Published in final edited form as:

Toxicol Appl Pharmacol. 2015 March 15; 283(3): 198–209. doi:10.1016/j.taap.2015.01.014.

Arsenic responsive microRNAs *in vivo* and their potential involvement in arsenic-induced oxidative stress

Xuefeng Ren^{a,b,*}, Daniel P. Gaile^c, Zhihong Gong^a, Wenting Qiu^d, Yichen Ge^a, Chuanwu Zhang^d, Chenping Huang^d, Hongtao Yan^d, James R. Olson^{a,b}, Terrance J. Kavanagh^e, and Hongmei Wu^{d,*}

^aDepartment of Social and Preventive Medicine, School of Public Health and Health Professions, the State University of New York, Buffalo, NY 14214

^bDepartment of Pharmacology and Toxicology, School of Biomedical Sciences, the State University of New York, Buffalo, NY 14214

^cDepartment of Biostatistics, School of Public Health and Health Professions, the State University of New York, Buffalo, NY 14214

^dSchool of Public Health, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China

^eDepartment of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98195

Abstract

Arsenic exposure is postulated to modify microRNA (miRNA) expression, leading to changes of gene expression and toxicities, but studies relating the responses of miRNAs to arsenic exposure are lacking, especially with respect to *in vivo* studies. We utilized high-throughput sequencing technology and generated miRNA expression profiles of liver tissues from Sprague Dawley (SD) rats exposed to various concentrations of sodium arsenite (0, 0.1, 1, 10 and 100 mg/L) for 60 days. Unsupervised hierarchical clustering analysis of the miRNA expression profiles clustered the SD rats into different groups based on the arsenic exposure status, indicating a highly significant association between arsenic exposure and cluster membership (*P*-value of 0.0012). Multiple miRNA expressions were altered by arsenic in an exposure concentration-dependent manner. Among the identified arsenic-responsive miRNAs, several are predicted to target Nfe2l2-regulated antioxidant genes, including glutamate-cysteine ligase (GCL) catalytic subunit (*GCLC*) and modifier subunit (*GCLM*) which are involved in glutathione (GSH) synthesis. Exposure to low concentrations of arsenic increased mRNA expression for *Gclc* and *Gclm*, while high concentrations significantly reduced their expression, which were correlated to changes in hepatic

© 2015 Published by Elsevier Inc.

*Corresponding Author: Xuefeng Ren, 276 Farber Hall, University at Buffalo, Buffalo, NY 14221, USA; Phone: (716), 829-5384, FAX: (716) 829-2979, xuefengr@buffalo.edu; Hongmei Wu, School of Public Health, Wenzhou Medical University, Wenzhou, 325035, China; Phone: (86) 577-86699216, FAX: (86) 577-86699122, hongmeiwwu@hotmail.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of interest: The authors declare that there are no conflicts of interest and nothing needs to be disclosed.

GCL activity and GSH level. Moreover, our data suggested that other mechanisms, e.g. miRNAs, rather than Nfe2l2-signaling pathway, could be involved in the regulation of mRNA expression of *Gclc* and *Gclm* post arsenic exposure *in vivo*. Together, our findings show that arsenic exposure disrupts the genome-wide expression of miRNAs *in vivo*, which could lead to the biological consequence, such as an altered balance of antioxidant defense and oxidative stress.

Keywords

Arsenic; High-throughput sequencing; MicroRNA (miRNA); Glutamate-cysteine ligase; Glutathione; Oxidative Stress

Introduction

Inorganic arsenic (iAs) is one of the strongest carcinogenic metalloids derived from the natural environment. It is widely accepted that exposure to iAs leads to carcinogenesis in multiple human tissues, including skin, lung and bladder, and possibly kidney and liver (IARC, 2012), the International Agency for Research on Cancer (IARC) has thus classified iAs^{III} as a group 1 carcinogen. However, the adverse effects of arsenic have not been limited to its carcinogenicities. Long-term exposure to iAs, mainly *via* drinking water provokes a number of additional health effects, such as diabetes mellitus, bladder, kidney and neurological effects, hypertension and cardiovascular diseases (Abernathy *et al.*, 2003; Elamin *et al.*, 2011).

Decades of effort has yielded great progress towards understanding the molecular mechanisms of iAs-induced health effects. Experimental results based on both *in vivo* and *in vitro* studies of arsenic exposure have suggested that an imbalance between antioxidant defense and the total burden of potentially harmful reactive biochemical species plays a role in arsenic-induced toxicity and carcinogenicity (Kitchin and Ahmad, 2003; Hughes and Kitchin, 2006; Hughes, 2009; Jomova *et al.*, 2011; Hall *et al.*, 2013). Among a number of antioxidant defense mechanisms, glutathione (GSH) is an abundant and ubiquitous intracellular antioxidant peptide and plays an essential function in cellular defense against oxidative stress caused by reactive oxygen species (ROS) and free radicals (Liu *et al.*, 2001; Lu, 2009). GSH is synthesized in the cytosol from its amino acid constituents *via* two sequential ATP-dependent reactions catalyzed by glutamate cysteine ligase (GCL) and glutathione synthetase (GS) (Deneke and Fanburg, 1989). GCL catalyzes the formation of gamma-glutamylcysteine (γ -GC) from L-glutamate and L-cysteine, which is generally considered the rate-limiting step in GSH biosynthesis (Fraser *et al.*, 2003; Mohar *et al.*, 2009). Therefore, GCL is a vital factor reflecting GSH biosynthesis capacity as well as the capacity for maintaining GSH homeostasis. Consequently, its expression and activity reflect susceptibility to oxidative stress. GCL is a heterodimer consisting of a catalytic subunit (GCLC) and a modifier subunit (GCLM) (Liu *et al.*, 2001; Lu, 2009). Studies have suggested that GSH concentration can be altered with iAs exposure *in vitro* and *in vivo* (Santra *et al.*, 2000a; Schuliga *et al.*, 2002) possibly by deregulation of the expression and activity of GCL (Schuliga *et al.*, 2002; Thompson *et al.*, 2009). Nuclear factor, erythroid 2-like 2 (NFE2L2; also known as Nrf2) is a transcription factor that regulates *GCLC* and

GCLM expression through its binding to antioxidant response elements (ARE) present in their 5'-promoter regions (Ma, 2013). Other important factors in the NFE2L2 signaling include Kelch-like ECH-associated protein 1 (KEAP1) and BTB and CNC homology 1 (BACH1). KEAP1 can act as an effective inhibitor of NFE2L2 signaling by facilitating its ubiquitin-dependent degradation (Sekhar *et al.*, 2002), while BACH1 competes with NFE2L2 at AREs to negatively regulate gene expression (Ma, 2013; Niture *et al.*, 2014). Recently, several studies have reported that arsenic can alter the expression of KEAP1 (Lau *et al.*, 2013) and BACH1 (Liu *et al.*, 2013), thus mediate the NFE2L2-regulated antioxidant pathway. However, previous study also suggested that the regulation of GCL by iAs exposure is independent of the NFE2L2 signaling pathway (Thompson *et al.*, 2009). This inconsistency in the regulation of GCL by arsenic has not yet been addressed.

Emerging evidence suggests that the toxic activity of arsenic may lie in its ability to induce massive aberrant gene expression and to dysregulate cell growth, proliferation, differentiation and antioxidant defense (Bustaffa *et al.*, 2014; Medeiros *et al.*, 2012). Studies have observed extensive changes in global gene expression in individuals following arsenic exposure (Andrew *et al.*, 2008; Bourdonnay *et al.*, 2009; Bustaffa *et al.*, 2014). Since epigenetic processes are major regulators of gene expression, these findings suggest that dysregulation of epigenetic processes could contribute mechanistically to arsenic-induced changes in gene expression and toxicities. Global DNA hypomethylation and focal DNA hypermethylation are both implicated in arsenic-induced malignant transformation *in vivo* and *in vitro* (Benbrahim-Tallaa *et al.*, 2005; Chanda *et al.*, 2006). Arsenic exposure has also been linked to altered histone modifications *in vitro* and in circulating leukocytes collected from humans with chronic arsenic exposure through drinking water (Jo *et al.*, 2009; Zhao *et al.*, 2010; Chu *et al.*, 2011; Ge *et al.*, 2013). While it has been proposed that arsenic can also modify microRNA (miRNA) expression and thus altering gene expression (Ren *et al.*, 2011; Li *et al.*, 2012; Martinez-Pacheco *et al.*, 2014), this has only been studied *in vitro* (Sturchio *et al.*, 2012; Rager *et al.*, 2013; Sturchio *et al.*, 2014). To our knowledge, there remains a lack of information regarding the effects of chronic arsenic exposure on miRNA expression *in vivo*. Various studies have begun to address a possible link between environmental factor-induced alterations in miRNA and consequent impacts on the expression of oxidative stress associated genes (Bollati *et al.*, 2010; Gielen *et al.*, 2012; Yamamoto *et al.*, 2013). Therefore, we investigated whether alterations in miRNA expressions induced by arsenic play a role in the regulation of the Nfe2l2 signaling pathway, and how this may affect antioxidant defense under the stress of arsenic exposure.

In terms of GCL and GSH alteration, liver is the major site of GSH biosynthesis, GSH is most abundant in liver of rodents and humans, and liver responds to oxidants sensitively (Hissin and Hilf, 1976; Liu and Dickinson, 2003; Valcheva-Kuzmanova *et al.*, 2004; Miltonprabu and Sumedha, 2014). Therefore, liver is employed to explore the expression alterations of GCL/GSH and its potential regulatory factors. In this study, we report that sub-chronic exposure of Sprague Dawley (SD) rats to sodium arsenite (iAs^{III}) was able to disrupt genome-wide miRNA expression in the liver, and significantly alter the expression of multiple miRNAs in an exposure concentration dependent manner. Reduced expressions of *Gclc* and *Gclm* at high concentrations of arsenic exposure are associated with a

significantly reduced hepatic GCL activity and GSH level. Several of the identified miRNAs are predicted to target *Gclc* and *Gclm* mRNAs, potentially regulating their expression in a manner that is independent of the Nfe2l2 pathway.

Materials and Methods

Chemical and Reagents

Chemicals used in this study included sodium arsenite, sucrose, Tris base, ethylenediamine tetra-acetic acid (EDTA), boric acid, L-serine, magnesium chloride hexahydrate (MgCl_2), 4-ethylmorpholine (NEM), triscarboxyethyl phosphine hydrochloride (TCEP), adenosine 5'-triphosphate disodium (ATP), L-glutamic acid, L-cysteine, 5-sulfosalicylic acid dihydrate (SSA), sodium hydroxide (NaOH), naphthalene-2,3-dicarboxaldehyde (NDA), dimethyl sulfoxide (DMSO) and gamma-glutamylcysteine (γ -GC). Chemicals were purchased either from Fisher Scientific International Inc. (Pittsburg, PA) or from Sigma-Aldrich (St. Louis, MO).

Animal Maintenance

All protocols were reviewed and received the approval of the Laboratory Animal Ethics Committee of Wenzhou Medical University (Wenzhou, China). Male Sprague Dawley (SD) rats were purchased from Shanghai Laboratory Animal Center, CAS (SLACCAS) (Shanghai, China). After an acclimation period of 2 weeks, the animals were transferred and housed in threes or fours in stainless steel wire cage without bedding in an specific pathogen free (SPF) animal facility with controlled temperature ($18\text{ }^{\circ}\text{C} - 26\text{ }^{\circ}\text{C}$), humidity ($50\% \pm 20\%$) and a 12 h light/12 h dark cycle. All animals were provided with a standard diet and acidified water *ad libitum*.

Sodium Arsenite Treatment and Liver Tissue Collection

After 2 weeks acclimation, 50 male SD rats weighing between 180–200g were randomly divided into five groups of 10 animals each, and provided with drinking water containing 0, 0.1, 1, 10, or 100 mg/L sodium arsenite. The consumption of drinking water was monitored every 3 days. All animals were maintained on their assigned arsenic exposure concentration until the termination of the experiment. Rats were examined every other day and any abnormal behaviors or symptoms were recorded. At the end of 60-days administration of sodium arsenite, rats were euthanized individually in a CO_2 chamber and fresh tissues including liver were isolated, rinsed with ice cold 0.9% saline solution, flash frozen in liquid nitrogen, and then stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Arsenic treatments at concentrations of study resulted in slight decrease in body weight after exposure though the decrease did not reach significant level in statistics (one-way ANOVA, $P = 0.097$). After exposure, the body weight of 100 mg/L group was 90.9% of the control, there was marginal difference in body weight between these 2 groups (Tukey HSD, $P = 0.071$). The liver coefficient (Liver weight/body weight *100) did not change significantly after arsenic exposure (one-way ANOVA, $P = 0.097$).

Liver Tissue RNA Extraction

Total RNA was obtained from 50–100 mg frozen liver tissue homogenized in Trizol, according to manufacturer's instructions (Life Technologies, Carlsbad, CA). Long and short RNA fractions were separated with an affinity resin column during clean up, according to the manufacturer's instructions (miRNeasy Mini Kit, Qiagen, MD). RNA was quantified by absorbance at 260 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE), further quantified using Picogreen (Life Technologies, Carlsbad, CA), and then stored at -80°C until analysis.

Small RNA Library Preparation and HiSeq Sequencing

The sequencing libraries of 3 randomly selected samples from each group were prepared with the TruSeq Small RNA kit (Illumina Inc.), from 1 μg total RNA (control group: RNA-014, RNA-022 and RNA-024; 0.1 mg/L group: RNA-074, RNA-075 and RNA-083; 1.0 mg/L group: RNA-094, RNA-101 and RNA-103; 10 mg/L group: RNA-111, RNA-121 and RNA-123; and 100 mg/L group: RNA-132, RNA-134 and RNA-141). Following manufacturer's instructions, the first step involves ligation of 5' and 3' RNA adapters to the mature miRNA's 5'-phosphate and 3'-hydroxyl groups, respectively. Following cDNA synthesis, the cDNA was then amplified with 13 cycles of PCR using a universal primer and a primer containing one of 15 index sequences. The 15 different indexed tags allow pooling of libraries and multiplex sequencing. Prior to pooling, each individual sample's amplified cDNA construct was visualized on a DNA-HS Bioanalyzer DNA chip (Agilent Technologies) for mature miRNA and other small RNA products (140–150 bp). Successful constructs were purified using a Pippen prep (Sage Inc.), using 125–160 bp product size settings with separation on a 3% agarose gel. The purified samples were validated for size, purity and concentration using a DNA-HS Bioanalyzer chip. Validated libraries were pooled with equal molar in Tris-HCl (10mM, pH 8.5), before sequencing on a HiSeq2000 (Illumina, Inc).

MiRNA Sequencing, Extraction, and Identification

Sequencing Libraries were clustered on an Illumina cBot 1.5.12.0, and then run on an Illumina HiSeq2000 in a 50 Cycle Single read flowcell. Once the sequencing was completed, samples were demultiplexed using Illumina's Casava v1.8.2, which converts the files from Illumina Qseq format into Fastq formatted files. Then, the fastq files were loaded into CLC Genomics Workbench version 5.5.1. Reads were trimmed of adapter sequences and controlled for quality. Reads less than 15 base pairs were discarded. Reads which passed filtering were then mapped to miRBase v19 (*Rattus Norvegicus*) and quantified *via* the CLC Genomics workbench.

Differential Expression Analysis for Hi-Seq miRNA Data

The miRNA agglomerated count data consists of 359 miRNAs made across 15 samples (i.e., three replicate observations for each of 5 different exposure levels) for which at least one sample had a non-zero count. The data were analyzed to identify differentially expressed miRNAs using statistical tools available in R (R-Core-Team, 2012). Mean count dispersion estimates were obtained using the Estimate Dispersions Function provided in DESeq library

(Anders and Huber, 2010) and by pooling across samples. The data were then variance stabilized, as recommended by the authors of the software. This last step provided a 359 by 15 matrix of variance stabilized 'expression values'.

The 15 sample miRNA profiles were clustered using the *hclust* hierarchical clustering function in R. The clustering was performed using the Manhattan distance metric in conjunction with Ward's minimum variance clustering method. The cluster tree was then cut to provide *k* clusters and a Fishers exact test was performed to determine if iAs exposure was associated with cluster membership. Values of *k* = 2, 3, 4, 5, 6 and 7 were considered. A minP permutation scheme was implemented to adjust the *p*-values for the multiplicity of testing across the 6 *k* values that were considered. The 15 sample miRNA profiles were subjected to a Principal Components Analysis (PCA) and the 15 samples were plotted according into their mappings into the space defined by the first two principal components. The principal component coefficients for the first and second principal components were tested for arsenic exposure related trends using Jonckheere's two sided trend test (Jonckheere, 1954). Sample RNA-083 might be an outlier so the PCA was repeated with respect to 14 sample miRNA profiles, excluding RNA-083.

Each of the 359 miRNAs were interrogated using Jonckheere's two sided trend test with the null hypothesis corresponding to no trend in observed 'expression values' as a function of increased exposure rates versus the alternatives of a decreasing or increasing trend. The 359 marginal *p*-values were adjusted to control the false discovery rate using the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Results were reported for all microRNAs with *q*-value of less than or equal to 0.20.

MiRNA Identification Targeting Molecules in Nfe2l2 Signaling Pathway

Utilizing microRNA target prediction tools developed by microRNA.org (Betel *et al.*, 2008; Betel *et al.*, 2010), we examine the identified arsenic-responsive miRNAs and their predicted target genes in Nfe2l2 Signaling Pathway, including *Gclc*, *Gclm*, *Nfe2l2*, *Bach1* and *Keap1*, for both rat and human genes. This predictive method uses machine learning and ranks the miRNA's target site by a down-regulation score, mirSVR score. On the basis of recommendation by the authors (Betel *et al.*, 2010), a mirSVR score lower than -0.1 was viewed as a score that indicates a true target. We thus only included the miRNAs that were classified as conserved with a good mirSVR score.

Quantitative Real-time PCR (qPCR) for the Identified MiRNAs and Data Analysis

RNA samples used for HiSeq2000 were reversely transcribed using the miScript reverse transcription kit (Qiagen, MD) based on the manufacturer's instructions. Real-Time PCR was performed to quantitate miRNA expression using Bio-Rad CFX96 Touch™ Real-Time PCR Detection System and RNA miScript SYBR Green PCR Kit (Qiagen, MD). The specific forward primers of miRNAs, including miR-26a, miR-148b, miR-151, miR-183, miR-192, and miR-423, are listed in Supplemental Table 1. RNA U6 was used as a control. Fold change estimates were calculated using the delta, delta *Ct* method normalizing to the average U6 value for the appropriate iAs concentration level. Jonckheere's two sided trend test was then performed with respect to the fold change estimates as a function of increasing

iAs concentration levels. Exact two-sided *p*-values for all 359 tests were calculated using 20,000 permutations of the iAs dosing categories relative to the sample data. Additionally, *q*-values were obtained for each of the 359 *p*-values via the application of the false discovery rate method of Benjamini and Hochberg (1995).

Quantitative Real-time PCR (qPCR) Analysis for the Genes in Nfe2l2 pathway and Data Analysis

Using same methods described above, we measured the mRNA expression of *Gclc*, *Gclm*, *Nfe2l2*, *Bach1* and *Keap1*. The qPCR primers of these five mRNAs are included in Supplemental Table 1. *Gapdh* mRNA was used as a control in the analysis.

GCL Activity Measurement

Liver tissues were minced on ice, then ground in a mortar and pestle filled with liquid nitrogen. Ground tissues were dispensed from mortars into 2 ml cryovials. After adding ice cold TES/SB buffer (containing 20 mM Tris, 1 mM EDTA, 0.25 M sucrose, 1 mM L-serine, 20 mM boric acid, pH 7.4), the collected tissues were vortexed and homogenized. The homogenates were centrifuged at $20,000 \times g$ for 30 minutes at 4 °C obtaining post-mitochondrial fractions that were used for GCL activity analyses. Protein concentration of supernatants was measured using the Bradford method (Bio-Rad, Hercules, CA). GCL activity was measured using our previously described method (Wu *et al.*, 2009). Briefly, one hundred μ l of supernatant, 100 μ l GCL reaction cocktail (400 mM Tris, 2 mM EDTA, 20 mM boric acid, 2 mM L-serine, 40 mM $MgCl_2$, 40 mM ATP and 40 mM L-glutamic acid, pH 7.4) and 100 μ l of 30 mM cysteine (optimized concentration determined in previous study) were added sequentially and incubated for 30 minutes at 37 °C. After the incubation, 100 μ l of 200 mM SSA were added to terminate the reaction. For GSH background measurement, 100 μ l of 200 mM SSA were added prior to the addition of GCL reaction cocktail and cysteine. The mixture was then centrifuged at $2,000 \times g$ for 10 minutes at 4 °C. Twenty μ l of supernatant were loaded into a black microplate, followed by the addition of 180 μ l of NDA solution (1:1:7 of 10 mM NDA: 0.5 M NaOH : 0.05 M Tris with a pH of 10.0, v/v/v), and the mixture was incubated in the dark at room temperature for 30 minutes. The fluorescence intensities of GSH-NDA and the sum of γ -GC-NDA and GSH-NDA were measured at 485 nm excitation and 538 nm emission, the difference being the γ -GC-NDA level. GCL activities were reported as nmol γ -GC/min/mg protein.

Analyses of GSH content

GSH content was measured following same protocol as described previously (Wu *et al.*, 2009). Briefly, the homogenates prepared as described above were added an equal volume of ice cold 10% SSA to precipitate all proteins and stabilize glutathione. The mixture was allowed to rest on ice for 10 minutes then centrifuged at $2,000 \times g$ for 10 minutes at 4 °C. The supernatant was used for GSH content measurement. Twenty-five μ l of the supernatant were mixed in a flat-bottomed black 96-well microplate, with 100 μ l of 0.2 M NEM (in 0.02 M KOH) and 10 μ l of 10 mM TCEP and incubated in the dark at room temperature for 15 minutes. Fifty μ l of 0.5 M NaOH were added to each cell, followed by the addition of 10 μ l of 10 mM NDA (in DMSO). The mixture was incubated in the dark for 30 minutes at room

temperature, after which, GSH-NDA fluorescence intensities were measured at 485 nm excitation and 538 nm emission on a fluorescence plate reader (Molecular Devices, CA, USA). GSH content was reported as $\mu\text{mol/g}$ tissue.

Data analysis

All tests were conducted with at least three rats/group and repeated at least three times. All results were expressed as mean $\pm$ SD. One-way ANOVA was used to compare the difference among groups. Data were analyzed using SAS 9.2 unless otherwise mentioned (SAS Institute Inc. Cary, NC, USA).

Results

Overall Assessment of miRNA HiSeq Data

HiSeq high-throughput sequencing technology was used to generate miRNA expression profiles in liver tissues of arsenic treated and control rats. Independent miRNA expression profiles were generated using high-throughput sequencing from liver tissue of 15 individual SD rats, with each of three animals/group exposed to a varied concentration of iAs^{III} for 60 days. The total reads of each sample were in the range of 3,000,000 to 16,000,000 (Figure 1). The annotated reads varied widely with 3.9% in the sample of RNA-083 and up to 61.1% in the sample of RNA-123. Sample RNA-083 within the 0.1 mg/L group had the lowest annotation and perfect matched reads even though its total read count was the highest (Figure 1).

MiRNA Expression Profiles in Rat Liver Tissue Are Modified by iAs^{III} Exposure

Figure 2 contains the results of hierarchical clustering of the variance stabilized miRNA 'expression' profiles of 15 samples. The 5 rectangle overlays indicate the membership of the $k=5$ clusters when the dendrogram is cut to that size. The gray scale bar at the base of the figure indicates the arsenic exposure for each of the samples. A visual inspection of these results suggests that there is an association between iAs exposure and cluster membership. Table 1 provides a summary of the cluster membership as a function of iAs exposure for $k=5$. The Fisher's exact test provided a p -value of 0.0012 with an adjusted (to account for considering $k=2, 3, 4, 5, 6$ and 7) p -value of 0.0029, indicating that such an association is highly significant (Supplemental Table 2). These results support the hypothesis that the steady state pattern of miRNA expression in the liver tissue can be modified by arsenic exposure.

Figure 3 contains the results of the PCA analysis of the miRNA profiles. The first two principal components accounted for 46% of the overall variability in the full set of 15 samples and 49% of the variability for the subset of the 14 samples when sample RNA-083 was excluded. Visual inspection of both principal components reveals a positive trend in the first principal component coefficients as a function of iAs exposure. The observed trends with respect to the first principal component are statistically significant as the Jonckheere trend tests yielded p -values of 0.021 and 0.0026 for the 15 sample and 14 sample PCAs, respectively. Analysis of the trend associated with the second principal component depended on the inclusion/exclusion of RNA-083. The trend with respect to the second principal

component was significant (p -value=0.016) when RNA-083 was included but was insignificant (p -value=0.4067) when it was excluded. These results support the hypothesis that the steady state pattern of miRNA expression in the liver tissue can be modified, in a concentration dependent manner, by arsenic exposure.

It should be noted that, as shown in the Figure 1, sample RNA-083 had a very high total counts but relatively low counts with respect to annotated and perfect matched reads. In both clustering and principal component analysis, the sample RNA-083 was an outlier and incorrectly assigned to other groups compared to the other two samples (RNA-074 and RNA-075) in the same concentration group.

Multiple miRNAs Are Differentially Expressed in Liver Tissue in Response to Arsenic Exposure

Table 2 provides the Jonckheere's trend test p -values and the Benjamini and Hochberg q -values for the 26 miRNAs with q -values less than 0.20 (i.e., corresponding to a false discovery rate of 20%). On the basis of q values, the top 4 up-regulated miRNAs and the top 2 down-regulated miRNAs were selected for verification by qPCR. Figures 4, 5, and 6 contain the RNA-seq and qPCR results for the six miRNAs (miR-151, miR-183, miR-148b, miR-192, miR-26a, and miR-423) which were selected for verification. The first column of each figure contains the RNA-seq normalized expression values as a function of iAs concentration while the second column contains the qPCR fold change estimates as a function of the same concentration levels. The expression levels resulting from Hi-Seq and qPCR of four of the six candidate miRNAs (miR-151, miR-183, miR-26a and miR-423) were correlated at a significant level of 0.05. MiR-148b provided a qPCR trend test p -value of 0.0672, which would have been significant at the 0.10 level. Only miR-192, with a p -value of 0.659, unquestionably failed qPCR verification. Although the trend is unimodal in general as showed in Figure 4 to 6, in a specific exposure concentration, the response of individual rat among a given group may vary. The miR-151 expression levels were significantly higher in the 0.1, 10.0 and 100.0 mg/L arsenic groups than in the control group with Hi-Seq results. In contrast, the miR-151 expression levels were significantly higher in the 1.0, 10.0 and 100.0 mg/L arsenic groups when samples were analyzed using qPCR. For miR-183, its expression was correlated tightly between Hi-Seq and qPCR analyses, and significantly increased in the 1.0, 10.0 and 100.0 mg/L arsenic groups compared to the control and 0.1 mg/L groups. At the p -value of 0.05 level, no significant changes of the expression of miR-148b and miR-192 were observed in comparison of arsenic exposure groups and control group with qPCR analysis.

Altered Hepatic GCL Activity and GSH Content Following Arsenic Exposure

Figure 7 showed that arsenic exposure altered hepatic GCL activity and GSH content in rats. At low concentrations, e.g. 0.1 mg/L, long term arsenic exposure significantly increased GCL activities by 86.1% compared to the control, whereas higher concentration treatment (10 and 100 mg/L) depleted GCL activities significantly to 63.8% and 52.8% of the control, respectively. Hepatic GSH contents were only slightly increased at the lower concentration group compared to control rats, but it was non-significant in either 0.1 or 1.0 mg/L exposure group. In comparison, exposure to high concentration of arsenic (10 and 100 mg/L)

significantly reduced GSH content by almost 2.5 fold, and no difference was observed between the 10 mg/L group and 100 mg/L group.

We further measured mRNA expression of *Nfe2l2*, *Gclc*, *Gclm*, *Keap1* and *Bach1* in rat liver following chronic arsenic exposure. As shown in the Figure 8, the expressions of *Gclc*, *Gclm*, *Keap1* and *Bach1* mRNAs following arsenic exposure showed a similar pattern, with an increase at the lower concentrations of iAs and a decrease at the higher concentrations. However, there was only a slight but non-significant change in mRNA expression of *Nfe2l2*, suggesting that *Nfe2l2* did not respond to arsenic-exposure *in vivo*.

Identification of miRNA Targets in Nfe2l2 Signaling Pathway

We analyzed the 26 arsenic-responsive miRNAs (Table 2) using prediction tools developed by miRNA.org to identify miRNAs that were predicted to regulate mRNA expression for *Nfe2l2*, *Gclc*, *Gclm*, *Keap1* and *Bach1*. We found that the predicted miRNAs regulating these genes are different between rats and human. Given the conserved nature of these identified miRNAs, it is possible that the arsenic-responsive miRNAs in rats could also be altered in human exposure to arsenic. We thus listed the predicted miRNAs targeting these genes both in rat and human in Table 3. Among these identified miRNAs, miR-25 has been predicted to target multiple genes, including *Gclc*, *Gclm* and *Bach1*. No arsenic-responsive miRNA was predicted to target *Keap1* in rat or human.

Discussion

A growing body of evidence has linked arsenic exposure to epigenetic modifications, which has been proposed to play a key role in arsenic-induced toxicities and carcinogenicity (Ren *et al.*, 2011). *In vitro*, studies support an association of arsenic exposure and altered miRNA expression (Wang *et al.*, 2011; Shan *et al.*, 2012). However, it is largely unknown how miRNA expression responds to chronic arsenic exposure *in vivo*. The research presented here showed that exposure to sodium arsenite for 60 days was able to alter the miRNA profiles in liver tissue of Sprague Dawley (SD) rats, and induce concentration-dependent changes of the expressions of multiple miRNAs. Following arsenic exposure, low concentrations increased and high concentrations reduced the expression of *Gclc* and *Gclm* mRNAs, which largely correlated with the alteration of hepatic GCL activity and GSH levels post arsenic exposure. Our data suggest that the changes in expression of *Gclc* and *Gclm* mRNAs were likely not due to changes in the *Nfe2l2* signaling pathway. Further analysis indicated that several of the identified arsenic-responsive miRNAs are predicted to target *Gclc* and *Gclm*, but these relationships are far from certain and warrant further studies. Together, this study suggests that changes of miRNA expression induced by arsenic exposure *in vivo* could play a role in arsenic-induced toxicity and carcinogenicity.

Hierarchical clustering of our experimental data suggests that the steady state pattern of miRNA expression in the liver tissue can be modified by arsenic exposure. Specifically, the clustering of the 15 liver samples formed 5 clusters largely based on arsenic concentrations. The Fishers Exact Test of the cluster composition yielded an adjusted *p*-value of 0.002, strongly supporting the hypothesis of association between miRNA expression and arsenic exposure.

Trend analyses were utilized to identify miRNAs with statistically significant concentration dependent differences in expression across treatment conditions. The Hi-Seq concentration dependent expression profiles for the top four miRNAs with significant up-regulated expression and top two miRNAs with significant down-regulated miRNAs post arsenic exposure were subjected to qPCR verification. The Hi-Seq and qPCR profiles were highly correlated for five of the six miRNAs (miR-151, miR-183, miR-26a, miR-423 and miR-148b). Only miR-192 failed the verification, as its expression was unaltered with arsenic treatment in qPCR analysis.

The miRNAs are a class of small RNAs that do not encode for proteins but are involved in the regulation of expression of multiple genes by inducing mRNA degradation or interfering with mRNA translation. This makes miRNA the largest class of gene regulators presently identified (Bartel, 2004). However, despite the significant progress made towards understanding the mechanisms of action of miRNA, much less is known about each individual miRNA. Among these five co-verified miRNAs (miR-151, miR-183, miR-26a, miR-423 and miR-148b), miR-151 and miR-183 were significantly up-regulated by arsenic exposure. It is reported that miR-151 expression was related to the ventricular arrhythmias vulnerability in rats (Zhang *et al.*, 2013). Additionally, miR-151 has been suggested to play a role in breast cancer metastasis and tumor cell proliferation in both human and *in vitro* studies (Chiyomaru *et al.*, 2012; Krell *et al.*, 2012; McNally *et al.*, 2013). The miR-183 has been suggested to influence the development and function in neurosensory organs (Li *et al.*, 2010; Li *et al.*, 2013; Lumayag *et al.*, 2013). Meanwhile, up-regulation of miR-183 has been linked to lung, breast, prostate and cervical cancers, and was associated with tumor cell migration, metastasis and invasion (Wang *et al.*, 2008; Sarver *et al.*, 2010; Ueno *et al.*, 2013). In contrast, miR-26a was significantly down-regulated by arsenic, and has been suggested to play roles in regulating the glucose metabolism, vascular smooth muscle cell (VSMC) function, pathological and physiological angiogenesis, and be probably involved in cardiovascular and cerebrovascular disorders and arthritis severity (Bai *et al.*, 2011; Chai *et al.*, 2013; Fu *et al.*, 2013; Icli *et al.*, 2013; Jiang *et al.*, 2014). Besides these roles, miR-26a is also generally thought to be a tumor suppressor (Chen *et al.*, 2011; Reuland *et al.*, 2012; Salvatori *et al.*, 2012; Yang *et al.*, 2013). The expression of circulating miR-423 is related to myocardial function (Goldraich *et al.*, 2014). Here, we showed that arsenic exposure significantly down-regulated the expression of miR-423 in rat liver tissue. Furthermore, accumulated evidence suggested that the miR-148 family plays important roles in nephropathy, diabetes, atherosclerotic lesions, and chronic fatigue syndrome (Bidzhekov *et al.*, 2012; Brenu *et al.*, 2012; Nielsen *et al.*, 2012; Serino *et al.*, 2012). Available studies have reported opposite effects of miR-148b in different types of cancer. Increased expression of miR-148b suppress cell growth of colorectal and pancreatic cancers (Song *et al.*, 2012; Zhao *et al.*, 2013), but it has also been linked to the development of ovarian carcinoma and melanoma (Chang *et al.*, 2012; Serino *et al.*, 2012). Together, it suggested that the biologic consequences of altered miRNAs induced by arsenic are complicated, and exactly how these changes in individual miRNA contribute to arsenic-induced toxicity warrants further study.

The mode of action of arsenicals is very complicated, and oxidative stress associated with arsenic exposure has been cited as a possible mechanism (Kitchin and Ahmad, 2003; Hughes and Kitchin, 2006; Hughes, 2009; Jomova *et al.*, 2011; Hall *et al.*, 2013). Maintaining the balance between antioxidant defenses and oxidants is critical for normal cell function. Arsenic-induced oxidative stress has been reported in numerous *in vitro* and *in vivo* studies (Kitchin and Conolly, 2010). Glutathione, as the most abundant non-protein sulfhydryl compound in cells, has been suggested to play an important role in arsenic metabolism and detoxification, and for antagonizing arsenic-induced oxidative stress (Flora, 2011). GSH conjugates are transported out of the cell and excreted resulting in the loss of GSH. Our study showed that sub-chronic exposure to high concentration of arsenic significantly diminished GSH levels, which is consistent with previous observations that chronic or acute exposure to high concentration iAs can deplete GSH in mice and rats, respectively (Maiti and Chatterjee, 2001; Flora *et al.*, 2008). Significant loss of GSH could be caused by the utilization of GSH through its binding to arsenic and its methylated metabolites. It could also be depleted secondarily to the production of lipid peroxidation products resulting from arsenic induced formation of ROS. In this study, GSH also can be suppressed, at least partly, as a result of decreased biosynthesis because hepatic GCL activities decreased substantially at high iAs exposures.

A previous study in mice suggested that lower arsenic concentration increased hepatic GSH level with 2 months of exposure, whereas 4 months of exposure decreased GSH level (Santra *et al.*, 2000b), suggesting a transient adaptation in GSH biosynthesis. The *de novo* biosynthesis of GSH is catalyzed by GCL and GS, and GCL is generally considered as the rate limiting enzyme. In the present study low concentration arsenic increased the expression of both *Gclc* and *Gclm*, while high concentration reduced the expression. Further, changes in *Gclc* and *Gclm* mRNA expression were parallel to the hepatic GCL activity. It thus suggests that following arsenic exposure altered mRNA expression of GCLC and GCLM results in consequent changes in GCL levels. An increased GCL mRNA and GCL activity with lower arsenic concentration exposure indicates an adaptive response of GCL expression and activity to this stressor. However, the elevated GCL activity did not result in an increase in GSH content in liver in the present study. One explanation could be that besides GCL, GS also contributes to the regulation of GSH biosynthesis under arsenic exposure condition in rats. A previous study showed that GS may also play an important role in the regulation of GSH biosynthesis (Choi *et al.*, 2000). Yet it is possible that hepatic GSH biosynthesis was induced, and the GSH induction was counteracted by increased GSH utilization and/or GSH excretion, and thus the hepatic GSH content remained unchanged. Hepatic GSH can be lost after formation of GSH conjugates which are then transported out of the liver and excreted. Alternatively, hepatic GSH may also be transported to other tissues in its original form. In multi-organ systems, the liver is generally the major site of GSH synthesis, and a portion of synthesized GSH is transported to plasma and taken up *via* γ -glutamyltransferase dependent transport by other tissues to help meet the requirements of these tissues. It is possible that when the biosynthesis of GSH in liver is induced, the organism increases the efflux and ensures the physiological functions of other tissues by maintaining sufficient GSH content in them. Measurement of GSH efflux may assist in clarifying this issue.

Numerous studies have reported that arsenic exposure can activate the Nfe2l2 signaling pathway in a variety of human cell lines (Lau *et al.*, 2013). Activated Nfe2l2 signaling was also linked to malignant transformation of human keratinocytes induced by arsenic (Pi *et al.*, 2008). The arsenic-induced activation of Nfe2l2 led to an increased intracellular GSH and elevated expression of Nfe2l2 targeting genes, including *Gclc* (Pi *et al.*, 2008). However, all of these studies have been done in cell models, and the effects *in vivo* remain unknown. In our study, arsenic exposure had no effect on the expression of *Nfe2l2*, but showed an increase in the lower concentration and a decrease in the higher concentration of *Keap1* and *Bach1*. Keap1 binds to Nfe2l2, which then leads to ubiquitination and degradation of Nfe2l2 (Kensler and Wakabayashi, 2010). The dissociation of Nfe2l2 from Keap1 under conditions of oxidative stress leads to the activation of the Nfe2l2 pathway (Itoh *et al.*, 1999). Bach1 competes with Nfe2l2 for antioxidant response element (ARE), thus leading to negative regulation of Nfe2l2 pathway (Dhakshinamoorthy *et al.*, 2005). Theoretically, while Keap1 represses Nfe2l2 protein at posttranslational level, and Bach1 simply competes for the ARE with Nfe2l2, neither of them affects the Nfe2l2 mRNA levels. However, the altered expressions of Keap1 and Bach1 could affect the transcriptional expression levels of downstream genes of Nfe2l2, for example, *Gclc* and *Gclm*. With increased KEAP1 and BACH1 levels and unaltered NFE2L2 expression, *Gclc* and *Gclm* mRNA expression could be inhibited *via* the Nfe2l2 regulatory pathway. Unexpectedly, in this study, *Gclc* and *Gclm* mRNA expression were increased at low concentrations, even though mRNA expression of *Keap1* and *Bach1* were also increased. Additionally, across all the arsenic concentration groups, hepatic *Gclc* and *Gclm* expression responded to arsenic biphasically while Nfe2l2 level remained stable. The unrelated alteration trend between GCL and its upstream regulators in Nfe2l2 pathway suggests that following arsenic exposure *in vivo*, hepatic GCL expression is independent of the Nfe2l2 pathway. Previously, a study in murine hepatocytes also suggested altered transcription of both *Gclc* and *Gclm* by arsenic exposure was independent of the Nfe2l2 signaling pathway (Thompson *et al.*, 2009). An additional study suggested the possibility that arsenic induced GCL expression was not regulated through the Nfe2l2 signaling pathway in primary mouse hepatocytes (Sumi *et al.*, 2007). Based on the above, there is a possibility that after exposure to arsenic *GCL* expression is not regulated by Nfe2l2 signaling pathway in liver tissue *in vivo* and *in vitro* in rodent species. MiR-148b and miR-25 were predicted to target *Gclc*. Both of them were up-regulated in a concentration-dependent manner following arsenic exposure. The expression changes in these two miRNAs were not completely parallel to the expression of *Gclc* mRNA, which showed higher expression at low concentration of iAs, but lower expression with higher concentration of iAs. It is not clear whether the changes in these miRNAs only are responsible for the changes of *Gclc* mRNA expression. With regard to *Gclm* mRNA, the expression of two miRNAs predicted to target it, miR-25 and miR-125b, were increased and decreased, respectively. It remains to be seen how and whether these two miRNAs interact to regulate the expression of *Gclm* following arsenic exposure. Further studies are warranted to decipher these unanswered questions, and to determine whether the observations made in this animal study translate to human exposures.

The current study has some limitations that need to be addressed in future studies. Arsenic was administered through the drinking water. Although our evaluation of drinking water

consumption by each group of rats showed no significant difference, the arsenic exposure level for individual rats could still vary, which may influence a precise dose-response relationship. Additionally, the exposure concentrations of this study were ranged from 0.1mg/L to 100 mg/L, spanning from the environmental exposure levels to relatively high concentrations. The effects of arsenic on miRNA expression were mainly observed in the high concentration groups. While it is not uncommon to use higher dose of arsenic in the animal studies, our results may not represent the responses in the low dose scenario but provide the mechanistic information in high exposure settings. Our analysis of mRNA expression was focused on Nfe2l2 relevant target genes. It will be of broader interest to the field if such analyses can undertake and incorporate the effects of miRNAs on global gene expression. These limitations will be addressed in our future work.

Conclusions

In summary, we show that sub-chronic arsenic exposure disrupts genome wide miRNA expression and induces a concentration-dependent effect on miRNA expression in liver tissue of rats. In future studies we plan to decipher further mechanisms and biological consequences of these altered miRNAs *in vivo* caused by chronic arsenic exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by Zhejiang Provincial Natural Science Foundation (Y2110648), China and a start-up fund (to X.R.) provided by University at Buffalo. Support was also provided by NIEHS grant R21ES022329 and R01ES022629 to X.R. and P30ES007033 to T.J.K. Scientific Research Foundation for the Returned Overseas Chinese Scholars (China State Education Ministry), Zhejiang Province Human Resources and Social Security Bureau, Wenzhou Medical College Scientific Research Foundation (QTJ10005), and Wenzhou Municipal Sci-Tech Bureau, China (H20110024) also provided the supports.

Abbreviations

BACH1	BTB and CNC homology 1
GCL	Glutamate-cysteine ligase
GCLC	Glutamate-cysteine ligase, catalytic subunit
GCLM	Glutamate-cysteine ligase, modifier subunit
GSH	Glutathione
iAs	Inorganic arsenic
IARC	International Agency for Research on Cancer
KEAP1	Kelch-like ECH-associated protein 1
miRNA	MicroRNA
NFE2L2	Nuclear factor, erythroid 2-like 2

qPCR Quantitative Real-time PCR
SD Sprague Dawley rats

References

- Abernathy CO, Thomas DJ, Calderon RL. Health effects and risk assessment of arsenic. *J Nutr.* 2003; 133:1536S–1538S. [PubMed: 12730460]
- Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol.* 2010; 11:R106. [PubMed: 20979621]
- Andrew AS, Jewell DA, Mason RA, Whitfield ML, Moore JH, Karagas MR. Drinking-water arsenic exposure modulates gene expression in human lymphocytes from a u.s. Population. *Environ Health Perspect.* 2008; 116:524–531. [PubMed: 18414638]
- Bai Y, Wang L, Sun L, Ye P, Hui R. Circulating microRNA-26a: Potential predictors and therapeutic targets for non-hypertensive intracerebral hemorrhage. *Medical Hypotheses.* 2011; 77:488–490. [PubMed: 21764522]
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004; 116:281–297. [PubMed: 14744438]
- Benbrahim-Tallaa L, Waterland RA, Styblo M, Achanzar WE, Webber MM, Waalkes MP. Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. *Toxicol Appl Pharmacol.* 2005; 206:288–298. [PubMed: 16039940]
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing". *Journal of the Royal Statistical Society, Series B (Methodological).* 1995; 57:289–300.
- Betel D, Koppal A, Agius P, Sander C, Leslie C. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. *Genome Biol.* 2010; 11:R90. [PubMed: 20799968]
- Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res.* 2008; 36:D149–D153. [PubMed: 18158296]
- Bidzhekov K, Gan L, Denecke B, Rostalsky A, Hristov M, Koepfel TA, Zerneck A, Weber C. microRNA expression signatures and parallels between monocyte subsets and atherosclerotic plaque in humans. *Thrombosis and Haemostasis.* 2012; 107:619–625. [PubMed: 22370758]
- Bollati V, Marinelli B, Apostoli P, Bonzini M, Nordio F, Hoxha M, Pegoraro V, Motta V, Tarantini L, Cantone L, Schwartz J, Bertazzi PA, Baccarelli A. Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. *Environ Health Perspect.* 2010; 118:763–768. [PubMed: 20061215]
- Bourdonnay E, Morzadec C, Sparfel L, Galibert MD, Jouneau S, Martin-Chouly C, Fardel O, Vernhet L. Global effects of inorganic arsenic on gene expression profile in human macrophages. *Molecular immunology.* 2009; 46:649–656. [PubMed: 19128835]
- Brenu EW, Ashton KJ, van Driel M, Staines DR, Peterson D, Atkinson GM, Marshall-Gradisnik SM. Cytotoxic lymphocyte microRNAs as prospective biomarkers for Chronic Fatigue Syndrome/ Myalgic Encephalomyelitis. *Journal of Affective Disorders.* 2012; 141:261–269. [PubMed: 22572093]
- Bustaffa E, Stoccoro A, Bianchi F, Migliore L. Genotoxic and epigenetic mechanisms in arsenic carcinogenicity. *Arch Toxicol.* 2014; 88:1043–1067. [PubMed: 24691704]
- Chai Z-T, Kong J, Zhu X-D, Zhang Y-Y, Lu L, Zhou J-M, Wang L-R, Zhang K-Z, Zhang Q-B, Ao J-Y, Wang M, Wu W-Z, Wang L, Tang Z-Y, Sun H-C. MicroRNA-26a Inhibits Angiogenesis by Down-Regulating VEGFA through the PIK3C2 alpha/Akt/HIF-1 alpha Pathway in Hepatocellular Carcinoma. *Plos One.* 2013;8.
- Chanda S, Dasgupta UB, Guhamazumder D, Gupta M, Chaudhuri U, Lahiri S, Das S, Ghosh N, Chatterjee D. DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol Sci.* 2006; 89:431–437. [PubMed: 16251483]

- Chang H, Zhou X, Wang ZN, Song YX, Zhao F, Gao P, Chiang Y, Xu HM. Increased expression of miR-148b in ovarian carcinoma and its clinical significance. *Mol Med Rep.* 2012; 5:1277–1280. [PubMed: 22344713]
- Chen L, Zheng J, Zhang Y, Yang L, Wang J, Ni J, Cui D, Yu C, Cai Z. Tumor-specific expression of microRNA-26a suppresses human hepatocellular carcinoma growth via cyclin-dependent and -independent pathways. *Mol Ther.* 2011; 19:1521–1528. [PubMed: 21610700]
- Chiyomaru T, Yamamura S, Zaman MS, Majid S, Deng G, Shahryari V, Saini S, Hirata H, Ueno K, Chang I, Tanaka Y, Tabatabai ZL, Enokida H, Nakagawa M, Dahiya R. Genistein suppresses prostate cancer growth through inhibition of oncogenic microRNA-151. *PLoS One.* 2012; 7:e43812. [PubMed: 22928040]
- Choi J, Liu RM, Kundu RK, Sangiorgi F, Wu W, Maxson R, Forman HJ. Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J Biol Chem.* 2000; 275:3693–3698. [PubMed: 10652368]
- Chu F, Ren X, Chasse A, Hickman T, Zhang L, Yuh J, Smith MT, Burlingame AL. Quantitative mass spectrometry reveals the epigenome as a target of arsenic. *Chem Biol Interact.* 2011; 192:113–117. [PubMed: 21075096]
- Deneke SM, Fanburg BL. Regulation of cellular glutathione. *Am J Physiol.* 1989; 257:L163–L173. [PubMed: 2572174]
- Dhakshinamoorthy S, Jain AK, Bloom DA, Jaiswal AK. Bach1 competes with Nrf2 leading to negative regulation of the antioxidant response element (ARE)-mediated NAD(P)H:quinone oxidoreductase 1 gene expression and induction in response to antioxidants. *J Biol Chem.* 2005; 280:16891–16900. [PubMed: 15734732]
- Elamin BK, Callegari E, Gramantieri L, Sabbioni S, Negrini M. MicroRNA response to environmental mutagens in liver. *Mutat Res.* 2011; 717:67–76. [PubMed: 21514310]
- Flora SJ. Arsenic-induced oxidative stress and its reversibility. *Free Radic Biol Med.* 2011; 51:257–281. [PubMed: 21554949]
- Flora SJ, Chouhan S, Kannan GM, Mittal M, Swarnkar H. Combined administration of taurine and monoisoamyl DMSA protects arsenic induced oxidative injury in rats. *Oxid Med Cell Longev.* 2008; 1:39–45. [PubMed: 19794907]
- Fraser JA, Kansagra P, Kotecki C, Saunders RD, McLellan LI. The modifier subunit of Drosophila glutamate-cysteine ligase regulates catalytic activity by covalent and noncovalent interactions and influences glutathione homeostasis in vivo. *J Biol Chem.* 2003; 278:46369–46377. [PubMed: 12954617]
- Fu X, Jin L, Wang X, Luo A, Hu J, Zheng X, Tsark WM, Riggs AD, Ku HT, Huang W. MicroRNA-26a targets ten eleven translocation enzymes and is regulated during pancreatic cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America.* 2013; 110:17892–17897. [PubMed: 24114270]
- Ge Y, Gong Z, Olson JR, Xu P, Buck MJ, Ren X. Inhibition of monomethylarsonous acid (MMA(III))-induced cell malignant transformation through restoring dysregulated histone acetylation. *Toxicology.* 2013; 312:30–35. [PubMed: 23891734]
- Gielen H, Remans T, Vangronsveld J, Cuypers A. MicroRNAs in Metal Stress: Specific Roles or Secondary Responses? *Int J Mol Sci.* 2012; 13:15826–15847. [PubMed: 23443096]
- Goldraich LA, Martinelli NC, Matte U, Cohen C, Andrades M, Pimentel M, Biolo A, Clausell N, Rohde LE. Transcoronary gradient of plasma microRNA 423-5p in heart failure: evidence of altered myocardial expression. *Biomarkers.* 2014; 19:135–141. [PubMed: 24506564]
- Hall MN, Niedzwiecki M, Liu X, Harper KN, Alam S, Slavkovich V, Ilievski V, Levy D, Siddique AB, Parvez F, Mey JL, van Geen A, Graziano J, Gamble MV. Chronic arsenic exposure and blood glutathione and glutathione disulfide concentrations in Bangladeshi adults. *Environ Health Perspect.* 2013; 121:1068–1074. [PubMed: 23792557]
- Hissin PJ, Hilf R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal Biochem.* 1976; 74:214–226. [PubMed: 962076]
- Hughes MF. Arsenic methylation, oxidative stress and cancer--is there a link? *Journal of the National Cancer Institute.* 2009; 101:1660–1661. [PubMed: 19933940]

- Hughes, MF.; Kitchin, KT. Arsenic, oxidative stress, and carcinogenesis. In: KK, S., editor. *Oxidative Stress, Disease and Cancer*. London, UK: Imperial College Press; 2006. p. 825-850.
- IARC. Arsenic, Metals, Fibres and Dusts. *IARC Monogr Eval Carcinog Risks Hum*. 2012;41–93.
- Icli B, Wara AKM, Moslehi J, Sun X, Plovie E, Cahill M, Marchini JF, Schissler A, Padera RF, Shi J, Cheng H-W, Raghuram S, Arany Z, Liao R, Croce K, MacRae C, Feinberg MW. MicroRNA-26a Regulates Pathological and Physiological Angiogenesis by Targeting BMP/SMAD1 Signaling. *Circulation Research*. 2013; 113:1231–U1127. [PubMed: 24047927]
- Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev*. 1999; 13:76–86. [PubMed: 9887101]
- Jiang C, Zhu W, Xu J, Wang B, Hou W, Zhang R, Zhong N, Ning Q, Han Y, Yu H, Sun J, Meng L, Lu S. MicroRNA-26a negatively regulates toll-like receptor 3 expression of rat macrophages and ameliorates pristane induced arthritis in rats. *Arthritis Research & Therapy*. 2014;16.
- Jo WJ, Ren X, Chu F, Aleshin M, Wintz H, Burlingame A, Smith MT, Vulpe CD, Zhang L. Acetylated H4K16 by MYST1 protects UROtsa cells from arsenic toxicity and is decreased following chronic arsenic exposure. *Toxicol Appl Pharmacol*. 2009; 241:294–302. [PubMed: 19732783]
- Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, Rhodes CJ, Valko M. Arsenic: toxicity, oxidative stress and human disease. *J Appl Toxicol*. 2011; 31:95–107. [PubMed: 21321970]
- Jonckheere AR. A distribution-free k-sample test again ordered alternatives. *Biometrika*. 1954; 41:133–145.
- Kensler TW, Wakabayashi N. Nrf2: friend or foe for chemoprevention? *Carcinogenesis*. 2010; 31:90–99. [PubMed: 19793802]
- Kitchin KT, Ahmad S. Oxidative stress as a possible mode of action for arsenic carcinogenesis. *Toxicol Lett*. 2003; 137:3–13. [PubMed: 12505428]
- Kitchin KT, Conolly R. Arsenic-induced carcinogenesis--oxidative stress as a possible mode of action and future research needs for more biologically based risk assessment. *Chem Res Toxicol*. 2010; 23:327–335. [PubMed: 20035570]
- Krell J, Frampton AE, Jacob J, Pellegrino L, Roca-Alonso L, Zeloof D, Alifrangis C, Lewis JS, Jiao LR, Stebbing J, Castellano L. The clinico-pathologic role of microRNAs miR-9 and miR-151-5p in breast cancer metastasis. *Mol Diagn Ther*. 2012; 16:167–172. [PubMed: 22489664]
- Lau A, Whitman SA, Jaramillo MC, Zhang DD. Arsenic-mediated activation of the Nrf2-Keap1 antioxidant pathway. *J Biochem Mol Toxicol*. 2013; 27:99–105. [PubMed: 23188707]
- Li G, Luna C, Qiu J, Epstein DL, Gonzalez P. Targeting of Integrin beta 1 and Kinesin 2 alpha by MicroRNA 183. *Journal of Biological Chemistry*. 2010; 285:5461–5471. [PubMed: 19940135]
- Li X, Kroin JS, Kc R, Gibson G, Chen D, Corbett GT, Pahan K, Fayyaz S, Kim J-S, van Wijnen AJ, Suh J, Kim S-G, Im H-J. Altered Spinal MicroRNA-146a and the MicroRNA-183 Cluster Contribute to Osteoarthritic Pain in Knee Joints. *Journal of Bone and Mineral Research*. 2013; 28:2512–2522. [PubMed: 23744481]
- Li X, Shi Y, Wei Y, Ma X, Li Y, Li R. Altered expression profiles of microRNAs upon arsenic exposure of human umbilical vein endothelial cells. *Environ Toxicol Pharmacol*. 2012; 34:381–387. [PubMed: 22728250]
- Liu D, Duan X, Dong D, Bai C, Li X, Sun G, Li B. Activation of the Nrf2 pathway by inorganic arsenic in human hepatocytes and the role of transcriptional repressor Bach1. *Oxid Med Cell Longev*. 2013; 2013:984546. [PubMed: 23738048]
- Liu RM, Choi J, Forman HJ. Oxidant-induced regulation of glutathione synthesis. *Curr Protoc Toxicol*. 2001 Chapter 6, Unit 6 7.
- Liu RM, Dickinson DA. Decreased synthetic capacity underlies the age-associated decline in glutathione content in Fisher 344 rats. *Antioxid Redox Signal*. 2003; 5:529–536. [PubMed: 14580307]
- Lu SC. Regulation of glutathione synthesis. *Mol Aspects Med*. 2009; 30:42–59. [PubMed: 18601945]
- Lumayag S, Haldin CE, Corbett NJ, Wahlin KJ, Cowan C, Turturro S, Larsen PE, Kovacs B, Witmer PD, Valle D, Zack DJ, Nicholson DA, Xu S. Inactivation of the microRNA-183/96/182 cluster

- results in syndromic retinal degeneration. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:E507–E516. [PubMed: 23341629]
- Ma Q. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*. 2013; 53:401–426. [PubMed: 23294312]
- Maiti S, Chatterjee AK. Effects on levels of glutathione and some related enzymes in tissues after an acute arsenic exposure in rats and their relationship to dietary protein deficiency. *Arch Toxicol*. 2001; 75:531–537. [PubMed: 11760813]
- Martinez-Pacheco M, Hidalgo-Miranda A, Romero-Cordoba S, Valverde M, Rojas E. mRNA and miRNA expression patterns associated to pathways linked to metal mixture health effects. *Gene*. 2014; 533:508–514. [PubMed: 24080485]
- McNally ME, Collins A, Wojcik SE, Liu J, Henry JC, Jiang J, Schmittgen T, Bloomston M. Concomitant dysregulation of microRNAs miR-151-3p and miR-126 correlates with improved survival in resected cholangiocarcinoma. *HPB (Oxford)*. 2013; 15:260–264. [PubMed: 23458262]
- Medeiros M, Zheng X, Novak P, Wnek SM, Chyan V, Escudero-Lourdes C, Gandolfi AJ. Global gene expression changes in human urothelial cells exposed to low-level monomethylarsonous acid. *Toxicology*. 2012; 291:102–112. [PubMed: 22108045]
- Miltonprabu S, Sumedha NC. Arsenic-induced hepatic mitochondrial toxicity in rats and its amelioration by diallyl trisulfide. *Toxicol Mech Methods*. 2014; 24:124–135. [PubMed: 24295472]
- Mohar I, Botta D, White CC, McConnachie LA, Kavanagh TJ. Glutamate cysteine ligase (GCL) transgenic and gene-targeted mice for controlling glutathione synthesis. *Curr Protoc Toxicol*. 2009 Chapter 6, Unit6 16.
- Nielsen LB, Wang C, Sorensen K, Bang-Berthelsen CH, Hansen L, Andersen M-LM, Hougaard P, Juul A, Zhang C-Y, Pociot F, Mortensen HB. Circulating Levels of MicroRNA from Children with Newly Diagnosed Type 1 Diabetes and Healthy Controls: Evidence That miR-25 Associates to Residual Beta-Cell Function and Glycaemic Control during Disease Progression (vol 2012, 896362, 2012). *Experimental Diabetes Research*. 2012
- Niture SK, Khatri R, Jaiswal AK. Regulation of Nrf2—an update. *Free Radic Biol Med*. 2014; 66:36–44. [PubMed: 23434765]
- Pi J, Diwan BA, Sun Y, Liu J, Qu W, He Y, Styblo M, Waalkes MP. Arsenic-induced malignant transformation of human keratinocytes: involvement of Nrf2. *Free Radic Biol Med*. 2008; 45:651–658. [PubMed: 18572023]
- R-Core-Team. [R: A Language and Environment for Statistical Computing]. R Foundation for Statistical Computing. Vienna, Austria: 2012. ISBN: 3-900051-07-0. <http://www.R-project.org/>
- Rager JE, Bailey KA, Smeester L, Miller SK, Parker JS, Laine JE, Drobna Z, Currier J, Douillet C, Olshan AF, Rubio-Andrade M, Styblo M, Garcia-Vargas G, Fry RC. Prenatal arsenic exposure and the epigenome: Altered microRNAs associated with innate and adaptive immune signaling in newborn cord blood. *Environ Mol Mutagen*. 2013
- Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L. An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. *Environ Health Perspect*. 2011; 119:11–19. [PubMed: 20682481]
- Reuland SN, Smith SM, Bemis LT, Goldstein NB, Almeida AR, Partyka KA, Marquez VE, Zhang Q, Norris DA, Shellman YG. MicroRNA-26a Is Strongly Downregulated in Melanoma and Induces Cell Death through Repression of Silencer of Death Domains (SODD). *J Invest Dermatol*. 2012
- Salvatori B, Iosue I, Mangiavacchi A, Loddo G, Padula F, Chiaretti S, Peragine N, Bozzoni I, Fazi F, Fatica A. The microRNA-26a target E2F7 sustains cell proliferation and inhibits monocytic differentiation of acute myeloid leukemia cells. *Cell Death Dis*. 2012; 3:e413. [PubMed: 23096114]
- Santra A, Maiti A, Chowdhury A, Mazumder DN. Oxidative stress in liver of mice exposed to arsenic-contaminated water. *Indian J Gastroenterol*. 2000a; 19:112–115. [PubMed: 10918716]
- Santra A, Maiti A, Das S, Lahiri S, Charkaborty SK, Mazumder DN. Hepatic damage caused by chronic arsenic toxicity in experimental animals. *J Toxicol Clin Toxicol*. 2000b; 38:395–405. [PubMed: 10930056]

- Sarver AL, Li L, Subramanian S. MicroRNA miR-183 functions as an oncogene by targeting the transcription factor EGR1 and promoting tumor cell migration. *Cancer Res.* 2010; 70:9570–9580. [PubMed: 21118966]
- Schuliga M, Chouchane S, Snow ET. Upregulation of glutathione-related genes and enzyme activities in cultured human cells by sublethal concentrations of inorganic arsenic. *Toxicol Sci.* 2002; 70:183–192. [PubMed: 12441363]
- Sekhar KR, Yan XX, Freeman ML. Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. *Oncogene.* 2002; 21:6829–6834. [PubMed: 12360409]
- Serino G, Sallustio F, Cox SN, Pesce F, Schena FP. Abnormal miR-148b expression promotes aberrant glycosylation of IgA1 in IgA nephropathy. *J Am Soc Nephrol.* 2012; 23:814–824. [PubMed: 22362909]
- Shan H, Zhang Y, Cai B, Chen X, Fan Y, Yang L, Liang H, Song X, Xu C, Lu Y, Yang B, Du Z. Upregulation of microRNA-1 and microRNA-133 contributes to arsenic-induced cardiac electrical remodeling. *Int J Cardiol.* 2012
- Song Y, Xu Y, Wang Z, Chen Y, Yue Z, Gao P, Xing C, Xu H. MicroRNA-148b suppresses cell growth by targeting cholecystikinin-2 receptor in colorectal cancer. *Int J Cancer.* 2012; 131:1042–1051. [PubMed: 22020560]
- Sturchio E, Colombo T, Boccia P, Carucci N, Meconi C, Minoia C, Macino G. Arsenic exposure triggers a shift in microRNA expression. *Sci Total Environ.* 2014; 472:672–680. [PubMed: 24317173]
- Sturchio E, Colombo T, Carucci N, Meconi C, Boccia P, Macino G. [Molecular biomarkers in workers and population exposed to inorganic arsenic: preliminary study in vitro]. *G Ital Med Lav Ergon.* 2012; 34:678–681. [PubMed: 23405750]
- Sumi D, Manji A, Shinkai Y, Toyama T, Kumagai Y. Activation of the Nrf2 pathway, but decreased gamma-glutamylcysteine synthetase heavy subunit chain levels and caspase-3-dependent apoptosis during exposure of primary mouse hepatocytes to diphenylarsinic acid. *Toxicol Appl Pharmacol.* 2007; 223:218–224. [PubMed: 17628625]
- Thompson JA, White CC, Cox DP, Chan JY, Kavanagh TJ, Fausto N, Franklin CC. Distinct Nrf1/2-independent mechanisms mediate As 3+-induced glutamate-cysteine ligase subunit gene expression in murine hepatocytes. *Free radical biology & medicine.* 2009; 46:1614–1625. [PubMed: 19328227]
- Ueno K, Hirata H, Shahryari V, Deng G, Tanaka Y, Tabatabai ZL, Hinoda Y, Dahiya R. microRNA-183 is an oncogene targeting Dkk-3 and SMAD4 in prostate cancer. *Br J Cancer.* 2013
- Valcheva-Kuzmanova S, Borisova P, Galunska B, Krasnaliev I, Belcheva A. Hepatoprotective effect of the natural fruit juice from *Aronia melanocarpa* on carbon tetrachloride-induced acute liver damage in rats. *Exp Toxicol Pathol.* 2004; 56:195–201. [PubMed: 15625789]
- Wang G, Mao W, Zheng S. MicroRNA-183 regulates Ezrin expression in lung cancer cells. *FEBS Lett.* 2008; 582:3663–3668. [PubMed: 18840437]
- Wang Z, Zhao Y, Smith E, Goodall GJ, Drew PA, Brabletz T, Yang C. Reversal and prevention of arsenic-induced human bronchial epithelial cell malignant transformation by microRNA-200b. *Toxicol Sci.* 2011; 121:110–122. [PubMed: 21292642]
- Wu H, White CC, Isanhart JP, McBride TJ, Kavanagh TJ, Hooper MJ. Optimization and application of glutamate cysteine ligase measurement in wildlife species. *Ecotoxicol Environ Saf.* 2009; 72:572–578. [PubMed: 18403016]
- Yamamoto M, Singh A, Sava F, Pui M, Tebbutt SJ, Carlsten C. MicroRNA expression in response to controlled exposure to diesel exhaust: attenuation by the antioxidant N-acetylcysteine in a randomized crossover study. *Environ Health Perspect.* 2013; 121:670–675. [PubMed: 23584289]
- Yang X, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, Gao XM, Qiao P, Zheng Y, Sheng YY, Wei JW, Zhou HJ, Ren N, Ye QH, Dong QZ, Qin LX. MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting IL-6-Stat3 pathway. *Hepatology.* 2013
- Zhang Y, Wang R, Du W, Wang S, Yang L, Pan Z, Li X, Xiong X, He H, Shi Y, Liu X, Yu S, Bi Z, Lu Y, Shan H. Downregulation of miR-151-5p contributes to increased susceptibility to

arrhythmogenesis during myocardial infarction with estrogen deprivation. PLoS One. 2013; 8:e72985. [PubMed: 24039836]

Zhao G, Zhang JG, Liu Y, Qin Q, Wang B, Tian K, Liu L, Li X, Niu Y, Deng SC, Wang CY. miR-148b functions as a tumor suppressor in pancreatic cancer by targeting AMPKalpha1. Mol Cancer Ther. 2013; 12:83–93. [PubMed: 23171948]

Zhao JY, Lu N, Yan Z, Wang N. SAHA and curcumin combinations co-enhance histone acetylation in human cancer cells but operate antagonistically in exerting cytotoxic effects. J Asian Nat Prod Res. 2010; 12:335–348. [PubMed: 20496190]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- Chronic arsenic exposure induces changes of hepatic miRNA expression profiles
- Hepatic GCL activity and GSH level in rats are altered following arsenic exposure
- Arsenic induced *GCL* expression change is independent to Nfe2l2-signaling pathway
- Expression of several miRNAs predicted to target *GCL* changed after arsenic exposure

Figure 1. Summary of Hi-Seq sequencing results

Total, annotated and precise matched reads of each sample were summarized. The ranges of total reads were from 3,000,000 to 16,000,000, and the annotated reads varied from 3.9% to more than 60% of total reads.

Figure 2. Unsupervised hierarchical clustering of miRNA expression data from liver tissue of 15 rats exposed to different concentrations of sodium arsenite

The miRNA profiles of 15 samples were clustered using the Manhattan distance metric in conjunction with Ward's minimum variance clustering method. The 5 rectangle overlays indicate the membership of the 5 clusters when the dendrogram is cut to that size. The gray scale bar at the base of the figure indicates the arsenic exposure for each of the samples. The association between iAs exposure and cluster membership is statistically significant (Fisher's exact test, P -value=0.0012), which strongly supports the hypothesis that the steady state pattern of miRNA expression in the liver tissue can be modified by arsenic exposure.

Figure 3. Principle components analysis (PCA) of miRNA expression data

The miRNA profiles of 15 samples were subjected to a PCA analysis. The 15 samples were plotted according to their mappings into the space defined by the first two principal components (Upper panel). Sample RNA-083 was considered to possibly be an outlier so the PCA was repeated with respect to 14 sample miRNA profiles, excluding RNA-083 (Lower panel). The first two principal components accounted for 46% and 49% of the overall variability in the full set of 15 samples and the subset excluding RNA-083, respectively. When the profiles of miRNAs were projected into the two dimensional space

defined by the first two principal components, the portion of the variability in the RNA-seq expression profiles that can be attributed to iAs exposure levels is significant enough to have observed weak clustering of the profile projections of samples with similar exposures.

Figure 4. RNA-seq and qPCR results for miR-151 and miR-183

The first column contains the RNA-seq normalized expression values as a function of iAs concentration while the second column contains the qPCR fold change estimates as a function of the same concentration levels. Both miR-151 and miR-183 were verified by qPCR at a nominal level of 0.05. The Jonkheere's trend test p -values are listed in the figure subheadings for all plots. The Benjamini and Hochberg q -values are listed for the RNA-seq data. The data were expressed as mean $\pm$ SD ($n=3$). Oneway ANOVA were applied to compare the differences in expression level between various arsenic concentration groups and control group (*, $P < 0.05$; **, $P < 0.01$). The P values were from ANOVA results.

Figure 5. RNA-seq and qPCR results for miR-148b and miR-192

The first column contains the RNA-seq normalized expression values as a function of iAs concentration while the second column contains the qPCR fold change estimates as a function of the same concentration levels. Neither miR-148b nor miR-192 was verified by qPCR at a nominal level of 0.05. The Jonkheere's trend test *p*-values are listed in the figure subheadings for all plots. The Benjamini and Hochberg *q*-values are listed for the RNA-seq data. The data were expressed as mean $\pm$ SD (*n*=3). One-way ANOVA were applied to compare the differences in expression level between various arsenic concentration groups and control group (*, *P* < 0.05; **, *P* < 0.01). The *P* values were from ANOVA results.

Figure 6. RNA-seq and qPCR results for miR-26a and miR-423

The first column contains the RNA-seq normalized expression values as a function of iAs concentration while the second column contains the qPCR fold change estimates as a function of the same concentration levels. Both miR-26a and miR-423 were verified by qPCR at a nominal level of 0.05. The Jonkheere's trend test *p*-values are listed in the figure subheadings for all plots. The Benjamini and Hochberg *q*-values are listed for the RNA-seq data. The data were expressed as mean $\pm$ SD ($n=3$). One-way ANOVA were applied to compare the differences in expression level between various arsenic concentration groups and control group (*, $P < 0.05$; **, $P < 0.01$). The *P* values were from ANOVA results.

Figure 7. Responses of hepatic GCL activity and GSH level to sub-chronic arsenic exposure in rats

Optimized experimental conditions were applied. Three rats were used for each concentration point. Low concentration (0.1 mg/L) arsenic exposure significantly increased GCL activities, whereas higher concentration treatment (10 and 100 mg/L) depleted GCL activities compared to the control. Hepatic GSH levels were significantly reduced with high concentration exposure (10 and 100 mg/L). * $P<0.05$; ** $P<0.01$

Figure 8. Expression of *Nfe2l2*, *Gclc*, *Gclm*, *Keap1* and *Bach1* mRNAs measured by qPCR
 Low concentration arsenic exposure increased the expressions of *Gclc*, *Gclm*, *Keap1* and *Bach1* mRNAs, whereas high concentrations reduced their expressions. No significant changes were observed for the expression of *Nfe2l2* mRNA following sub-chronic arsenic exposure. * $P<0.05$; ** $P<0.01$

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Cluster membership for k = 5 as a function of iAs exposure

[iAs]	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5
0 mg/L	3	0	0	0	0
0.1 mg/L	0	2	1	0	0
1.0 mg/L	1	0	0	2	0
10 mg/L	0	0	0	3	0
100 mg/L	0	0	0	1	2

Table 2

p-values and *q*-values of the 26 miRNAs which had a *q*-value less than or equal to 0.20 (i.e., corresponding to a false discovery rate of 20%).

	rno-miRNA	<i>p</i>-value	<i>q</i>-value
Expression Increased	rno-miR-183	1.00E-04	0.018
	rno-miR-872	1.00E-04	0.018
	rno-miR-148b	3.00E-04	0.0199
	rno-miR-151	5.00E-04	0.0199
	rno-miR-126a	3.00E-04	0.0199
	rno-miR-192	5.00E-04	0.0199
	rno-miR-25	0.0015	0.0442
	rno-miR-532	0.0016	0.0442
	rno-miR-331	0.002	0.0513
	rno-miR-194-1	0.0035	0.0824
	rno-miR-497	0.0038	0.0824
	rno-miR-99b	0.0062	0.1171
	rno-miR-96	0.0085	0.1453
	rno-miR-450a	0.0128	0.1836
	rno-miR-672	0.0133	0.1836
Expression Decreased	rno-miR-26a	4.00E-04	0.0199
	rno-miR-34c	4.00E-04	0.0199
	rno-miR-423	5.00E-04	0.0199
	rno-miR-702	0.0016	0.0442
	rno-miR-6321	0.0015	0.0442
	rno-miR-20a	0.0039	0.0824
	rno-miR-425	0.0052	0.1037
	rno-miR-664-1	0.0077	0.1382
	rno-miR-125b-1	0.0103	0.1681
	rno-miR-19a	0.0108	0.1686
	rno-miR-339	0.0119	0.178

Note: *p*-values were obtained *via* the application of Jonckheere's trend test and were adjusted (i.e., *q*-values were obtained) using the method of Benjamini Hochberg.

Table 3

Predicted miRNAs targeting molecules in Nfe2l2 signaling pathway.

Genes	Rat			Human		
	Conserved miRNA	Response to Arsenic in Rats	miSVR score	Conserved miRNA	Response to Arsenic in Rats	miSVR score
Gcle	mo-miR-148b	Increase	-0.2133	hsa-miR-497	Increase	-0.5256
	mo-miR-25	Increase	-0.1279			
Gclm				hsa-miR-183	Increase	-1.1342
				hsa-miR-192	Increase	-1.0096
	mo-miR-25	Increase	-0.3019	hsa-miR-25	Increase	-0.2177
				hsa-miR-425	Decrease	-0.2333
	mo-miR-125b	Decrease	-0.1315	hsa-miR-125	Decrease	-0.1669
Nfe2l2	mo-miR-20a	Decrease	-0.1867	hsa-miR-20a	Decrease	-0.1973
	mo-miR-194	Increase	-0.19	hsa-miR-425	Decrease	-0.2215
Keap1						
Bach1	mo-miR-25	Increase	-0.121	hsa-miR-25	Increase	-0.1245
				hsa-miR-194	Increase	-0.195