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Research Article

Inorganic arsenic as a developmental toxicant: *In utero* exposure and alterations in the developing rat lungs

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In the present study, we characterize the toxic effects of *in utero* arsenic exposure on the developing lung. We hypothesize that *in utero* exposure to inorganic arsenic through maternal drinking water causes altered gene and protein expression in the developing lung, indicative of downstream molecular and functional changes. From conception to embryonic day 18, we exposed pregnant Sprague-Dawley rats to 500 ppb arsenic (as arsenite) *via* the drinking water. Subtracted cDNA libraries comparing control to arsenic exposed embryonic lungs were generated. In addition, a broad Western blot analysis was performed to identify altered protein expression. A total of 59 genes and 34 proteins were identified as being altered. Pathway mapping and analysis showed that cell motility was the process most affected. The most likely affected pathway was alteration in integrin signaling through the β-catenin pathway, altering c-myc. The present study shows that arsenic induces alterations in the developing lung. These data may be useful in the elucidation of molecular targets and biomarkers of arsenic exposure during lung development and may aid in understanding the etiology of arsenic induced adult respiratory disease and lung cancers.

Keywords: Arsenic / Lung development / Microarray / Protein array

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1 Introduction

Inorganic arsenic is a potent human carcinogen. Chronic environmental arsenic exposure through consumption of geologically contaminated drinking water has been correlated with increased incidence of and mortality due to internal cancers of the lung, skin, kidney, urinary bladder, and liver [1 – 4]. In addition, reports from human studies in Chile, Bangladesh, and the West Bengal region of India show that chronic exposure to arsenic *via* drinking water is correlated with increased incidence of chronic cough, chronic bronchitis, shortness of breath, and obstructive or restrictive lung disease [5 – 8]. Taken together, these studies argue unequivocally that the lung is targeted by arsenic, producing both carcinogenic and noncarcinogenic endpoints.

Growth and development requires the temporal and spatial coordinated expression of genes and gene products. During this critical time, *in utero* and early postnatal exposure to toxicants has the potential to affect gene expression, altering organ structure, and physiological function. However, only limited attention has been paid to the effects of environmentally relevant exposures to toxicants during these critical periods of development. The effects of human exposure to arsenic during these sensitive developmental times have recently been reported by Smith *et al*. [8]. Exposures to high levels of arsenic, either *in utero* or during early childhood development led to an increased risk of lung cancers and chronic lung disease in adults. In a mouse model of transplacental carcinogenesis, arsenic exposure (42.5 and 85 ppm) during gestation days 8 – 18 lead to significant increases in tumor incidence and multiplicity in the lung and several other organs in adult offspring [9]. While the doses used in the previous mouse studies are high compared to environmental exposure levels, they do show that tumor formation can occur in an animal model of *in utero* arsenic exposure. While arsenic has long been recognized as a human carcinogen, the noncancerous health effects of arsenic ingestion in the drinking water can also lead to significant disease, including cardiovascular disease, arteriosclerosis, diabetes,

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and chronic pulmonary disease. However, the molecular targets for these alterations after exposure to environmentally relevant levels of arsenic are not known.

To date, there are few studies detailing alterations in the developing fetus induced by maternal arsenic exposure. Acute, high dose i.p. injections of arsenic (30–45 mg/kg) during gestation have been associated with neural tube defects and corresponding aberrant gene expression of developmentally important transcription factors in the neural tube, including Hox 3.1 and Pax3 [10]. These doses of arsenic also triggered upregulation of bcl-2 and p53 gene expression in the neural tube, indicative of inhibition of cellular proliferation [11].

In the present study, we examined gene and protein expression changes in developing lungs of arsenic exposed fetal rats. We generated subtracted cDNA libraries from day 18 fetal lungs of rat pups exposed to 500 ppb arsenic (using arsenite) *in utero* beginning at conception. These doses are environmentally relevant and have been observed in areas of endemic exposure, including Chile [8] and the West Bengal region of India [6].

Our results show that arsenic caused aberrant expression of 93 genes and proteins. Analysis revealed that pathways involved in β-catenin signaling were affected. In addition, pathway analysis indicated that c-myc may play a central role in the *in utero* responses to arsenic. Taken together, these alterations demonstrate that arsenic causes alterations in gene and protein expression in the embryonic rat lung, following *in utero* exposure through maternal drinking water. Our results may be useful in the elucidation of molecular targets and biomarkers of inorganic arsenic exposure during lung development and may give insight into the etiology of arsenic-induced respiratory disease and lung cancers.

2 Materials and methods

2.1 Reagents

Tissue Protein Extraction Reagent (T-PER), Halt Protease Inhibitor Cocktail Kit, and Restore Western Blot Stripping Buffer were obtained from Pierce Biotechnology (Rockford, IL). High range and full range Rainbow M_r markers were obtained from Amersham Biosciences (Piscataway, NJ). Sodium arsenite was obtained from J.T. Baker (Phillipsburg, NJ). RNA Stat-60 RNA isolation reagent was purchased from Tel-Test Incorporated (Friendswood, TX). All other laboratory chemicals were purchased from Sigma Chemical (St. Louis, MO), and were secured in the highest purity available.

2.2 Animal breeding and treatments

Male and female Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed in a humidity controlled room, maintained at 22°C on a 12 h light–dark cycle and were given standard rat chow and water *ad libitum*. Rat chow used in our experiments was found to contain 75 ng/g of total arsenic. Based on daily intake of food and water this would increase the intake of arsenic in the exposure group by about 7% over the expected 500 ppb levels. Female rats were weighed nightly and estrus cycles were monitored using an Estrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA). Upon reaching estrus female rats were mated overnight and pregnancy was confirmed the following morning by the presence of sperm in a vaginal lavage sample. Immediately after mating, pregnant animals were randomly placed in control or 500 ppb arsenic (sodium arsenite) drinking water treatment groups. Arsenic solutions were replaced once daily and arsenic concentration was validated by ICP MS (data not shown). Mothers were euthanized with CO_2 at embryonic day 18 (day 1 was the second morning after mating). Embryonic lungs were removed from day 18 fetal rats by microdissection and were immediately snap frozen in liquid nitrogen. Pup lung weights and body weights were recorded. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Arizona.

2.3 Preparation of subtracted cDNA library

Total RNA was extracted from lung tissue of a single control and treated day 18 fetal rat using RNA Stat-60. RNA was ethanol precipitated and mRNA was subsequently isolated using oligo-dT selection with the NucleoTrap mRNA Mini Purification Kit (Clontech, Palo Alto, CA). Synthesis and amplification of first strand cDNA from mRNA was carried out using the SMART PCR cDNA Synthesis Kit (Clontech). Subsequent preparation of subtracted cDNA libraries was performed using the PCR-Select cDNA Subtraction Kit (Clontech). The subtractive hybridizations performed were: (i) treated (500 ppb arsenate or arsenite) cDNA subtracted with an excess of control cDNA and (ii) control cDNA subtracted with an excess of treated cDNA. Following cDNA subtractions, cDNA fragments were cloned into TOP-10 chemically competent *Escherichia coli* (Invitrogen, Carlsbad, CA). All cDNA clones were sequenced in both directions by the Arizona Research Laboratories Genetic Analysis and Technology Core Sequencing Facility at the University of Arizona using an Applied Biosystems (Foster City, CA) ABI Prism 3700 DNA Analyzer. Sequences were processed to remove poor quality sequence data and vector sequences using the FAKtory DNA Sequence Fragment Assembly System [12]. Sequences were subsequently blasted with BLAST at NCBI. Genes were classified by function using the BioRag database (bioresource for array genes) found at http://www.biorag.org.

Table 1. Real-time PCR primers

Gene	Primer	Accession no.	Sequence (5′ – 3′)
Beta Actin	Forward	NM_007393	ACCCAGGCATTGCTGACAGG
Beta Actin	Reverse	NM_007393	TGGACAGTGAGGCCAGGATG
Sprouty 2	Forward	NM_001012046	TGCTCCAATGACGACGAGGA
Sprouty 2	Reverse	NM_001012046	TAAGGCAACCCTTGGCTGGA
Collagen III a1	Forward	NM_032085	TGGGATCCAATGAGGGAGAA
Collagen III a1	Reverse	NM_032085	CTCATGGCCTTGCGTGTTTG

Table 2. Arsenite induced changes in fetal lung and body weights

Treatment	*N*	Pup weight (g)	Lung weight (g)	Lung weight/body weight
Control	46	1.591 ± 0.015	0.057 ± 0.001	0.036 ± 0.001
Arsenite	54	1.486 ± 0.017***	0.050 ± 0.002***	0.033 ± 0.001*

Comparison of day 18 fetal lungs in control *versus* 500 ppb arsenic (as sodium arsenite) dosed mothers. Statistical evaluation by two-tailed unpaired Student's *t*-test *** $p < 0.0005$, * $p < 0.05$.

2.4 Real-time PCR studies

Isolation of total RNA from animal tissues was carried out utilizing the RNA stat 60 reagent and homogenization with a Tissue-Tearor (BioSpec Products, Bartlesville, OK). Two ethanol precipitations were performed to ensure RNA purity. Total RNA isolation from cell culture samples was performed as described above. DNase treatment of total RNA for real-time PCR was also carried out as described above. Reverse transcription of 3.0 μg total RNA was performed using the Ambion EndoFree RT Kit, according to manufacturer protocol. Primer sets were designed against the first 300 bp of the 3′ end of the gene of interest using the Primer 3 program from the Massachusetts Institute of Technology: http://www. genome.wi.mit.edu/cgi-bin/primer/primer3_www_slow.cgi. Primers are listed in Table 1 and were purchased from Integrated DNA Technologies (Coralville, IA). Real-time PCR reactions were performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and were carried out using a Rotor Gene RG-3000 instrument and Rotor Gene Version 4.6 software (Corbett Research, Mortlake, NSW, Australia). Each 10 μL reaction contained: 1 μM each forward and reverse primers, 6.25 mM supplemental $MgCl_2$, 2.0 μL 1:25 diluted cDNA from RT reaction, 1 × QuantiTect SYBR Green PCR Master Mix, and 0.25 × SYBR Green I (purchased as 10 000 × concentrate). Amplification conditions utilized 95°C for 15 min followed by 45 PCR cycles as follows: 95°C for 15 s, 58°C for 15 s, 72°C for 20 s, followed by a final incubation for 45 s at 72°C and a subsequent melt from 72–99°C at 1°C intervals, 5 s *per* interval. Real-time PCR analysis of fetal lung cDNA samples was performed in triplicate, using cDNA samples from individual pups, each pup being from a different litter ($N = 3$ control, $N = 4$ treated). All gene expressions were normalized to expression of β-actin. Prior to export of real-time PCR data, slopes were corrected using the slope correct function of the Rotor Gene software.

2.5 Western immunoblotting

Total protein was isolated from snap frozen fetal lung tissue using 5 μL/mg tissue of T-PER with 10 μL/mL Halt Protease Inhibitor Cocktail and 1 mM sodium orthovanadate, according to manufacturer protocols. Protein quantitation was performed using the Bicinchonic Acid Kit (Sigma). Protein samples were analyzed by Becton Dickinson using their Powerblot analysis, which stains using 995 well-characterized antibodies. (BD Biosciences, San Diego, CA). Of these, 716 of the antibodies are known to react with rat proteins, 16 are known not to react with rat, and 263 are untested in rat. A total of 853 individual or modified proteins were detected. Protein samples were run on multiple lanes, blotted, and immunostained. Levels of proteins were determined using densitometric analysis. Proteins from three arsenic treated and three control pups were run independently. Significance was assigned only if all three treated samples responded similarly when tested against all three controls.

2.6 Data analysis

Analysis of the altered genes and proteins was performed using MetaCore from GeneGo (San Diego, CA). This is a curated analysis that allows for determination of the most likely cellular processes, pathways, transcriptional regulators, and receptors that are altered. Statistical analysis of lung weights and lung to body weight ratios were compared using Student's *t*-test, conducted with Graph Pad Prism 3.0

Table 3. Arsenic-altered genes

	Symbol	Gene name		Symbol	Gene name
↑	Actb	β-Actin	↓	Nr1d2	Nuclear receptor subfamily 1, group D, member 2
↓	Akr1a1	Aldo – keto reductase family 1, member A1	↓	Ogn	Osteoglycin (Ogn)
↑	AMELX	Amelogenin	↑	Opn3	Similar to opsin 3
↓	Atp5d	ATP synthase, H+ transporting, mitochondrial F1 complex, δ-subunit	↑	Pcgf4	Polycomb group ring finger 4
↓	Atxn10	Ataxin 10	↑	Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1
↑	Atxn2	Ataxin 2	↓	Pnrc1	Proline-rich 2
↑	Birc1b	Baculoviral IAP repeat-containing 1b	↑	Ppp1cb	Protein phosphatase 1, catalytic subunit, beta isoform
↓	Ca2	Carbonic anhydrase 2	↑	Psmd12	Proteasome (prosome, macropain) 26S subunit, nonATPase, 12
↓	Casp2	Caspase 2	↓	Rabggtb	RAB geranylgeranyl transferase, b subunit
↓	Cdc73	Cell division cycle 73, Paf1/RNA polymerase II complex component	↑	Rpl26	Ribosomal protein L26
↓	Col3a1	Collagen α 1 type III	↑	Rpl27a	Ribosomal protein L27a
↑	Coq7	Demethyl-Q 7	↑	Rpl6	Ribosomal protein L6
↓	Cox5a	Cytochrome *c* oxidase, subunit Va	↑	Rps11	Ribosomal protein S11
↓	Crry	Complement receptor related protein	↑	Rps16	Ribosomal protein 16S
↓	Ddx5	DEAD box polypeptide 5	↓	Rps2	Ribosomal protein S2
↓	Dhx33	DEAH box polypeptide 33	↑	Rps5	Ribosomal protein S5
↑	Eef1g	Eukaryotic translation elongation factor 1 g	↑	Scl4a1ap	Solute carrier family 4 (anion exchanger), member 1, adaptor protein
↓	Ehbp1	EH domain binding protein 1	↓	sep15	Selenoprotein
↓	Grb10	Growth factor receptor bound protein	↑	Skp1a	S-phase kinase-associated protein 1A
↑	H2ai	Similar to histone 1, H2ai	↓	Spry2	Sprouty homolog 2
↑	Hba-a1	Hemoglobin α, adult chain 1	↑	srp14	Signal recognition particle 14
↑	Hbb	Hemoglobin β-chain complex	↓	srprb	Signal recognition particle receptor, B subunit
↓	Hspa9a	Heat shock 70kDa protein 9A	↓	Ssb	Sjogren syndrome antigen B
↓	Khdrbs1	KH domain containing, RNA binding, signal transduction associated 1	↓	Strn3	Striatin, calmodulin binding protein 3
↓	Lamc1	Laminin γ 1	↑	Tfpi	Tissue factor pathway inhibitor
↓	Lbr	Lamin B receptor	↓	Tnni1	Troponin I, skeletal, slow 1
↑	LUC7-like 2	Luc7l2	↓	Tubb2	Tubulin, β 2c
↓	Map1lc3b	Microtubule-associated protein 1 light chain 3 beta	↓	unr	Upstream of NRAS
↑	Mss4	Guanine nucleotide-releasing protein (mss4)	↓	14-3-3 epsilon	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ε
↓	Nap1l1	Nucleosome assembly protein 1-like 1			

An expanded Table 3, which includes functional descriptions of the genes, can be found in the Supporting Information.

(Graph Pad Software, San Diego, CA). Statistical analysis of real-time PCR data were performed using two-tailed Student's *t*-tests, requiring $p < 0.05$ for statistical significance.

3 Results

3.1 Arsenic induced changes in fetal growth

Treatment with 500 ppb arsenic (as sodium arsenite) resulted in decreased body weight and lung weight in day 18 fetal rats (Table 2). In particular, arsenic caused significant ($p < 0.0005$) reduction of fetal body weight by 6.6% and lung weights by 13.3%. Arsenic exposure also caused significant ($p < 0.05$) reduction of lung to body weight ratios by 7.2%. No changes in maternal weight gain or food and water consumption were observed between control and treated pregnant animals during the course of this study (data not shown). Additionally, there were no alterations in litter size or in the number of fetal resorptions between treatment groups (data not shown).

3.2 cDNA library preparation and sequencing

The cDNA libraries isolated from subtractive hybridization of control *versus* arsenic treated day 18 fetal rat lungs con-

Table 4. Arsenic-altered proteins

Fold change	Symbol	Gene name	Fold change	Symbol	Gene name
4.00	CAV1	Caveolin 1	0.34	LRRFIP1	leucine rich repeat (in FLII) interacting protein 1
2.46	CAV3	Caveolin 3	0.31	MAPK8	mitogen-activated protein kinase 8 (pT183/pY185) Phospho-Specific-41KD
0.10	CDC42	Cell division cycle 42 (GTP binding protein)	2.17	MSH6	MutS homolog 6 (*E. coli*)
6.06	CHAF1A	Chromatin assembly factor 1, subunit A	0.44	OPTN	Optineurin
1.95	CLTC	Clathrin, heavy polypeptide (Hc)	0.53	PDI	Procollagen-proline, 2-oxoglutarate 4-dioxygenase
3.24	CTTN	Cortactin	3.50	PPP1R8	Protein phosphatase 1, regulatory (inhibitor) subunit 8
0.25	DHFR	Dihydrofolate reductase	1.73	PRKR2B	Protein kinase, cAMP-dependent, regulatory, type II, beta (pS114)
0.17	DUSP3	Dual specificity phosphatase 3	1.87	Ptk2b	Protein tyrosine kinase 2 beta
0.25	EPB49	Erythrocyte membrane protein band 4.9 (dematin)	2.05	PTPN6	Protein tyrosine phosphatase, nonreceptor type 6
7.46	EZR	Ezrin	0.34	PXN	Paxillin
1.95	FEN1	Flap structure specific endonuclease 1	0.34	Ran	RAN, member RAS oncogene family
17.45	Gphn	Gephyrin	1.89	RBBP4	Retinoblastoma binding protein 4
0.51	Hap1	Huntingtin-associated protein 1	2.64	SC65	Synaptonemal complex protein SC65
0.07	Homer1	Homer homolog 1 (Drosophila)	1.63	Sptlc1	Serine palmitoyltransferase, long chain base subunit 1
2.63	HSPBP1	Hsp70-interacting protein	6.69	UBE2E1	Ubiquitin-conjugating enzyme E2E 1 (UBC4/5 homolog, yeast)
1.57	ITGB3	Integrin b3	3.29	WRN	Werner syndrome
1.69	ITPR3	Inositol 1,4,5-triphosphate receptor, type 3	0.57	YES1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1

An expanded Table 4 can be found in the Supporting Information.

tained 326 clones. From these clones, 281 sequences were obtained with approximately 160 representing unique genes. Such redundancy in subtracted cDNA libraries is consistent with that seen in the literature [13]. After applying an *e*-value match score maximum of e^{-50}, 129 unique genes were identified with 59 clones representing genes of known function. Genes from arsenic subtracted libraries are listed in Table 3. An expanded Table 3, which includes functional descriptions of the genes, can be found in the Supporting Information. Major functional areas described by this list of genes include: matrix, cytoskeletal, and adhesion genes, transcriptional regulators, ribosomal genes, and protein modification and turnover genes. Subtractive hybridization selects for low abundance genes that are differentially expressed in two samples. Therefore, the effect of arsenic is listed as either increasing or decreasing expression, based on abundance in controls and treated animals.

3.3 Real-time PCR validation studies

Quantitative real-time PCR was used to validate differential expression of select genes identified in the subtracted libraries. The following genes were analyzed: collagen type III α1 chain and sprouty-2. Primer sequences are given in Table 1. Data for real-time were consistent with the differential expression data. Sprouty-2 and collagen 3a1 were both downregulated by two-fold with 500 ppb arsenic exposure *in vivo*.

3.4 Differentially expressed proteins

Proteins from three treated and three control pups were analyzed using BD Powerblot (Table 4). In order for the proteins to be considered as altered, changes in expression had to occur between each treated sample when compared against each individual control. A total of 34 proteins were determined to be altered by the *in utero* exposure to arsenic (Table 4). Alterations in serine/threonine phosphorylation were also detected in three unidentified proteins. A majority of the proteins that were misregulated are associated with cell motility, adhesion, and cytoskeleton. (See expanded Table 4 in the Supporting Information). The Powerblot analysis contained antibodies against only four of the arsenic altered gene products. (ataxin 2, 14-3-3ε, caspase 2, HSP70). While the protein levels tended to follow the downregulation of the gene expression, none of these four protein levels reached the strict significance criteria used for the Powerblot analysis.

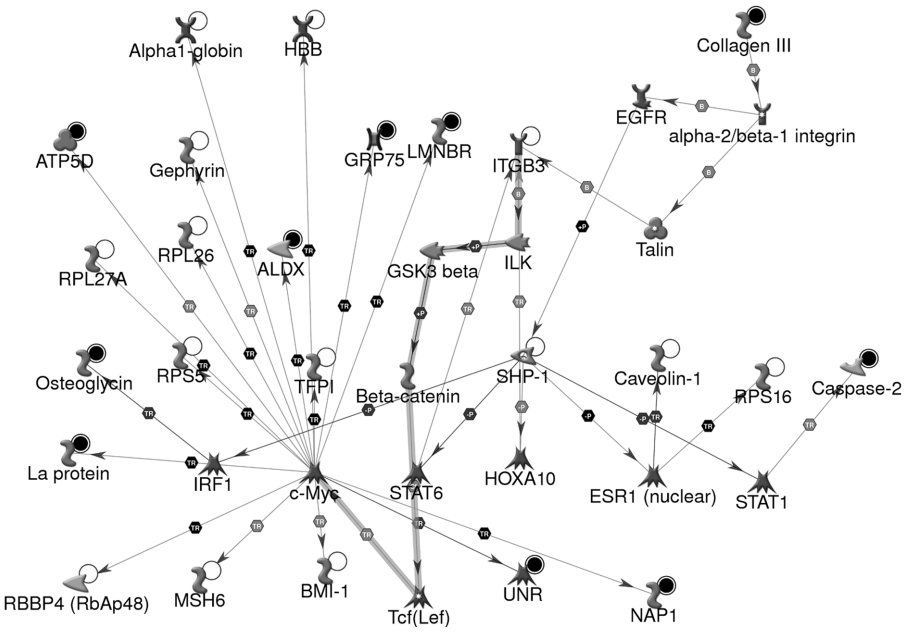

Figure 1. Pathway analysis using all altered genes and proteins. The network shown represents the most statistically probable pathways and elements when analyzed from receptor activation to signaling. One canonical pathway, β-catenin, activated through ITGB3 is shown by the broad line. This would ultimately result in altered expression of c-myc, which is a central transcription factor involved with a high percentage of the altered genes and proteins. Genes and proteins that were identified and are in the network are highlighted by large circles. Under expression is represented by black circles (*i.e.*, collagen III) and over expression by white circles (ITGB3). Interactions between elements are defined by the interconnecting lines, *e.g.*, phosphorylation between GSK3 β and β-catenin.

3.5 Pathway analysis

Both genes and proteins that were identified as being differentially expressed by arsenic were entered into a curated data analysis program, MetaCore from GeneGo. This program utilizes published data to determine the most probable pathways, diseases, cellular processes, and transcriptional regulation that are defined by the altered expression. For the genes and proteins that were identified, the statistically most likely disease is lung cancer and the most probable cellular function that has been altered is cell motility. Using the receptor pathway analysis, MetaCore identified matrix and extracellular signaling through the β-catenin pathway as a highly likely site of action described by the altered genes and proteins (Fig. 1). Finally, the altered genes and proteins interacted mostly with transcription factors c-myc (22 genes/proteins), HNF-4α (16), and p53 (14).

4 Discussion

Alterations in the developing rat lung induced by environmentally relevant doses of arsenic (500 ppb as arsenite) were analyzed to assess a potential role for this metalloid in alteration of lung development. The changes observed were chronicled by subtractive hybridization and by Powerblot analysis of proteins.

Environmental arsenic exposure in drinking water has been linked to an increase in respiratory disease. Recently, Smith *et al*. [8] have reported in a human population from Chile that *in utero* and early postnatal exposures to high environmental levels (800 – 900 ppb) greatly increase the incidence on lung disease associated mortalities later in life. Standard mortality ratios (SMR) from lung cancers, bronchiectasis, and other chronic lung diseases were increased five- to ten-fold with early postnatal exposure alone. Combined *in utero* and postnatal exposures increased the SMR even higher. Our data reported here using 500 ppb arsenic exposures are therefore relevant for providing potential mechanisms that can lead to human diseases.

Analysis of altered genes and proteins in our rat model has indicated that the β-catenin pathway and c-myc may be important targets of *in utero* arsenic exposure. The wnt signaling pathway and c-myc have been shown to be important regulators of lung development as well as playing a role in lung cancers and chronic lung diseases. β-Catenin is required for appropriate proximal/distal lung fate during lung morphogenesis [14] and c-myc is important for appropriate cell expansion [15]. Both are also overexpressed in lung cancer [16 – 17]. Additionally, β-catenin and c-myc gene expression were found to be altered in the livers of mice following *in utero* exposure to 85 ppm arsenite [18]. While the pathway mining suggests β-catenin pathways and c-myc as important molecular targets, their involvement must be verified in additional experiments.

Additional genes of particular interest to our research group were the arsenic induced modulation of extracellular matrix (ECM) genes, cell motility genes, and those involved with regulation of fetal growth. A number of these genes and proteins were modulated by arsenic in the present study. Collagen type III is an interstitial collagen found within the alveolar walls and pulmonary vasculature [19]. Expression of the gene encoding collagen III peaks at embryonic day 12 in the mouse, with its expression becoming gradually reduced until birth [20]. Decreased immunos-

taining of this collagen is found in the alveoli in a mouse model of metalloproteinase-induced emphysema [19]. This particular emphysema model is independent of changes in elastin and illustrates that reduced expression of collagen III can contribute to reduced lung function through induction of emphysematous changes. We have also previously reported misregulation of this and other matrix genes following arsenic exposure in adult mice [21].

Aberrant airway remodeling is a hallmark of many diseases including emphysema, asthma, idiopathic pulmonary fibrosis, tuberculosis, and bronchiectasis [22 – 26]. It consists of an array of persistent tissue structural changes that occur through a process of injury and dysregulated repair that may lead to airway chronic inflammation and altered ECM deposition in the airway wall, leading to airflow obstruction [23, 27 – 30]. In addition to matrix, cell migration is a critical step in tissue remodeling [31] and abnormalities in cell migration have the potential to lead to respiratory disease.

Changes that we have seen are consistent with arsenic induced chronic obstructive pulmonary disease (COPD) incidence in exposed humans. Environmental arsenic exposure in drinking water has been linked to an increase in respiratory disease [32 – 35]. Reduced collagen expression and altered cell motility and evidence of its association with emphysematous changes in the lung [19] may also be consistent with arsenic induced COPD. Therefore, the effects of arsenic on the developing lungs in the present study and the physiological consequences thereafter should ultimately be assessed by physiological and morphological analyses conducted over a developmental time course.

Sprouty-2 is another gene that is involved in fetal growth that was modulated by arsenic exposure in the present study. Sprouty-2 is necessary for lung development, lung maturation, and regulation of cellular proliferation in the developing lung and is crucial for embryonic survival. A loss of function mutation of sprouty-2 in *Drosophila* caused excessive branching and embryonic death [36]. Cultured mouse embryos injected with sprouty-2 antisense oligonucleotides showed a significant increase in tracheal branching and increased expression of the proliferating cell nuclear antigen, along with increased expression of the lung maturation markers, surfactant protein genes SP-A, SP-B, and SP-C [37]. The main target of sprouty-2 seems to be epithelial cells, where its expression reduces cellular proliferation [38]. Downregulation of sprouty-2 by arsenic in the present study is thus likely to be physiologically significant and merits further study.

In the present study, a general decrease in fetal growth and fetal lung growth were observed on day 18 fetal rats following 500 ppb arsenic (as arsenite) exposure during gestation (Table 2). These findings are consistent with previous studies showing reduced birth weight of pups exposed to arsenic *in utero* [10, 39] and are also novel, as changes in fetal lung growth have not been documented following gestational arsenic exposure. Neither changes in maternal weight gain during pregnancy were observed between control and arsenic treated mothers, nor were there any changes in number of pups *per* litter or number of fetal resorptions (data not shown).

Arsenic is at best a weak mutagen. Therefore, several epigenetic mechanisms have been proposed as sites of action for arsenic. Alteration of DNA methylation is one of the main epigenetic modifications in humans that controls gene expression [40]. The methylation status of genes is long lived and heritable. Alteration in the methylation of genes will result in long term effects on gene expression. Therefore, *in utero* alteration of methylation status of genes important for the control or the development of chronic diseases could lead to adult diseases later in life. Exposure to arsenic has been shown to alter DNA methylation. Both global hypomethylation [41] and specific gene hypo-(c-myc, c-Ha-ras) and hypermethylation (p16, p53) have been reported following arsenic exposure [42 – 44]. It is believed that arsenic exerts its effect of methylation status due to its metabolism. Inorganic arsenic (III) is methylated to mono- and dimethyl forms using methyltransferases and requires *S*-adenosyl-methionine as a cofactor. Altering the activity in this pathway could affect DNA methylation status, resulting in altered gene expression. Additionally, arsenic has also been shown to affect DNA repair processes [45]. Our analysis has identified alterations of MSH6, a protein associated with recognition of DNA mismatches and mismatch repair. The levels of this protein were increased in arsenic exposed animals. This may be indicative of increased repair. Alternatively, DNA repair proteins have also been associated with regulation of apoptosis. Therefore, this increase may be a response to additional cellular stress caused by the arsenic exposures.

Arsenic methylation provides a site for interaction between arsenic exposure and dietary methyl donors. Many micronutrients are essential for DNA synthesis and DNA repair. Among these, folate is one of the more extensively studied. Not only is folate important in proper nucleotide synthesis, but it also plays an important role in DNA methylation [46]. Thus dietary folate could provide an intervention strategy for reducing the adverse *in utero* exposure effects of arsenic exposure.

We have used a combination of alterations in mRNA and protein expression to determine altered pathways and sites of action of arsenic. We feel that using information from these two sources strengthens our results. Alterations in levels of expression of genes or proteins can potentially occur independent of each other. For example, gene expression could be unchanged, but alterations in protein degradation could affect the levels of the protein. Therefore using both mRNA and protein levels provides a wider range of potential sites of action and more confidence in the pathway analysis.

Finally, it should be pointed out that the levels of exposure in these experiments, while found in the environment,

are quite high. It will be important to retest these results using lower doses of arsenic consumption. In addition, we did not directly measure the levels of arsenic found in the fetal lungs. However, based on work reported by Devesa *et al*. [47] we would expect to find that the levels of arsenic in the fetal lung would be about 1% of the administered arsenic, or about 5 ppb, mostly in the form of dimethylarsenic.

In conclusion, the findings presented in this study implicate arsenic as a developmental toxicant in the rat lung. Reduction in fetal weight, lung weight and lung to body weight ratios, and altered expression of cellular differentiation markers demonstrate that arsenic is causing changes in fetal growth and development, following *in utero* exposure from conception to embryonic day 18. These alterations may ultimately result in fetal lungs that are premature, while also being highly differentiated and branched. It is also possible that the observed reduction in lung growth was a compensatory response to increased differentiation. Targets of *in utero* arsenic exposure in the developing lung appear to be the developing ECM and the processes of cellular differentiation and branching morphogenesis. The arsenic-induced toxic and growth alterations presented in this study demonstrate the effects of arsenic in the developing lung, findings which merit further mechanistic and physiological studies.

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The authors have declared no conflict of interest.

5 References

[1] NRC, *National Research Council Report: Arsenic in Drinking Water*, National Academy Press, Washington, DC 1999.

[2] Bates, M. N., Smith, A. H., Hopenhayn-Rich, C., Arsenic ingestion and internal cancers: A review. *Am. J. Epidemiol.* 1992, *135*, 462 – 476.

[3] Chen, C. J., Chuang, Y. C., Lin, T. M., Wu, H. Y., Malignant neoplasms among residents of a blackfoot disease-endemic area in Taiwan: High-arsenic artesian well water and cancers. *Cancer Res.* 1985, *45*, 5895 – 5899.

[4] Chen, C. J., Chuang, Y. C., You, S. L., Lin, T. M., Wu, H. Y., A retrospective study on malignant neoplasms of bladder, lung and liver in blackfoot disease endemic area in Taiwan. *Br. J. Cancer* 1986, *53*, 399 – 405.

[5] Milton, A. H., Hasan, Z., Rahman, A., Rahman, M., Non-cancer effects of chronic arsenicosis in Bangladesh: Preliminary results. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* 2003, *38*, 301 – 305.

[6] Mazumder, D. N., Haque, R., Ghosh, N., De, B. K., *et al*., Arsenic in drinking water and the prevalence of respiratory effects in West Bengal, India. *Int. J. Epidemiol.* 2000, *29*, 1047 – 1052.

[7] Mazumder, D. N. G., Chronic arsenic toxicity: Clinical features, epidemiology, and treatment: Experience in West Bengal. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* 2003, *38*, 141 – 163.

[8] Smith, A. H., Marshall, G., Yuan, Y., Ferreccio, C., *et al*., Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic *in utero* and in early childhood. *Environ. Health Perspect.* 2006, *114*, 1293 – 1296.

[9] Waalkes, M. P., Ward, J. M., Liu, J., Diwan, B. A., Transplacental carcinogenicity of inorganic arsenic in the drinking water: Induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice. *Toxicol. Appl. Pharmacol.* 2003, *186*, 7 – 17.

[10] Wlodarczyk, B., Bennett, G. D., Calvin, J. A., Craig, J. C., Finnell, R. H., Arsenic-induced alterations in embryonic transcription factor gene expression: Implications for abnormal neural development. *Dev. Genet.* 1996, *18*, 306 – 315.

[11] Wlodarczyk, B. J., Bennett, G. D., Calvin, J. A., Finnell, R. H., Arsenic-induced neural tube defects in mice: alterations in cell cycle gene expression. *Reprod. Toxicol.* 1996, *10*, 447 – 454.

[12] Miller, S., Myers, E., *The FAKtory DNA sequence fragment analyzer.* Department of Computer Science, The University of Arizona. 1999; http://www.cs.arizona.edu/research/reports.html.

[13] Cooper, P., Mueck, B., Yousefi, S., Potter, S., Jarai, G., cDNA-RDA of genes expressed in fetal and adult lungs identifies factors important in development and function. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2000, *278*, L284 – L293.

[14] Mucenski, M. L., Wert, S. E., Nation, J. M., Loudy, D. E. *et al*., β-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. *J. Biol. Chem.* 2003, *278*, 40231 – 40238.

[15] Cohen, J. C., Scott, D. K., Miller, J., Zhang, J., *et al*., Transient *in utero* knockout (TIUKO) of c-myc affects lung and intestinal development in the mouse. *BMC Dev. Biol.* 2004, *4*, 4.

[16] Mazieres, J., He, B., You, L., Xu, Z., Jablons, D. M., Wnt signaling in cancer. *Cancer Lett.* 2005, *222*, 1 – 10.

[17] Zajac-Kaye, M., Myc oncogene: A key component in cell cycle regulation and its implication for lung cancer. *Lung Cancer* 2001, *34*, S43 – S46.

[18] Liu, J., Xie, Y., Ducharme, D. M. K., Shen, J., *et al*., Global gene expression associated with hepatocarcinogenesis in adult male mice induced by *in utero* arsenic exposure. *Environ. Health Perspect*. 2006, *114*, 404 – 411.

[19] Foronjy, R. F., Okada, Y., Cole, R., D'armiento, J., Progressive adult-onset emphysema in transgenic mice expressing human MMP-1 in the lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2003, *284*, L727 – L737.

[20] Mariani, T. J., Reed, J. J., Shapiro, S. D., Expression profiling of the developing mouse lung: Insights into the establishment of the extracellular matrix. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2002, *26*, 541 – 548.

[21] Lantz, R. C., Hays, A. M., Role of oxidative stress in arsenic-induced toxicity. *Drug Metab. Rev.* 2006, *38*, 791 – 804.

[22] Jeffery, P. K., Remodeling in asthma and chronic obstructive lung disease. *Am. J. Respir. Crit. Care Med.* 2001, *164*, S28 – S38.

[23] Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., Holgate, S. T., Airway remodeling in asthma: New insights. *J. Allergy Clin. Immunol.* 2003, *111*, 215 – 225.

[24] Dheda, K., Booth, H., Huggett, J. F., Johnson, M. A., *et al*., Lung remodeling in pulmonary tuberculosis. *J. Infect. Dis.* 2005, *192*, 1201 – 1209.

[25] Niimi, A., Torrego, A., Nicholson, A. G., Cosio, B. G., *et al*., Nature of airway inflammation and remodeling in chronic cough. *J. Allergy Clin. Immunol.* 2005, *116*, 565 – 570.

[26] Reynolds, H. Y., Gail, D. B., Kiley, J. P., Interstitial lung diseases – where we started from and are now going. *Sarcoidosis Vasculitis Diffuse Lung Dis.* 2005, *22*, 5 – 12.

[27] Ramos-Barbon, D., Ludwig, M. S., Martin, J. G., Airway remodeling: Lessons from animal models. *Clin. Rev. Allergy Immunol.* 2004, *27*, 3 – 21.

[28] Nakano, Y., Muller, N. L., King, G. G., Niimi, A., *et al*., Quantitative assessment of airway remodeling using high-resolution CT. *Chest* 2002, *122*, 271S – 275S.

[29] McParland, B. E., Macklem, P. T., Pare, P. D., Airway wall remodeling: Friend or foe? *J. Appl. Physiol.* 2003, *95*, 426 – 434.

[30] Holgate, S. T., Holloway, J., Wilson, S., Bucchieri, F., *et al*., Epithelial-mesenchymal communication in the pathogenesis of chronic asthma. *Proc. Am. Thorac. Soc.* 2004, *1*, 93 – 98.

[31] Dosanjh, A., Zuraw, B., Endothelin-1 (ET-1) decreases human bronchial epithelial cell migration and proliferation: Implications for airway remodeling in asthma. *J. Asthma* 2003, *40*, 883 – 886.

[32] von Ehrenstein, O. S., Mazumder, D. N., Yuan, Y., Samanta, S., *et al*., Decrements in lung function related to arsenic in drinking water in West Bengal, India. *Am. J. Epidemiol.* 2005, *162*, 533 – 541.

[33] Tsai, S., Wang, T., Ko, Y., Mortality for certain diseases in areas with high levels of arsenic in drinking water. *Arch. Environ. Health* 1999, *54*, 186 – 193.

[34] Smith, A. H., Goycolea, M., Haque, R., Biggs, M. L., Marked increase in bladder and lung cancer mortality in a region of northern Child due to arsenic in drinking water. *Am. J. Epidemiol.* 1998, *147*, 660 – 669.

[35] Borgono, J. M., Vicent, P., Venturino, H., Infante, A., Arsenic in the drinking water of the city of Antofagasta: Epidemiological and clinical study before and after the installation of a treatment plant. *Environ. Health Perspect*. 1977, *19*, 103 – 105.

[36] Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y., Krasnow, M. A., Sprouty encodes a novel antagonist of FGF signaling that patterns apical branching of the Drosophila airways. *Cell* 1998, *92*, 253 – 263.

[37] Tefft, J. D., Lee, M., Smith, S., Leinwand, M., *et al*., Conserved function of mSpry-2, a murine homolog of Drosophila sprouty, which negatively modulates respiratory organogenesis. *Curr. Biol.* 1999, *9*, 219 – 222.

[38] Mailleux, A. A., Tefft, D., Ndiaye, D., Itoh, N., *et al.*, Evidence that SPROUTY2 functions as an inhibitor of mouse embryonic lung growth and morphogenesis. *Mech. Dev.* 2001, *102*, 81 – 94.

[39] Shalat, S. L., Walker, D. B., Finnell, R. H., Role of arsenic as a reproductive toxin with particular attention to neural tube defects. *J. Toxicol. Environ. Health* 1996, *48*, 253 – 272.

[40] Esteller, M., Herman, J. C., Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *J. Pathol.* 2002, *196*, 1 – 7.

[41] Chen, Y., Zhao, Y. H., Wu, R., Differential regulation of airway mucin gene expression and mucin secretion by extracellular nucleotide triphosphates. *Am. J. Respir. Cell Mol. Biol.* 2001, *25*, 409 – 417.

[42] Lu, G., Cai, Q., Zhang, W., Effects of inorganic arsenicals on methylation of p16 CpG islands and the expression of p16 gene in BEP2D cells. *Chin. Med. J.* 2001, *81*, 1238 – 1241.

[43] Mass, M. J., Wang, L., Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: A model for a mechanism of carcinogenesis. *Mut. Res.* 1997, *386*, 263 – 277.

[44] Zhong, C. X., Mass, M. J., Both hypomethylation and hypermethylation of DNA associated with arsenite exposure in cultures of human cells identified by methylation-sensitive arbitrarily-primed PCR. *Toxicol. Lett.* 2001, *122*, 223 – 234.

[45] Andrew, A. S., Burgess, J. L., Meza, M. M., Demidenko, E., *et al*., Arsenic exposure is associated with decreased DNA repair *in vitro* and in individuals exposed to drinking water arsenic. *Environ. Health Perspect*. 2006, *114*, 1193 – 1198.

[46] Friso, S., Choi, S. W., Gene-nutrient interactions and DNA methylation. *J. Nutr.* 2002, *132*, 2382S – 2387S.

[47] Devesa, V., Adair, B. M., Liu, J., Waalkes, M. P., *et al*., Arsenicals in maternal and fetal mouse tissues after gestational exposure to arsenite. *Toxicology* 2006, *224*, 147 – 155.