

Activation of Hedgehog Signaling by the Environmental Toxicant Arsenic May Contribute to the Etiology of Arsenic-Induced Tumors

Dennis Liang Fei¹, Hua Li¹, Courtney D. Kozul¹, Kendall E. Black¹, Samer Singh¹, Julie A. Gosse¹, James DiRenzo¹, Kathleen A. Martin^{1,2}, Baolin Wang³, Joshua W. Hamilton^{1,4}, Margaret R. Karagas⁵, and David J. Robbins^{1,6}

Abstract

Exposure to the environmental toxicant arsenic, through both contaminated water and food, contributes to significant health problems worldwide. In particular, arsenic exposure is thought to function as a carcinogen for lung, skin, and bladder cancer via mechanisms that remain largely unknown. More recently, the Hedgehog signaling pathway has also been implicated in the progression and maintenance of these same cancers. Based on these similarities, we tested the hypothesis that arsenic may act in part through activating Hedgehog signaling. Here, we show that arsenic is able to activate Hedgehog signaling in several primary and established tissue culture cells as well as *in vivo*. Arsenic activates Hedgehog signaling by decreasing the stability of the repressor form of GLI3, one of the transcription factors that ultimately regulate Hedgehog activity. We also show, using tumor samples from a cohort of bladder cancer patients, that high levels of arsenic exposure are associated with high levels of Hedgehog activity. Given the important role Hedgehog signaling plays in the maintenance and progression of a variety of tumors, including bladder cancer, these results suggest that arsenic exposure may in part promote cancer through the activation of Hedgehog signaling. Thus, we provide an important insight into the etiology of arsenic-induced human carcinogenesis, which may be relevant to millions of people exposed to high levels of arsenic worldwide. *Cancer Res*; 70(5); 1981–8. ©2010 AACR.

Introduction

More than 100 million people are currently exposed to drinking water containing inorganic arsenic at the level above 0.13 $\mu\text{mol/L}$ (10 $\mu\text{g/L}$), the maximum contaminant level set by the WHO (1). Contaminant levels of inorganic arsenic as high as $\geq 10 \mu\text{mol/L}$ can be found in drinking water throughout the world. Chronic arsenic ingestion causes numerous human health problems (1). In particular, arsenic exposure has a strong association with human can-

cers (2), including those derived from the lung, skin, bladder, and possibly other sites (1). Consistent with these findings, chronic low-level arsenic treatment has been shown to promote cell proliferation (3) and transform cells *in vitro* (4). Although arsenic exposure clearly contributes to carcinogenesis, the underlying mechanisms have only recently been described (1).

The secreted protein Hedgehog was first described as a key factor in metazoan development, determining cell fate, promoting proliferation or differentiation, and acting as a survival factor or a guidance molecule (5). Emerging results now suggest that Hedgehog signaling may also play a fundamental role in the maintenance function of adult tissues undergoing continuous proliferation and differentiation (6) perhaps by regulating the small pool of stem/progenitor cells that regulate these processes (7). Consistent with this important role in fetal development and adult tissue maintenance, deregulation of Hedgehog signaling leads to a variety of human cancers (8, 9), some of which are commonly associated with arsenic exposure. There are three mammalian Hedgehog family members, Sonic Hedgehog, Indian Hedgehog, and Desert Hedgehog, which are thought to function primarily by engaging a common signaling pathway. These proteins, which we collectively call Hedgehog, initiate their biological effects through binding to the cell surface receptor Patched. Such binding relieves the inhibitory effect of Patched on a seven-transmembrane protein Smoothed, resulting in modulation of the GLI

Authors' Affiliations: Departments of ¹Pharmacology and Toxicology and ²Surgery, Dartmouth Medical School, Hanover, New Hampshire; ³Department of Genetic Medicine, Weill Medical College of Cornell University, New York, New York; ⁴Bay Paul Center in Comparative Molecular Biology & Evolution, Marine Biological Laboratory, Woods Hole, Massachusetts; and ⁵Section of Biostatistics and Epidemiology, Department of Community and Family Medicine, Dartmouth Medical School; ⁶Norris Cotton Cancer Center, Lebanon, New Hampshire

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Current address for J.A. Gosse: Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469.

Corresponding Author: David J. Robbins, Department of Pharmacology and Toxicology, Dartmouth Medical School, Rensselaer 614, Hanover, NH 3755. Phone: 603-650-1716; Fax: 603-650-1129; E-mail: david.j.robbins@dartmouth.edu.

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transcription factors (GLI1, GLI2, and GLI3; refs. 10, 11). GLI1 is a pure transcriptional activator and is itself a Hedgehog target gene, the expression level of which is thought to be the most reliable marker for Hedgehog pathway activity (12). GLI2 and GLI3 function as both positive and negative regulators of the Hedgehog pathway depending on their proteolytic status. Full-length GLI2 and GLI3 are transcriptional activators (GLI-A; refs. 13, 14). However, in the absence of Hedgehog, GLI2 and GLI3 are actively converted into partially proteolyzed transcriptional repressors (GLI-R) through a processing mechanism regulated by the proteasome (15, 16). The activity of the full-length GLIs is also regulated by controlling their protein stability via their complete proteosomal degradation (17). Ultimately, the overall ratio between GLI-A and GLI-R defines the levels of Hedgehog pathway activation (10, 11).

As there are many similarities in outcomes between individuals chronically exposed to arsenic and those harboring mutations that result in deregulated Hedgehog signaling (1, 8, 9), we hypothesized that arsenic might act to regulate Hedgehog signaling. Here, we provide evidence suggesting that arsenic exposure results in constitutive Hedgehog signaling and that this activation occurs at concentrations relevant to human exposure. Moreover, using an established bladder cancer patient cohort, we show a strong positive association between arsenic exposure and high-level Hedgehog signaling, underscoring the physiologic relevance of our findings. This is the first report implicating the Hedgehog signaling pathway as a physiologically relevant biological target for arsenic, which may begin to explain some of the underlying carcinogenesis found in humans exposed to environmental arsenic.

Materials and Methods

Cell culture, reagents, and arsenic treatment. All cells were grown and maintained as described previously (18–20). Cell growth media used in these experiments have undetectable levels of background arsenic (data not shown). Sonic Hedgehog conditioned medium was collected from HEK293 cells stably expressing murine Sonic Hedgehog (21). Conditioned medium from the parental HEK293 cells was used as the negative control. For the experiments involving chronic arsenic treatment, sodium arsenite (Sigma) was added to NIH3T3 cells to final concentrations of 0, 0.5, 1, or 5 $\mu\text{mol/L}$ for 1 to 8 weeks. For other assays, sodium arsenite was added to cells for 24 h. Cyclopamine (Toronto Research Chemicals) or vehicle control (DMSO) was added to 8-week-old chronic arsenic-treated NIH3T3 cells in low serum medium (0.5% serum) for 24 h before harvesting cells for RNA isolation. Hedgehog reporter assays were done using a GLIBS-Luc reporter construct or a miniTK-luciferase control plasmid as described previously (22).

Clonogenic cell survival assay. NIH3T3 cells were seeded into six-well dishes at 500 per well and allowed to grow overnight in normal growth medium. Cells were then exposed to 0 to 20 $\mu\text{mol/L}$ sodium arsenite for 24 h and washed once with PBS followed by the addition of fresh growth medium.

After 5 days post-arsenic treatment, cells were stained with 0.4% (w/v) Giemsa dye (in 70% methanol; Sigma) for 90 min. Clonogenicity was defined as colony numbers formed as a percent of control untreated cells.

RNA extraction, reverse transcription, and real-time PCR. RNA was extracted using Tri Reagent (Molecular Research Center) followed by a column cleanup step using a RNeasy kit (Qiagen). cDNA was synthesized from 2 μg total RNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative PCR was done using a 7500 Fast Real-time PCR System with inventoried Taqman gene expression assay probes (Applied Biosystems) as described previously (22).

Immunoblot. GLI1 was enriched from one confluent 150 mm dish of cells using Sepharose beads conjugated with a Gli-binding oligonucleotide, or nonspecific oligonucleotide, as described previously (16). In all other cases, proteins were directly extracted in 2 \times Laemmli sample buffer. Immunoblotting was done as described previously (23). Antibodies used were as follows: rabbit polyclonal GLI1 (affinity-purified, raised against a synthetic peptide corresponding to amino acids residue 802–817 of human GLI1, which has no homology with GLI2 or GLI3, and characterized in Supplementary Fig. S1), rabbit polyclonal GLI3 (24), and mouse monoclonal $\alpha\text{TUBULIN}$ (Calbiochem). In certain cases, densitometry analysis was done on developed X-ray films using ImageQuant (version 5.2). The quantitation results were expressed as ratios of the intensity score of the anti-GLI3 bands versus the corresponding intensity score of the anti-TUBULIN band. The ratio was used to compare the relative GLI3 levels with or without chronic arsenic exposure. The immunoblot for $\alpha\text{TUBULIN}$ was used as a loading control in all cases.

Mice. All mouse studies were done in accordance with the policies of the Dartmouth Institutional Animal Care and Use Committee. Seven-week-old C57BL/6J male mice (The Jackson Laboratory) were housed on an AIN-76A diet (*ad libitum*; Harlan-Teklad) and Carefresh bedding in autoclaved cages. After 2 weeks, these mice were given drinking water (*ad libitum*, changed weekly) with or without addition of 1.3 $\mu\text{mol/L}$ sodium arsenite for 5 weeks as described previously (25). Tissues were collected from these mice and stored in RNA later (Ambion) before RNA extraction in Tri Reagent using a motor-driven homogenizer.

Bladder cancers and GLI1 immunohistochemistry. The study group was composed of newly diagnosed, histologically confirmed bladder cancer patients on whom we obtained the original diagnostic formalin-fixed tumor block. Subjects were interviewed as part of case-control study that involved testing a tap water sample from the participants' homes for arsenic concentrations using high-resolution inductively coupled plasma mass spectrometry (26). Diagnostic slides underwent a standardized histopathology re-review by the study pathologist, who selected the tumor block and outlined regions for cutting 0.6 mm tissue cores for tissue microarrays. Immunohistochemistry was done as described previously (27) using an affinity-purified rabbit polyclonal GLI1 antibody (1:300) characterized in Supplementary Fig. S1. The GLI1 score from each bladder tumor sample was

examined by a research pathologist, who was blinded to the arsenic exposure and tumor invasiveness data sets. The GLI1 scoring criteria were based on an estimate of the percentage of tumor cells with nuclear GLI1 staining: <1%, 1% to 5%, 5% to 25%, and 25% to 50% (Supplementary Fig. S2). No tumors with >50% nuclear GLI1 staining have been observed. Although we noted that the intensity of nuclear GLI1 staining appeared to positively correlate with the percentage of positively stained cells, this observation was not used as a factor for scoring. A second GLI1 antisera (Abcam) was used to confirm these immunohistochemistry results (Supplementary Fig. S1). The specificity of this second antisera was characterized previously (22).

Statistics. All experiments were independently done at least three times. Statistical significance was determined by Student's *t* test. For the analysis of tissue microarrays, we compared the proportion of tumors that stained positively for GLI1 among those with high versus low water arsenic concentrations (above and below the median for the study) using a χ^2 test. In all analyses, two-tailed *P* values ≤ 0.05 were considered statistically significant.

Results

To test the hypothesis that arsenic activates Hedgehog signaling, we first evaluated the ability of sodium arsenite to activate a well-characterized Hedgehog reporter construct in cultured cells originating from several tissues that are known arsenic targets (1, 28). This Hedgehog reporter construct was transiently transfected into primary bovine aorta endothelial cells (BAEC) and immortalized bronchial epithelial cells (BEAS 2B) followed by arsenic treatment for 24 h. Sodium arsenite (1 $\mu\text{mol/L}$; 77 $\mu\text{g/L}$) gave rise to a statistically significant 1.7-fold increase in Hedgehog activity in both cell types, whereas treatment with 5 $\mu\text{mol/L}$ arsenic resulted in a 2.1- or 8.7-fold increase in Hedgehog activity in BEAS 2B or BAEC, respectively (Fig. 1A). The activity of a constitutively expressed *Renilla* luciferase construct was not significantly changed in these arsenic-exposed cells (data not shown), consistent with these doses of arsenic not inducing a general toxic effect on these cells. Next, we examined the ability of arsenic to activate Hedgehog signaling in NIH3T3 fibroblasts, a cell line commonly used to study Hedgehog signaling (18). We first determined the tolerable doses of sodium arsenite for NIH3T3 cells using a clonogenic assay, in which the ability of arsenic-treated cells to proliferate and form colonies was tested. The clonogenicity of NIH3T3 cells appears unaffected when treated with up to 5 $\mu\text{mol/L}$ sodium arsenite, with an overall IC_{50} of ~ 9.5 $\mu\text{mol/L}$ (Fig. 1B). Similar to the results observed with BAEC and BEAS 2B cells, 5 $\mu\text{mol/L}$ arsenic activated Hedgehog signaling 2.6-fold above background in NIH3T3 cells (Fig. 1C, black column). The activation of the Hedgehog reporter gene by arsenic appears specific to Hedgehog pathway activation, as a similar construct lacking GLI-binding sites failed to respond to arsenic exposure (Fig. 1C, gray column). These results suggested that activation of Hedgehog signaling was induced by arsenic and that this activation could occur at arsenic

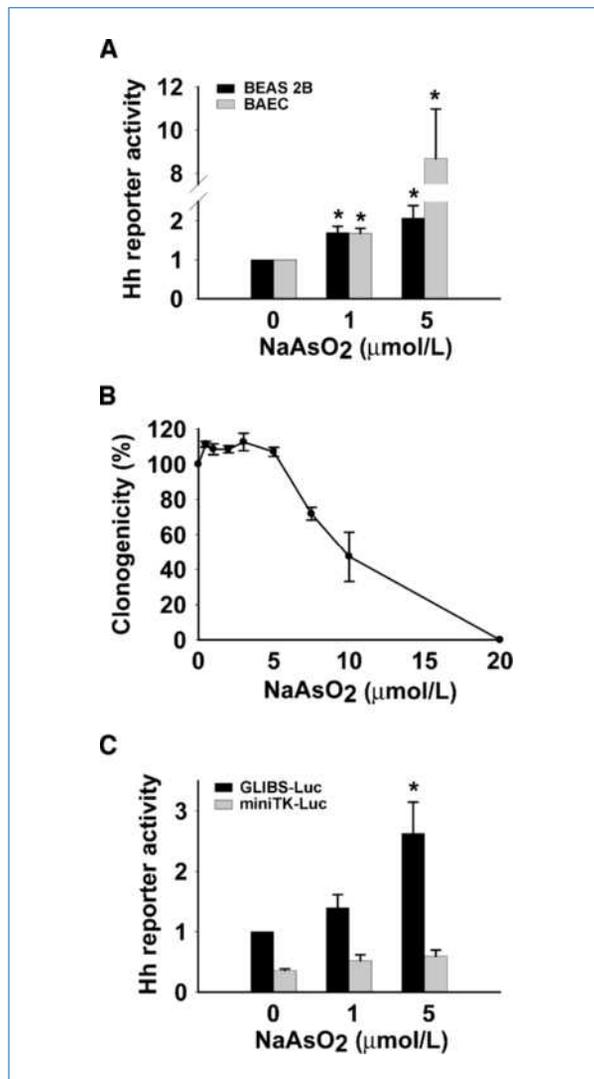


Figure 1. Arsenic activates Hedgehog signaling in various cells. A, a Hedgehog reporter construct (GLIBS-Luc) was transfected into primary bovine aorta endothelial cells (BAEC) and immortalized human lung epithelial cells (BEAS 2B). Luciferase activity was measured 24 h after arsenic treatment and normalized to *Renilla* activity. Normalized luciferase activity in vehicle-treated cells was set to 1. B, clonogenic assay in NIH3T3 cells exposed to the indicated doses of arsenic for 24 h. Data are expressed as clonogenicity, which is the number of colonies formed as a percentage of the untreated control cells. C, GLIBS-Luc or a control construct (miniTK-Luc) was transfected into NIH3T3 cells followed by measurement of luciferase activity 24 h after arsenic treatment and normalized to *Renilla* activity. Normalized luciferase activity in vehicle-treated GLIBS-Luc-transfected cells was set to 1. Bars, SE from three independent experiments. *, statistically significant changes compared with vehicle-treated cells.

levels similar to those found at contamination levels relevant to human exposure (1).

We next examined the ability of arsenic to activate Hedgehog signaling when cells were exposed to low levels of arsenic in a chronic manner, which is thought to more closely mimic

human exposure in arsenic-contaminated areas (1, 2). NIH3T3 cells were cultured either in standard growth medium or the same medium containing 0.5, 1, or 5 $\mu\text{mol/L}$ sodium arsenite for 1 to 8 weeks. As an endogenous indicator of Hedgehog pathway activation, the expression of the Hedgehog target gene *GLI1* was examined in cells treated with arsenic for 1, 3, or 5 weeks using a quantitative real-time reverse transcription-PCR assay. After 1 week of arsenic exposure, 1 and 5 $\mu\text{mol/L}$ arsenic-treated cells showed a modest increase in the expression of *GLI1*, 1.4- and 2.2-fold, respectively, relative to control cells that were not exposed to arsenic (Fig. 2A). This activation became more robust as the time of arsenic treatment increased. Interestingly, although 1-week exposure of cells to 0.5 $\mu\text{mol/L}$ arsenic did not cause any increase in *GLI1* induction, longer exposure (≥ 3 weeks) resulted in a 1.7-fold *GLI1* induction. This concentration of arsenic is lower than the maximum contaminant level (0.63 $\mu\text{mol/L}$) allowed in potable water in the United States before 2002 (2). Thus, *GLI1* expression was activated by chronic arsenic treatment in a dose- and time-dependent manner. To strengthen this analysis, we examined the expression of a panel of Hedgehog target genes

in NIH3T3 cells exposed to sodium arsenite for 8 weeks. This panel consists of those genes normally upregulated by Hedgehog [*GLI1*, *patched 1* (*PTCH1*), *patched 2* (*PTCH2*), and *secreted phosphoprotein 1* (*SPP1*); refs. 5, 29, 30] as well as a gene that is repressed by Hedgehog in cultured fibroblasts [*secreted frizzled-related protein 2* (*SFRP2*); ref. 31]. Four of five of these genes (*GLI1*, *PTCH1*, *SPP1*, and *SFRP2*) showed dose-dependent changes consistent with activation of Hedgehog signaling (Fig. 2B). Arsenic effects on NIH3T3 cells were verified by a dose-dependent induction of *heme oxygenase 1* (*HO1*) expression, which is a well-established arsenic target (32). As these analyzed genes are all well-characterized Hedgehog target genes, their expression serve as physiologically relevant markers of Hedgehog pathway activation. We also independently verified the ability of chronic low-dose arsenic to activate Hedgehog target genes in a pluripotent mesenchymal mouse embryonic cell line C3H/10T1/2 (data not shown).

To further validate the observed arsenic-induced changes in *GLI1* expression, we next examined the change in *GLI1* protein in cells chronically exposed to arsenic. To detect endogenous *GLI1* in lysates of these NIH3T3 cells, we first

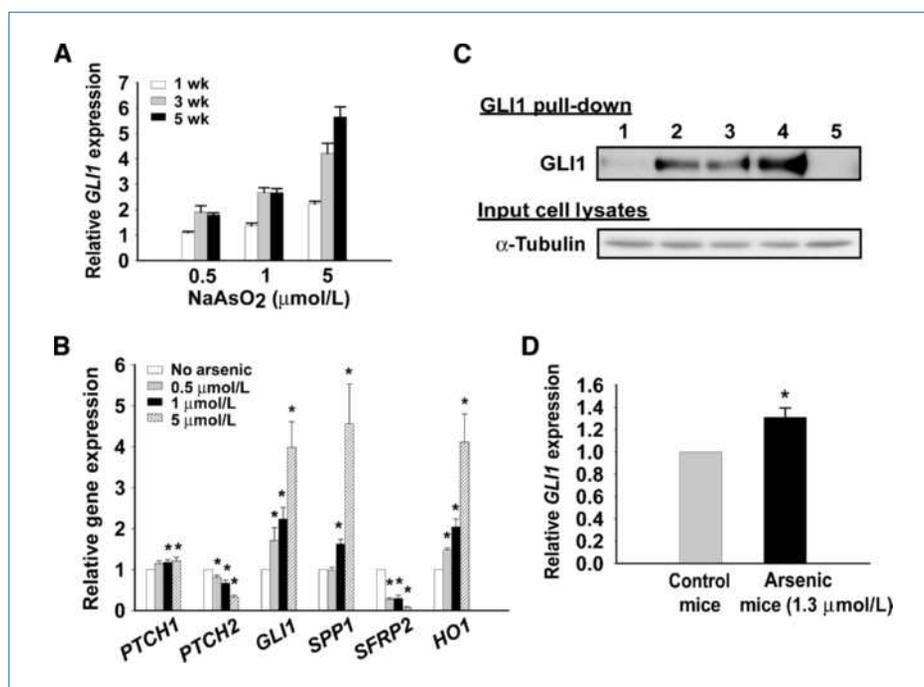


Figure 2. Chronic low-dose arsenic exposure activates Hedgehog signaling *in vitro* and *in vivo*. A, NIH3T3 cells were cultured in medium with or without 0.5, 1, or 5 $\mu\text{mol/L}$ sodium arsenite. Real-time PCR was done to determine the expression of the Hedgehog target gene *GLI1* relative to expression of 18S rRNA after 1, 3, or 5 wk of arsenic treatment. A representative experiment of *GLI1* expression in arsenic-treated cells relative to untreated control cells is shown. B, RNA was extracted from NIH3T3 cells treated with or without 0.5, 1, or 5 $\mu\text{mol/L}$ sodium arsenite for 8 wk. The expression levels of indicated genes were examined by real-time PCR relative to the expression of 18S rRNA. The level of expression for each indicated gene in untreated control cells was set to 1. Bars, SE from five independent experiments. *, significant changes compared with control cells. C, immunoblot for *GLI1* was done after enrichment of the protein using Sepharose beads conjugated with a *Gli1*-binding oligonucleotide (lanes 1-4) or nonspecific oligonucleotide (lane 5). Lane 1, control cells; lane 2, 0.5 $\mu\text{mol/L}$ arsenic-exposed cells; lane 3, 1 $\mu\text{mol/L}$ arsenic-exposed cells; lanes 4 and 5, 5 $\mu\text{mol/L}$ arsenic-exposed cells. The immunoblot for $\alpha\text{TUBULIN}$ in lysates before *Gli1* pull-down served as an indicator of equal protein input. D, 9-wk-old C57BL/6J male mice were given drinking water with or without 1.3 $\mu\text{mol/L}$ sodium arsenite for 5 wk followed by harvesting of RNA from the kidneys. Real-time PCR was done to determine *GLI1* expression relative to expression of 18S rRNA. Expression of *GLI1* was set to 1 in control mice that were not exposed to arsenic via drinking water. Six control mice and six arsenic-exposed mice were used in each experiment. Bars, SE from three independent experiments. *, statistically significant changes.

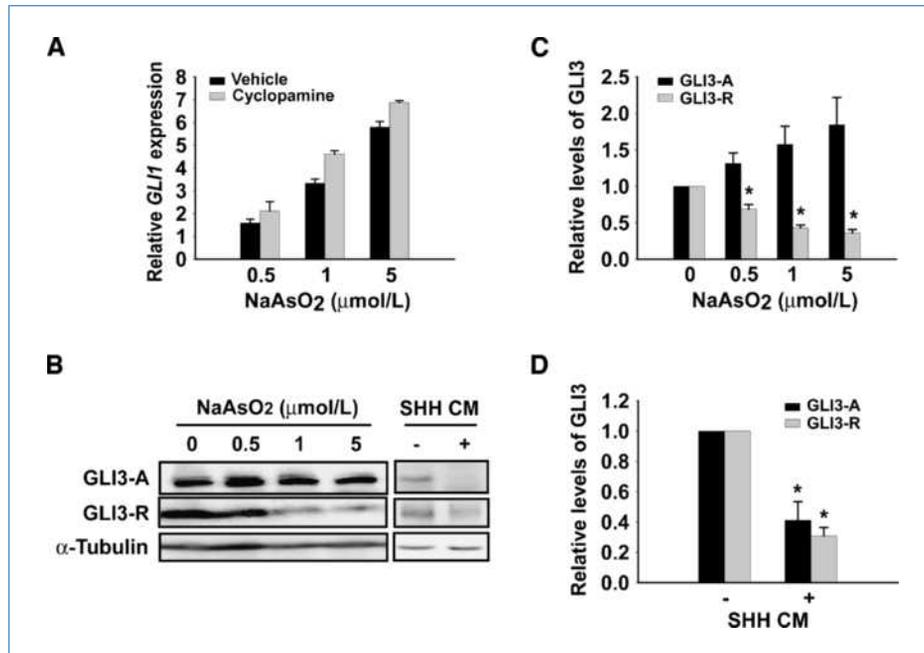


Figure 3. Chronic arsenic exposure reduces the level of GLI3 repressor. A, chronic arsenic-exposed NIH3T3 cells were treated with 5 $\mu\text{mol/L}$ cyclopamine or vehicle for 24 h. Real-time PCR was done to determine the expression of the Hedgehog target gene *GLI1* relative to expression of 18S rRNA. A representative experiment of *GLI1* expression in arsenic-treated cells, relative to untreated control cells, is shown. B, immunoblot for full-length GLI3 activator (GLI3-A) and GLI3 repressor (GLI3-R) was done in total cell lysates from NIH3T3 cells exposed to the indicated doses of arsenic for 8 weeks or Sonic Hedgehog conditioned medium (SHH CM) or control medium for 24 h. C and D, levels of GLI3-A and GLI3-R were quantified by densitometry analysis. Levels of GLI3-A and GLI3-R were set to 1 in control cells that were not exposed to arsenic. Bars, SE from three independent experiments. *, statistically significant changes compared with control cells.

enriched for GLI1 using Sepharose beads conjugated with oligonucleotides encoding a defined GLI-binding site (16) followed by immunoblotting with GLI1 antisera. Using this approach, we observed a dramatic increase in GLI1 protein in response to increasing amount of arsenic exposure, with the strongest induction occurring in 5 $\mu\text{mol/L}$ arsenic-exposed cells (Fig. 2C, lane 4). Sepharose beads conjugated with nonspecific DNA oligonucleotides were used to control the specificity of these pull-downs (Fig. 2C, lane 5).

Our findings were also confirmed in a mouse model of chronic arsenic exposure (25). Nine-week-old C57BL/6J mice were exposed to 1.3 $\mu\text{mol/L}$ sodium arsenite for 5 weeks in their drinking water. These animals were sacrificed and various tissues were harvested. Total RNA was extracted from these tissues, and the expression of *GLI1* was analyzed by real-time reverse transcription-PCR. A statistically significant increase in the relative expression of *GLI1* was observed from the kidneys of arsenic-exposed mice but not from other tissues examined (Fig. 2D; data not shown). This arsenic-induced increase in Hedgehog signaling is likely an underestimate of the extent of activation, as only a limited group of adult cells in any tissue may be capable of elaborating a Hedgehog response. We did not observe any signs of arsenic-induced toxicity in these animals over these 5-week studies (data not shown). Thus, these results suggest that chronic arsenic exposure is also able to activate Hedgehog signaling *in vivo* at least in a subset of tissues.

To begin to understand the mechanism of Hedgehog pathway activation on arsenic exposure, we determined if the arsenic activation of Hedgehog signaling required Smoothened function, which is a key regulator in the Hedgehog pathway (5). Consistent with the pivotal role of Smoothened in Hedgehog signaling, several small-molecule Smoothened modulators have been described (33). Moreover, Smoothened appears to be the most common small-molecule target identified in numerous high-throughput screens, making it a likely arsenic target. Somewhat surprisingly, the Smoothened antagonist cyclopamine was unable to attenuate arsenic-induced Hedgehog signaling in NIH3T3 cells (Fig. 3A). Cyclopamine decreased overall *GLI1* expression in both chronic arsenic-exposed cells and control cells to a similar extent (data not shown). Thus, the fold induction of *GLI1* expression by arsenic was not inhibited. As a control for the effectiveness of this drug, Sonic Hedgehog induced *GLI1* expression was completely abolished by cyclopamine (Supplementary Fig. S3). This result argued for a dispensable role of Smoothened in arsenic-induced Hedgehog signaling, thus ruling out an indirect contribution of Hedgehog in arsenic-mediated pathway activation. Hedgehog signaling ultimately results in changes in the levels and activation status of GLI1-3 (11). Thus, we hypothesized that arsenic might be activating Hedgehog signaling by modulating the processing of GLI2 or GLI3. Interestingly, arsenic attenuated the level of GLI3-R in a dose-dependent manner but

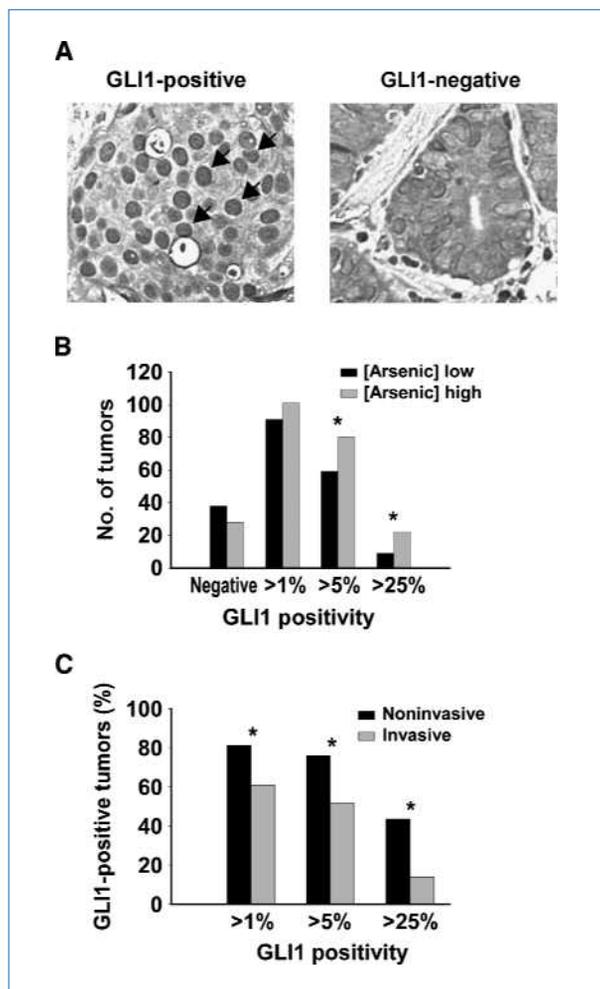


Figure 4. High-level Hedgehog signaling positively correlates with arsenic exposure in human bladder cancer. **A**, immunohistochemical staining and scoring were done on a tumor array consisting of 265 bladder cancers using an antibody against GLI1. A representative immunohistochemical staining for GLI1 from a positive case (left) and a negative case (right) of bladder cancer is shown. A subset of GLI1-positive cancer cells is indicated by arrows. **B**, tumors were grouped into “high” or “low” arsenic categories based on the subject’s drinking water arsenic level (high: arsenic concentration \geq median; low: arsenic concentration $<$ median). Different levels of GLI1 positivity were plotted against the number of tumors in each category. *, statistically significant changes when comparing the distribution of different levels of GLI1-positive tumors with GLI1-negative tumors in each study category. **C**, all tumors were divided into categories of noninvasive tumors and invasive tumors. Different GLI1 classifications were plotted against the percent of GLI1-positive tumors in each category. *, statistically significant changes when comparing the percentage of GLI1-positive tumors in each category.

had little effect on the level of GLI3-A (Fig. 3B and C). Because arsenic treatment also modestly induced the expression of *GLI3* (Supplementary Fig. S4), we speculate that GLI3-R might be selectively degraded on arsenic exposure. Consistent with this speculation, arsenic is known to regulate the stability of other proteins in both proteosomal- and lysosomal-dependent manner (20, 34). Loss of GLI3-R in the context

of similar GLI3-A levels would result in a net increase in Hedgehog activity, similar to what we have observed. However, the effect of arsenic on GLI3 appears different from the way Hedgehog regulates GLI3 in these cells. Hedgehog inhibited GLI3 protein processing into GLI3-R and also decreases *GLI3* mRNA at the same time (24, 35), resulting in a loss of both GLI3-A and GLI3-R (Fig. 3B and D). We were unable to detect endogenous GLI2 from these cells. However, the decreases in *GLI2* transcript observed in response to arsenic are consistent with GLI3-A driving arsenic-mediated activation of Hedgehog signaling (Supplementary Fig. S4).

A series of epidemiologic studies have shown an association between arsenic exposure and bladder cancer (1, 36, 37). Moreover, Hedgehog signaling has also been implicated in bladder cancer progression (38, 39). Thus, to determine a putative linkage between chronic arsenic exposure and Hedgehog signaling in carcinogenesis, we used tumors from a large population-based bladder cancer patient cohort (26). We examined GLI1 protein level as a readout for Hedgehog pathway activity in 265 bladder cancers cases using a quantitative immunohistochemistry protocol that estimated the percentage of tumor cells with nuclear GLI1 staining (Fig. 4A). We defined bladder cancers as negative for GLI1 staining if $<1\%$ of cancer cells contained nuclear GLI1, whereas $>1\%$, $>5\%$, and $>25\%$ nuclear GLI1 delineated different relative levels of GLI1 positivity. The number of cases with each GLI1 score is shown in Supplementary Table S1. Household tap water arsenic concentrations from 97% of the study subjects were measured, ranging from 0.004 to 160.5 $\mu\text{g/L}$, with the median concentration being 0.326 $\mu\text{g/L}$. We classified patients into two categories of “high” or “low” water arsenic exposure using the median arsenic concentration as the cut-point. In general, the numbers of GLI1-positive tumors were higher in patients with high arsenic exposure, whereas GLI1-negative tumors resided more in the low arsenic category (Fig. 4B). For example, when using the highest level of GLI1 quantitation observed ($>25\%$) as the definition of GLI1 positivity, 22 bladder tumors were positive among patients with high water arsenic levels versus 9 among those with low levels ($P = 0.0087$; Fig. 4B). We also examined the distribution of GLI1-positive cancers in two major clinical categories, noninvasive and invasive bladder cancers, as these two tumor types have different clinical management and prognostic criteria (40). Seventy-one percent of the cancer cohort we used consisted of noninvasive tumors, with the remaining samples being invasive tumors. We observed that a higher percentage of GLI1-positive cancers were noninvasive regardless of their classification of GLI1 positivity (Fig. 4C). In particular, there were 3-fold more GLI1-positive tumors (44% versus 14%) in the noninvasive category when only tumors with highest GLI1 positivity were taken into account. These results suggest that activation of Hedgehog signaling by arsenic may play a preferential role in the formation of noninvasive bladder cancers. Indeed, the association between GLI1 positivity and arsenic exposure was also more pronounced in noninvasive tumors compared with invasive tumors (data not shown). Thus, these results suggest a highly significant positive association between the levels of arsenic exposure and Hedgehog pathway

activity in bladder cancer, showing the relevance of this work to human health.

Discussion

We show here that the environmental toxicant arsenic activates the Hedgehog signaling pathway. The activation of this important signaling pathway requires levels of arsenic relevant to human exposure and occurs in various cell types *in vitro* as well as *in vivo*. Activation of Hedgehog signaling occurs in response to acute and chronic arsenic exposure, in a manner that is time and dose dependent, and via a mechanism that does not appear to affect cell viability. The chronic effects of arsenic are particularly relevant to human disease, as most arsenic-related health concerns occur in individuals exposed to environmental levels of arsenic for a long period (1, 2). We suggest a model whereby arsenic activates Hedgehog signaling by targeting GLI3-R for proteolytic degradation, in a manner that appears independent of Smoothened. Our results also show a strong association between exposure to arsenic and activation of high-level Hedgehog signaling in a cohort of bladder cancer patients, particularly those with noninvasive bladder cancers, showing the significance of our findings to human health.

Although arsenic is able to activate Hedgehog signaling in many cell types, the level of activation observed is relatively low compared with those initiated by Hedgehog (Supplementary Fig. S3). However, constitutive low-level Hedgehog activation over long periods might contribute to significant health problems as illustrated in individuals diagnosed with Gorlin's syndrome (41). These individuals harbor loss of function mutations in one copy of *PTCH1*, which results in constitutive low-level activation of Hedgehog signaling (42). These individuals exhibit an increased risk for several health problems, including certain types of cancer. Chronic exposure to arsenic also results in an increased risk to a variety of tumors, including those derived from skin, lung, and bladder (1). Our results support the hypothesis that chronic activation of Hedgehog signaling by arsenic might contribute to the development of a subset of these tumors. Activation of Hedgehog signaling is thought to act as a survival factor in tumor cells, with the extent of Hedgehog pathway activation approximating tumor progression (43). Recent evidence has also suggested that ectopic Hedgehog activation is important to create the microenvironment required for efficient tumor growth (44). Consistent with the frequent requirement of Hedgehog activity in carcinogenesis, the Hedgehog target gene *GLII* is itself an oncogene. Overexpression of *GLII* results in a transformed phenotype *in vitro* (45) and is sufficient to drive the formation of many cancers when overexpressed in animal models, including those associated with arsenic exposure (17, 46, 47). Therefore, we speculate that the ability of arsenic to function as a carcinogen might act in part through its ability to activate Hedgehog signaling, helping to create a microenvironment permissive to tumor development.

Urine is the major route of arsenic excretion in humans, making the bladder a susceptible target for the adverse effects of arsenic (1). Several epidemiologic studies have sug-

gested an increased risk of bladder cancer with chronic arsenic exposure (1, 36, 37). However, the mechanism underlying this increased risk remains unknown. Our results suggest that activation of Hedgehog signaling by arsenic might contribute to the etiology of arsenic related bladder cancers. It has been suggested previously that Hedgehog signaling may contribute to the development of bladder cancers (38, 39). Deletions in chromosome 9q are the most common genetic alteration and earliest marker for bladder cancers (48). Noticeably, *PTCH1* resides in the region of 9q often lost in human bladder cancers (38, 48). Furthermore, gene amplification of a region of chromosome 12q13-q15, which encodes *GLII*, has also been found in a subset of bladder cancers (49). Interestingly, loss of *PTCH1* and amplification of *GLII* would all result in Hedgehog pathway activation. Consistent with the Hedgehog pathway playing an important role in the progression of bladder cancer, *PTCH1* heterozygous mice exhibit a predisposition to carcinogen-induced bladder cancer (50). We have shown here that *GLII* levels are elevated in ~75% of bladder cancers (Supplementary Table S1; 198 of 265 cases) and that an environmental toxicant known to function as a risk factor for bladder cancer can also activate Hedgehog signaling, albeit through a different mechanism. Combined, these results suggest that the Hedgehog signaling pathway plays an important role in bladder cancer progression and that its activation can occur via distinct mechanisms: loss of *PTCH1*, amplification of *GLII*, or degradation of GLI3-R in response to chronic exposure to arsenic.

In conclusion, our study provides for the first time evidence that links activation of the Hedgehog pathway with arsenic exposure. This pathway activation occurs in both acute and chronic manners and occurs at environmentally relevant levels of arsenic. Furthermore, we show that arsenic exposure correlates with high-level Hedgehog activation in bladder cancer samples isolated from a large cohort of such patients. Thus, high-level Hedgehog signaling may provide a diagnostic marker for those bladder cancer patients exposed to arsenic and provides a novel mechanism of how arsenic might function in carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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