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Prenatal exposure of mice to tungstate is associated with decreased transcriptome-expression of the putative tumor suppressor gene, DMBT1: implications for childhood leukemia

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Abstract

Background. Two concurrent, childhood leukemia clusters have been identified in the southwestern United States at Fallon, Nevada, and Sierra Vista, Arizona. Additionally, Fallon, Nevada has also experienced concurrent contamination by atmospheric tungsten particles. The etiology of leukemia is not known. Hypothesized risk factors for leukemia are environmental exposure, genetic predisposition, and viral infection. Additionally, strong evidence supports a prenatal origin. Our objective is to generate testable hypotheses towards elucidating the probable, multi-factorial etiology of leukemia by identifying the exposures unique to Fallon, Nevada, and held in common with Sierra Vista, Arizona, then exposing C57BL/6 mice, while *in utero*, to these chemicals to ascertain their leukemogenic potential. Utilizing advances in medical geology to analyze tree rings, surface dust, lichens and atmospheric particulate matter, we have identified tungsten and arsenic as potentially relevant to leukemogenesis. **Methods.** We utilized microarray (Affymetrix 430A 2.0 mouse) and real-time RT-PCR of Dmbt1 transcriptome-expression in spleen tissue collected from four-week-old C57BL/6 mouse pups (N = 6–8/group/gender) exposed, while *in utero*, to tungstate, arsenite, tungstate/arsenite and longitudinal controls at 20% of the normalized exposure a human mother would receive during gestation at mean environmental concentrations. **Results.** Prenatal exposure to tungstate is associated with a 37 $\pm$ 1.2-fold (p = 0.012) decrease in DMBT1 transcriptome-expression in mice expressing DMBT1 at high levels. Additionally, prenatal exposure to tungstate/arsenite significantly altered a cytokine–cytokine receptor interaction pathway associated with lymphocyte activation and a network associated with hematological/immunological disease. **Conclusion.** Because DMBT1 protein products are known to aggregate viruses and possibly regulate immune response, additional research is warranted to determine the potential that prenatal exposure to tungstate or tungstate/arsenite has to increase susceptibility to viruses and to induce leukemogenesis.

Key words: acute lymphocytic leukemia, arsenite, cancer, childhood leukemia, DMBT1, tungstate, tungsten

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1 INTRODUCTION

Fallon, Nevada has concurrently experienced a uniquely significant childhood leukemia cluster (Steinmaus *et al.* 2004) and atmospheric contamination by tungsten particles (Table 1). Because the etiology of leukemia is not known, this suggested positive correlation between temporal elevations in tungsten levels, and the incidence of childhood leukemia strongly warrants renewed scrutiny of the effect of tungsten on

Table 1. Summary of the unique portion of the Fallon, Nevada profile for concentrations of metals in the environment

End measure	Concentrations in Fallon, Nevada		Concentrations of controls	
	Tungsten	Cobalt	Tungsten	Cobalt
ICP–MS of ambient PM[a]			Control communities: Lovelock, Fernley, Yerington, Reno	
	March: 0.57–8.56 ng m^{-3}	March: 0.66–1.68 ng m^{-3}	March: 0.12–0.16 ng m^{-3}	March: 0.37–0.58 ng m^{-3}
	Nov: 0.10–40.9 ng m^{-3}	Nov: 0.04–7.5 ng m^{-3}	Nov: 0.02–0.09 ng m^{-3}	Nov: 0.04–0.13 ng m^{-3}
INAA of surface dust[b]	Background: 20–30 ppm	Background: 15–25 ppm	Grid pattern outside of Fallon taken 2–5 km apart	
	Hotspot: 934 ppm	Hotspot: 98 ppm	0–10 ppm	10–15 ppm
ICP–MS of lichens[c]			20 km outside of Fallon in each cardinal direction	
	24.95 ppm	3.95 ppm	1.99 ppm	1.79 ppm
ICP–MS of tree-rings[d]	Prior to cluster (1989–1992):		Control communities: Lovelock, Fernley, Yerington	
	39 ppb	94 ppb	39 ppb	35 ppb
	During cluster (1993–2004):			
	96 ppb	87 ppb	39 ppb	35 ppb

ICP–MS – Inductively coupled plasma mass spectroscopy; INAA – instrumental neutron activation analysis; PM – particulate matter. a. Sheppard *et al*. (2006), values are medians; b. Sheppard *et al*. (2007*a*); c. Sheppard *et al*. (2007*c*); d. Sheppard *et al*. (2007*b*), values are medians

physiological systems to ultimately determine the level of seriousness associated with tungsten contamination.

Tungsten compounds have been reported to be toxicologically and biologically inert in *Escherichia coli*, except for effects on clpB and osmY stress promotergenes and inhibition of enzymes with nucleic-acid substrates (Tajima 2003). Additionally, tungsten has been reported to cross the placenta in murines (Wide *et al.* 1986); to compromise fetal ossification (Nadeenko *et al.* 1978); and to be preferentially retained in bone tissue (Wase 1955; Mullen *et al.* 1976; Ando *et al*. 1989). Leukemogenic risk factors are hypothesized to be environmental exposure; genetic predisposition (primarily the prenatal formation of chimeral genes resulting from chromosomal translocations); and viral infection with a probable multi-factorial etiology (reviewed in Belson *et al*. 2007; Rubin *et al.* 2007). We are profiling and comparing the environmental exposures in two concurrent, childhood leukemia clusters in the Southwestern United States at Fallon, Nevada (CDC 2003; CDC 2006; Rubin *et al*. 2007; Steinberg *et al.* 2007) and Sierra Vista, Arizona (Humble and Flood 2002; CDC 2006). Utilizing prenatal exposures in a whole system (C57BL/6 mice), we are modeling those exposures shared in common by the leukemia clusters. We hypothesize that prenatal exposure to an environmental chemical, held in common by communities experiencing elevated rates of leukemia, but not experienced by geographically similar control communities, results in genetic changes associated with increased susceptibility towards virus-induced leukemogenesis.

We present a summary of our environmental profiling of the metal exposures unique to Fallon, Nevada (Table 1). The unique portion of the atmospheric, metallic particulate matter is significantly elevated concentrations of cobalt and tungsten, which co-vary as a function of time. While the residents are exposed to elevated concentrations of arsenic, this alone is not unique to Fallon, as compared to geographically similar communities. The temporal time study, utilizing dendrochemistry of tree rings, indicates that not only are tungsten concentrations elevated as compared to control communities, medians of 96 ppb vs. 39 ppb respectively (p = 0.04), but that tungsten concentrations were about the same as the control communities, 39 ppb, a decade prior to the onset of the childhood leukemia cluster. The concentration of cobalt in Fallon has been consistently elevated over the past 15 years as compared to the control communities. The combinations of elevated concentrations of tungsten with arsenic as well as tungsten with cobalt may be relevant to leukemogenesis. Along with elevated concentrations of atmospheric tungsten, preliminary results in Sierra Vista indicate that atmospheric arsenic is also periodically elevated (data not shown); therefore, we chose to use tungsten, arsenic, and tungsten/arsenic in a combinative study. Arsenic is a known carcinogen, but is not known to be a leukemogen.

2. MATERIALS AND METHODS

2.1 Mouse exposures

2.1.1 Mouse exposure study, Part I

Ten C57BL/6 male and female, five-week-old mice were purchased through an IACUC approved protocol and randomly assigned to one of four groups to receive ammonium paratungstate ($(NH_4)_{10}H_2W_{12}O_{42}$, Spectrum; selected for solubility in saline) as an aerosol (13 ng/m^3), delivered utilizing a nebulizer attached to a nose-only IN-TOX chamber (Albuquerque, NM) for one hour a day, five days a week for one year, and/or as a water supplement (100 μg/m^3), or to a no-exposure group. The concentrations are a tabulated mean from Fallon, NV and Sierra Vista, AZ, based on water and tree-core samples collected at the point in time that the study began. Both Fallon and Sierra Vista have elevated concentrations of tungsten in the atmospheric particulate matter, and Fallon also has elevated tungsten levels in the drinking water. Because the primary exposure route is unknown, we utilized both gastric and pulmonary routes. Complete blood counts with a differential were obtained (Nijmeijer *et al*. 2001) from an automated HEMAVET 850 Multispecies Hematology Analyzer.

2.1.2 Mouse exposure study, Part II

C57BL/6 eight-week-old female mice, purchased through an IACUC-approved protocol, were tagged with an identification chip and randomly assigned to an exposure group to receive sodium tungstate ($Na_2O_4W{\cdot}2H_2O$, Acros Organics), sodium arsenite ($AsNaO_2$, Acros Organics), both agents, or neither through aerosol and water at 20% of the environmental concentrations and normalized from the quantity that a pregnant woman would inhale/ingest during a 40-week gestation in Fallon, Nevada. Exposure concentrations for tungstate were 187.517 g/L as an aerosol delivered as described above, and 0.1403 mg/L in water consumed *ad libitum*. Exposure concentrations for arsenite were 0.604 g/L as an aerosol and 0.00875 mg/L in water. All groups were housed in sterile, micro-isolation rooms. The female mice were exposed on a daily basis for one week, mated over the weekend with C57BL/6 male mice of equivalent age, and then exposures were continued until parturition. After weaning, the pups were sorted by gender, and their immune organs (bone marrow, spleen, thymus and liver) were harvested and preserved in RNALater (Ambion).

2.2 Transcriptome studies

Total RNA was extracted from W/As and control spleen tissue, utilizing a commercially available kit (BioRad). Because the average child does not develop leukemia, and because we did not want to average out the differential expression of constituent genes involved in a potential translocation should a small population of pre-leukemic clones be present, we did not pool the samples. Affymetrix 430A 2.0 mouse-gene chip arrays were hybridized and read with an Agilent/Affymetrix 2500A scanner. Raw data (.CEL files) were analyzed with GeneSpring v7.0 software containing the GC-RMA algorithm (Wu *et al*. 2004). Transcript signals were loaded into BRB ArrayTools (http://linus.nci.nih.gov/BRB-arraytools.html) and filtered based on the variance of each transcript. The log ratios of the variance were compared to the median of all transcript variances and significant (p = 0.001) transcripts selected. Onto-Express (http://www.geneontology.org/GO.tools.microarray.shtml) was utilized for ontology and BioRag for pathway analyses from which DMBT1 was selected for further RT–PCR studies. Network evaluation was conducted with Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Mountain View, CA, USA), in which mapping to a network was scored ($-\log_{10}(p\text{-value})$) and ranked. The RT-PCR data were generated utilizing a DMBT1 assay (Applied Biosystems, ABI) and ABI 7000 and 7300 SDS equipment utilizing GAPDH for determining baseline expression and evaluated with the Delta Delta–Cycle$_{Threshold}$ method.

2.3 STATISTICAL ANALYSES

Statistical analyses were conducted with Excel's XLStat 2007 and the data presented as mean ± standard error of the mean (SEM). Significance was determined with a Kruskal–Wallis test and a two-tailed Student t-test adjusted for multiple comparisons with significance accepted when $p < 0.05$.

3. RESULTS

3.1 Hematological profile

Leukemia affects the production of blood cells, resulting in an altered hematological profile such as anemia,

thrombocytopenia, neutropenia and, primarily, leukocytosis comprising immature cells called blasts. We exposed young adult, male and female mice ($N = 10$/group), to ammonium paratungstate through aerosol and/or water. No blasts were observed in the peripheral blood samples, suggesting that exposure to tungsten-alone is not a direct leukemogen in the young adult. However, tungsten exposure through water, aerosol, and both exposure routes altered blood parameters (primarily increasing the incidence of thrombocytopenia in both genders and dual symptoms of anemia/thrombocytopenia in males) during the first 84 days of exposure, which resolved as the mice grew older (data not shown).

3.2 TRANSCRIPTOME CHANGES

In utero exposure to tungstate/arsenite altered the expression of 177 genes (154 mapped and 22 unmapped) greater than 10-fold. Approximately 20 of these genes may be significant above what is expected due to chance. Analysis of ontology demonstrated that the majority of the differentially expressed genes were associated with hydrolase activity (elastases, kallikreins and trypsins), with the majority of these genes being down-regulated 100-fold, with the exception of cathepsin M which was up-regulated 12.6-fold. The biological processes most significantly represented in this pool of genes were cellular, including cell differentiation and proliferation (DMBT1, Phospholipase and Fibroblast Growth Factor) and cytoskeletal organization (MyosinVb, Piccolo, Filaggrin, and Expressed Sequence AV006891). Pathway analysis of these 177 genes with differential expression greater than 10-fold yielded 15 pathways which included Mek/Erk/Wnt pathways, antigen presentation and processing pathways, and cytokine interactions involving lymphocytic signaling (Table 2). Of these differentially expressed genes, we are particularly interested in the gene named Deleted in Malignant Brain Tumors 1 (DMBT1): anti-

Table 2. Significantly altered pathways associated with *in utero* exposure to tungstate/arsenite

Pathway title	Significance*	Gene bank	Gene symbol	Gene title	Fold change
MAPK signaling	0.000	AA517858	Fgf9	Fibroblast growth factor9	+38.1
		NM_011107	Pla2g1b	Phospholipase A2, group IB, pancreas	–35.7
		BM940281	Mapk8	Mitogen activated protein kinase/c-JunN-terminal kinase 1	+10.4
Erk1/Erk 2	0.004	NM_008456	Klk5	Kallikrein 5/nerve growth factor	–15.6
		BM120341	Itgb1	Integrin beta 1 (fibronectin receptor beta)/CD29	+6.24
		NM_008284	Hras1	Harvey rat sarcoma virus oncogene 1	–2.39
		AB057663 & NM_029780	Raf1	v-raf-1 leukemia viral oncogene 1	–2.4/–1.01
		AW553456	Map2k2	Mitogen activated protein kinase kinase 2	–2.55
		NM_021462	Mknk2	MAP kinase-interacting serine/threonine kinase 2	–3.98
		NM_021461	Mknk1	MAP kinase-interacting serine/threonine kinase 1	–21.5/–1.36
Wnt signaling	0.017	NM_021457	Fzd1	Frizzled homolog 1 (*Drosophila*)	–7.33
		NM_139059	Csnk1d	Casein kinase 1, delta	–15.0
ATM signaling	0.004	BC002033	Rad50	RAD50 homolog (*S. cerevisiae*)	+6.91
		NM_013752	Nbn	Nibrin	–1.64
		NM_007499	ATM	Ataxia telangiectasia mutated homolog (human)	–1.71
Antigen processing and presentation	0.017	NM_009952	Creb1	cAMP responsive element binding protein 1	–13.4
		NM_008198	H2-Bf	Histocompatibility 2, complement component factor B	–4.16
		BF319868	Grp58	Glucose-regulated protein	–2.41
		NM_011175	Lgmn	Legumain	–2.26
		BE691515	Tap2	Transporter2, ATP-binding cassette, sub-family B (MDR/TAP)	–2.37
		NM_010654	Klrd1	Killer cell lectin-like receptor, subfamily D, member 1	–2.23
		AF106008	Klrc1	Killer cell lectin-like receptor, subfamily C, member 1	–2.00
Cytokine–cytokine receptor interaction	0.017	NM_021782	Il21	*Mus musculus*, similar to interleukin 21 (LOC386435)	+13.7
		NM_009403	Tnfsf8	Tumor necrosis factor (ligand) superfamily, member 8/ Lymphocyte activation antigen CD30 (verified with RT-PCR)	+29.5

* Significance determined for the population of genes up-regulated greater than ten-fold.

gen presentation and processing pathways were significantly altered. DMBT1's protein products aggregate microorganisms. Furthermore, DMBT1 may function as a tumor suppressor and, additionally, may regulate immune response.

We verified the gene microarray expression of DMBT1, and then conducted a real-time RT–PCR investigation of DMBT1 expression in male and female C57BL/6 pups (N = 6–8/group/gender) exposed while *in utero* to tungstate, arsenite, tungstate/arsenite and longitudinal controls through aerosol and water. The amplification plots of DMBT1 expression demonstrated that DMBT1 is not constitutively expressed in spleen tissue. Four pups from each group demonstrated no expression of DMBT1, except for the arsenic group, which demonstrated expression in every sample tested. Prenatal exposure to arsenic demonstrated a strong bimodal distribution, and most other exposure groups (longitudinal controls and tungstate/arsenite, but not tungstate-alone) demonstrated a similar tendency. Some spleen samples expressed DMBT1 at high levels (the amplification plots formed a sigmoid curve) and some samples expressed at low levels (only the linear portion formed). When ranked by expression level, the low-expressing samples demonstrated no difference in expression level between groups, but the high-expressing samples demonstrated a significant difference according to type of *in utero* exposure (Figure 1). A Kruskal–Wallis test did not find a significant difference between the exposed pups and the controls, as whole populations. However, the differences observed in the high expressers, hypothesized to occur because of individual alleles (Blackburn *et al*. 2007; Mollenhauer *et al.* 2002*a*) or G0/G1 arrest and cell growth reduction (Kang *et al*. 2005), was significant for prenatal exposure to tungsten (Figure 2).

Networks containing DMBT1 were focused on the gene Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4), which codes for an enzyme involved in metal ion binding (Table 3) and centered on the down-regulation of the transcription factor E2F1 (Table 4). Additionally, Ubinuclein 1, which competes with an Epstein–Barr virus (EBV) early gene for AP-1 binding sites (Aho *et al*. 2000), was down-regulated. A network associated with immunological/hematological disease (Table 4) indicates impaired immune defense against dsRNA, virus E3 proteins, Hepatitis C Virus and repair of DNA lesions in hematopoietic tissues.

4. DISCUSSION

We hypothesize that prenatal exposure to an environmental chemical, held in common by communities experiencing elevated rates of leukemia, but not experienced by geographically similar control communities, results in genetic changes associated with increased susceptibility towards virus-induced leukemogenesis. We have demonstrated with multiple measures that Fallon is contaminated with ambient particles of tungsten. We have demonstrated that tungsten, alone, does not induce leukemia in the adult mouse, but we have

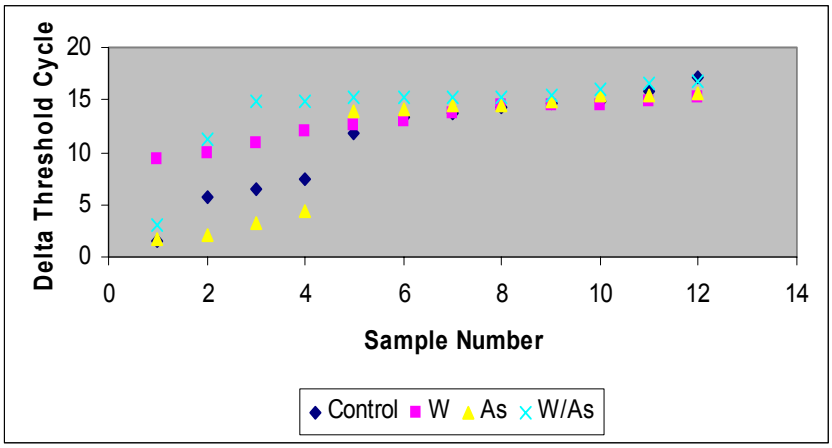

Figure 1. Expression of DMBT1 ranked by delta-threshold-cycle depicting populations of C57BL/6 mouse pups expressing DMBT1 at high and low levels. Statistical analysis of the ΔΔCt for the entire group as a whole demonstrated no significant difference (p = 0.058) from the controls; however, the difference between As and W/As was significant.

Table 3. Networks associated with diseases and disorders resulting from genes differentially expressed > ten-fold

Network	Disease/disorder (function)	Molecules in network[a]	Score	Focus genes
1	Cancer, neurological disease (lipid metabolism)	ANGPTL2, Ap1, CEL, CIT, CLPS, Creb, CREB1, CSNK1D, Cyclin A, Histone h3, HMGCR, KLF6, LRAT, P8, Pdgf, PDGF BB, PITX2, Pkc(s), PNLIP, PNLIPRP1, PNLIPRP2, PP2A, Rac, Ras, REG3A, RIT2, RPS6KB2, Rsk, SLC2A3, Sod, SOD2, SOX2, TP53, triacylglycerol lipase, VAMP1	43	22
2	Cancer, developmental disorder (cellular movement)	ACAA1, AKT1, BAIAP2, CACNA1C, CPA1, CTRB1, CTSB, CUBN, CYFIP2, ELA1, GAP43, GOSR2, GRP, ILF2, MAP3K14, MMP8, MMP9, NR2F2, NUAK1, OTT, PCLO, PPARBP, PPARG, PRSS1 (includes EG:5644), PRSS2 (includes EG:5645), PRSS3 (includes EG:5646), PTF1A, RABEP1, RAC1, RARB, RBPJL, retinoic acid, RNASE1 (includes EG:6035), Rxr, SPINK1 (includes EG:6690)	23	15
3	Cancer (cellular growth and proliferation, tissue development)	ANKRD25, ATAD2, beta-estradiol, C5, CDKN1A, CDKN2A, CPA2, CUZD1, DDR1, dehydroisoandrosterone, E4F1, ELA2A, estriol, FLG, HLA-DMB, HUNK, IFNG, KCNMA1, KCNMB1, KCNMB2, KCNMB4, KCNU1 (includes EG:16532), KLF6, MAPK3, PENK, PHIP, PLK2, PTX3, REG1B, RLN2, SLPI, STAT3, TNFSF8, TP53INP1, ZNF148	22	13
4	Cancer (cell signaling, drug metabolism)	3,7,12-trihydroxycoprostane, 4-androstene-3,17-dione, AMY2, ANGPTL4, ARNT2, CYP3A, CYP3A4, desoxycorticosterone, DMBT1, ERBB2, FOXA3, hydrogen peroxide, IL6, KLK1 (includes EG:16612), KLK1B5, lithocholic acid, MAP3K14, Ncoa-Nr1i2-Rxra, NR1I2, NR3C1, POR, progesterone, PSMC5, PSMD7, PXR ligand-PXR-retinoic acid-RXRα, REG1A, SLC7A11, SLCO1B3, SPAG1, SPINT2, tauroursodeoxycholic acid, UGT1A1, UGT1A6, ursodeoxycholic acid, ZG16	22	13
16	Cancer, cell morphology, hematological disease	MLL, PHF20, WDR5	2	1

a. Genes colored red were up-regulated and genes colored green were down-regulated in the gene microarray analysis. Genes in black capitals were differentially expressed less than 10-fold.

Table 4. Networks containing the gene DMBT1 or associated with hematological disease resulting from genes differentially expressed > five-fold

Network	Disease/condition (function)	Molecules[a]	Score	Genes
6	Cancer, hepatic system disease, cellular growth and proliferation	ARNT2, BNC1, CCDC80, CEBPA, CYP2C40, DMBT1,↓E2F1, EBF3, FOXA2, GZMK, ↑KRAS, ↓LDB2, ↓LITAF, LPPR4, LSM2, ↑MAP3K4, ↑MMD, ↑MMP8, PCLO, PFN1, POLA2, PTX3, ↓SERPINB1, ↓SLC2A4, ↓SMN1, SNN, SOD2, SOX4, SPINT2, TDO2, TNF, TNFSF9, UBN1, ↓ZFP36L1, ZG16	23	16
8	Hematological disease, immunological disease, cell death	ABCB1B, ACAA1, ANGPTL4, ANKRD25, ATG7, Cbp/p300, CDKN2A, cyclic AMP, DCTN4, dehydroepiandrosterone sulfate, ↓DUSP2, ↓DUT (includes EG:1854), ↓GABARAP, GH1, HMGA2, HMGC52, hydrogen peroxide, ↓IRF3, LGP2, ↓LIPE, MCHR1, PHF20, ↓PPAP2A, PPARG, ↓RAD23A, ↓RPL21, SLC31A2, SLC7A11, SLCO1, SLCO1B3, SOD2, ↑TP53INP1, ↓UQCRH, ↓WDR5, ZMYND19	9	14

a. Genes colored red were up-regulated and genes colored green were down-regulated in the gene microarray analysis. Genes in black capitals were differentially expressed less than five-fold.

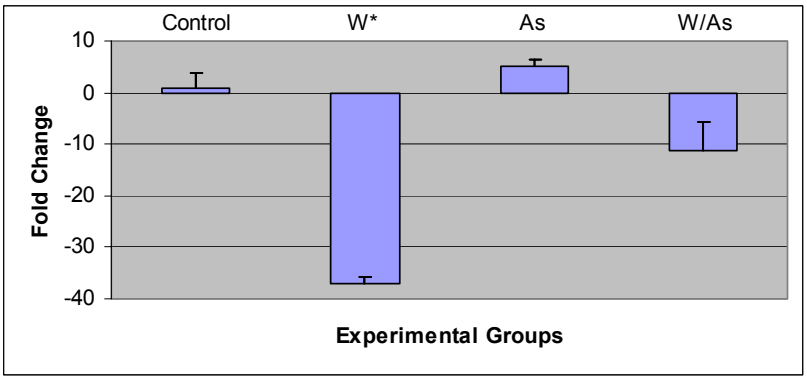

Figure 2. Fold-changes for DMBT1 expression in the high-expressing population of C57BL/6 pups. The statistical comparison of high-expressers indicates that *in utero* exposure to tungstate decreased expression by almost 37 ± 1.2-fold (p = 0.012), arsenic increased expression by greater than 5 ± 1.2-fold (not significant, p = 0.158), and the combination of W/As decreased expression by more than 11 ± 5.8-fold (N = 2).

also verified reports that tungsten ores may alter blood parameters (Idiiatullina 1981; Misiewicz 1983), and have discovered that this hematopoietic influence is greatest while the mice are young. We demonstrated that *in utero* exposure to tungstate is associated with significantly reduced transcription of the gene, Deleted in Malignant Brain Tumors 1 (DMBT1), whose protein products aggregate bacteria and viruses, by 37 $\pm$ 1.2-fold (p = 0.012) in mice that express DMBT1 at high levels, the implications of which are discussed below. We have suggested that this tungstate-induced down-regulation of DMBT1 may be mediated by Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4) and the transcription factor E2F1. Additionally, we found that *in utero* exposure to tungstate/arsenite generated differential expression for seven of the 13 genes identified as being consistently altered in acute lymphoblastic leukemias (Mullighan *et al*. 2007), one of which is ATM and is involved in a significantly altered pathway.

4.1 Expression of DMBT1

The DMBT1 gene was first identified by its homozygous deletion in glioblastoma multiforme (Rasheed *et al*. 1995; Albarosa *et al.* 1996), and as a candidate tumor suppressor by its presence in a locus (10q25.3-q26.1) identified by loss of heterozygosity in malignant brain tumors (Mollenhauer *et al*. 1997), and subsequently in other forms of cancer in epithelial tissues such as breast, colorectal, esophageal/gastric, pancreatic, and lung cancers (Mori *et al*. 1999; Takeshita *et al.* 1999; Wu *et al.* 1999; Hustinx *et al.* 2004; Robbe *et al*. 2005; Blackburn *et al*. 2007). However, some investigations have demonstrated elevated expression of DMBT1 in slow-growing, differentiated tumors (Kang *et al.* 2005). We discovered variable levels of DMBT1 expression in spleen tissue from C57BL/6 four-week-old pups in all exposure groups and in both genders. Variable levels of DMBT1 expression have been attributed to individual alleles (Mollenhauer *et al*. 2002*a*; Blackburn *et al*. 2007), antigenic stimulus (Bikker *et al*. 2004; Hartshorn *et al.* 2006) or G0/G1 arrest and cell growth reduction during differentiation (Kang *et al*. 2005).

Several groups (Takeshita *et al.* 1999; Wu *et al*. 1999; Petersen *et al*. 2000; Mollenhauer *et al*. 2002*b*), investigating the association of DMBT1 alleles and lack of expression in tumors, concluded that mutations in DMBT1 occur at low frequency in cancer. If allelic differences are associated with variable DMBT1 expression, it is most likely within the promoter region, but largely remains uninvestigated. We believe microbial stimulus is not the cause of the dynamic expression of DMBT1 within the controls, because we housed our mice in sterile, micro-isolation rooms. The most likely explanation for the variable levels of DMBT1 expression is provided by a study in a gastric cell line, which demonstrates that extracellular signal-related kinase (ERK) and protein kinase C (PKC) mediated the down-regulation of DMBT1 expression and the initiation of cell differentiation. Additionally, inhibition of proliferation, either with an ERK inhibitor or high-density seeding, was associated with marked increase in DMBT1 expression (Kang *et al.* 2005).

4.2 Tungstate-associated suppression of DMBT1

The differentiation hypothesis predicts that the tungstate-associated suppression of DMBT1 (Figure 2) in cells which may be receiving signals to differentiate, may encourage epithelial cells to ignore signals to exit the cell cycle, but instead encourages them to continue to proliferate. Exposure to tungstate may encourage tumorigenesis of epithelial tissues as previous research has suggested (Wei *et al.* 1985).

Additionally, if prenatal exposure to tungstate, which decreases the high-level expression of DMBT1 (Figure 2), also results in decreased levels of protein product available to aggregate microorganisms, such as the human herpes virus-4 (EBV) (which target B-cells for proliferation), more may escape aggregation to infect B lymphocytes. As the total number of EBV-infected cells increases, the probability increases that one of the cells targeted for proliferation by EBV may be a preleukemic B-cell harboring a translocation generated spontaneously during *in utero* growth and which has managed to escape apoptosis through down-regulation of ATM (Table 2).

Although the dams were exposed to Na_2WO_4 dissolved into physiological saline, and brought to neutral pH or dissolved into drinking water, the actual tungsten molecule formed under physiological conditions which may be associated with the down-regulation of DMBT1 transcription, is not known. Under some physiological conditions (i.e. neutral pH), the tungstate anion may polymerize with itself, forming polyoxotungstates (POTs) around a phosphate or other metal cation, such as molybdenum or cobalt. Arsenite-POTs have also been synthesized. POTs demonstrate various physiological effects, dependent upon the nature of the central atom, and most likely as a consequence of the high anionic valency of the molecule (e.g. $(PW_{11}O_{39})^{7-}$ at neutral pH), coupled with a relatively small molecular radius. These physiological effects associated with phosphorous-POTs include inhibition of factors IIa and Xa in the presence of anti-thrombin III and inhibition of anion-transport in erythrocytes and porcine sperm. The WO_4^{2-} ion did not exhibit these traits (reviewed in Tajima 2005).

4.3 Suggested mechanism of tungstate-associated suppression of DMBT1

The cancer networks (Table 3) primarily involve cell-to-cell interactions and signal transduction. Networks containing DMBT1 suggest that tungstate-induced down-regulation of DMBT1 may be mediated by the gene CYP3A4 (Table 3), which codes for an enzyme involved in metal–ion binding, and the down-regulation of the transcription factor E2F1 (Table 4). While CYP3A4 is not reported to bind tungsten, it is consistent with the wide range of xenobiotics that CYP3A4 is able to process. Both the ten-fold network (Table 3) and five-fold network (Table 4) connect DMBT1 through the 1.6-fold up-regulation of Map3K14 (Chen *et al*. 2003).

MAP3K14 has been shown to be regulated by the E2F1 transcription factor (Stanelle *et al.* 2002), which was down-regulated 1.5-fold and which regulates cell cycle progression through the G1/S phase transition (La Thangue 1994). Mice deficient in E2F1 demonstrate a more rapid and increased entry into the S phase upon antigenic stimulus (Salam *et al.* 2004). Such biological consequences may also include reduced DMBT1 expression, whose increased expression is associated with exit from the cell cycle (Kang *et al.* 2005). This network also suggests other down-stream consequences which may be mediated by transcription factor CEBPA (CCAAT/enhancer binding protein alpha), and which interacts with UBN1 (Ubinuclein 1), both of which were down-regulated (Table 4). UBN1 competes with EB1, an EBV early gene, for AP-1 binding sites in the reactivation of latent EBV infection (Aho *et al*. 2000). Although antimony- and silicon-POTs have potent antiviral activity, this may be only for concurrent exposures. While dual treatment of a Burkitt's lymphoma-derived cell line with antimony-POT and an EBV-reactivation agent (5-iodo-2'-deoxyuridine, IUdR) decreased the expression of the EBV early antigen as compared to IUdR controls, pretreatment with antimony-POT 24-hours prior to reactivation, enhanced the expression of the EBV early antigen (Souyri-Caporale *et al.* 1984). The cancer network with DMBT1 (Table 4) suggests that a prenatal exposure to tungstate may down-regulate Ubinuclein 1, possibly potentiating the enhanced expression of the EBV early antigen.

5 CONCLUSION

We have reported that prenatal exposure to tungstate is associated with a decrease in transcriptional expression of DMBT1 by 37 $\pm$ 1.2-fold (p = 0.012) in mice

expressing DMBT1 at high levels. This association alone does not indicate that prenatal exposure to tungstate is leukemogenic or carcinogenic. However, it does indicate, along with the possible alterations in the ATM pathway and network connections associated with EBV interactions, and possibly HCV and HV-3, that further research is warranted to ascertain the leukemogenic ability of tungstate/arsenite exposures.

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REFERENCES

Aho, S., Buisson, M., Pajunen, T., Ryoo, Y.W., Giot, J.F., Gruffat, H. *et al.* (2000) Ubinuclein, a novel nuclear protein interacting with cellular and viral transcription factors. *J. Cell Biol.*, **148** (6), 1165–76

Albarosa, R., Colombo, B.M., Roz, L., Magnani, I, Pollo, B., Cirenei, N. *et al*. (1996) Deletion mapping of gliomas suggest the presence of two small regions for candidate tumor-suppressor genes in a 17-cM interval on chromosome 10q. *Am. J. Hum. Genet.*, **58** (6), 1260–7

Ando, A., Ando, I., Hiraki, T. and Hisada, K. (1989) Relation between the location of elements in the periodic table and various organ-uptake rates. *Int. J. Rad. Appl. Instrum. B*, **16** (1), 57–80

Belson, M., Kingsley, B. and Holmes, A. (2007) Risk factors for acute leukemia in children: a review. *Environ. Health Perspect*., **115** (1) 138–45

Bikker, F.J., Ligtenberg, A.J., End, C., Renner, M., Blaich, S., Lyer, S. *et al*. (2004) Bacteria binding by DMBT1/SAG/gp-340 is confined to the VEVLXXXXW motif in its scavenger receptor cysteine-rich domains. *J. Biol. Chem*., **279** (46), 47699–703

Blackburn, A.C., Hill, L.Z., Roberts, A.L., Wang, J., Aud, D., Jung, J. *et al*. (2007) Genetic mapping in mice identifies DMBT1 as a candidate modifier of mammary tumors and breast cancer risk. *Am. J. Pathol*., **170** (6), 2030–41

CDC (2003) *A Cross-sectional Exposure Assessment of Environmental Exposures in Churchill County, Nevada*. US Centers for Disease Control and Prevention. Available at www.cdc.gov/nceh/clusters/fallon/default.htm

CDC (2006) *Biosampling Case Children with Leukemia (Acute Lymphocytic and Myelocytic Leukemia) and a Reference Population in Sierra Vista, Arizona*. US Centers for Disease Control and Prevention. Available at www.cdc.gov/NCEH/clusters/sierravista/default.htm

Chen, D., Xu, L.G., Chen, L., Li, L., Zhai, Z. and Shu, H.B. (2003) NIK is a component of the EGF/heregulin receptor signaling complexes. *Oncogene*, **22** (28), 4348–55

Hartshorn, K.L., Ligtenberg, A., White, M.R., Van Eijk, M., Hartshorn, M., Pemberton. L. *et al*. (2006) Salivary agglutinin and lung scavenger receptor cysteine-rich glycoprotein 340 have broad anti-influenza activities and interactions with surfactant protein D that vary according to donor source and sialylation. *Biochem. J*., **393** (2), 545–53

Humble, W. and Flood, T. (2002) Health consultation: review of environmental data in air, drinking water, and soil. Arizona Department of Health Services. Available at www.azdhs.gov/phs/oeh/pdf/sierra_vista_sept12.pdf

Hustinx, S.R., Cao, D., Maitra, A., Sato, N., Martin, S.T., Sudhir, D. *et al*. (2004) Differentially expressed genes in pancreatic ductal adenocarcinomas identified through serial analysis of gene expression. *Cancer Biol. Ther*., **3** (12), 1254–61

Idiiatullina, F.K. (1981) [Data on a hygienic standard for wolfram in the air]. *Gig. Sanit.* [in Russian], **9**, 79–81

Kang, W., Nielsen, O., Fenger, C., Leslie, G., Holmskov, U. and Reid, K.B. (2005) Induction of DMBT1 expression by reduced ERK activity during a gastric mucosa differentiation-like process and its association with human gastric cancer. *Carcinogenesis*, **26** (6), 1129–37

La Thangue, N.B. (1994) DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. *Trends Biochem. Sci*,. **19** (3), 108–14

Misiewicz, A. (1983) [Effect of air containing benzine, wolfram, titanium, cobalt and vanadium on peripheral blood]. *Med. Pr.,* [in Polish], **34** (3), 251–7

Mollenhauer, J., Wiemann, S., Scheurlen, W., Korn, B., Hayashi, Y., Wilgenbus, K.K. *et al.* (1997) DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. *Nat. Genet*., **17** (1), 32–9

Mollenhauer, J., Helmke, B., Muller, H., Kollender, G., Krebs, I., Wiemann, S. *et al*. (2002*a*) An integrative model on the role of DMBT1 in epithelial cancer. *Cancer Detect. Prev.*, **26** (4), 266–74

Mollenhauer, J., Muller, H., Kollender, G., Lyer, S., Diedrichs, L., Helmke, B. *et al.* (2002*b*) The SRCR/SID region of DMBT1 defines a complex multi-allele system representing the major basis for its variability in cancer. *Genes, Chromosomes and Cancer*, **35** (3), 242–55

Mori, M., Shiraishi, T., Tanaka, S., Yamagata, M., Mafune, K., Tanaka, Y. *et al*. (1999) Lack of DMBT1 expression in oesophageal, gastric and colon cancers. *Br. J. Cancer*, **79** (2), 211–3

Mullen, A.L., Bretthauer, E.W. and Stanley, R.E. (1976) Absorption, distribution and milk secretion of radionucleotides by the dairy cow – V. Radiotungsten. *Health Phys*., **31** 417–24

Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D. *et al*. (2007) Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature*, **446** (7137), 758–64

Nadeenko, V.G., Lenchenko, V.G., Genkina, S.B. and Arkhipenko, T.A. (1978) [Effect of tungsten, molybdenum, copper and arsenic on the intrauterine development of the fetus]. *Farmakol Toksikol*., **41** (5), 620–623 (in Russian)

Nijmeijer, B.A., Mollevanger, P., van Zelderen-Bhola, S.L, Kluin-Nelemans, H.C., Willemze, R., Falkenburg, J.H. (2001) Monitoring of engraftment and progression of acute lymphoblastic leukemia in individual NOD/SCID mice. *Exp. Hematol*., **29** (3), 322–9

Petersen, S., Rudolf, J., Bockmuhl, U., Deutschmann, N., Dietel, M. and Petersen, I. (2000) Analysis of the DMBT1 gene in carcinomas of the respiratory tract. *Int. J. Cancer*, **88** (1), 71–6

Rasheed, B.K., McLendon, R.E., Friedman, H.S., Friedman, A.H., Fuchs, H.E., Bigner, D.D. *et al.* (1995) Chromosome 10 deletion mapping in human gliomas: a common deletion region in 10q25. *Oncogene,* **10** (11), 2243–6

Robbe, C., Paraskeva, C., Mollenhauer, J., Michalski, J.C., Sergi, C. and Corfield, A. (2005) DMBT1 expression and glycosylation during the adenoma–carcinoma sequence in colorectal cancer. *Biochem. Soc. Trans*., **33** (4), 730–2

Rubin, C.S., Holmes, A.K., Belson, M.G., Jones, R.L., Flanders, W.D., Kieszak, S.M. *et al*. (2007) Investigating childhood leukemia in Churchill County, Nevada. *Environ. Health Perspect*., **115** (1), 151–7

Salam, M.A., Matin, K., Matsumoto, N., Tsuha, Y., Hanada, N. and Senpuku, H. (2004) E2f1 mutation induces early onset of diabetes and Sjogren's syndrome in nonobese diabetic mice. *J. Immunol*., **173** (8), 4908–18

Sheppard, P.R., Ridenour, G., Speakman, R.J. and Witten, M.L. (2006) Elevated tungsten and cobalt in airborne particulates in Fallon, Nevada: possible implications for the childhood leukemia cluster. *Applied Geochemistry*, **21** (1), 152–65

Sheppard, P.R., Speakman, R.J., Ridenour, G., Glascock, M.D., Farris, C. and Witten, M.L. (2007*a*) Spatial patterns of tungsten and cobalt in surface dust of Fallon, Nevada. *Environ. Geochem. Health,* **29** (5), 405–12

Sheppard, P.R., Speakman, R.J., Ridenour, G. and Witten, M.L. (2007*b*) Temporal variability of tungsten and cobalt in Fallon, Nevada. *Environ. Health Perspect*., **115** (5), 715–9

Sheppard, P.R., Speakman, R.J., Ridenour, G., Witten, M.L. (2007*c*) Using lichen chemistry to assess airborne tungsten and cobalt in Fallon, Nevada. *Environ. Monit. Assess*., **130** (1–3), 511–8

Souyri-Caporale, M., Tovey, M.G., Ono, K., Jasmin, C. and Chermann, J.C. (1984) Modulation by the polyoxotungstate HPA-23 of Epstein–Barr virus early antigen expression in Raji cells treated with iododeoxyuridine. *J. Gen. Virol*., **65** (4), 831–5

Stanelle, J., Stiewe, T., Theseling, C.C., Peter, M. and Putzer, B.M. (2002) Gene expression changes in response to E2F1 activation. *Nucleic Acids Res*., **30** (8), 1859–67

Steinberg, K.K., Relling, M.V., Gallagher, M.L., Greene, C.N., Rubin, C.S., French, D. *et al*. (2007) Genetic studies of a cluster of acute lymphoblastic leukemia cases in Churchill County, Nevada. *Environ. Health Perspect*., **115** (1), 158–64

Steinmaus, C., Meng, L., Randall, L.T. and Smith, A.H. (2004) Probability estimates for the unique childhood leukemia cluster in Fallon, Nevada, and risks near other U.S. military aviation facilities. *Environ. Health Perspect*., **112** (6), 766–71

Tajima, Y. (2003) The effects of tungstophosphate and tungstosilicate on various stress promoters transformed in *Escherichia coli*. *J. Inorg. Biochem*., **94** (1–2), 155–60

Tajima, Y. (2005) Polyoxotungstates reduce the beta-lactam resistance of methicillin-resistant *Staphylococcus aureus*. *Mini Rev. Med. Chem*., **5** (3), 255–68

Takeshita, H., Sato, M., Shiwaku, H.O., Semba, S., Sakurada, A., Hoshi, M. *et al.* (1999) Expression of the DMBT1 gene is frequently suppressed in human lung cancer. *Jpn. J. Cancer Res.,* **90** (9), 903–8

Wase, A.W. (1955) Absorption and distribution of radio-tungstate in bone and soft tissues. *Arch. Biochem. Biol. Phys*., **61**, 272–77

Wei, H.J., Luo, X.M. and Yang, S.P. (1985) Effects of molybdenum and tungsten on mammary carcinogenesis in SD rats. *J. Natl. Cancer Inst*., **74** (2), 469–73

Wide, M., Danielsson, B.R.G. and Dencker, L. (1986) Distribution of tugstate in pregnant mice and effect in embryonic cells in vitro. *Environ. Res*., **40**, 487–98

Wu, W., Kemp, B.L., Proctor, M.L., Gazdar, A.F., Minna, J.D., Hong, W.K. *et al*. (1999) Expression of DMBT1, a candidate tumor suppressor gene, is frequently lost in lung cancer. *Cancer Res*., **59** (8), 1846–51

Wu, Z., Irizarry, R.A., Gentleman, R., Martinez-Murillo, F. and Spencer, F. (2004) A model-based background adjustment for oligonucleotide expression arrays. *J. Amer. Stat. Assoc*., **99**, 909–17