as *Cdkn1a*(↑), *Mapre*(↑), *Re9L1*(↑), *Sash1*(↑), *Cdk9*(↓) and *Hus1*(↓), were identified to be altered by both As and Cd (Figure 7). These genes reflect different mechanistic aspects of cell cycle control. For example, in response to stress, the p53-downstream mediator *Cdkn1a* inhibits cell cycle progression between G1-S and G2-M

checkpoints by interacting with cdk/cyclin complexes (Kuribayashi and El-Deiry, 2008). Studies assessing metal exposure across a diverse array of *in vivo* and *in vitro*-based models suggest As and Cd to induce RNA expression of Cdkn1a and p53 as well as other p53-downstream mediators in association with increased cell cycle inhibition (Yu *et al.*, 2008; Yu *et al.*, 2010). Furthermore, previous RNA (RT-PCR and microarray) and protein expression studies have identified Cdkn1a to be induced by Cd associated with increased developmental toxicity and NTD formation (Wlodarczyk *et al.*, 1996; Fernandez *et al.*, 2003; Robinson *et al.*, 2009b; Robinson *et al.*, 2010b). Therefore, this study provides further evidence of metal-induced alterations in cell cycle control as a mechanism of toxicity and suggests disruption of cell cycle regulation to be associated with increased developmental toxicity in embryos undergoing neurulation.

In addition, we observed overlapping effects between As and Cd on genes associated with the GO biological process term Response to UV (Figure 3), as well as enrichment of related GO categories DNA repair (Cd, Figure 3; As, Figure 5) and Response to DNA Damage (Cd, Figure 3). Genes significantly altered with As or Cd ($p < 0.0001$) involved in these categories showed robust changes in comparison with other enriched GO biological processes for both metals (Figure 5), suggesting these genes are sensitive indicators of metal-induced response in association with developmental toxicity. Genes within the GO category Response to UV, impacted by As and/or Cd, included genes involved in DNA repair (Ercc5, Xpa, Rev1), cell cycle arrest (Cdkn1a, Hus1) and apoptosis (Pmaip1). Shared dose-dependent increases in genes such as Ercc5, previously characterized to be a primary component of nucleotide excision repair due to DNA damage (Castorena-Torres *et al.*, 2008) suggests both of these metals may disrupt DNA processes in the developing embryo in association with increased toxicity. Furthermore, gene expression responses relative to DNA damage response in this study correlate and support previous classical assessments of DNA damage (e.g. Comet assay, TUNEL assay) caused by As and Cd during embryonic development (Fernandez *et al.*, 2003; Unis *et al.*, 2009).

Initial studies suggest exposure to metals during embryonic development results in increased oxidative stress (Paniagua-Castro *et al.*, 2007) and RNA changes in oxidative stress-related genes such as metallothioneins and Hmox1 (Kultima *et al.*, 2006; Robinson *et al.*, 2010b). While GO analyses performed of all genes specifically altered by As or Cd (Figure 3) did not reveal enrichment of GO biological processes, such as “response to stress”, enrichment analysis in clusters of genes specifically displaying upregulated response showed overrepresentation of stress response related associations (Cluster I, As and Cluster IV, Cd). Additionally, GO categories, such as glutathione metabolism were identified to be enriched within these clusters (Figure 5, 6). At the gene level, specific targets such as Mt1, responsible in maintaining cellular homeostasis with known roles in ROS detoxification were observed to be upregulated by As and Cd in a monotonic dose dependent manner at 8h (Figure 7). Epidemiological and animal-based studies suggest Mt1 to be a biomarker of human As and Cd exposure (Chang *et al.*, 2006; Liu *et al.*, 2007). Furthermore, Mt1 RNA expression correlates with embryonic Cd uptake in exposed mouse embryos and is an indicator of embryonic sensitivity to Cd-induced developmental toxicity (Robinson *et al.*, 2009a; Robinson *et al.*, 2010b). In this study, we provide dose-response evidence regarding the upregulation of Mt1 as well as other stress-related genes due to metal exposure in developing mouse embryos undergoing neurulation.

Additionally, we observed that both As and Cd alter genes related to apoptosis (programmed cell death) in a dose-dependent manner (Figure 5–7). Genes related to apoptosis (46 total) were shown to be upregulated with As (primarily with 10mg/kg) (Cluster I, Figure 5B). With Cd, significant alterations in apoptosis genes (12 total), including 7 genes associated with negative regulation (Son, Htt, Mapk8, Polb, Birc6, Mapk8ip1, Rtn4) were evident in

Cluster I (Figure 6B). Interestingly, these genes were upregulated with 0.5 and 2mg/kg and downregulated with 4mg/kg Cd (Figure 6C). The non-monotonic nature of the dose-response relationship within this subset of genes may implicate differing mechanisms associated with exposure-levels of Cd which do or do not induce developmental toxicity (e.g. NTDs). Upregulation of genes within this subset suggests upregulation of processes that inhibit cell death at non-toxic doses. For example, *Birc6*, which was upregulated within this subset, was previously identified to inhibit caspase activation and subsequent apoptotic signaling (Bartke *et al.*, 2004) with doses of 0.5 and 2mg/kg which do not induce NTDs. In comparison, downregulation of *Birc6* with 4mg/kg Cd suggests a shift in regulation of apoptotic gene expression in promoting apoptosis in association with increased NTD formation. These observations correlate with previous studies assessing high dose effects of Cd (C57, GD8, 4mg/kg) using similar experimental design indicating morphological and molecular apoptotic events (i.e. increased cleaved caspase 3) to occur in the neural tube of Cd-exposed embryos during the neurulation period (Webster and Messerle, 1980; Fernandez *et al.*, 2003; Robinson *et al.*, 2009b). Furthermore, Robinson *et al.* (2010b) suggests that the expression of cleaved caspase 3 at 12h associates with increased NTD incidence in Cd-exposed sensitive (C57BL/6J) and resistant (SWV) embryos (Robinson *et al.*, 2010b). In this study, we provide preliminary context of dose-response related mechanisms of metal-induced apoptosis in whole mouse embryos in support of previous investigations.

Glucose and Alcohol Metabolism Pathways as a Unique Mechanism of As-Induced Developmental Toxicity

Uniquely in this study, we observed genes involved in sugar metabolism-related categories (glycolysis, and glucose, alcohol, pyruvate, monosaccharide metabolism-related categories) to be disrupted by As exposure in a dose-dependent manner (Figure 3–5). These GO biological processes represented some of the most robust changes, in terms of magnitude of impact (Figure 4). For example, the absolute average FC of 22 genes linked with the GO biological process glucose metabolic process was similar to categories previously identified to be linked with environmental stress-related pathways (response to UV, apoptosis, regulation of cell cycle), indicating genes within this category to be prime candidates for markers of As developmental toxicity. Preliminary dose-response information from this study may inform future studies examining As-induced alterations in glucose metabolism pathways within the developing embryo and the relationship with developmental toxicity and NTDs.

Conclusion

These preliminary studies used As and Cd as model teratogens to examine dose-dependent metal effects on gene expression in C57BL/6J mouse embryos during neurulation to describe alterations in genes and associated biological processes with developmental toxicity and NTD formation. We provide evidence of As and Cd to disrupt genes involved in environmental stress-related pathways in a dose-dependent manner, such as cell cycle arrest, DNA damage response, oxidative stress and apoptosis-related biological processes. We have also identified unique responses associated with As exposure in its ability to significantly alter multiple genes involved in glucose metabolism. Within the aforementioned shared and unique biological processes and related pathways, we have identified gene expression alterations commonly altered as well as gene expression alterations specific for As or Cd that may serve as biomarkers of metal teratogenicity. Global assessment of RNA changes induced by As and Cd across multiple genes and pathways expand initial observations of specific targets observed in previous studies in relation with developmental toxicity and propose future assessments of specific functional interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Analysis of Genes Altered by Arsenic and Cadmium

This flow diagram represents the statistical approach used to explore dose- and time-dependent effects of As and Cd exposure on gene expression and corresponding GO biological processes. Linear models assessing for dose (As or Cd) and time effects were used to identify genes that were significantly altered by metal exposure (ANOVA, B_{Dose} , $p < 0.0001$). Enrichment analyses of GO biological processes were conducted for all genes significantly differentially expressed due to As or Cd exposure to identify biological processes altered by metals. Quantitative analysis of enriched GO biological processes was employed using GO-Quant, enabling dose- and time-dependent calculations in genes within GO subsets. In addition, K-means cluster gene analysis was used to assess unique dose-response relationships due to As (or Cd) exposure and corresponding enrichment of GO biological processes. We assessed the 116 genes altered by both As and Cd for unique dose-response relationships using K-means cluster gene analysis, and we determined the quantitative relationship within these clusters across dose and time (average FC). Enrichment of transcription factor binding sites and associated GO biological processes were also evaluated.

Figure 2. Dose Dependent Metal-Induced Gene Expression Alterations

(A) The venn diagram illustrates 1960 and 775 genes identified to be significantly altered by As and Cd, respectively (F-test, $p < 0.0001$), and overlapping genes intersecting between these two populations (116 total). Using principal components analysis (PCA), we determined similarities in gene expression alterations between exposure groups across all genes altered by As and/or Cd (B). Within genes identified to be significantly altered by As (C) and Cd (D) (F-test, $p < 0.0001$), post-hoc analyses (t-test) were completed to identify the total number of differentially expressed genes with each dose group in comparison with their respective concurrent control (8h or 12h). The percentage of genes differentially expressed (t-test, $p < 0.05$) are displayed in comparison to the total number of genes identified to be significantly altered across all dose groups (black bar). Additionally, the proportion of up (red) and down (green)-regulated genes are indicated by color.

Figure 3. Enrichment of GO Biological Processes Altered by As and Cd

Using a cross scatter-plot, we illustrate overlapping and non-overlapping enriched GO biological processes altered by As and Cd. Genes significantly impacted by As and Cd ($p < 0.0001$), were separately analyzed for enrichment of GO biological processes (MAPPFinder). Assessing over 4500 GO biological processes, we identified 159 (As) and 38 (Cd) GO biological processes meeting our criteria of enrichment, ($p < 0.02$, Z score > 2 , and ≥ 3 genes altered). Using the product of the $-\log(p\text{-value})$ and Z score, we obtained a measure of ranking the significance of GO categories to allow direct comparisons between As and Cd. The dashed line represents the minimum criteria to be identified as significant (~ 2.6). Symbols are differentiated by GO hierarchy. Overlapping altered GO biological processes are represented as having enrichments scores > 2.6 for both As and Cd (Quadrant I). Enriched categories unique to As or Cd are identified in Quadrant II and IV, respectively.

Figure 5. Enriched GO Biological Processes Altered by As Dependent on Dose-Response Relationship

K-means cluster analysis was conducted on all 1960 genes found to be significantly altered by As ($p < 0.0001$) (TIGR MEV). Two relationships were determined, Cluster I and II. Separate GO analyses were conducted for each cluster. Significant GO gene categories (Biological Process Level 4) determined using both MappFinder (M) and/or DAVID (D) are shown. Enriched categories were determined by the following criteria: p -value (< 0.02), Z score > 2 and a minimum of 3 genes for MappFinder and $p < 0.05$, > 2 genes altered for DAVID. The total number of genes altered within each GO category as determined by MappFinder or DAVID is shown in parentheses. The absolute average ratio of gene expression response for genes within each enriched GO category was calculated using GO-Quant across dose and time and depicted by color using TIGR MEV (Figure 4C). Color coding was used to differentiate between GO biological process families within the GO hierarchy and to make comparisons between As and Cd (Figure 4 and 5).

Figure 6. Enriched GO Biological Processes Altered by Cd Dependent on Dose-Response Relationship

K-means cluster analysis was conducted on all 775 genes identified to be significantly altered with Cd. In general, four relationships were determined, clusters I-IV. Separate GO analyses were conducted for each cluster. Significant GO gene categories (Biological Process Level 4) determined using both MappFinder (M) and/or DAVID (D) are shown. Enriched categories were determined by the following criteria: p-value (<0.02), Z score >2 and a minimum of 3 genes for MappFinder and p<0.05, >2 genes altered for DAVID as indicated by letters M or D. The number of genes altered within each GO category as determined by MappFinder or DAVID are shown in parentheses. Color coding was used to differentiate between GO biological process families within the GO hierarchy and to make comparisons between As and Cd (Figure 4 and 5).

Figure 7. Dose Dependent Gene Expression Alterations Altered by both As and Cd
 Genes identified to be altered by As and Cd ($p < 0.0001$) were analyzed using K-means clustering analysis across dose and time. Three dose-response relationships were identified (I-III) (A). Quantitative analysis of each cluster was conducted by calculating the average FC associated with all genes within each cluster for As (B) or Cd (C) across dose and time. Transcription factor binding motifs (TFBS) were identified using oPPOSUM (D). TFBS (Z score > 2.5 , ≥ 10 genes represented) were selected as enriched (*). Approximately 70% of commonly altered genes were linked with the TFBS database. In addition, TFBS analysis was conducted within each cluster (I-III) to identify if overrepresented TFBS were enriched within each cluster. Associations with selected GO biological processes were determined using DAVID. All enriched TFBS and GO associations are displayed for each gene directly to the right of its name.