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Arsenic inhibits hedgehog signaling during P19 cell differentiation

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Abstract

Arsenic is a toxicant found in ground water around the world, and human exposure mainly comes from drinking water or from crops grown in areas containing arsenic in soils or water. Epidemiological studies have shown that arsenic exposure during development decreased intellectual function, reduced birth weight, and altered locomotor activity, while *in vitro* studies have shown that arsenite decreased muscle and neuronal cell differentiation. The sonic hedgehog (Shh) signaling pathway plays an important role during the differentiation of both neurons and skeletal muscle. The purpose of this study was to investigate whether arsenic can disrupt Shh signaling in P19 mouse embryonic stem cells, leading to changes muscle and neuronal cell differentiation. P19 embryonic stem cells were exposed to 0, 0.25, or 0.5 μ M of sodium arsenite for up to 9 days during cell differentiation. We found that arsenite exposure significantly reduced transcript levels of genes in the Shh pathway in both a time and dose-dependent manner. This included the Shh ligand, which was decreased 2- to 3-fold, the *Gli2* transcription factor, which was decreased 2- to 3-fold, and its downstream target gene *Ascl1*, which was decreased 5-fold. GLI2 protein levels and transcriptional activity were also reduced. However, arsenic did not alter GLI2 primary cilium accumulation or nuclear translocation. Moreover, additional extracellular SHH rescued the inhibitory effects of arsenic on cellular differentiation due to an increase in GLI binding activity. Taken together, we conclude that arsenic exposure affected Shh signaling, ultimately decreasing the expression of the Gli2 transcription factor. These results suggest a mechanism by which arsenic disrupts cell differentiation.

Keywords

arsenic; P19 cells; stem cells; Sonic Hedgehog; Gli2; cell differentiation

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Introduction

Arsenic is a naturally occurring metalloid that exists in bedrock, sediments and soils. Drinking water derived from groundwater has been reported as the most common source of human exposure to inorganic arsenic. According to the U.S. Environmental Protection Agency and the World Health Organization, the drinking water standard for arsenic is 0.01mg/L (10 ppb). Even with this limit, residents in several counties, including Bangladesh, China, India, Pakistan, Taiwan, Hungary, Argentina, and Mexico, who are still at risk of elevated arsenic exposure due to high concentrations of arsenic in ground water (Naujokas *et al.*, 2013). An increasingly important source of exposure to inorganic arsenic is food, such as cereals, crops, and vegetables, which are cultured in arsenic-rich areas where farmers use groundwater for irrigation or in areas with historic arsenic-based pesticide usage. Both epidemiological and animal studies showed that arsenic exposure causes adverse developmental effects such as fetal loss, infant death, low intelligence quotient scores, reduced birth weight, and reduced weight gain (Calderon *et al.*, 2001; Rodriguez *et al.*, 2002; Wang *et al.*, 2006a; Huyck *et al.*, 2007; Rahman *et al.*, 2007; Li *et al.*, 2009; Bloom *et al.*, 2014), suggesting that arsenic exposure impacts development.

In addition to the reductions in body weight and changes in neuronal function, arsenic exposure has been shown to decrease muscle and neuron development. For example, exposure to micromolar levels of arsenite reduced neurite production, outgrowth, and complexity in differentiating rat PC12 cells (Frankel *et al.*, 2009), while in Neuro-2a neuroblastoma cells, arsenic inhibited neurite outgrowth by inhibiting the LKB1 AMPK pathway (Wang *et al.*, 2010). Arsenic also induced neural tube defects in mouse embryos (Włodarczyk *et al.*, 1996). Exposure to arsenic trioxide exposure in 0.1–0.5 μ M range decreased myogenic differentiation in C2C12 mouse myoblast cells and during muscle regeneration after injury in mice by inhibiting Akt-regulated signaling (18). Similarly, C2C12 mouse myoblast cells to be exposed to 20 nM sodium arsenite showed delayed myotube formation due to reductions in transcription factors such as myogenin and myocyte enhancer factor 2C (Mef2C). (Steffens *et al.*, 2011). These results suggest that arsenic inhibits skeletal muscle and neuronal differentiation without affecting proliferation.

Sonic hedgehog (Shh) signaling is an evolutionarily conserved pathway that is needed for somite formation, patterning of embryonic structures, and neural progenitor cell survival (Litingtung and Chiang, 2000; Mill *et al.*, 2003; Anderson *et al.*, 2012). Studies have shown that Shh signaling is essential for myogenesis in zebrafish since without *Shh* signaling, adaxial cells are delayed in terminal differentiation (Coutelle *et al.*, 2001). In mouse cells, hedgehog signaling regulates both cardiac and skeletal myogenesis, as the transcription factor of hedgehog signaling, Gli2, has been shown to regulate and activate *Mef2c* and *MyoD* expression, which are transcription factors needed for myogenic differentiation of progenitor cells (Voronova *et al.*, 2012; Voronova *et al.*, 2013). *Shh* signaling is also critical for neuronal development. Studies have shown that lack of Shh signaling disrupts dorso-ventral patterning within the neural tube in mice (Chiang *et al.*, 1996; Murdoch and Copp, 2010). In zebrafish, inhibition of Shh signaling by using mutants that lack essential components of the Shh pathway, like Smoothen (*Smo*) and *Gli*, reduces neural progenitor cell proliferation in the dorsal mesencephalon (Feijoo *et al.*, 2011). Moreover, mice with

mutations in *Shh* and *Gli* show a delay in motor neuron differentiation in spinal cord, suggesting that Shh signaling is also important in neurogenesis (Oh *et al.*, 2009).

The key proteins of Shh pathway are zinc-finger transcription factors family termed *Gli*. *Gli2* is the primary transcription factor of Shh signaling pathway. It has two different activities based on post-translational modification, in which the full length protein acts as activator and the truncation of its C-terminus acts as repressor. *Gli1* acts as a minor activator and is involved in cellular growth and cell cycle progression (Sun *et al.*, 2014), and *Gli3* is a transcriptional repressor, but its expression is very low (Hui and Angers, 2011). In the absence of SHH, the membrane receptor Patched (PTCH) inhibits the activity of Smoothened (SMO), a 7-pass transmembrane protein. GLI2 protein is transferred to the primary cilium and forms a complex with KIF7 and Suppressor of Fused (SUFU). The complex then binds to GSK3 β and PKA to phosphorylate GLI2, leading to the cleavage of GLI2 into a repressive form and inactivating the pathway (Kim *et al.*, 2009). When SHH is present, it binds to PTCH on the membrane, resulting in the loss of inhibitory effect between PTCH and SMO. SMO is then phosphorylated by GRK2 and translocated to primary cilium, which cause the accumulation of SMO in primary cilium. Accumulation of SMO in primary cilium leads to the dissociation of SUFU and GLI2, releasing full length of GLI2, and therefore allowing the active GLI2 to translocate into the nucleus and induce cell differentiation (Ingham and McMahon, 2001; Jiang and Hui, 2008).

Arsenic trioxide is used as a chemotherapeutic agent, and it has been recently shown to inhibit the Hedgehog pathway in cancer cells by inhibiting GLI proteins. For example, arsenic trioxide decreased Hh-induced ciliary accumulation of GLI2 and increased its rate of degradation (Kim *et al.*, 2010). Another study found that in the Ewing sarcoma cell line, arsenic trioxide inhibited hedgehog signaling by inhibiting GLI1 protein transactivation (Beauchamp *et al.*, 2011). Other studies have shown that arsenic trioxide exposure in several cancer cell lines decreased the transcription of GLI1, GLI2 and PTCH1, leading to the inhibition of cell growth (Nakamura *et al.*, 2013; Yang *et al.*, 2013). Taken together, arsenic trioxide inhibits Shh signaling in cancer cells at the level of GLI transcription factors. Thus, we were interested in whether arsenic can also alter Shh signaling in embryonic stem cells, thereby reducing cellular differentiation.

In our previous study, arsenic exposure inhibited both skeletal muscle and neuronal cell differentiation in P19 embryonic stem cells by reducing the expression of *MyoD* and *NeuroD1*, transcription factors needed for myogenesis and neurogenesis respectively (Hong and Bain, 2012). However, the mechanism by which arsenic inhibits cell fate determination is not fully understood. In the present study, we examined whether sodium arsenite affects sonic hedgehog signaling during cell differentiation. Our findings indicate that sodium arsenite specifically decreased *Gli2* expression and transcriptional activity, thereby reducing the levels of several of its downstream targets. When additional recombinant SHH protein was added, SHH rescued arsenic's inhibitory effects on cell differentiation. Taken together, our results indicate that arsenic inhibit cell differentiation into myotubes and neurons by inhibiting sonic hedgehog signaling.

Material and methods

P19 cell culture and differentiation

The mouse embryonal carcinoma P19 cell line (ATCC, Manassas, VA) was maintained in α -MEM supplemented with 7.5% bovine calf serum (Hyclone, Logan, UT), 2.5% fetal bovine serum (Mediatech, Manassas, VA), 1% L-glutamine, and 1% penicillin/streptomycin (designated as growth medium) at 37°C in a humidified incubator containing 5% CO₂. To induce differentiation, P19 cells were aggregated by the hanging drop method with some modifications (Wang and Yang, 2008). Briefly, P19 cells were trypsinized and suspended in growth medium containing 1% DMSO with 0, 0.25, or 0.5 μ M sodium arsenite at a cell density of 500 cells/ 20 μ l or drop. Hanging drops were incubated for 2 days (day 2) to let cells undergo aggregation. After 2 days, each individual drop was transferred to a 96-well ultralow attachment plate containing fresh differentiation medium with or without sodium arsenite. After 3 days of culture (day 5), the embryoid bodies were transferred to a 0.1% gelatin coated 48-well plate containing fresh differentiation medium with or without sodium arsenite. Medium was then renewed every 48 hours until cells were harvested.

Developing stable Gli reporter gene transfectants

P19 cells were transfected with a *Gli*-luciferase lentiviral reporter (pCignal Lenti-TRE-Reporter) following supplier's instructions (SABiosciences, CA) to develop a stably transfected cell line. Briefly, P19 cells were seeded in 24 well cell culture plate (2×10^3 cells per well) for 1 day to avoid over confluency. Cells were then transfected with Cignal lentiviral particles for 20 hours and recovered in normal growth medium afterward. To isolate stable transfectants, 2 μ g/mL puromycin was added into each well for a total of 14 days, while passaging the cells every two days. To assess arsenic's effects on hedgehog signaling, the stable cells were plated at 5000 cells/well in a 12 -well plate and exposed to 0, 0.25, or 0.5 μ M sodium arsenite for 48 hours (n= 3). Additionally, the stably transfected cells were exposed to 0 or 0.5 μ M arsenite while being differentiated into mature embryoid bodies via the hanging drop method as above (n= 3). Luciferase activity was measured using One-Glu luciferase assay kit (Promega, Madison, WI).

qPCR

After 2 or 5 days of exposure, ninety six aggregating embryoid bodies (1 plate) were collected as one replicate (n=3 per treatment group per day). Total RNA were extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO) and reverse transcribed using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. Real-time qPCR was conducted using RT² SYBR green master mix (Qiagen, Valencia, CA) and appropriate primers as shown in Table 1. A standard curve was generated via 5 concentration points (10^{-3} to 10^{-7} ng of cDNA) to quantify expression levels and assess reaction efficiency. Samples were run in triplicate and gene expression data was analyzed and normalized with the Gapdh housekeeper using the comparative threshold (Ct) method (Livak and Schmittgen, 2001).

Immunoblotting

Cells were treated with 0, 0.25, or 0.5 μM arsenite and harvested at days 2 and days 5 as described above ($n=3$ per group per day). Collected cells were placed in RIPA lysis buffer (Thermo Scientific, Waltham, MA) with protease and phosphatase inhibitors (Thermo Scientific). Cell culture medium was also collected from day 5 embryoid bodies to collect exosomes. Briefly, medium was passed through a $0.2\mu\text{m}$ filter and incubated with ExoQuick-TC isolation reagent overnight at 4°C (Systems Biosciences, Mountain View, CA). Exosomes were isolated by centrifugation per manufacturer's instructions and lysed in RIPA buffer with protease and phosphatase inhibitors. Protein concentrations were quantified using Bio-Rad's DC protein assay kit. Immunoblots were performed according to standard methods. Dilutions for primary antibodies were as follows: anti-GLI2 (1:200, Santa Cruz, #SC-271786), anti-GSK3 β (1:1000, Cell Signaling, #9315), anti-phospho-GSK3 β (Ser9) (1:1000, Cell Signaling, #9323), anti-SHH (1:1000, Cell Signaling, #2207), and GAPDH (1:1000, IMGEX, #IMG-5019A). Secondary antibodies and dilutions were as follows: goat anti-mouse-HRP (1:2000, Santa Cruz, #SC-2005) and goat anti-rabbit-HRP (1:2000, #SC-2004). The amount of protein was detected by chemiluminescence (Luminol, Santa Cruz) using BioRad's Chemidoc imaging system. Average densitometry values were determined using BioRad's Quantity One software and were normalized by GAPDH as loading control.

Immunohistochemistry

Differentiating P19 cells were exposed to 0, 0.25, and 0.5 μM sodium arsenite as described above. After 2 days and 5 days of cells aggregation, 96 embryoid bodies were harvested as one replicate ($n=3$ per day per group). Embryoid bodies were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections ($5\mu\text{m}$) were placed on slides, deparaffinized and rehydrated in graded ethanol. Slides were blocked and incubated with specific primary antibodies for one hour (GLI2 (C-10) 1:400, Santa Cruz, #SC-271786) and incubated with secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA). DAPI (Invitrogen) was used to stain nuclei. Immunofluorescence was measured on a Ti Eclipse Inverted Microscope (Nikon, Melville, NY). Overall fluorescence intensity was quantified and normalized with nuclear intensity using Image J to obtain the average GLI2 expression per cell (Barbachano *et al.*, 2010). Relative quantification units were calculated after normalizing with the intensity values to the control groups. For the later stages of differentiating cells, embryoid bodies exposed to arsenic as described above were transferred to 0.1% gelatin coated glass chambers ($n = 4$ per treatment per group). Cells were cultured for 7 more days with the renewal of medium every two days. Cells were fixed with 4% of PFA at room temperature for 10 minutes, permeabilized and blocked with 0.2% Triton-X-100 and 1% BSA respectively. Cells were stained with the appropriate primary antibody for 1 hour (TUJ1 1:200, Millipore #MAB1637; NANOG 1:100, GeneTex #GTX100863; GLI2 1:100, Abcam #AB26056; Acetylated-Tubulin 1:5000, Sigma #T7451) and incubated with Alexa Fluor 488, 568 conjugated secondary antibody (Invitrogen, 1:2000) and DAPI. Fluorescence was measured via Nikon Ti Eclipse Inverted Microscope.

SHH rescue

SHH-N terminal peptide (1 or 2.5 $\mu\text{g/mL}$) (R&D System, Minneapolis, MN) was dissolved in growth medium and P19 cell differentiation was conducted via the standard method described above, with or without 0.5 μM sodium arsenite. After five days, aggregated embryoid bodies were transferred onto a 48 well 1% gelatin coated plate and cultured for additional 4 days. Eight individual differentiated embryoid bodies per treated group were imaged on day 9 ($n=8$ per concentration). The length and area of differentiating and proliferating cells was quantified using ImageJ.

Stable GLI-luciferase reporter cells were plated at 5000 cells/well in a 12 -well plate and exposed to 0 or 0.5 μM sodium arsenite with or without 1 $\mu\text{g/mL}$ SHH-N terminal peptide for 24 hours ($n= 3$). Luciferase activity was measured using One-Glu luciferase assay kit.

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. Data were analyzed by ANOVA followed by Tukey's HSD Test or t-test, as appropriate. Statistical significance was achieved at $p<0.05$ (*) or $p<0.01$ (**).

Results

Arsenic decreases Sonic hedgehog pathway related gene expression

To determine what signaling pathways were involved in arsenic's ability to reduce stem cell differentiation into skeletal myotubes and sensory neurons, we examined mRNA expression of key proteins in pathways known to be involved in cellular differentiation. P19 cells treated with 0, 0.25 μM and 0.5 μM of sodium arsenite were harvested at days 4 and 6 of differentiation. Key transcripts examined included *Bmp4*, *Fgf4*, *Fgf8*, *Hes1* expression for the Notch pathway, and *Shh* and *Gli2* for the Shh pathway. During the process of embryoid body formation, *Shh*, *Gli2*, and *Bmp4* expression increased by 2.5-, 6-, and 2.5-fold, respectively (Figures 1A–C), *Hes1* and *Fgf4* expression decreased by 3- and 8-fold respectively (Figures 1D and E), and *Fgf8* expression did not change (Figure 1F). Arsenic exposure reduced transcript levels of both *Shh* expression (2-fold) and *Gli2* expression (1.5-fold), respectively, during embryoid body formation (Figure 1A and B), but did not change the levels of any of the other transcription factors. To further examine Shh pathway related gene expression, P19 cells exposed to 0 or 0.5 μM sodium arsenite were harvested at 2, 5, 7 and 9 days of differentiation. Transcript levels of *Shh*, the transcription factor *Gli2*, and three downstream genes: *Gli1*, *Ptch1*, and *Ascl1* were determined. Over the time of differentiation, both *Shh* and *Gli2* were significantly increased by 5 days of differentiation and expressed at high levels until day 9, supporting the role of Shh signaling in P19 cell differentiation. Arsenic exposure significantly reduced the expression of Shh and Gli2 transcripts by 2.5-fold and 3-fold, respectively, on day 5. These reductions were maintained at days 7 and 9, indicating that the inhibitory effects of arsenic on cell differentiation are related to Shh signaling (Figure 2A and B). Three *Gli2* downstream target genes were also examined. There was no difference in *Gli1* and *Ptch1* expression level when exposed to arsenic (Figure 2C and D). *Gli1* expression was down regulated during early embryoid body

formation and remained reduced throughout the differentiation period (Figure 2C). *Ptch1* levels remained relatively constant from days 0–9 (Figure 2D), suggesting that it has only a minor role in cell differentiation. *Ascl1* is another *Gli2* downstream gene, and studies have shown that an increase in its expression can promote neuronal differentiation (Voronova *et al.*, 2011). During P19 cell differentiation, *Ascl1* expression was induced dramatically in controls cells, while arsenic exposure decreased *Ascl1* expression by 2.5, 7, and 4-fold on days 5, 7, and 9, respectively (Figure 2E). Taken together, the data suggests that arsenic decreases Shh pathway gene expression, thus inhibiting cell differentiation.

Arsenic exposure does not alter the levels of SHH expression nor GLI2 accumulation in the primary cilium

Since the genes whose expression was reduced after arsenic exposure were the Shh signal itself and the *Gli2* transcription factor, we first examined SHH protein levels. Day 5 embryoid bodies exposed to 0 or 0.5 μ M arsenic were collected along with the culture medium to assess secreted SHH levels contained in exosomal vesicles. In day 5 embryoid bodies, we were able to visualize both precursor and processed SHH, but arsenic exposure did not alter either one of their levels (Figure 3A). The secreted precursor and processed SHH was at a much higher molecular weight, likely in the cholesterol bound form (Porter *et al.*, 1996). However, its expression in the exosomes also remained constant between control and arsenic exposed cells (Figure 3B). Shh signaling activation causes ciliary accumulation of GLI2, resulting in GLI2 nuclear translocation, which further activates Shh signaling downstream genes (Chen *et al.*, 2009; Kim *et al.*, 2009). We investigated if arsenic inhibited GLI2 accumulation in primary cilium by differentiating P19 cells for 9 days in 0 or 0.5 μ M arsenic, and co-staining them with the primary cilium marker acetylated-tubulin and GLI2. There is no difference between control and arsenic exposed cells (Figure S1).

Arsenic decreases GLI2 protein levels

GLI protein families are the key transcription factors in Shh signaling. Therefore, we determined whether GLI2 protein levels were altered after exposure to arsenic. GLI2 protein exists in a full length active (~160 kDa) form and in a repressor form (~78 kDa) after C-terminal truncation (Pan *et al.*, 2006a). Western blot confirms that the active full-length form of GLI2 is decreased when exposed to arsenic in day 5 embryoid bodies (Figure 3C and D), although the antibody used cannot detect the truncated repressor form. We also examined GLI2 levels by immunohistochemistry. In day 2 embryoid bodies, GLI2 protein is expressed, but most of it remains outside the nucleus. There is also no difference in expression between control and arsenic exposed embryoid bodies (Figure 4). By day 5, the majority of GLI2 is translocated to the nuclei in both groups. However, overall GLI2 expression is significantly reduced by 50% in the arsenic-exposed cells (Figure 4).

Arsenic inhibits GLI activity

Since arsenic reduced the levels of GLI2 protein, we wanted to determine if the activity of GLI2 was also inhibited after arsenic exposure. P19 cells were used to generate a stable transfectant containing the GLI protein binding element adjacent to a luciferase reporter gene. Cells were exposed to 0.25 and 0.5 μ M of sodium arsenite for 48 hours prior to

assessing activity. The results show that GLI activity was significantly decreased by 40% when exposed to 0.5 μ M arsenite (Figure 5A). We further determined if GLI activity was inhibited during cell differentiation by forming embryoid bodies from the transfected cells. GLI activity was also reduced by 35% in the arsenic-exposed embryoid bodies, indicating that arsenic decreases Shh signaling activity when P19 cells are undergoing differentiation (Figure 5B).

Arsenic does not disrupt GSK3 β phosphorylation

In order to generate truncated GLI2 protein, GSK3 β phosphorylates GLI2, leading it to be partially processed by proteasomes (Aza-Blanc *et al.*, 1997). GSK3 β kinase activity can be inhibited by phosphorylation of its serine 9 site (Godemann *et al.*, 1999). Therefore, we examined if arsenic disrupted GSK3 β kinase activity by determining the level of total and phosphorylated GSK3 β . However, there were no significant changes between control and arsenic treated day 5 embryoid bodies (Figure 6).

Induction of the Shh pathway rescues arsenic's inhibitory effects on cell differentiation

To determine if stimulating the Shh pathway activity rescues the arsenic-mediated reduction in cell differentiation, 1 μ g/mL of mouse recombinant SHH N-terminal domain protein was added into growth medium during cell differentiation and morphological changes were observed in the cells after 9 days of exposure. As shown in figure 7A, cells around the original embryoid bodies within the dashed line were defined as undifferentiated cells and were confirmed with NANOG staining (data not shown). The control group had less than 50% of undifferentiated cells and more spindle-shaped mesenchymal cells and myocytes that are migrating out from the embryoid bodies (Figure 7A). When cells were exposed to SHH only, neither migration distance nor percentage of differentiating cells changed, but there were more myocytes around the embryoid bodies. The cells exposed only to 0.5 μ M arsenic had less differentiating cells and shorter migration distance. When cells were co-treated with 0.5 μ M arsenic and SHH, there were more differentiated cells outside of the embryoid bodies and fewer undifferentiated cells compared to the arsenic-only group, indicating that SHH protein rescues the arsenic-induced inhibition on cell differentiation (Figure 7B).

In order to further confirm the rescue, P19 cells were co-treated with 2.5 μ g/mL of recombinant SHH N-terminal domain protein and 0.5 μ M of sodium arsenite during cell differentiation. After 12 days of differentiation, the number of neuronal cells was determined by stained with neuron specific marker TUJ1. The arsenic-treated cells have less neurons, but when cells are co-treated with SHH, many more neuronal cells are seen, suggesting that SHH attenuates the inhibitory effects caused by arsenic (Figure 7C).

To examine the mechanism by which SHH rescues the arsenic-induced inhibition of cellular differentiation, stable GLI protein binding element-luciferase cells were exposed to 0.5 μ M arsenite with or without 1 μ g/mL SHH for 24 hours. GLI activity was significantly decreased by 20% when exposed to 0.5 μ M arsenite (Figure 8). However, when the cells were co-incubated with arsenic and SHH, relative GLI activity was equivalent to the controls (Figure

8). This data suggests that the rescue of cellular differentiation by SHH is due to increases in GLI activity.

Discussion

In the present study, the mechanisms by which arsenic disrupts during cell differentiation in P19 embryonic stem cells were examined, focusing on cell signaling pathways. Arsenic specifically reduces the expression of genes in the Shh signaling pathway, such as *Shh*, *Gli2* and *Ascl1*. GLI2 protein levels and transcriptional activity were also decreased by arsenic during cell differentiation. The inhibition of activity is not caused by changes in SHH protein levels, GLI2 nuclear translocation, GLI2 primary cilium accumulation, or GLI2 degradation. However, the arsenic-mediated reduction in cell differentiation can be recovered by treating the cells with additional recombinant SHH protein due to an increase in GLI activity. These results suggest that Shh signaling is a target of arsenic during cellular fate determination.

It has been previously shown that arsenic can inhibit cellular differentiation in P19 embryonic stem cells (Hong and Bain, 2012) as well as in human neuronal stem cells and rat cardiac myoblasts (Steffens *et al.*, 2011; Ivanov and Hei, 2013; Sumi *et al.*, 2013). However, except for the P19 cells, these other cells are progenitor cells which have already been restricted to certain cell fates. After screening several growth signaling pathways including the Notch, Bmp, Fgf, and Shh pathways, arsenic exposure decreased the expression of *Shh* and its transcription factor *Gli2* in P19 cells, but did not affect key proteins in the other pathways.

Hes1 is a bHLH transcriptional factor which is directly regulated by Notch signaling and has been shown to negatively regulate cell differentiation (Kageyama *et al.*, 2005). Its expression was reduced during cell differentiation study, but was not altered by arsenic exposure. *Fgf4* and *Fgf8* signals are important growth factors during embryogenesis, with *Fgf4* expression increasing early in embryogenesis and *Fgf8* expression increasing slightly later (Wang *et al.*, 2006b; Lanner and Rossant, 2010). *Bmp4* belongs to the TGF β superfamily, which is both critical to induce embryonic cell differentiation and is also upregulated during cardiomyocyte differentiation (Monzen *et al.*, 1999; Bragdon *et al.*, 2011). Our qPCR data of those genes shows patterns consistent with those studies above. Although the transcripts of these decreased and increased, respectively, during differentiation, their expression was again not impacted by arsenic.

The down-regulation of Shh signaling pathway was also examined over a longer time period. *Gli1* and *Gli2* are both transcription factors in Shh signaling, but their expression patterns are different based upon whether the cells are proliferating or whether they are differentiating. *Gli1* expression is down regulated after forming embryoid bodies, indicating that it has a minor role in cell differentiation. This is similar to several studies in mice, in which *Gli2* appears to be the primary transcriptional activator, while *Gli1* is not required for embryonic development (Mo *et al.*, 1997; Ding *et al.*, 1998; Bai *et al.*, 2002). Indeed, in P19 cells, *Gli2* is upregulated rather than *Gli1* after 5 days of cell differentiation (Voronova *et al.*, 2013), which is similar to what is seen in our study. Moreover, reports have shown that

Gli2 regulates *Pax3* and *MyoD* expression during skeletal and cardiac myogenesis (Petropoulos *et al.*, 2004; Voronova *et al.*, 2013). In our previous study, Pax3 and MyoD were down regulated when exposed to arsenic. Therefore, it is not surprising that arsenic only inhibits *Gli2* expression. This is also similar to reductions in *Gli2* transcripts in several cancer cell lines, leading to the inhibition of cellular growth (Nakamura *et al.*, 2013; Yang *et al.*, 2013). *Ascl1* is a bHLH transcription factor that has been reported to convert embryonic fibroblasts to functional neurons and to induce neurogenesis in P19 cells (Farah *et al.*, 2000; Vierbuchen *et al.*, 2010), and is a target of *Gli2* during *Gli2*-induced neurogenesis (Voronova *et al.*, 2011). *Ascl1* was also inhibited by arsenic in the same pattern as *Gli2*. Taken together, these results indicate that the arsenic-mediated inhibitory effect on cell differentiation is due to the inhibition of the Shh signaling pathway.

There are several potential protein targets of arsenic within the Shh pathway between the SHH ligand itself and the *Gli2* transcription factor. In vertebrates, SHH protein is a morphogen that controls central nervous system and limb development in a tightly regulated dose- and time-dependent manner (Yang *et al.*, 1997; Briscoe and Ericson, 2001; Ahn and Joyner, 2004; Harfe *et al.*, 2004). The *Shh* gene has cis-regulatory elements that lie up to 1000kB upstream of its transcriptional start site, and it appears that each tissue has a means for intrinsic or self-regulation (Dessaud *et al.*, 2007; Bastida *et al.*, 2009; Benazet *et al.*, 2009; Anderson and Hill, 2014). SHH protein is processed by cleavage, and the final product modified with cholesterol and palmitate moieties, as well as with heparin sulfate (Beachy *et al.*, 2010). The modifications allow SHH to form multimeric complexes, to be anchored to the cell membrane, or to vesicle membranes, and the modifications also correlate with its signaling potency (Chen *et al.*, 2004; Grover *et al.*, 2011). Our results show that SHH protein expression is very robust in both the P19 cells themselves as well as in secreted, exosomal vesicles, although arsenic exposure does not alter SHH protein expression. This may be a reason why our SHH rescue required so much protein compared to other studies. In typical cell culture experiments, the concentration of additional, exogenous SHH required to induce neuronal and muscle differentiation from ES cells varies from 0.1 to 0.5µg/mL (Dutton *et al.*, 1999; Elia *et al.*, 2007; Salero and Hatten, 2007). We exposed P19 cells to concentrations ranging from 0.2 to 2.5µg/mL, but the SHH-mediated rescue only occurred at concentrations of at least 1 µg/mL.

PTCH1 is a transmembrane protein that is the receptor of SHH ligand and acts as a repressor in the absence of SHH signaling (Agren *et al.*, 2004). However, *Ptch1* expression can also be regulated in a Shh-independent manner (Agren *et al.*, 2004). Our data indicates that *Ptch1* expression is not significantly changed by arsenic and its expression does not change during the cell differentiation period. Along with PTCH1, other plasma membrane proteins can also bind to SHH. For example, Hedgehog interacting protein (HHIP) appears to compete directly with PTCH for SHH binding, resulting in an inhibition of SHH signaling activity (Chuang and McMahon, 1999). We do not know whether HHIP levels are increased, but this may be a reason why SHH levels are not altered, but Shh signaling is reduced.

Shh signaling results in active GLI2 formation when GLI2 dissociates from the Suppressor of fused (SUFU) in the primary cilium due to the accumulation of Smoothened (SMO) (Murdoch and Copp, 2010). In the absence of SHH, SMO is prevented from entering the

primary cilium, leading to GLI2 protein processing and degradation by the proteasome (Kim *et al.*, 2009). Investigators have shown that in cancer cells, arsenic trioxide decreased the Hh-induced ciliary accumulation of GLI2 (Beauchamp *et al.*, 2011). However, we co-stained differentiating day 9 cells exposed to arsenic with acetylated-tubulin, a primary cilium marker, and GLI2, and found no effect on the ciliary accumulation of GLI2 when exposed to arsenic (Figure S1).

In the primary cilium, GLI2 can be processed to a repressive form if its C-terminus is truncated (Sasaki *et al.*, 1999). The antibody used in the GLI2 immunoblotting and immunohistochemistry assays recognizes amino acids 841–1140 of GLI2, which should identify only active GLI2. Whether it can identify the repressive form is unknown, but this is unlikely because the processing determinant domain (PDD) of GLI2 is between amino acids 648–915 (Pan and Wang, 2007) and, although processing of GLI2 into the truncated repressor form occurs *in vivo*, the repressor form is typically undetectable in cell culture conditions (Pan *et al.*, 2006b). We can determine reductions in active GLI2 protein levels (Figure 3), but cannot visualize any cleaved repressive proteins on our blots. Phosphorylation of GLI2 by GSK3 β is a critical step for GLI2 to be truncated into repressive form or cleaved by the proteasome. Our data shows no inhibition of GSK3 β kinase activity when exposed to arsenic, indicating that arsenic did not affect the GSK-mediated phosphorylation. However, more evidence is needed to determine whether arsenic targets other kinases, such as protein kinase A and casein kinase (Hui and Angers, 2011), thus increasing the phosphorylation and proteosomal degradation of GLI2 protein.

After active GLI2 is released from the primary cilium, it acts as a transcription factor. Our immunohistochemistry data indicates that GLI2 does not accumulate in the nucleus until day 5 of embryoid body formation, which indicates that the Shh signaling pathway starts between day 2 and 5. This supports our qPCR results that *Gli2* and other downstream transcription factors have significantly higher expression after day 5. However, the relative nuclear translocation between control and arsenic exposed embryoid bodies were not changed. This indicates that nuclear translocation was not altered; only the total GLI2 level. But, this reduction in total GLI2 protein does correlate with the reductions in GLI2 activity seen in the reporter gene assays. In addition, exogenous SHH can rescue the loss of differentiation in P19 cells after arsenic exposure by increasing GLI activity.

Beyond the increase in GLI activity, there are several other mechanisms of regulating Shh pathway signaling. For example, we have previously published that β -catenin, a key transcription factor of Wnt signaling, is down regulated in P19 cells that are exposed to arsenic (Hong and Bain, 2012). It is reported that in P19 cells, short hairpin RNAs targeting β -catenin transfectants also decreased *Gli2* expression during cell differentiation (Wong *et al.*, 2013). Indeed, it is well-known that crosstalk exists between the Wnt and Shh pathways (Winkler *et al.*, 2014). Recent studies suggested that epigenetic regulation including DNA methylation, histone modification and microRNA (miRNAs) expression can be mediated by arsenic exposure (Ren *et al.*, 2011). Indeed, arsenic is well-known to cause oxidative stress in cells, which can alter DNA and histone modifications (reviewed in Chervona and Costa, 2012). *Gli2* expression can also be decreased by oxidative stress in both animal models and in cultured astrocytes (Xia *et al.*, 2012; Ji *et al.*, 2012). Studies have shown that microRNAs

participate in development and cell differentiation through regulating growth related gene expression (Kim, 2005; Hou *et al.*, 2012; Kawahara *et al.*, 2012). Moreover, a recent study shows that miR-326 represses Shh signaling activity by directly targeting *Gli2* expression in embryonic lung epithelium cells (Jiang *et al.*, 2014). Taken together, arsenic-caused inhibitory effects on Shh signaling might also be due to changes in epigenetic regulation.

Results of the present study demonstrate that arsenic exposure reduces GLI2 transcript and protein expression and GLI transcriptional activity, thereby reducing P19 cell differentiation into neuronal and muscle cells. Although SHH levels are not changed, additional SHH ligand rescues the inhibitory effects caused by arsenic. We excluded other possible targets in Shh signaling such as GLI2 primary cilium accumulation, GLI2 nuclear translocation, and extracellular SHH expression. Our studies provide the insight into the mechanism by which arsenic alters during cell differentiation. However, additional targets of arsenic may still need to be elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Shh	sonic hedgehog
PTCH	patched
SMO	smoothened
SUFU	suppressor of fused
Bmp	bone morphogenetic protein
Fgf	fibroblast growth factor
Gsk3β	glycogen synthase kinase 3 β

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Highlights

- Arsenic exposure decreases sonic hedgehog pathway-related gene expression
- Arsenic decreases GLI2 protein levels and transcriptional activity in P19 cells
- Arsenic exposure does not alter the levels of SHH expression and GLI2 primary cilium accumulation
- Induction of the Shh pathway rescues arsenic's inhibitory effects on cell differentiation

Figure 1. Arsenic decreased hedgehog pathway gene expression during cell differentiation

P19 cells treated with 0, 0.25 μ M and 0.5 μ M of sodium arsenite were harvested at days 4 and 6 of embryoid body formation (n=3 per day and concentration). The expression of key genes in differentiation signaling pathways were examined. Values were normalized with *Gapdh* as the housekeeping gene and average fold changes were compared to undifferentiated P19 cells (day 0). Statistical differences were determined by Student's t-test (*; $p < 0.05$). Error bars represent $\pm$ SEM from three biological replicates.

Figure 2. P19 cells exposed to arsenic during differentiation repress hedgehog pathway related gene expression in a time dependent manner

RNA was extracted from differentiating P19 cells, with or without 0.5 μ M sodium arsenite exposure, on days 0, 2, 5, 7, and 9. Transcript levels of sonic hedgehog signaling pathway genes (*Gli1*, *Gli2*, *Ptch1*, *Shh*, *Ascl1*) were determined by qPCR analysis (n=3 per group per day). Values were normalized to *Gapdh* and all expression fold changes were compared to day 0 control cells. Statistical differences were determined by Student's t-test (*; $p < 0.05$, **; $p < 0.01$).

Figure 3. Arsenic reduces GLI2 protein levels, but not SHH levels

P19 cells were induced to differentiate for 5 days while being exposed to 0, 0.25, or 0.5 μ M arsenic. Protein was extracted from both the cells and secreted exosomes. SHH levels in the cells were assessed by immunoblotting (n=3 per treatment group) and were normalized using GAPDH (A). SHH levels in the exosomes were examined by immunoblotting (n=2 per group) (B). Full length GLI2 levels were also examined by immunoblotting, with results normalized to GAPDH (n=3 per group) (C). GLI2 protein levels were analyzed by densitometry, were averaged, and are expressed as the fold-change from control cells $\pm$ standard deviation (D). Statistical differences were determined by Student's t-test (*, $p < 0.05$).

Figure 4. Arsenic decreases GLI2 protein levels in day 5 embryoid bodies

After exposure to 0 or 0.5 μ M arsenic, day 2 and day 5 embryoid bodies were embedded in paraffin and GLI2 expression examined by immunofluorescence (A). Overall day 5 fluorescence intensity was quantified as integrated intensity per cell (n=5 slides per treatment group) (B). Results were normalized to the control group and statistical differences were determined by Student t-test (*; $p < 0.05$).

Figure 5. Arsenic decreases GLI reporter activity in P19 cells

P19 cells were stably transfected with a GLI binding element-luciferase plasmid. Stable transfectants were then exposed to 0, 0.25, and 0.5 μM sodium arsenite for 48 hours and luciferase activity was assessed by luminometry (A). The stable strain was also differentiated into embryoid bodies for 5 days while being exposed to 0 or 0.5 μM arsenite, and luciferase activity measured (B). Luminescence values were averaged, normalized to the controls, and are expressed as a relative reporter activity value $\pm$ SEM ($n=3$ per concentration). Statistical differences were determined by ANOVA analysis followed by Tukey's HSD test or by Student's t-test (*; $p<0.05$).

Figure 6. Arsenic exposure does not affect GSK3β phosphorylation

Days 5 embryoid bodies were exposed to 0, 0.25 and 0.5 μM of arsenic and were harvested for protein extraction. GLI2, GSK3β and phospho-GSK3β (Ser9) expression was examined by immunoblotting. Shown is a representative blot from 3 replicates (n=3).

Figure 7. Additional Shh rescues arsenic's inhibitory effects on cell differentiation

P19 cells were aggregated via hanging drops with or without 1 μ g/mL recombinant SHH protein and 0.5 μ M of sodium arsenite. Embryoid bodies were transferred to 0.1% gelatin coated plates after 5 days and were differentiated for an additional 4 days to observe morphological changes. The embryoid body is pictured in the top right corner, while the dashed line delineates the boundary between differentiated and undifferentiated cells (confirmed by NANOG expression). The longest distance of migration is shown as a green bar from a representative image (A). The percentage of undifferentiated cells compared to

total migration area was calculated for each group, and statistical differences were determined by ANOVA followed by Tukey's (*; $p < 0.05$). Error bars represent $\pm$ S.D. from ten replicates (n=10) (B). P19 cells were differentiated for 12 days with or without 2.5 $\mu\text{g/mL}$ recombinant SHH protein and 0.5 μM sodium arsenite. Cells were fixed on day 12 and TUJ1 expression was examined by immunofluorescence (C).

Figure 8. Shh rescues GLI activity after arsenic exposure

GLI-luciferase transfected P19 cells were exposed to 0 or 0.5 μM arsenite with or without 1 μg/mL recombinant Shh protein for 24 hours. Luciferase activity was assessed by luminometry. Luminescence values were averaged, normalized to the controls, and are expressed as a relative reporter activity value $\pm$ SEM (n=3 per concentration). Statistical differences were determined by ANOVA analysis followed by Tukey's HSD test (*; $p < 0.05$).

Table 1

Primer sequences for quantitative PCR

Gene Name	Forward	Reverse
<i>Ascl1</i>	ACTTGAACCTCTATGGCGGGTT	CCAGTTGGTAAAGTCCAGCAG
<i>Bmp4</i>	AGAACTGCCGTCGCCATTCATCTAT	AGTTGAGGTGATCAGCCAGTGGAA
<i>Fgf4</i>	GGTGAGCATCTTCGGAGTG	GTAGGATTCGTAGGCGTTGTAG
<i>Fgf8</i>	GCTCATTGTGGAGACCGATAC	CCAGCACGATCTCTGTGAATAC
<i>Gli1</i>	CCAAGCCAACCTTTATGTCAGGG	AGCCCGCTTCTTTGTTAATTGA
<i>Gli2</i>	ATCAGCCAACCAAGAGACAGCAGA	TGGGCAGCCTCCATTCTGTTTATA
<i>Hes1</i>	GCCTCTGAGCACAGAAAGTCATCA	CTTGGAATGCCGGGAGCTATCTTT
<i>Ptch1</i>	AAAGAACTGCGCAAGTTTTTG	CTTCTCTATCTTCTGACGGGT
<i>Shh</i>	GCCAAGCAACCTGCTGAAAGTCTA	TTTACACTTGCGGGAAAGCAGGAG