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Arsenite exposure in human lymphoblastoid cell lines induces autophagy and coordinated induction of lysosomal genes

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Abstract

Chronic exposure to inorganic arsenic is associated with diverse, complex diseases, making the identification of the mechanism underlying arsenic-induced toxicity a challenge. An increasing body of literature from epidemiological and *in vitro* studies has demonstrated that arsenic is an immunotoxicant, but the mechanism driving arsenic-induced immunotoxicity is not well established. We have previously demonstrated that in human lymphoblastoid cell lines (LCL), arsenic-induced cell death is strongly associated with the induction of autophagy. In this study we utilized genome-wide gene expression analysis and functional assays to characterize arsenic-induced effects in seven LCL that were exposed to an environmentally relevant, minimally cytotoxic, concentration of arsenite (0.75 μ M) over an eight-day time course. Arsenic exposure resulted in inhibition of cellular growth and induction of autophagy (measured by expansion of acidic vesicles) over the eight-day exposure duration. Gene expression analysis revealed that arsenic exposure increased global lysosomal gene expression, which was associated with increased functional activity of the lysosome protease, cathepsin D. The arsenic-induced expansion of the lysosomal compartment in LCL represents a novel target that may offer insight into the immunotoxic effects of arsenic.

Keywords

Autophagy; Arsenic; Lysosome; Microarray; Lymphoblastoid

INTRODUCTION

Arsenic exposure is associated with considerable human morbidity and mortality. Among its many targets, the human immune system has been shown to sustain damage from exposure to inorganic arsenic. Raqib et al. investigated the immunosuppressive effects of arsenic in a population of pregnant women in rural Bangladesh, reporting that arsenic exposure in the pregnant mothers was associated with the incidence of maternal fever and diarrhea during pregnancy, and acute respiratory infections in the infants (Raqib et al., 2009). Maternal arsenic exposure has also been associated with decreased thymus size in infants who were exposed to arsenic *in utero* (Moore et al., 2009). Soto-Pena et al., studying children in Mexico reported that arsenic exposure was associated with diminished proliferative response

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of T-lymphocytes to phytohemagglutinin (PHA) stimulation, together with additional evidence of immunotoxicity (Soto-Pena et al., 2006).

We recently demonstrated that in a human lymphoblastoid cell line (LCL), arsenic exposure induced cytotoxicity that was closely associated with the induction of autophagy, a process by which specific proteins, organelles and bulk cytoplasm are delivered to lysosomes for degradation. In this experimental model of immune cell targeting by arsenic, frankly cytotoxic concentrations of arsenic induced a cell death that was not associated with apoptosis, but was characterized by hallmarks of autophagy that included increased protein levels of microtubule associated protein 1 light chain 3 B type II (LC3B-II), as well as an increased number of autophagic vesicles and lysosomes measured by electron microscopy and by flow cytometry (Bolt et al., 2010). Because that study was aimed at understanding the cellular mechanisms associated with arsenic-induced cell death in immune cells, relatively high concentrations (6 μ M) and short exposure durations (96 hours) were used. In light of the importance of autophagy as a basic cellular process, as well as its specific importance to antigen-presenting cells in the immune system (also modeled by LCL) we were interested to know whether a lower, more environmentally relevant concentration of inorganic arsenic used in a longer exposure duration was also associated with autophagy in LCL. Here we report the analysis of LCL from seven individuals exposed *in vitro* to levels of arsenite commonly encountered in the environment.

MATERIALS AND METHODS

Reagents

Sodium arsenite (dissolved in MilliQ H₂O) was purchased from Sigma Aldrich (St. Louis, MO).

Cell Culture and Exposure Conditions

Human Epstein-Barr virus immortalized lymphoblastoid cell lines GM18564, GM19209, GM18853, GM18550, GM18504, GM18532, and GM18561 were purchased from Coriell Cell Repository (Camden, NJ). Cultures were maintained in RPMI media supplemented with L-glutamine, 15% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA). Cells were grown in culture flasks and cultures were maintained between the cell concentrations of 350,000–2,000,000 cells/ml at 5 % CO₂ and 37 °C. Cell cultures were seeded to 350,000–500,000 cells/ml in supplemented media and dosed with sodium arsenite at the indicated concentration and exposure length. Cells were harvested by centrifugation and subsequently processed and analyzed by particular methods mentioned below.

Cell Proliferation

In order to measure the effect of arsenic on cell proliferation, trypan blue positive and negative cells were counted using a Vi-Cell Series Cell Viability Analyzer (Beckman Coulter Inc., Brea, CA). Each cell line was cultured for 8-days with or without exposure to 0.75 μ M sodium arsenite. Total cell count per sample was determined by the number of viable cells (trypan blue negative) per milliliter multiplied by the total volume of cells in each culture. Percent viability for each sample was calculated by the number of trypan blue negative cells divided by the total number of cells (trypan blue positive and negative cells), represented as a percent. Cell population doubling time was determined with the following equation: $D_t = ((\text{total days of growth (D)}) \times \text{Log}2/\text{Log} (\# \text{ of cells Time } D/\text{number of cells Time } 0)) \times 24$. Mean doubling time and standard error of the mean (SEM) for each sample were calculated from three independent experiments. Mean doubling time ratio between the eight-day arsenite exposed samples versus the control samples for each cell line was

calculated to measure the effect arsenic on cell proliferation. *Statistical analysis:* T-test (unpaired) comparisons were performed for the mean doubling time and percent trypan blue negative cells values between control and eight-day arsenite exposed samples separately for each cell line at a significance threshold of $P < 0.05$ using Microsoft Excel, MAC 2008 (Redmond, WA).

Lysotracker Red Dye (LRD) Staining

Cells (1×10^6) were harvested on day 8 under control or 0.75 μ M sodium arsenite exposure conditions, centrifuged, resuspended in 20 nM LRD (Invitrogen, Carlsbad, CA), and incubated at 37 °C for 30 min. Cells were then washed once in 1x phosphate buffered saline (PBS) and resuspended in 500 μ l PBS to a final cell concentration of 2×10^6 cells/ml. Samples were analyzed by flow cytometry on a LSR II Flow Cytometer (BD Biosciences, Sparks, MD). Fluorescence of LRD was collected through the 610/20 nm bandpass filter. Data were analyzed using FACS Diva (BD Biosciences, Sparks, MD) software. Mean relative fluorescence units (RFU) and SEM of LRD fluorescence were calculated for each sample from three independent experiments. LRD ratios were calculated by dividing the mean RFU for the eight-day arsenite exposed sample by the mean RFU for the control sample for each cell line. *Statistical Analysis:* T-test (unpaired) comparisons were performed to compare the mean RFU of LRD fluorescence between control and eight-day arsenite exposed samples for each cell line separately at a significance threshold of $P < 0.05$ using Microsoft Excel, MAC 2008 (Redmond, WA). To determine if arsenite-induced growth inhibition was correlated with either cytotoxicity or LRD fluorescence, we performed correlation analyses (Spearman's), comparing doubling time ratios with either cytotoxicity (percentage of trypan blue positive cells) or LRD ratios at a significance cutoff of $P < 0.05$ using PASW Statistics version 18.0 (SPSS/IBM, Chicago, Illinois, USA).

RNA Isolation

Cells (5×10^6) were harvested by centrifugation after exposure to 0.75 μ M sodium arsenite for 0, 1, 2, 4, 6, or 8 days. RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). On column DNase digestion was performed using the RNase-Free DNase Set (Qiagen, Valencia, CA). RNA was quantitated with the Nanodrop 1000 spectrometer at an absorbance of 260 nm (Nanodrop Products, Wilmington, DE).

Microarray Gene Expression Analysis

RNA quality for each sample was evaluated using A260/A280 ratio (> 2.0) and RNA 6000 Nano Chips in the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The expression profiles were generated using the Affymetrix GeneChip Human Gene 1.0 ST oligonucleotide arrays according to manufacturer's protocol (Affymetrix, Santa Clara, CA), which simultaneously measures expression of 28,869 genes across the genome. Affymetrix CEL files were imported into GeneSpring GX v.11.0 (Agilent Technologies, Santa Clara, CA) analysis software. Data were normalized using robust multichip analysis (RMA). *Statistical Analysis:* To specifically identify gene expression changes regulated by arsenite exposure that occurred in genes whose products are localized to the lysosome we used the Gene Ontology (GO) category, lysosome (GO:0005764) from AmiGO gene ontology (Ashburner et al., 2000). The GO lysosome gene list was imported into GeneSpring GX and 224 of the total 940 annotated genes from this category were present on the Affymetrix array platform.

This filtered 224 gene list was used as the input for statistical testing of gene expression differences between days of arsenite exposure. Gene expression differences between days of arsenite exposure (0,1,2,4,6,8) was evaluated using a one-way ANOVA analysis with a false discovery rate threshold of 0.05 as implemented in GeneSpring GX v. 11.0. Tukey's post-

hoc test was used to evaluate pair-wise comparisons of treatment days for genes with significant expression differences.

To compare the proportion of up- and down-regulated lysosomal genes to the proportion in the unfiltered (by functional ontology) genome, we also performed a similar one-way ANOVA (FDR < 0.05) analysis for differences in expression between days of arsenite exposure on all genes with expression levels greater than the 20th percentile.

TFEB (alone) gene expression was analyzed for differences between days of arsenite treatment using a one-way ANOVA analysis with Tukey's post hoc test, with a significance threshold of $P < 0.05$.

Real-Time PCR Validation of Microarray Data

To validate microarray gene expression data we measured mRNA expression by real-time polymerase chain reaction (RT-PCR) for the genes transcription factor EB (TFEB), lysosomal-associated membrane protein 2 (LAMP2) and lysosomal-associated membrane protein 3 (LAMP3). TaqMan primer-probe sets for TFEB, LAMP2, LAMP3, and the constitutively expressed gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems Inc. (Foster City, CA). RT-PCR was performed using TaqMan One-Step RT-PCR Master Mix and the Applied Biosystems 7900 RT-PCR System (Applied Biosystems Inc., Foster City, CA) following manufacture's protocol. We analyzed mRNA expression levels from control and day 8 arsenite exposed RNA samples (same samples used for the microarrays) from each of the 7 cell lines. Each reaction was performed in quadruplicate. TFEB, LAMP2, and LAMP3 values were normalized to GAPDH values for each sample. Mean fold change in gene expression was calculated for each arsenite sample verses the untreated control sample (fold change 1.0) using the $\Delta\Delta C_T$ method.

Cathepsin D Activity Assay

Cathepsin D activity levels were measured using the Cathepsin D Activity Assay Kit (Bio Vision, Mountain View, CA) following manufacture's protocol. Briefly, cells (1×10^6) were harvested from vehicle control and 8-day arsenite (0.75 μ M) exposed samples and lysed with 200 μ l lysis buffer. 25 μ l of cell lysate from each sample was added to a 384 well plate along with reaction buffer and substrate and incubated at 37 °C for 2 hr. Fluorescence was measured using a SpectraMax microplate spectrofluorometer and SoftMax Pro software (Molecular Devices, Sunnyvale, CA) at an excitation/emission of ex: 320 nm/em: 460 nm. Each sample was analyzed in triplicate and the mean RFU and SEM was calculated for each sample. *Statistical Analysis:* T-test (unpaired) comparisons between control and 8 day arsenite-exposed LCL were performed separately for each cell line using Microsoft Excel, MAC 2008 (Redmond, WA).

RESULTS

Arsenite impact on LCL proliferation and cytotoxicity

Control LCL cultures derived from seven human donors exhibited population doubling times ranging from 49.4 hours to 64.1 hours (Table 1). We chose an arsenite exposure level of 0.75 μ M (about 56 parts per billion) because preliminary experiments suggested that this exposure level produced minimal LCL cell death, as well as because this arsenic concentration is commonly encountered in drinking water, and is within the arsenic concentration range measured in human blood from arsenic-exposed individuals (Hall et al., 2006). Exposure to 0.75 μ M arsenite for 8 days slowed cell growth in all seven lines, but we observed individual variability in the severity of this effect. The most sensitive LCL, 18853, sustained an increased doubling time of 2.3-fold compared to control, from 57 hours to 134

hours, while the most resistant LCL, 18564 sustained a doubling time increase of only 1.1 fold, from 49 hours to 56 hours. None of the LCL doubled more rapidly with arsenite exposure. The reduction in population doubling rate was not accompanied by substantial increases in cell death (Figure 1). While arsenite exposure resulted in decreased LCL viability (decreased percent trypan blue negative cells) that was statistically significant in four of the seven LCL, the magnitude of arsenite-induced cell death was not large. Transforming the raw trypan blue-negative LCL percentages shown in Figure 1 into viability in arsenite-exposed cultures as a percent of control cultures, arsenite exposure resulted in percent of control viability that ranged from 91% to 99%. The degree of arsenite-induced cytotoxicity among the seven LCL was positively correlated to the degree of proliferative inhibition (Spearman's $r = 0.873$, $P = 0.01$).

Arsenite induces expansion of acidic vesicles consistent with autophagy

We have previously shown that increased fluorescence of the acidic vesicle-accumulating dye lysotracker red dye in arsenite-exposed LCL is concurrent with cardinal markers of autophagy (Bolt et al., 2010). The seven LCL were assayed using LRD following culture for 8 days under control conditions or exposure to arsenite (0.75 μ M). In each LCL (Table 2), arsenite exposure resulted in an increased level of LRD fluorescence, ranging from a 1.3 fold increase to a 2.2 fold increase. Similar to its effect on cytotoxicity, the extent to which arsenite induced the expansion of the vesicular acidic compartment varied among the LCL. Interestingly, there was also a positive correlation between fold-increase in LRD fluorescence and the proliferative inhibition induced by arsenic (Spearman's $r = 0.80$, $P = 0.03$).

Gene Expression Analysis

The LRD data suggested that lysosomes, or their derivative autophagic structures, autolysosomes, were increased in size or number or both after 8 days of arsenite exposure in LCL. In order for this expansion of lysosomes to be sustained over 8 days it seemed likely that the expression of genes with products localized to the lysosome (for convenience, hereafter called "lysosomal genes") would be up-regulated to support this process. In order to test this hypothesis we used genome-wide microarray analysis of gene expression in each LCL measured at 0, 1, 2, 4, 6, and 8 days of arsenite (0.75 μ M) exposure. Using all genes from all species assigned to the "lysosome" gene ontology category (940 genes) we screened for the subset of those 940 genes present on the Affymetrix Human Gene 1.0 ST array, producing a list of 224 lysosomal genes for which we could measure gene expression. We tested whether the expression of these genes was altered by arsenic exposure using one-way ANOVA analysis and Tukey's post-hoc test, with a false discovery rate threshold set at 0.05. ANOVA analysis identified 40 statistically significantly dysregulated genes. Tukey's post hoc testing of pairs of conditions revealed that 38 of the 40 genes were significantly differentially expressed between day 0 (control) LCL and one or more time-points during arsenite exposure (Table 3). There was a strong bias toward arsenite causing induction of these genes compared to repression. Comparing day 0 to day 8 expression, 95% of the 40 significant genes were up-regulated. This strong bias toward up-regulation was specific to the lysosomal genes. In comparison to the lysosomal genes, one-way ANOVA analysis of all genes (above the 20th percentile in expression) for gene expression differences between days at a 5% false discovery rate threshold produced 2367 significant genes, of which only 32% were up-regulated on day 8 relative to day 0 (data not shown).

The fact that a substantial fraction of lysosomal genes was induced by arsenite suggested that a master regulator of gene expression could be involved. One such gene has been described, transcription factor EB (TFEB), which trans-activates gene expression in target genes containing the CLEAR cis-acting element, a characteristic of many lysosomal genes

(Sardiello et al., 2009). We evaluated the effect of arsenite on TFEB expression using the microarray data. In every LCL, TFEB expression was induced by arsenite exposure. Temporally, the increase in TFEB expression (Figure 2) paralleled the increase in lysosomal gene induction (Table 3), with a small increase on days 1 and 2, and a larger, statistically significant increase in expression on days 4 through 8.

Real-Time PCR validation of microarray data

To ensure that the microarray data were not systematically inaccurate in defining gene expression changes we chose two genes with a low level induction (transcription factor EB (TFEB) and lysosome associated membrane protein 2 (LAMP2)) and one gene with a high level of induction (lysosome associated membrane protein 3 (LAMP3)) measured by microarray, and quantified their gene expression by RT-PCR in the same RNA samples in which microarray analysis was performed, comparing control to 8 day arsenite-exposed LCL. Microarray analysis of LAMP3 demonstrated robust induction from arsenite exposure, with fold-changes ranging from 1.3 – 4.9 for the seven LCL (Table 4). LAMP3 gene induction was confirmed in six of seven LCL by RTPCR. In one LCL, RT-PCR failed to confirm LAMP3 induction, measuring no change in gene expression between arsenite-exposed and control LCL. Not surprisingly this involved the LCL with the lowest fold-change measured by microarray (1.3 fold). TFEB and LAMP2 demonstrated a less robust induction from arsenite exposure in the microarray study, with fold change ranging from 1.1 – 1.6. LAMP2 gene induction was confirmed by RTPCR in four of seven LCL. In the three LCL in which the induction of LAMP2 measured by microarray was not confirmed, the RTPCR expression levels were not in stark disagreement with microarray measurements, with RT-PCR fold changes in those non-concordant LCL ranging from 0.9 – 1.0, compared to microarray-based fold changes in those LCL ranging from 1.3 – 1.4. RT-PCR analysis of TFEB mRNA levels confirmed day 8 induction in six of seven LCL. Taken together we found no evidence to suggest that the global gene induction of lysosomal genes or of TFEB suggested by microarray was a systematic artifact of the technology.

Functional correlate of lysosomal gene expression

In microarray data (Table 3) comparing control LCL to day 8 arsenite-exposed LCL, mRNA for the lysosomal protease cathepsin D was statistically significantly ($P=0.014$) elevated 1.4-fold by arsenite exposure. In order to evaluate the functional correlate of this, we evaluated cathepsin D activity using a fluorescence-based proteolytic activity assay specific for this protease. Cathepsin D activity was determined for each of the seven cell lines for control and eight-day arsenite exposed cells. We observed an increase in cathepsin D activity in LCL following 8 days of arsenite exposure, with mean fluorescence fold-increase ranging from 1.2 – 2.4 fold in the seven cell lines analyzed (Figure 3). This arsenite-induced activity increase was consistent with the average 1.4 fold level of induction of cathepsin D mRNA measured by microarray analysis.

DISCUSSION

Several epidemiological studies have identified adverse effects on the immune system as a consequence of inorganic arsenic exposure in human populations. These studies are corroborated by *in vitro* and *ex vivo* analyses of human leukocytes that collectively implicate altered cytokine production, impaired phagocytosis, blunted antibody response, and impaired proliferative response resulting from arsenic exposure. Notwithstanding these studies no clear mechanism of action has emerged to explain how environmental arsenic exposure brings about these effects. In this study we add to the mechanistic explanation of arsenic immunotoxicity by establishing several key features of the response of LCL from seven individuals to inorganic arsenic exposure.

Exposure to 0.75 μ M arsenite increases population doubling time in LCL in a manner that is not explained by frank cell death. In the two most sensitive LCL, population doubling time following 8 days of arsenite exposure was greater than 200% of controls, however viable (trypan blue negative) cell percentages for these two LCL were reduced following arsenite exposure by only 6.2% and 9.0% (from 94.5% to 88.3% for LCL 550 and from 96.8% to 87.8% in LCL 853). Thus while some loss of cells from the population due to cell death probably contributed to the observed increase in population doubling time, it is likely that arsenite exposure resulted in altered kinetics of passage through the cell cycle. This is an interesting feature of the LCL model as it mirrors the inhibition of leukocyte proliferation observed in arsenic-exposed human lymphocytes (Biswas et al., 2008; Soto-Pena et al., 2006).

Two processes that were differentially impacted by arsenite exposure in the seven LCL were significantly correlated with the differential inhibition of cell proliferation between the LCL. The extent of arsenite-induced cell death, while not of great magnitude, was correlated with the extent of proliferative inhibition. Thus the LCL that sustained the greatest proliferative inhibition from arsenite tended to have the most arsenite-induced cell death. One explanation of this is that a central process may be responsible for both effects, and that cell death may be an end stage of a process that initially slows transit through the cell cycle. In a similar way, the extent of lysosomal expansion, a marker of autophagy, is correlated with the proliferative inhibition amongst the LCL such that the LCL with the greatest arsenite-induced proliferative inhibition had the greatest lysosomal expansion. It is interesting in this regard that the induction of autophagy has been reported to be associated with both growth arrest as well as cell death (Komata et al., 2004; Pattingre et al., 2005). More detailed mechanistic studies will be necessary to determine whether autophagy is functioning as an effector pathway for these toxic effects, or alternatively as a stress-compensatory pathway attempting to restore homeostasis to the arsenite-exposed LCL. Nevertheless, the prospect that the extent of autophagy induction may be a biomarker for individual susceptibility to arsenite is exciting.

A novel characteristic of the arsenite induced acidic vesicle expansion reported here is the globally increased mRNA levels of lysosomal genes that could be coordinated by arsenite-induced expression of TFEB, a master regulator of lysosomal gene expression. While we did not examine the cytoplasmic to nuclear translocation of TFEB required for its trans-activating activity, the global induction that we observed is similar to that reported by Sardiello et al., in TFEB-overexpressing cells (Sardiello et al., 2009). In that report, the authors provided evidence that the TFEB-mediated induction of lysosomal gene expression may be triggered by accumulation of degradation-targeted molecules in lysosomes. That possibility is particularly interesting in the context of arsenic toxicology, given work establishing that trivalent arsenic exposure is capable of protein damage, and of eliciting the unfolded protein response, processes that would be expected to present damaged proteins to the lysosome for degradation (Binet et al., 2010; Ramadan et al., 2009).

To our knowledge this is the first report of arsenic exposure modulating the regulation of genes encoding lysosomal constituents. The lysosome, historically considered a dead end for damaged cellular contents, is emerging as a critical player that is actively maintaining cellular homeostasis. Its pivotal role in autophagy links the lysosome to regulating levels of key cellular signaling proteins and to carcinogenic transformation (Komatsu et al., 2010; Mathew et al., 2009). In antigen presenting cells such as LCL autolysosomes play a key role in antigen processing and subsequent loading onto MHC molecules (Munz, 2010). Within this rapidly evolving understanding of the lysosome's function, its novel role as an arsenic target offers new paths in which to pursue mechanistic explanations of arsenic immunotoxicology.

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Abbreviations used

LCL	Lymphoblastoid cell line(s)
LRD	Lysotracker red dye

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Figure 1.

Cell viability after eight-day arsenite exposure. LCL were exposed to vehicle control or 0.75 uM arsenite for eight days and cell viability was calculated by trypan blue exclusion cell counts. Bars represent the mean percentage of trypan blue negative cells in each cell line, error bars represent SEM, for three independent measurements. * $P < 0.05$ Vs. control.

Figure 2. TFEB RNA measurements over the eight-day arsenite exposure. Graph represents the normalized, log2-transformed intensity values for TFEB gene expression measured by microarray. Data points represent mean value, error bars represent (+/-) SEM. * $P < 0.05$ Vs. control, ** $P < 0.001$ vs. control.

Figure 3.

Change in cathepsin D enzymatic activity levels after arsenite exposure. LCL were exposed to vehicle control or 0.75 μ M arsenite for eight days and enzymatic activity of cathepsin D was quantified. Bars represent the mean RFU per 1×10^6 cells, error bars represent SEM, of triplicates for each cell. * P < 0.05 Vs. control, ** P < 0.001 Vs. control.

Table 1

LCL population doubling time over 8 days under control conditions or exposed to arsenite (0.75 μ M). Values shown are mean (SEM) doubling time in hours.

Cell Line	Control (C) Doubling Time	+ Arsenite (As) Doubling Time	As/C Doubling Time Ratio
564	49.4 (0.5)	55.7 (0.2) **	1.1
853	57.1 (0.9)	133.5 (14.3) *	2.3
550	62.6 (1.0)	137.1 (13.3) *	2.2
209	64.1 (1.6)	100.0 (4.6) *	1.6
504	60.2 (0.8)	76.3 (2.7) *	1.3
561	53.6 (1.0)	86.3 (1.8) **	1.6
532	50.1 (0.1)	67.5 (0.9) **	1.3

* $P < 0.05$,

** $P < 0.001$.

Table 2

Lysotracker red dye fluorescence following 8 days under control conditions or exposed to arsenite (0.75 uM). Values shown are mean (SEM) fluorescence units.

Cell Line	Control (C) LRD Fluorescence	+Arsenite (As) LRD Fluorescence	As/C Ratio
564	1429.0 (38.2)	1806.3 (1.8) *	1.3
853	1150.0 (111.4)	2546.0 (108.2) *	2.2
550	1436.3 (68.9)	2445.0 (211.6) *	1.7
209	1596.0 (15.7)	2424.0 (38.6) **	1.5
504	1666.7 (85.3)	2412.0 (69.6) *	1.4
561	1334.7 (43.2)	2845.0 (78.3) **	2.1
532	1225.7 (98.4)	2032.0 (97.9) *	1.7

*
P < 0.05,

**
P < 0.001.

Table 3

Statistically significant lysosomal genes, differentially expressed between day 0 and one or more days of arsenite exposure. Black cells indicate that a particular gene is differentially expressed on that day in comparison to day 0.

Corrected p-value	Gene Symbol	Gene Description	Day 1	Day 2	Day 4	Day 6	Day 8
0.009	LAMP3	lysosomal-associated membrane protein 3					
0.000	ASAHI	N-acylsphingosine amidohydrolase (acid ceramidase) 1					
0.000	TRIP10	thyroid hormone receptor interactor 10					
0.000	TPP1	tripeptidyl peptidase I					
0.001	GM2A	GM2 ganglioside activator					
0.000	SLC15A4	solute carrier family 15, member 4					
0.002	ADA	adenosine deaminase					
0.000	CTSA	cathepsin A					
0.000	NPC2	Niemann-Pick disease, type C2					
0.027	IDS	iduronate 2-sulfatase (Hunter syndrome)					
0.000	CLCN7	chloride channel 7					
0.003	GNS	glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID)					
0.014	SLC17A5	solute carrier family 17 (anion/sugar transporter), member 5					
0.027	IGF2R	insulin-like growth factor 2 receptor					
0.000	SRGN	serglycin					
0.010	IDS LOC727913	iduronate 2-sulfatase (Hunter syndrome) similar to iduronate 2-sulfatase (Hunter syndrome)					
0.032	PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain) member 1					
0.008	IL4I NUP62	interleukin 4 induced 1 nucleoporin 62kDa					
0.000	LAMP1	lysosomal-associated membrane protein 1					
0.014	CTSD	cathepsin D					
0.001	CTSS	cathepsin S					
0.011	ARL8B	ADP-ribosylation factor-like 8B					
0.030	IFI30 PIK3R2	interferon, gamma-inducible protein 30 phosphoinositide-3-kinase, regulatory subunit 2 (beta)					
0.003	LAMP2	lysosomal-associated membrane protein 2					
0.033	CD63						
0.001	HEXA	hexosaminidase A (alpha polypeptide)					

Corrected p-value	Gene Symbol	Gene Description	Day 1	Day 2	Day 4	Day 6	Day 8
0.001	VPS11	vacuolar protein sorting 11 homolog (S. cerevisiae)					
0.005	NEU1	sialidase 1 (lysosomal sialidase)					
0.013	ARL8A	ADP-ribosylation factor-like 8A					
0.010	VPS18	vacuolar protein sorting 18 homolog (S. cerevisiae)					
0.001	SGSH	N-sulfoglucosamine sulfohydrolase (sulfamidase)					
0.005	LITAF	lipopolysaccharide-induced TNF factor					
0.030	LAPTM5	lysosomal associated multispinning membrane protein 5					
0.003	FNBP1	formin binding protein 1					
0.000	CHID1	chitinase domain containing 1					
0.033	USP6	ubiquitin specific peptidase 6 (Tre-2 oncogene)					
0.008	AP3M1	adaptor-related protein complex 3, mu 1 subunit					
0.007	MAN2B1	mannosidase, alpha, class 2B, member 1					

Table 4

Control (C) Vs. Day 8 (As) gene expression validation by RT-PCR of TFEB, LAMP2, and LAMP3.

Cell Line	TFEB		LAMP2		LAMP3	
	Fold Δ As/C Array	Fold Δ As/C RT-PCR	Fold Δ As/C Array	Fold Δ As/C RT-PCR	Fold Δ As/C Array	Fold Δ As/C RT-PCR
564	1.5	1.0	1.3	0.9	4.9	5.2
561	1.4	1.6	1.4	1.0	4.0	7.6
853	1.3	1.4	1.5	1.4	2.7	6.3
550	1.3	1.1	1.1	0.9	1.3	1.0
209	1.4	1.4	1.4	1.3	1.7	1.8
532	1.6	1.3	1.3	1.4	2.4	2.7
504	1.5	1.1	1.6	1.1	2.4	2.2