Published in final edited form as:

Epigenomics. 2010 February 1; 2(1): 87–104. doi:10.2217/epi.09.45.

Effects of arsenic exposure on DNA methylation and epigenetic gene regulation

John F Reichard[†] and Alvaro Puga

Abstract

Arsenic is a nonmutagenic human carcinogen that induces tumors through unknown mechanisms. A growing body of evidence suggests that its carcinogenicity results from epigenetic changes, particularly in DNA methylation. Changes in gene methylation status, mediated by arsenic, have been proposed activate oncogene expression or silence tumor suppressor genes, leading to long-term changes in activity of genes controlling cell transformation. Mostly descriptive, and often contradictory, studies have demonstrated that arsenic exposure is associated with both hypo- and hyper-methylation at various genetic loci *in vivo* or *in vitro*. This ambiguity has made it difficult to assess whether the changes induced by arsenic are causally involved in the transformation process or are simply a reflection of the altered physiology of rapidly dividing cancer cells. Here, we discuss the evidence supporting changes in DNA methylation as a cause of arsenic carcinogenesis and highlight the strengths and limitations of these studies, as well areas where consistencies and inconsistencies exist.

Keywords

arsenate; arsenic; arsenite; cancer; carcinogenesis; DNMT; epigenetics; glutathione; hypermethylation; hypomethylation; oxidative stress; S-adenosylmethionine; SAM; transsulfuration

Arsenic is an element of major health concern because substantial epidemiologic evidence links inorganic arsenic exposure to a variety of human cancers [201,202]. Chronic low-dose dietary arsenic exposure is causally linked to cancers of the skin, bladder, liver and lung [1-4], and human exposures during gestation are associated with elevated incidence of lung and bladder cancer decades later during adulthood [4-6]. It is estimated that over 100 million people worldwide are exposed to carcinogenic levels of arsenic [201], the vast majority owing to the consumption of drinking water taken from arsenic contaminated aquifers. Populations affected by arsenic- contaminated drinking water span the globe with significant exposures identified in Bangladesh, India, Taiwan, China, Mexico, Argentina, Chile, Europe and regions of North America.

The mechanism by which arsenic mediates carcinogenesis remains a subject of debate, with evidence supporting several plausible etiologies, including disruption of signaling cascades [7], elevated levels of oxidative stress [8,9], chromosomal aberrations [10] and epigenetic

© 2010 Future Medicine Ltd

[†]Author for correspondence: Department of Environmental Health & Center for Environmental Genetics, University of Cincinnati College of Medicine, 3223 Eden Avenue, PO Box 670056, Cincinnati, OH 45267-0056, USA Tel.: +1 513 558 0712 Fax: +1 513 558 0925 John.Reichard@uc.edu.

Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

changes [2,11]. Arsenic does not cause point mutations in standard mutagenicity assays and is generally considered to be nongenotoxic [12,13]. Uncertainty regarding possible mechanisms of arsenic carcinogenicity persists because arsenic has generally been found to be noncarcinogenic in laboratory animals when administered as a single agent [14,15], with the possible exception of transplacental exposures in mice [16]. In rodents, arsenic is neither a tumor initiator nor a tumor promoter, though it does act as a co-carcinogen by synergistically enhancing tumorigenicity of other carcinogens such as UV irradiation [17]. Thus, inorganic arsenic is generally not carcinogenic in animal models when administered as a single agent and studies in humans are complicated by the fact that arsenic exposures in human populations can occur in association with exposures to other potential carcinogens.

Epigenetics

The term epigenetics was coined by Nanney [18] to describe systems of cellular heredity based on processes other than changes in DNA sequence. Epigenetic mechanisms allow cells to rapidly alter long-term transcriptional activity, thereby permitting coordinated changes in gene expression without permanently altering the sequence of DNA. Chromatin structures limit access of the transcriptional apparatus to genes, thereby interfering with transcriptional activity. Epigenetic restructuring of chromatin can add or remove these obstructive complexes to stably repress or activate genes, respectively. Environmental factors that disrupt epigenetic programming cause aberrant changes in gene expression that predispose to a broad array of diseases, including cancer, hypertension, Type 2 diabetes and congenital malformations [19].

The epigenetic effects of arsenic have been an area of intense research interest since it was reported by Mass and Wang that arsenic causes hypermethylation of the *p53* gene [20]. Since their report, changes in DNA methylation have been the most intensively studied epigenetic phenomena resulting from arsenic exposure, while changes in histone modification and microRNA expression have begun to receive increasing attention.

DNA methylation is a fundamental determinant of chromatin structure. In general, the extent of methylation at CpG islands, DNA sequence clusters rich in CG dinucleotides in the vicinity of gene promoters, correlates with the long-term silencing of gene transcription [21,22]. By repressing gene expression, DNA methylation provides an essential level of control over genes regulating cell differentiation, proliferation and organism development, and these changes can be passed on to daughter cells during replication [23]. Global disruption of DNA methylation is lethal to mammals, and even focal disruption at imprinted loci can produce serious developmental abnormalities. Changes in promoter CpG island methylation can significantly change cell behavior. DNA hypermethylation has been implicated in the loss of tumor suppressor gene expression [24-26]. Conversely, it was noted more than 20 years ago that oncogenic transformation goes hand-in-hand with genome-wide hypomethylation [27,28]. Hypomethylation appears to contribute to malignant transformation by predisposing cells to chromosomal defects and rearrangements leading to genetic instability [29,30] and spontaneous mutations [31,32]. These observations suggest that genome hypomethylation precedes and likely predisposes to transformation. Thus, both DNA hypermethylation and hypomethylation appear to independently contribute to cancer development and progression.

Mechanisms of arsenic mediated epigenetic disruption

Several mechanisms are theorized to underlie epigenetic changes in DNA methylation by arsenic. Central to each is an effect of arsenic on the activity of DNA methyltransferase (DNMT) enzymes. DNMTs catalyze the transfer of a methyl group from *S*-adenosylmethionine (SAM) onto the C5' position of cytosine at CpG dinucleotides to produce 5-methylcytosine [33]. Inhibition of DNMT activity by arsenic appears to cause whole-genome and localized

gene specific demethylation. By contrast, reports that arsenic causes localized DNA hypermethylation are more challenging to explain mechanistically.

Metabolism of arsenic

The cytotoxicity and metabolism of arsenic is a function of its oxidation state and methylation status [14]. In animals, absorbed pentavalent arsenate (As^{+5}) is rapidly reduced to trivalent arsenite (As^{+3}) primarily in the blood and liver, and is subsequently distributed to tissues throughout the body [34,35]. The bulk of circulating arsenic undergoes biotransformation in hepatocytes where arsenite undergoes a series of sequential oxidative-methylation and reduction steps yielding several methylation products (Figure 1) [36]. This methylation of inorganic arsenic to dimethylarsenic acid (DMA) facilitates excretion [37], but in the process consumes both SAM and glutathione (GSH) [38].

The reduction of arsenate (As^{+5}) to arsenite (As^{+3}) is a nonspecific reaction carried out by multiple mammalian enzymes [39], including purine nucleoside phosphorylase [40], glyceraldehyde-3-phosphate dehydrogenase [41] and ornithine carbamoyl transferase [42]. These enzymes appear to facilitate arsenate reduction by converting arsenate into arsenylated metabolites (arsenate esters or anhydrides) that are much more readily reduced to arsenite by GSH than is inorganic arsenate [39]. Regardless of the particular enzymatic pathway, GSH is consumed in support of arsenate reduction. Once reduced, arsenite methyltransferase (AS3MT) catalyzes the addition of a methyl group to arsenite in what appears to be two sequential chemical reactions: methylation of trivalent arsenicals using SAM as the methyl donor and oxidation to pentavalent arsenate. AS3MT is capable of using several different reductants to support the methylation of As^{+3} , including dithiothreitol, glutaredoxine and thioredoxin, the latter of which may be the most effective [43,44]. Following oxidative methylation, monomethylarsonic acid (MMA^{+5}) is again reduced prior to the addition of another methyl group. This reduction is reportedly carried out by GSH S-transferase Ω (GSTO) [45,46] and by AS3MT [47]. In humans, 10–20% of arsenic is excreted as MMA^{+5} and 60–80% as DMA^{+5} [48]. Thus, the methylation of inorganic arsenic utilizes multiple equivalents of GSH and SAM per molecule of arsenic.

Methyltransferase competition for SAM

Consumption of SAM during the metabolism of arsenic has been proposed as an important consequence of exposure. In the presence of excessive arsenic, this theory suggests that SAM, which would otherwise be used to carry out normal cellular methylation reactions including DNA methylation, is utilized by AS3MT to methylate arsenic leading to depletion of SAM and accumulation of S-adenosylhomocysteine (SAH) (Figure 2) [49–51]. Hence, SAM depletion caused by extensive arsenic metabolism may impose cofactor limitations on the activities DNMTs and presumably other cellular methyltransferases including histone methyltransferases. Furthermore, high-level SAH accumulation negatively regulates SAM-dependent methyltransferase activity by feedback inhibition, further contributing to DNMT inhibition [52,53]. It is notable that arsenic is primarily metabolized in the liver, thus, the inhibition of DNMT activity due to AS3MT-mediated arsenic metabolism is limited primarily to the liver, and possibly other AS3MT-expressing cell types. For this reason, the effect of arsenic on cysteine transsulfuration may be more important for most cell types.

Arsenic exposure in the presence of folate nutritional deficiency may facilitate genomic hypomethylation. Since methylation of inorganic arsenic uses SAM as a methyl donor, diets deficient in folic acid, methionine, choline and vita-min B12 can limit the synthesis and reutilization of SAM. SAH is recycled back to SAM in a multi step process that proceeds via the folic acid-dependent enzyme 5-methyltetra hydrofolatehomocysteine (HCY) methyltransferase (MTR) and betaine HCY methyl transferase (BHMT), and the cycle is

completed when methionine is conjugated with ATP to regenerate SAM by methionine adenosyl transferase (MAT). MTR-mediated SAM regeneration is the primary pathway for recycling SAM and requires the presence of tetrahydrofolate (from folic acid) and cobalamin (vitamin B12) as enzyme cofactors. These cofactors are obtained from dietary sources and therefore SAM synthesis is susceptible to dietary deficiencies of these essential nutrients as well as methionine [54,55]. Dietary deficiencies that limit the supply of SAM have been linked to epi genetic changes in gene expression and predispose to the transgenerational occurrence of disease [56].

Oxidative stress & transsulfuration in cysteine cycle disruption

It has been proposed that changes in cellular redox status can elicit developmental events, such as cell differentiation, by influencing epigenetic programming [57,58]. This theory proposes that GSH synthesis is bio chemically linked to epigenetic processes through the transsulfuration pathway (Figure 2). HCY is a major source of cysteine for GSH biosynthesis. When increased GSH production is required to offset increases in oxidative stress, induction of the enzyme cystathione β -synthase (CBS) shunts HCY from the methionine cycle into the transsulfuration pathway (Figure 2). In response to arsenic, the expression of enzymes responsible for GSH biosynthesis are rapidly induced [59,60] including those of the transsulfuration pathway [51]. Thus, during periods of high oxidative stress HCY is shunted into the transsulfuration pathway, which limits the availability of recycled SAM necessary for chromatin methylation, resulting in genome-wide DNA hypomethylation [52,61,62].

Arsenic is a well-characterized inducer of oxidative stress. Under physiologic conditions arsenite spontaneously reacts with cellular sulfhydryls, including cysteine residues of proteins, nonprotein dithiols and GSH. In the liver arsenic reduction and oxidative-methylation reactions consumes multiple equivalents of GSH, contributing to oxidation of intracellular GSH [63]. Furthermore, arsenic stimulates the generation of reactive oxygen species during its metabolism through the activation of NADH oxidase [11]. Thus, arsenic rapidly depletes intracellular GSH [64], thereby increasing the oxidation state of exposed cells. Recovery from an arsenic insult is greatly diminished in cells possessing a mutation that impairs GSH synthesis, underscoring the importance of GSH in the protection of arsenic-induced oxidative stress [59].

To date, a causal relationship between activation of the transsulfuration pathway by arsenic and DNA hypomethylation has not been rigorously tested. However, a report by Coppin *et al.* suggested that long-term arsenic exposure shunts HCY to the transsulfuration pathway resulting in DNA hypomethylation [51]. In their investigation, the authors treated RPWE-1 cells with 5 μ M arsenite for 16 weeks. RPWE-1 cells are a malignantly transformed prostate epithelial cell line with a low capacity to methylate arsenic. Hence, in these cells, significantly reduced SAM depletion is not attributable to AS3MT-mediated oxidative methylation. Throughout the 16-week treatment, arsenite-treated cells displayed an adaptive increase in the expression of enzymes responsible for transsulfuration GSH synthesis. Over the course of the 16 week treatment, an adaptive increase in intracellular GSH concentration (up to fivefold) was accompanied by consistently decreased SAM levels (~20-30%). Concomitantly, DNA methylation was reportedly decreased up to 90% 4 weeks after the initiation of arsenic treatment. It may be important that DNA methylation levels were not reported either at the end of the study nor periodically throughout the 16-week treatment. Surprisingly, the study did not show a reciprocal correlation between SAM levels and GSH synthesis, which would have supported the observed change in DNA methylation status. In addition, the approach used for the DNA demethylation assay did not target specific genes and was not linked to changes in expression of particular genes. Despite these limitations, the evidence from this work suggests that transsulfuration may contribute to arsenic-mediated depletion of SAM and promote DNA

hypomethylation. It would be interesting to know if blocking the utilization of HCY for GSH synthesis could block DNA hypomethylation.

DNMT activity & expression

In mammals, DNA methylation is catalyzed by three methyltransferase enzymes: DNMT1, DNMT3A and DNMT3B; each with differing activities for maintenance and *de novo* DNA methylation. DNMT1 is the methyltransferase predominantly responsible for copying methylation patterns during DNA replication and therefore is primarily responsible for propagating epigenetic DNA methylation to daughter cells [65,66]. By contrast, the DNMT3 family appears to establish *de novo* methylation patterns during embryogenesis. This division of labor is not entirely dichotomous; however, since DNMT1 activity is also required for *de novo* methylation at non-CpG cytosines [67] and perhaps to a limited extent within CpG-rich sites [68,69].

Arsenic appears to inhibit DNMT activity by either reducing expression or inactivating the enzyme. Zhao *et al.* have demonstrated that chronic exposure of cells to low-dose arsenite for 18 weeks inhibited DNA methyltransferase activity in cell lysates by 40% [49]. Interestingly, DNMT1 mRNA expression in the same cells was increased nearly 50%. The loss of DNMT activity in the lysates could result from either a reduction of the protein level or irreversible inactivation of the enzyme. Because the relative amounts of the different DNMT proteins present in the lysates were not evaluated, it remains to be determined whether arsenic-mediated loss of methyltransferase activity reflects changes in DNMT protein levels or enzyme inactivation. However, the loss of activity is not attributable to SAM restriction since enzyme activity was quantified *in vitro* using standardized concentrations of SAM.

Follow-up work published by the same group using AS3MT-deficient RWPE-1 cells found that, even in the absence of significant arsenic methylation, 30 weeks of continuous arsenic exposure caused genomic hypomethylation [70]. In this study, Benbrahim-Tallaa *et al.* reported that 7 weeks of arsenic treatment decreased DNMT activity by as much as 60%; this level of activity persisted throughout the remainder of the 30 week treatment [70]. Although the relative amounts of DNMTs present in cell lysates were not evaluated, RT-PCR analysis demonstrated that DNMT mRNA expression levels did not change. This model is of particular interest because the lack of AS3MT in RWPE-1 cells indicates that arsenic methyltransferase-mediated SAM depletion is not responsible for DNMT inhibition, suggesting that arsenic-mediated DNA hypomethylation may be a consequence of a loss of enzymatically active DNMT rather than competition for cofactor(s) with arsenic methylation.

While Zhao *et al.* demonstrated that DNMT expression was increased by arsenic and Benbrahim-Tallaa reported that the levels remained unchanged; Cui *et al.* reported that exposure of HepG2 cells to arsenite for 48 h decreased DNMT1 mRNA expression [71]. Importantly, expression changes were not entirely dose dependent; rather, DNMT1 expression was strongly repressed by low concentrations of arsenite (2 and 5 μ M) and unaffected by 10 μ M, while DNMT enzymatic activity in nuclear extracts was lost across all arsenic concentrations. In our laboratory, we have observed that treatment of human keratinocyte-derived HaCaT cells with arsenic concentrations up to 5 μ M for 72 h significantly inhibited the expression of DNMT1 and DNMT3A (DNMT3B was undetected); enzyme activity and protein expression levels were not assayed [50].

Taken together, these data paint an uncertain picture for the effect of arsenic on DNMT expression. Although it appears that DNMT activity is reduced by arsenic, the mechanism responsible for this inhibition remains to be determined. The influence that cell type and treatment protocol might have on DNMT expression may be a major contributor to the uncertainty. The ability of arsenic to affect methyltransferase activity might extend beyond

simple cofactor inhibition or gene repression. Direct inactivation or degradation of arsenic-adducted proteins is a possibility since DNMTs are sulfhydryl-rich proteins; DNMT1, 3a and 3b contain 41, 28 and 25 cysteines, respectively. A systematic investigation of arsenic effects on DNMT activity, expression and stability is necessary to fill these knowledge gaps. Regardless of the outcome, it would still be a challenge to reconcile DNMT inhibition with studies reporting hypermethylation of CpG islands.

Global versus localized epigenetic reprogramming by arsenic

If arsenic acts as a sink for GSH and SAM, then sustained arsenic exposure would be expected to deplete SAM to an extent that other methyltransferases, including DNMT, would be inhibited leading to loss of DNA methylation. While plausible, studies claiming to substantiate this model have deficiencies and are contradicted by other studies linking arsenic exposure to DNA hypermethylation [72-76]. Thus, the effect that arsenic has on DNA methylation is questionable since it is difficult to reconcile opposing results found in various reports. The diversity of model systems, treatment protocols and end points used by different research groups to assay DNA methylation contribute to the uncertainty, making it necessary to evaluate these reports in the context of their methodologies (Table 1).

Mass and Wang were the first to propose that arsenic altered the epigenetic status of chromatin. After treating A459 human adenocarcinoma cells with 2 μ M arsenite for 2 weeks, these investigators observed that arsenic increased the methylation state of DNA [20]. This finding was based on assays that evaluated both gene-specific methylation and global DNA methylation. These investigators focused on the *p53* gene and demonstrated a sevenfold increase in promoter methylation. Likewise, SssI methyl incorporation assays [77] demonstrated a significant increase in global DNA methylation following arsenic treatment. The authors did not examine whether these epigenetic changes were associated with changes in *p53* gene transcription, and did not extend the methylation studies to genes other than *p53*. The authors speculated that hypermethylation is a result of elevated DNA methyltransferase stimulated by an elevated SAM level that occurs in response to increased demand [20].

Shortly after Mass and Wang published their findings, the laboratory of Michael Waalkes published the first of a series of reports linking arsenic treatment to loss of DNA methylation. In this first report, TRL 1215 rat liver epithelial cells cultured in up to 0.5 μ M arsenite for 18 weeks became tumorigenic when inoculated into nude mice, whereas similarly passaged control cells were nontumorigenic [49]. The critical tumorigenic change apparently occurred at a point between 8 weeks of exposure, when injected cells were incapable of forming tumors, and week 18, when injected cells were tumorigenic. Other markers indicative of cell transformation that could have been used to help delineate the timeframe of transformation (e.g., growth in soft agar) during this intervening 10 week period of culture were not evaluated. Genome methylation studies demonstrated that prolonged arsenic exposure for 12, 18 and 22 weeks caused substantial DNA demethylation; however, the status of DNA methylation at the apparently nontumorigenic 8 week time point was not reported. Hence, it is not clear in this model if methylation changes are early events predisposing to subsequent transformation or if hypomethylation temporally coincides with transformation.

Both hypomethylation and hypermethylation have been associated with cell differentiation and cancer cell transformation [22]. When these changes occur concurrently, as they appear to do following chronic arsenic exposure, methylation changes at sites regulating expression of individual genes may be more relevant to carcinogenesis than the global trend in DNA methylation. The localized nature of these changes strongly implies that these changes are mediated by mechanisms that are targeted in nature and not the result of nonspecific mechanisms. It has been argued that arsenic-mediated global DNA hypomethylation underlies

increased oncogene expression and contributes to malignant transformation. At the same time, several studies have demonstrated hypermethylation of tumor suppressor genes. The possibility that both events occur simultaneously was demonstrated by treating TRL 1215 cells with arsenic for 18 weeks. Tumorigenicity of these cells, when injected into nude mice, was correlated with DNA hypomethylation and elevated c-Myc expression [78]. These transformed cells also had increased expression of cell-cycle genes, including *PCNA* and cyclin D1 [78-80]. Interestingly, transformation was also associated with repression of genes known to be silenced by promoter CpG methylation, including p21, metallothionein-1 and several inflammatory and tumor suppressor genes. Treating these arsenic-transformed cells with the DNA methyltransferase inhibitor 5-aza-deoxycytidine for 72 h restored p21 and metallothionein-1 expression to levels at or above those in nontransformed control cells [80], suggesting that during arsenic-mediated malignant transformation promoter hypermethylation occurs in the presence of widespread global genome hypomethylation. To date, this possibility has not been directly investigated, though in the context of cell differentiation and carcinogenesis these findings are not surprising.

The liver is clearly a target organ for arsenic toxicity in humans and is an important site for the metabolism of dietary arsenic. To investigate the relationship between global DNA methylation and gene-specific changes during arsenic-mediated transformation, Chen *et al.* administered drinking water containing moderately-high arsenic concentrations (45 ppm) to adult 129/SvJ mice for 48 weeks. At the end of the treatment, global hepatic DNA methylation was decreased as determined by both HPLC measurement of 5-methyldeoxycytosine content and the SssI methyl incorporation assay [81]. Gene-expression analysis revealed deregulation of a number of genes, including elevated expression of cyclin D1 and estrogen receptor (*ER*)- α . Both genes were evaluated for changes in promoter CpG methylation by sequencing bisulfite-modified DNA. Of 13 CpG sites in the *ER*- α promoter, eight demonstrated a significant decrease in methylation status following chronic arsenite treatment. In comparison, the cyclin D1 promoter was unmethylated and remained so following arsenic treatment. Thus, it does not appear that increases in cyclin D1 expression related to arsenic exposure depend on promoter methylation; however, cyclin D1 effects attributed to arsenic may have a basis in the increased expression of *ER*- α due to epigenetic reprogramming [82].

A major difficulty in the analysis of the epigenetic outcome of arsenic exposure is that the effects appear to be influenced by the particular model system used. This difficulty can be illustrated by the studies of Cui *et al.* where the same methods and genes were used to evaluate DNA methylation changes at different arsenic concentrations and in different model systems [71,74]. In one investigation, human-derived HepG2 and Huh-7 liver cancers cell lines were exposed to 2 and 10 μ M arsenite for 72 h. In these cells, 2 μ M arsenic caused a loss of DNA methylation within the promoters of *p16^{INK4a}* (*CDKN2A*), *RASSF1A*, E-cadherin (*CDH1*) and *GSTP1*, and the extent of demethylation was similar to that obtained by treating cells with 5-azadeoxycytidine [71]. Similar results were found in T24 (human bladder carcinoma), HCT116 (colon carcinoma) and HL-60 (human promyelocytic leukemia) cell lines, suggesting that these changes are not unique to a particular cell line. By contrast, 10 μ M arsenic had little effect on promoter methylation in these cells, a result that was attributed to cytotoxicity. In a separate report, the same authors administered drinking water containing 100 ppm arsenate to A/J mice for 18 months. Treatment doubled the occurrence of poorly differentiated lung adenocarcinomas from 0.6 to 1.2 tumors/mouse. By contrast to the hypomethylation observed in HepG2 and Huh-7 cells, tumor-containing lung tissue from arsenic-treated mice had dose-dependent hypermethylation of the *p16^{INK4a}* and *RASSF1A* promoters [74]. The extent of DNA methylation of normal lung tissue in arsenic-treated animals did not change relative to unexposed controls.

Arsenic is not generally considered to be a complete carcinogen [15,83]; rather, it is a cocarcinogen requiring an accompanying second agent to induce tumors in adult animals [84,85]. For this reason, most carcinogenesis studies in rodent species have employed some initiating event. Xie *et al.* evaluated the capacity of arsenic to achieve epigenetic changes as a cocarcinogen in Tg.AC mice in conjunction with phorbol 12-myristate 13-acetate (TPA) skin-painting [86]. Tg.AC mice are unique because they harbor multiple copies of a transgene consisting of the v-Ha-ras structural gene fused to a ζ -globin hemoglobin promoter. This mouse line is a validated dermal model for testing skin carcinogenesis in as little as 6 months, compared with the traditional 2-year National Toxicology Program bioassay [87]. Xie administered organic and inorganic arsenicals to Tg.AC mice in drinking water for 17 weeks. A total of 4 weeks after the initiation of arsenic treatment, the tumor promoter TPA was applied to the skin for a 2 week period. At the end of the 17 week study period, global DNA hypomethylation was associated with all forms of arsenic tested (As^{+3} , As^{+5} , MMA and DMA). Interestingly, despite the fact that arsenic is a well documented skin carcinogen and TPA is an inducer of skin papillomas in Tg.AC mice, the authors did not report any dermal response in conjunction with TPA treatments. Presumably, the 17 week study design may have been too short to permit papilloma detection [88,89]. Microarray-based whole-liver transcriptome analysis of treated mice revealed that each form of arsenic produced dissimilar but overlapping patterns of gene expression. Unfortunately, the authors did not investigate whether the observed differences in gene expression were associated with differences in promoter-region DNA methylation.

Epigenetic effects of *in utero* arsenic exposure

Based on observations that gene imprinting is critical for fetal development, it has been proposed that gestational disruption of normal epigenetic programming by arsenic predisposes to congenital defects and cancer later in life. Epidemiologic evidence links *in utero* and early-life human exposures to arsenic with increased risk of cancer mortality during adulthood [4, 6]. Likewise, gestational arsenic exposure has been associated with developmental and long-term disease consequences, including pulmonary and cardiovascular diseases, fetal loss and birth defects [4-6,90-92]. Since arsenic readily crosses the placenta and concentrates in the fetus [93-95], maternal arsenic exposure might disrupt epigenetic gene imprinting during susceptible periods of fetal development. Theoretically, this disruption of normal epigenetic programming might lead to aberrant changes in gene expression, which in turn influences fetal development and long-term risk of disease [56,96].

Chemical exposures during sensitive periods of gestation can increase the risk of tumors in the offspring. In mice, gestational exposure to arsenic reportedly increases the incidence of malignant tumors of the liver and lungs in mature offspring [16,97,98]. This transplacental carcinogenesis was investigated by continuously exposing pregnant C3H mice to drinking water containing 0, 42.5 or 85 ppm arsenite during gestational days 8 through to 18. The incidence is strongly affected by gender such that male offspring had a greater incidence of hepatocellular carcinoma at 74 weeks after birth, whereas female offspring had an increased occurrence of pulmonary carcinoma at 90 weeks after birth. Although a promising model for arsenic tumorigenesis, a recent report from a collaborating laboratory using the same mouse strain and a similar exposure regimen reported very different outcomes [99]. This subsequent study found no increase in liver tumors in either male or female mice 52 weeks after *in utero* exposure. More difficult to reconcile with the original study is the observation that exposure of offspring to drinking water containing 85 ppm of arsenic in addition to the *in utero* exposure actually protected against liver tumors. Since the 52-week study design of latter work was significantly shorter than the original study, the possibility remains that if the animals had been maintained for the full treatment periods the outcomes may have been more similar.

Based on initial observations that gestational exposure of pregnant C3H mice induced tumors in adult offspring, follow-up studies have attempted to investigate associated epigenetic changes. The long-term effect of gestational arsenic exposure on DNA methylation was assessed in livers of male mice obtained 36 weeks after birth [82]. As with previous studies published by this research team, dams were exposed to 85 ppm arsenic in drinking water during gestational days 8 through to 18. Global and gene-specific DNA methylation changes were determined in normal-appearing liver tissue obtained from 5 control mice and 5 arsenic-exposed mice with hepatocellular carcinoma. *In utero* arsenic exposure was not associated with any change in global DNA methylation or with changes in cyclin D1 promoter methylation. By contrast, the promoter region of the *ER-α* demonstrated significantly reduced methylation at 12 of 13 CpG sites. These changes were consistent with those reported by Chen *et al.* in adult 129/SvJ mice exposed to drinking water containing 45 ppm of arsenic for 48 weeks [81] with the exception that Chen *et al.* reported that arsenic produced global DNA demethylation and marked liver histological changes, including steatosis and hypertrophy, but without detectable tumors. It is possible that the differences in the extent of global demethylation as well as differences in carcinogenesis may be attributed to the timing of exposure (*in utero* exposure vs exposure in mature animals).

A subsequent report addressed the more immediate effect of gestational exposure by assessing DNA methylation status in newborn pups. Following a 10 day gestational exposure to drinking water containing 85 ppm arsenic, livers were obtained from newborn pups shortly after birth [100]. As with adult mice, global methylation status did not predict localized CpG methylation status: there was no change in global DNA methylation, whereas randomly targeted CpG-rich regions became demethylated. These data, and those of the previous study [82] suggest that *in utero* arsenic exposure causes targeted gene-specific changes rather than global genomic demethylation.

Epigenetic effects of arsenic in humans

As with the preceding animal studies, attempts to investigate the epigenetic effects of arsenic in humans also have had limitations. Subject recruitment, exposure assessment, tissue sample procurement and normalization for confounding factors are just a few of the complications unique to human studies. Evaluated individually, the existing reports of epigenetic changes mediated by arsenic in exposed human populations highlight the need for further investigation.

Chanda *et al.* investigated the association between DNA methylation changes and arsenic exposure in human cancers [72]. Peripheral whole-blood samples were collected from 158 residents of West Bengal, India, who were chronically exposed to arsenic-contaminated drinking water. Subjects were stratified on the basis of estimated drinking water exposure over the course of at least 6 months. The methylation status of two tumor suppressor genes was investigated using a study design having two phases. The first phase demonstrated that chronic arsenic exposure was associated with the hypermethylation of the *p53* promoter in 96 subjects. The second phase demonstrated hypermethylation of the *p16^{INK4a}* promoter in a different set of 62 subjects. These results appear to be in line with the cell culture studies demonstrating hypermethylation of *p53* [20], and hypermethylation of *p16^{INK4a}* in arsenic-induced lung adenomas [74]; however, they contrast with the finding that arsenic induced hypomethylation of *p16^{INK4a}* and *RASSF1A* in human liver tumor cells resulting in increased expression of these genes [71]. Recently, Majumdar *et al.* published a follow-up investigation to the 2006 Chanda study [101]. This latter study stratified subjects on the basis of estimated drinking water exposure and determined DNA methylation status in peripheral blood cells. The results were partially consistent with the gene-specific methylation changes of the original study in that the second highest exposure level was associated with global hypermethylation, but the highest levels of arsenic exposure demonstrated a trend towards hypomethylation. To explain this

discrepancy, the authors proposed that low-dose arsenic might induce DNMT3A leading to subsequent DNA hypermethylation, whereas the high-dose exposures cause SAM depletion and DNMT inhibition. Since DNMT activity and expression were not investigated, the extent to which changes in DNMT activity actually contribute to changes in DNA methylation remains speculative.

Several limitations complicate the interpretation of these studies. The minimum cut-off for subject inclusion was a 6-month exposure to contaminated drinking water, but epigenetic changes were not stratified on the basis of exposure time. Since the time-frame necessary to achieve measurable epigenetic changes in blood is unknown, short exposure periods might have falsely limited detection of early changes, leading to an underestimation of epigenetic changes. Furthermore, DNA methylation of the *p53* and *p16^{INK4a}* promoters was assayed in different sets of subjects using different techniques. Thus, it cannot be concluded that hypermethylation of one gene correlates with hypermethylation of the other gene in the same individuals. Identification of signature genes undergoing congruent methylation and demethylation in the same cells and tissues should be a major focus of future investigations.

Marsit *et al.* examined 351 bladder tumors from arsenic-exposed residents of New Hampshire, USA [73]. In this study, subjects were designated as either arsenic-exposed or control on the basis of toenail arsenic measurements, and methylation levels were determined for promoter regions of *p16^{INK4a}*, *RASSF1A* and *PRSS3*. The study found no change at the *p16^{INK4a}* promoter but observed significant increase in methylation at the *RASSF1A* and *PRSS3* promoters. These results are partially consistent with those of Cui *et al.* mentioned earlier, who found dose-dependent hypermethylation of *RASSF1A* and *p16^{INK4a}* in lung adenomas of A/J mice [74]. As with A/J mouse lung tumors, *RASSF1A* DNA methylation in bladder tumors increased with higher tumor grade.

Influence of nutritional factors on arsenic-mediated epigenetic changes

Diets deficient in folate, methionine, vitamin B12 and choline restrict the availability of substrates necessary for SAM synthesis and may inhibit DNA methylation [54,55] contributing to oncogene activation [102,103]. Since arsenic methylation consumes both SAM and GSH, arsenic exposure in the presence of dietary methyl deficiencies may exacerbate DNA hypomethylation. To test this hypothesis, Okoji *et al.* fed adult C57BL/6J mice a diet deficient in methionine, choline and folate. At the end of a 21-day pretreatment period, four dosage groups were administered arsenic-containing drinking water for an additional 130 days. Necropsied livers demonstrated a dose-dependent global DNA hypomethylation. Gene-localized DNA methylation was quantified at two sites upstream of the *Ha-ras* gene: one was a low CpG-density region (segment 474/976) and the other a high CpG-density region (segment 962/1487). In the low CpG region, arsenite treatment plus methyl deficiency decreased the level of methylation at five of 11 restriction sites cleaved by methylation-sensitive restriction enzymes (*EcoRII*, *StuI*, *AluI*, *AvaII* and *XhoI*) and increase methylation at one site (*HhaI*). Notably, although the activities of these six restriction enzymes are sensitive to 5-methyl cytosine, only two are sensitive to cytosine methylation at CpG dinucleotides (*XhoI* and *HhaI*), whereas the other four are inhibited by methylation of cytosines outside the CpG dinucleotide (*EcoRII*, *AluI*, *StuI* and *AvaII*). The majority of methylation changes were detected by enzymes sensitive to methylation of non-CpG dinucleotides within the low density CpG region upstream of the *Ha-ras* transcription start site. Thus, the majority of methylation changes occurred on non-CpG cytosines. By contrast, high-density CpG island regions upstream of *Ha-ras* transcription start site remained unmethylated regardless of treatment conditions [104]. Since the authors did not associate the effects of arsenic or methyl deficiency with gene expression, it is not possible to determine whether these changes had a measurable physiological effect on *Ha-ras* expression. Other studies have noted that there is not a clear-

cut correlation between *Ha-ras* promoter methylation and mRNA expression [105]. In parts of the world where environmental exposure to arsenic is endemic, poor folate nutritional status may promote arsenicotic skin lesions (melanosis, leukomelanosis and keratosis) and cancer through the disruption of normal DNA methylation. To test this hypothesis, Pilsner *et al.* recruited 294 Bangladeshi men and women and stratified them on the basis of dietary folate status and arsenic exposure. Surprisingly, the results demonstrated that arsenic had no effect on global DNA methylation in the low-folate group but it had a dose-dependent correlation with DNA hypermethylation in the high-folate group [106]. A follow-up study compared arsenic-exposed individuals with and without skin lesions, matched for arsenic exposure, sex and age. Consistent with the previous study, arsenic exposure was associated with a global increase in DNA methylation in peripheral blood samples. Stratification by nutritional folate status revealed that DNA hypermethylation correlated with arsenic exposure only in subjects with adequate folate status, but this association was only applicable to control cases, or rather, subjects without skin lesions. Hence, the authors concluded that arsenic exposure was positively associated with genomic methylation only if folate status is adequate [107]. To explain these counter intuitive findings, the authors proposed a rationale similar to that of Mass and Wang, discussed earlier, wherein adequate folate is permissive for an adaptive increase in genomic methylation, possibly through increased DNMT activity.

Conclusion

Despite an accumulating number of investigations indicating that arsenic affects the epigenetic status of cells and tissues, there is surprisingly little consensus regarding what exactly are the changes that occur. Both increased and decreased DNA methylation has repeatedly been reported at both the genome-wide and gene-specific levels (Table 1). The reasons for this diversity of findings may be attributable in some cases to technical experimental details since arsenic has different physiologic effects depending on dose and duration of exposure. However, it appears that assays of genome-wide methylation status generally demonstrate hypomethylation more often than not (with the exception of *in utero* exposures), while assays of individual genes can demonstrate either hypermethylation or hypomethylation, depending on the tissue, cell type and gene examined. Future investigations capitalizing on genomic technologies should provide a powerful tool for obtaining a more comprehensive picture of arsenic epigenetic effects. The use of high-throughput deep sequencing and microarray technologies should be useful for identifying signature genes that undergo hypermethylation and hypomethylation as a result of arsenic exposure.

In general, it is difficult to reconcile the incongruent findings reported by the diverse array of model systems discussed here. Even among models sharing similar treatments techniques, findings tend to be divergent and difficult to interpret. It is clear that a simple mechanistic model, such as repression of DNMT activity by SAM depletion, is overly simplistic [49,48, 71]. Rather, DNA methylation patterns suggest that arsenic may cause both hyper- and hypomethylation simultaneously, in all likelihood on a gene-by-gene basis with an overall trend towards global demethylation. Although it may seem counterintuitive that hypermethylation can occur at one gene while hypomethylation occurs at another, it is likely that this is in fact the case. The fact that investigators have chosen to study a wide array of different genes as an index of DNA methylation status makes it difficult to compare the effects of arsenic amongst the various model systems. In this regard, it is a rare occurrence when different groups used the same genes to index DNA methylation status. In addition, most authors typically use a single technique to evaluate methylation changes, which contributes to the confusing array of conclusions, since different methods of determining DNA methylation analysis provide different types of information with different biases and artifacts [108]. Awareness of these limitations and the application of appropriate methods for the desired research aim may help to resolve some of these issues. In addition, by capitalizing on current genomic technologies,

future investigations will be able to correlate DNA and transcriptional changes so that model systems can be compared globally with sequence-level resolution.

In this review we have focused on arsenic effects on DNA methyltransferases; however, chromatin structure also depends on a complex network of histone modifying enzymes, including histone methyltransferases. If cofactor restriction is indeed a primary mechanism mediating DNMT inhibition, then it stands to reason that a multitude of other methyltransferases may also be inhibited; presumably this would include histone methyltransferases as well. Inhibition of histone methyltransferase by SAM depletion could profoundly affect both gene activation and repression. For example, demethylation of trimethylated lysine-27 in histone H3 (H3K27me₃) coincides with a generalized increase in gene activation [109,110] and loss of DNA methylation [111]. Likewise, loss of H3K4me₃ is associated with gene repression and DNA imprinting. Thus, a more nuanced view of the role that SAM depletion may play may be necessary to explain the apparently incongruous findings reported to date. Since histones are proteins that constantly turn over, changes in histone methylation patterns will prove to be the next important target of arsenic intoxication [112].

Future perspective

Recent technological advancements including methylation-sensitive deep sequencing and microarrays now provide useful tools for characterizing epigenetic changes globally with sequence-level sensitivity. In parallel, application of similar techniques to the study of gene expression provides a comprehensive snapshot of gene-expression changes. Together, using a variety of bioinformatic approaches, chromatin methylation changes can be integrated with gene-expression changes to map the inter-relationships between chromatin methylation and gene-expression patterns in arsenic target tissues. Over the next few years next-generation sequencing techniques should help to address questions surrounding genome-wide versus regional changes in DNA methylation. Furthermore, other epigenetic mechanisms influencing gene expression, such as histone modification and microRNA, have scarcely been investigated.

More daunting are the questions surrounding the model system in which to investigate the epigenetic effects of arsenic. Investigators attempting to address these concerns are faced with dilemmas that accompany their choice of exposure assessment, sample procurement and end points to measure. From an environmental standpoint, a major question is how best to model relevant human exposures. In the laboratory, the applicability of animal model systems to human exposures remains uncertain. Although the next several years might bring some consensus to the field, it is currently difficult to perceive how any current model best reflects disease occurrence associated with human arsenic exposure. Certainly, however, if it is established that epigenetic changes consistently observed in an animal model also occurring in target tissues of humans, this would represent a major step forward.

Executive summary

Arsenic reportedly causes both DNA hypermethylation & hypomethylation

- A difficulty in analyzing the epigenetic outcomes of arsenic exposure is that they appear to be influenced by the model system and treatment conditions.

Proposed mechanisms underlying the effect of arsenic on DNA methylation

- Methyltransferase competition for *S*-adenosylmethionine (SAM). SAM depletion by arsenic metabolism may impose cofactor restriction on DNA methyltransferase (DNMT) enzymes.
- Shunting of homocysteine to the transsulfuration pathway during oxidative stress may contribute to SAM depletion.

- Inhibition of DNMT activity and expression promotes DNA hypomethylation.

Arsenic may affect global & regional DNA methylation differently

- Regional promoter hypermethylation occurs in the presence of global hypomethylation.

Gestational arsenic exposure appears to be associated with developmental & long-term disease consequences

- *In utero* arsenic exposure has provided inconsistent epigenetic results.
- Gestational exposure may preferentially cause targeted gene-specific changes rather than significant global changes.

Human studies highlight areas for further investigation

- Human studies are limited by issues including subject recruitment, exposure assessment, tissue procurement and confounding factors.
- Arsenic exposure is positively associated with global DNA hypermethylation only when nutritional folate status is adequate.
- Inconsistencies exist between the epigenetic effects of arsenic in humans and in animal models.

Conclusion

- The diversity of model systems, treatment protocols and end points used by different research groups to assay DNA methylation contributes to the uncertainty encountered when reconciling opposing results among various reports.
- It is not clear if methylation predisposes to subsequent transformation or if epigenetic changes are a phenotypic reflection of cell transformation.
- The localization of epigenetic changes to specific gene promoters implies that these changes are targeted in nature and not the result of nonspecific mechanisms.
- Identification of signature genes consistently undergoing congruent changes in methylation should be a major focus of future investigations.
- In future studies changes in gene methylation status need to be presented in the context of gene expression patterns.
- The extent to which changes in DNMT activity contribute to changes in DNA methylation remains to be thoroughly investigated.

Acknowledgments

Research in the authors' laboratory is supported by NIEHS grants R01 ES06273, R01 ES10807 and the NIEHS Center for Environmental Genetics grant P30 ES06096.

Bibliography

Papers of special note have been highlighted as:

- of interest
- ■ of considerable interest

1. Chen CJ, Kuo TL, Wu MM. Arsenic and cancers. *Lancet* 1988;1(8582):414–415. [PubMed: 2893213]

2. Smith AH, Hopenhayn-Rich C, Bates MN, et al. Cancer risks from arsenic in drinking water. *Environ. Health Perspect* 1992;97:259–267. [PubMed: 1396465]
3. Hopenhayn-Rich C, Biggs ML, Fuchs A, et al. Bladder cancer mortality associated with arsenic in drinking water in Argentina. *Epidemiology* 1996;7(2):117–124. [PubMed: 8834549]
4. Marshall G, Ferreccio C, Yuan Y, et al. Fifty-year study of lung and bladder cancer mortality in Chile related to arsenic in drinking water. *J. Natl Cancer Inst* 2007;99(12):920–928. [PubMed: 17565158]
5. Smith AH, Marshall G, Yuan Y, et al. Increased mortality from lung cancer and bronchiectasis in young adults after exposure to arsenic *in utero* and in early childhood. *Environ. Health Perspect* 2006;114(8):1293–1296. [PubMed: 16882542]
6. Yuan Y, Marshall G, Ferreccio C, et al. Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950 to 2000. *Am. J. Epidemiol* 2007;166(12):1381–1391. [PubMed: 17875584]
7. Kumagai Y, Sumi D. Arsenic: signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. *Annu. Rev. Pharmacol. Toxicol* 2007;47:243–262. [PubMed: 17002598]
8. Gebel TW. Genotoxicity of arsenical compounds. *Int. J. Hyg. Environ. Health* 2001;203(3):249–262. [PubMed: 11279822]
9. Hei TK, Filipic M. Role of oxidative damage in the genotoxicity of arsenic. *Free Radic. Biol. Med* 2004;37(5):574–581. [PubMed: 15288115]
10. Hughes MF. Arsenic toxicity and potential mechanisms of action. *Toxicol. Lett* 2002;133(1):1–16. [PubMed: 12076506]
11. Huang C, Ke Q, Costa M, Shi X. Molecular mechanisms of arsenic carcinogenesis. *Mol. Cell. Biochem* 2004;255(1–2):57–66. [PubMed: 14971646]
12. Rossman TG. Mechanism of arsenic carcinogenesis: an integrated approach. *Mutat. Res* 2003;533(1–2):37–65. [PubMed: 14643412]
13. Klein CB, Leszczynska J, Hickey C, Rossman TG. Further evidence against a direct genotoxic mode of action for arsenic-induced cancer. *Toxicol. Appl. Pharmacol* 2007;222(3):289–297. [PubMed: 17316729]
14. Cohen SM, Arnold LL, Eldan M, Lewis AS, Beck BD. Methylated arsenicals: the implications of metabolism and carcinogenicity studies in rodents to human risk assessment. *Crit. Rev. Toxicol* 2006;36(2):99–133. Excellent review of the relative metabolism, disposition and toxicity of inorganic and organic arsenic in various animal species. [PubMed: 16736939]
15. Kitchin KT. Recent advances in arsenic carcinogenesis: modes of action, animal model systems, and methylated arsenic metabolites. *Toxicol. Appl. Pharmacol* 2001;172(3):249–261. [PubMed: 11312654]
16. Waalkes MP, Liu J, Ward JM, Diwan BA. Animal models for arsenic carcinogenesis: inorganic arsenic is a transplacental carcinogen in mice. *Toxicol. Appl. Pharmacol* 2004;198(3):377–384. [PubMed: 15276417]
17. Rossman TG, Uddin AN, Burns FJ. Evidence that arsenite acts as a cocarcinogen in skin cancer. *Toxicol. Appl. Pharmacol* 2004;198(3):394–404. [PubMed: 15276419]
18. Nanney DL. Epigenetic control systems. *Proc. Natl Acad. Sci. USA* 1958;44(7):712–717. [PubMed: 16590265]
19. Tang WY, Ho SM. Epigenetic reprogramming and imprinting in origins of disease. *Rev. Endocr. Metab. Disord* 2007;8(2):173–182. [PubMed: 17638084]
20. Mass MJ, Wang L. Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene p53 in human lung cells: a model for a mechanism of carcinogenesis. *Mutat. Res* 1997;386(3):263–277. [PubMed: 9219564]
21. Fouse SD, Shen Y, Pellegrini M, et al. Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. *Cell Stem Cell* 2008;2(2):160–169. Comprehensively maps the genome-wide methylation patterns in embryonic stem cells and demonstrates that CpG methylation patterns other regulatory mechanisms. [PubMed: 18371437]
22. Meissner A, Mikkelsen TS, Gu H, et al. Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 2008;454(7205):766–770. Describes the generation of DNA

- methylation maps covering most CpG islands and compares methylation changes during cell differentiation and among high- and low-density CpG promoters. [PubMed: 18600261]
23. Herceg Z. Epigenetics and cancer: towards an evaluation of the impact of environmental and dietary factors. *Mutagenesis* 2007;22(2):91–103. [PubMed: 17284773]
 24. de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB. The short arm of chromosome 11 is a 'hot spot' for hypermethylation in human neoplasia. *Proc. Natl Acad. Sci. USA* 1988;85(15):5693–5697. [PubMed: 2840671]
 25. Laird PW, Jaenisch R. DNA methylation and cancer. *Hum. Mol. Genet* 1994;3(Spec. No.):1487–1495. [PubMed: 7849743]
 26. Palii SS, Robertson KD. Epigenetic control of tumor suppression. *Crit. Rev. Eukaryot. Gene Expr* 2007;17(4):295–316. [PubMed: 17725495]
 27. Gama-Sosa MA, Slagel VA, Trewyn RW, et al. The 5-methylcytosine content of DNA from human tumors. *Nucleic Acids Res* 1983;11(19):6883–6894. [PubMed: 6314264]
 28. Dials ES, Cheah MS, Rowitch D, Hoffman RM. Extent of DNA methylation in human tumor cells. *J. Natl Cancer Inst* 1983;71(4):755–764. [PubMed: 6578371]
 29. Almeida A, Kokalj-Vokac N, Lefrancois D, et al. Hypomethylation of classical satellite DNA and chromosome instability in lymphoblastoid cell lines. *Hum. Genet* 1993;91(6):538–546. [PubMed: 8340107]
 30. Trinh BN, Long TI, Nickel AE, Shibata D, Laird PW. DNA methyltransferase deficiency modifies cancer susceptibility in mice lacking DNA mismatch repair. *Mol. Cell. Biol* 2002;22(9):2906–2917. [PubMed: 11940649]
 31. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature* 1998;395(6697):89–93. [PubMed: 9738504]
 32. Maloisel L, Rossignol JL. Suppression of crossing-over by DNA methylation in *Ascomobolus*. *Genes Dev* 1998;12(9):1381–1389. [PubMed: 9573054]
 33. Bestor TH. The DNA methyltransferases of mammals. *Hum. Mol. Genet* 2000;9(16):2395–2402. [PubMed: 11005794]
 34. Vahter M, Envall J. *In vivo* reduction of arsenate in mice and rabbits. *Environ. Res* 1983;32(1):14–24. [PubMed: 6617606]
 35. Csanaky I, Gregus Z. Role of glutathione in reduction of arsenate and of γ -glutamyltranspeptidase in disposition of arsenite in rats. *Toxicology* 2005;207(1):91–104. [PubMed: 15590125]
 36. Vahter M. Mechanisms of arsenic biotransformation. *Toxicology* 2002;181–182:211–217.
 37. Hughes MF, Kenyon EM, Edwards BC, Mitchell CT, Razo LM, Thomas DJ. Accumulation and metabolism of arsenic in mice after repeated oral administration of arsenate. *Toxicol. Appl. Pharmacol* 2003;191(3):202–210. [PubMed: 13678653]
 38. Drobna Z, Waters SB, Devesa V, Harmon AW, Thomas DJ, Styblo M. Metabolism and toxicity of arsenic in human urothelial cells expressing rat arsenic (+3 oxidation state)-methyltransferase. *Toxicol. Appl. Pharmacol* 2005;207(2):147–159. [PubMed: 16102566]
 39. Gregus Z, Roos G, Geerlings P, Nemeti B. Mechanism of thiol-supported arsenate reduction mediated by phosphorolytic-arsenolytic enzymes: II. Enzymatic formation of arsenylated products susceptible for reduction to arsenite by thiols. *Toxicol. Sci* 2009;110(2):282–292. [PubMed: 19478237]
 40. Gregus Z, Nemeti B. Purine nucleoside phosphorylase as a cytosolic arsenate reductase. *Toxicol. Sci* 2002;70(1):13–19. [PubMed: 12388830]
 41. Nemeti B, Gregus Z. Glutathione-dependent reduction of arsenate by glycogen phosphorylase a reaction coupled to glycogenolysis. *Toxicol. Sci* 2007;100(1):36–43. [PubMed: 17693425]
 42. Nemeti B, Gregus Z. Glutathione-supported arsenate reduction coupled to arsenolysis catalyzed by ornithine carbamoyl transferase. *Toxicol. Appl. Pharmacol* 2009;239(2):154–161. [PubMed: 19248796]
 43. Thomas DJ, Li J, Waters SB, et al. Arsenic (+3 oxidation state) methyltransferase and the methylation of arsenicals. *Exp. Biol. Med* 2007;232(1):3–13. Discusses the various enzymatic roles of arsenite methyltransferase in the metabolism of arsenic.
 44. Thomas DJ, Waters SB, Styblo M. Elucidating the pathway for arsenic methylation. *Toxicol. Appl. Pharmacol* 2004;198(3):319–326. [PubMed: 15276411]

45. Zakharyan RA, Sampayo-Reyes A, Healy SM, et al. Human monomethylarsonic acid (MMA[V]) reductase is a member of the glutathione-S-transferase superfamily. *Chem. Res. Toxicol* 2001;14(8):1051–1057. [PubMed: 11511179]
46. Aposhian HV, Zakharyan RA, Avram MD, Sampayo-Reyes A, Wollenberg ML. A review of the enzymology of arsenic metabolism and a new potential role of hydrogen peroxide in the detoxication of the trivalent arsenic species. *Toxicol. Appl. Pharmacol* 2004;198(3):327–335. [PubMed: 15276412]
47. Waters SB, Devesa V, Fricke MW, Creed JT, Styblo M, Thomas DJ. Glutathione modulates recombinant rat arsenic (+3 oxidation state) methyltransferase-catalyzed formation of trimethylarsine oxide and trimethylarsine. *Chem. Res. Toxicol* 2004;17(12):1621–1629. [PubMed: 15606138]
- 48■■. Vahter M. Effects of arsenic on maternal and fetal health. *Annu. Rev. Nutr* 2009;29:381–399. Thorough review of the literature surrounding the gestational effects of arsenic exposure in humans. [PubMed: 19575603]
49. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP. Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. *Proc. Natl Acad. Sci. USA* 1997;94(20):10907–10912. [PubMed: 9380733]
50. Reichard JF, Schnekenburger M, Puga A. Long term low-dose arsenic exposure induces loss of DNA methylation. *Biochem. Biophys. Res. Commun* 2007;352(1):188–192. [PubMed: 17107663]
51. Coppin JF, Qu W, Waalkes MP. Interplay between cellular methyl metabolism and adaptive efflux during oncogenic transformation from chronic arsenic exposure in human cells. *J. Biol. Chem* 2008;283(28):19342–19350. [PubMed: 18487201]
52. Caudill MA, Wang JC, Melnyk S, et al. Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine β -synthase heterozygous mice. *J. Nutr* 2001;131(11):2811–2818. [PubMed: 11694601]
53. Hoffman DR, Marion DW, Cornatzer WE, Duerre JA. S-adenosylmethionine and S-adenosylhomocysteine metabolism in isolated rat liver. Effects of l-methionine, l-homocysteine, and adenosine. *J. Biol. Chem* 1980;255(22):10822–10827. [PubMed: 7430157]
54. Counts JL, Sarmiento JJ, Harbison ML, Downing JC, McClain RM, Goodman JL. Cell proliferation and global methylation status changes in mouse liver after phenobarbital and/or choline-devoid, methionine-deficient diet administration. *Carcinogenesis* 1996;17(6):1251–1257. [PubMed: 8681439]
55. Davis CD, Uthus EO. DNA methylation, cancer susceptibility, and nutrient interactions. *Exp. Biol. Med. (Maywood)* 2004;229(10):988–995. [PubMed: 15522834]
- 56■■. Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. *Nutrition* 2004;20(1):63–68. Focuses on the influence of early nutrition on DNA methylation at certain genomic regions. [PubMed: 14698016]
- 57■. Hitchler MJ, Domann FE. An epigenetic perspective on the free radical theory of development. *Free Radic. Biol. Med* 2007;43(7):1023–1036. Discusses the literature supporting the influence that glutathione and oxygen have over development and morphogenesis, and DNA methylation to S-adenosylmethionine availability. [PubMed: 17761298]
58. Allen RG, Balin AK. Oxidative influence on development and differentiation: an overview of a free radical theory of development. *Free Radic. Biol. Med* 1989;6(6):631–661. [PubMed: 2666278]
59. Kann S, Estes C, Reichard JF, et al. Butylhydroquinone protects cells genetically deficient in glutathione biosynthesis from arsenite-induced apoptosis without significantly changing their prooxidant status. *Toxicol. Sci* 2005;87(2):365–384. [PubMed: 16014739]
60. Zheng XH, Watts GS, Vaught S, Gandolfi AJ. Low-level arsenite induced gene expression in HEK293 cells. *Toxicology* 2003;187(1):39–48. [PubMed: 12679051]
61. Lertratanangkoon K, Orkiszewski RS, Scimeca JM. Methyl-donor deficiency due to chemically induced glutathione depletion. *Cancer Res* 1996;56(5):995–1005. [PubMed: 8640792]
62. Lertratanangkoon K, Wu CJ, Savaraj N, Thomas ML. Alterations of DNA methylation by glutathione depletion. *Cancer Lett* 1997;120(2):149–156. [PubMed: 9461031]
63. Spuches AM, Kruszyna HG, Rich AM, Wilcox DE. Thermodynamics of the As(III)-thiol interaction: arsenite and monomethylarsenite complexes with glutathione, dihydrolipoic acid, and other thiol ligands. *Inorg. Chem* 2005;44(8):2964–2972. [PubMed: 15819584]

64. Kann S, Huang MY, Estes C, et al. Arsenite-induced aryl hydrocarbon receptor nuclear translocation results in additive induction of phase I genes and synergistic induction of phase II genes. *Mol. Pharmacol* 2005;68(2):336–346. [PubMed: 15894712]
65. Hermann A, Gowher H, Jeltsch A. Biochemistry and biology of mammalian DNA methyltransferases. *Cell. Mol. Life Sci* 2004;61(19–20):2571–2587. [PubMed: 15526163]
66. Jeltsch A. On the enzymatic properties of Dnmt1: specificity, processivity, mechanism of linear diffusion and allosteric regulation of the enzyme. *Epigenetics* 2006;1(2):63–66. [PubMed: 17965604]
67. Grandjean V, Yaman R, Cuzin F, Rassoulzadegan M. Inheritance of an epigenetic mark: the CpG DNA methyltransferase 1 is required for *de novo* establishment of a complex pattern of non-CpG methylation. *PLoS ONE* 2007;2(11):E1136. [PubMed: 17989773]
68. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM. Predicting aberrant CpG island methylation. *Proc. Natl Acad. Sci. USA* 2003;100(21):12253–12258. [PubMed: 14519846]
69. Jair KW, Bachman KE, Suzuki H, et al. *De novo* CpG island methylation in human cancer cells. *Cancer Res* 2006;66(2):682–692. [PubMed: 16423997]
70. Benbrahim-Tallaa L, Waterland RA, Styblo M, Achanzar WE, Webber MM, Waalkes MP. Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. *Toxicol. Appl. Pharmacol* 2005;206(3):288–298. [PubMed: 16039940]
71. Cui X, Wakai T, Shirai Y, Yokoyama N, Hatakeyama K, Hirano S. Arsenic trioxide inhibits DNA methyltransferase and restores methylation-silenced genes in human liver cancer cells. *Hum. Pathol* 2006;37(3):298–311. [PubMed: 16613325]
72. Chanda S, Dasgupta UB, Guhamazumder D, et al. DNA hypermethylation of promoter of gene p53 and p16 in arsenic-exposed people with and without malignancy. *Toxicol. Sci* 2006;89(2):431–437. [PubMed: 16251483]
73. Marsit CJ, Karagas MR, Danaee H, et al. Carcinogen exposure and gene promoter hypermethylation in bladder cancer. *Carcinogenesis* 2006;27(1):112–116. [PubMed: 15987713]
74. Cui X, Wakai T, Shirai Y, Hatakeyama K, Hirano S. Chronic oral exposure to inorganic arsenate interferes with methylation status of p16^{INK4a} and RASSF1A and induces lung cancer in A/J mice. *Toxicol. Sci* 2006;91(2):372–381. [PubMed: 16543296]
75. Chen WT, Hung WC, Kang WY, Huang YC, Chai CY. Urothelial carcinomas arising in arsenic-contaminated areas are associated with hypermethylation of the gene promoter of the death-associated protein kinase. *Histopathology* 2007;51(6):785–792. [PubMed: 17953697]
76. Jensen TJ, Novak P, Eblin KE, Gandolfi AJ, Futscher BW. Epigenetic remodeling during arsenical-induced malignant transformation. *Carcinogenesis* 2008;29(8):1500–1508. [PubMed: 18448484]
77. Balaghi M, Wagner C. DNA methylation in folate deficiency: use of CpG methylase. *Biochem. Biophys. Res. Commun* 1993;193(3):1184–1190. [PubMed: 8323540]
78. Chen H, Liu J, Zhao CQ, Diwan BA, Merrick BA, Waalkes MP. Association of c-myc overexpression and hyperproliferation with arsenite-induced malignant transformation. *Toxicol. Appl. Pharmacol* 2001;175(3):260–268. [PubMed: 11559025]
79. Chen H, Liu J, Merrick BA, Waalkes MP. Genetic events associated with arsenic-induced malignant transformation: applications of cDNA microarray technology. *Mol. Carcinog* 2001;30(2):79–87. [PubMed: 11241755]
80. Liu J, Brahim-Tallaa L, Qian X, et al. Further studies on aberrant gene expression associated with arsenic-induced malignant transformation in rat liver TRL1215 cells. *Toxicol. Appl. Pharmacol* 2006;216(3):407–415. [PubMed: 16876216]
81. Chen H, Li S, Liu J, Diwan BA, Barrett JC, Waalkes MP. Chronic inorganic arsenic exposure induces hepatic global and individual gene hypomethylation: implications for arsenic hepatocarcinogenesis. *Carcinogenesis* 2004;25(9):1779–1786. [PubMed: 15073043]
82. Waalkes MP, Liu J, Chen H, et al. Estrogen signaling in livers of male mice with hepatocellular carcinoma induced by exposure to arsenic *in utero*. *J. Natl Cancer Inst* 2004;96(6):466–474. [PubMed: 15026472]
83. Bates MN, Smith AH, Hopenhayn-Rich C. Arsenic ingestion and internal cancers: a review. *Am. J. Epidemiol* 1992;135(5):462–476. [PubMed: 1570813]

84. Rossman TG, Uddin AN, Burns FJ, Bosland MC. Arsenite is a cocarcinogen with solar ultraviolet radiation for mouse skin: an animal model for arsenic carcinogenesis. *Toxicol. Appl. Pharmacol* 2001;176(1):64–71. [PubMed: 11578149]
85. Germolec DR, Spalding J, Yu HS, et al. Arsenic enhancement of skin neoplasia by chronic stimulation of growth factors. *Am. J. Pathol* 1998;153(6):1775–1785. [PubMed: 9846968]
86. Xie Y, Trouba KJ, Liu J, Waalkes MP, Germolec DR. Biokinetics and subchronic toxic effects of oral arsenite, arsenate, monomethylarsonic acid, and dimethylarsinic acid in v-Ha-ras transgenic (Tg.AC) mice. *Environ. Health Perspect* 2004;112(12):1255–1263. [PubMed: 15345372]
87. Tennant RW, Spalding J, French JE. Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. *Mutat. Res* 1996;365(1–3):119–127. [PubMed: 8898993]
88. Spalding JW, Momma J, Elwell MR, Tennant RW. Chemically induced skin carcinogenesis in a transgenic mouse line (TG.AC) carrying a v-Ha-ras gene. *Carcinogenesis* 1993;14(7):1335–1341. [PubMed: 8330346]
89. Thompson KL, Rosenzweig BA, Tsong Y, Sistare FD. Evaluation of *in vitro* reporter gene induction assays for use in a rapid prescreen for compound selection to test specificity in the Tg.AC mouse short-term carcinogenicity assay. *Toxicol. Sci* 2000;57(1):43–53. [PubMed: 10966510]
90. Aschengrau A, Zierler S, Cohen A. Quality of community drinking water and the occurrence of spontaneous abortion. *Arch. Environ. Health* 1989;44(5):283–290. [PubMed: 2554824]
91. Cherry N, Shaikh K, McDonald C, Chowdhury Z. Stillbirth in rural Bangladesh: arsenic exposure and other etiological factors: a report from Gonoshasthaya Kendra. *Bull. World Health Organ* 2008;86(3):172–177. [PubMed: 18368203]
92. Kwok RK, Kaufmann RB, Jakariya M. Arsenic in drinking-water and reproductive health outcomes: a study of participants in the Bangladesh Integrated Nutrition Programme. *J. Health Popul. Nutr* 2006;24(2):190–205. [PubMed: 17195560]
93. Lindgren A, Danielsson BR, Dencker L, Vahter M. Embryotoxicity of arsenite and arsenate: distribution in pregnant mice and monkeys and effects on embryonic cells *in vitro*. *Acta Pharmacol. Toxicol. (Copenh.)* 1984;54(4):311–320. [PubMed: 6730986]
94. Concha G, Vogler G, Lezcano D, Nermell B, Vahter M. Exposure to inorganic arsenic metabolites during early human development. *Toxicol. Sci* 1998;44(2):185–190. [PubMed: 9742656]
95. Devesa V, Adair BM, Liu J, et al. Arsenicals in maternal and fetal mouse tissues after gestational exposure to arsenite. *Toxicology* 2006;224(1–2):147–155. [PubMed: 16753250]
96. Liu L, Li Y, Tollefsbol TO. Gene–environment interactions and epigenetic basis of human diseases. *Curr. Issues Mol. Biol* 2008;10(1–2):25–36. [PubMed: 18525104]
97. Waalkes MP, Ward JM, Liu J, Diwan BA. Transplacental carcinogenicity of inorganic arsenic in the drinking water: induction of hepatic, ovarian, pulmonary, and adrenal tumors in mice. *Toxicol. Appl. Pharmacol* 2003;186(1):7–17. [PubMed: 12583988]
98. Waalkes MP, Liu J, Ward JM, Diwan BA. Mechanisms underlying arsenic carcinogenesis: hypersensitivity of mice exposed to inorganic arsenic during gestation. *Toxicology* 2004;198(1–3):31–38. [PubMed: 15138027]
99. Ahlborn GJ, Nelson GM, Grindstaff RD, et al. Impact of life stage and duration of exposure on arsenic-induced proliferative lesions and neoplasia in C3H mice. *Toxicology* 2009;262(2):106–113. [PubMed: 19450653]
100. Xie Y, Liu J, Brahim-Tallaa L, et al. Aberrant DNA methylation and gene expression in livers of newborn mice transplacentally exposed to a hepatocarcinogenic dose of inorganic arsenic. *Toxicology* 2007;236(1–2):7–15. [PubMed: 17451858]
101. Majumdar S, Chanda S, Ganguli B, Mazumder DN, Lahiri S, Dasgupta UB. Arsenic exposure induces genomic hypermethylation. *Environ. Toxicol.* 2009 Epub ahead of print.
102. Goodman JI, Watson RE. Altered DNA methylation: a secondary mechanism involved in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol* 2002;42:501–525. [PubMed: 11807181]
103. Kisseljova NP, Kisseljov FL. DNA demethylation and carcinogenesis. *Biochemistry (Mosc.)* 2005;70(7):743–752. [PubMed: 16097937]
104. Okoji RS, Yu RC, Maronpot RR, Froines JR. Sodium arsenite administration via drinking water increases genome-wide and Ha-ras DNA hypomethylation in methyl-deficient C57BL/6J mice. *Carcinogenesis* 2002;23(5):777–785. [PubMed: 12016150]

105. Borrello MG, Pierotti MA, Tamborini E, et al. DNA methylation of coding and non-coding regions of the human *H-RAS* gene in normal and tumor tissues. *Oncogene* 1992;7(2):269–275. [PubMed: 1549348]
106. Pilsner JR, Liu X, Ahsan H, et al. Genomic methylation of peripheral blood leukocyte DNA: influences of arsenic and folate in Bangladeshi adults. *Am. J. Clin. Nutr* 2007;86(4):1179–1186. [PubMed: 17921400]
107. Pilsner JR, Liu X, Ahsan H, et al. Folate deficiency, hyperhomocysteinemia, low urinary creatinine, and hypomethylation of leukocyte DNA are risk factors for arsenic-induced skin lesions. *Environ. Health Perspect* 2009;117(2):254–260. [PubMed: 19270796]
108. Shen L, Waterland RA. Methods of DNA methylation analysis. *Curr. Opin. Clin. Nutr. Metab. Care* 2007;10(5):576–581. [PubMed: 17693740]
109. Zhou X, Sun H, Ellen TP, Chen H, Costa M. Arsenite alters global histone H3 methylation. *Carcinogenesis* 2008;29(9):1831–1836. [PubMed: 18321869]
110. Jensen TJ, Wozniak RJ, Eblin KE, Wnek SM, Gandolfi AJ, Futscher BW. Epigenetic mediated transcriptional activation of WNT5A participates in arsenical-associated malignant transformation. *Toxicol. Appl. Pharmacol* 2009;235(1):39–46. [PubMed: 19061910]
111. Pan G, Tian S, Nie J, et al. Whole-genome analysis of histone H3 lysine 4 and lysine 27 methylation in human embryonic stem cells. *Cell Stem Cell* 2007;1(3):299–312. [PubMed: 18371364]
112. Uthus EO, Davis C. Dietary arsenic affects dimethylhydrazine-induced aberrant crypt formation and hepatic global DNA methylation and DNA methyltransferase activity in rats. *Biol. Trace Elem. Res* 2005;103(2):133–145. [PubMed: 15772437]

■ Websites

201. IARC. Some Drinking-Water Disinfectants and Contaminants, Including Arsenic. Vol. 84. Who Press; Geneva, Switzerland: 2004. IARC Monographs on the Evaluation of Carcinogenic Risks to Human IARC Monographs. ISBN-13 9789283212843, ISBN-10 9283212843 <http://monographs.iarc.fr/ENG/Monographs/vol84/index.php>
202. ATSDR. Toxicological Profile for Arsenic. Department of Health and Human Services, Public Health Service; GA, USA: 2007. Toxicological profile for Arsenic. www.atsdr.cdc.gov/toxprofiles/tp2.html

Figure 1. Oxidative methylation of arsenite

Arsenate (As^{+5}) reduction is catalyzed by several mammalian enzymes of various function. Methylation of trivalent arsenic is carried out by AS3MT. Methylation of arsenic by AS3MT also results in its oxidation from trivalent to pentavalent arsenic. Following oxidative methylation, pentavalent arsenic is reduced back to trivalent arsenic by GSTO. AS3MT: Arsenic (+3 oxidation state) methyltransferase; GSTO: Glutathione *S*-transferase Ω .

Figure 2. Biochemistry of one-carbon metabolism and interactions with arsenic metabolism

*Nutrients obtained through dietary sources.

†Enzymes with mRNA expression that is induced by arsenic.

§Enzymes with mRNA expression that is repressed by arsenic.

AHCY: Adenosylhomocysteinase; AS3MT: Arsenic (+3 oxidation state) methyltransferase; B6: Vitamin B6; B12: Vitamin B12; BHMT: Betaine-homocysteine methyltransferase; CBS: Cystathionine-b-synthase; CHDH: Choline dehydrogenase; CTH: Cystathionase; DNMT: DNA methyltransferase; gGCL: Glutamate-cysteine ligase; GSS: Glutathione synthetase; GSTO: Glutathione S-transferase W; HCY: Homocysteine; MAT: Methionine adenosyltransferase; MTHFR: 5,10-methylene-tetrahydrofolate reductase (NADPH); MTR: 5-methyltetrahydrofolatehomocysteine methyltransferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SHMT: Serine hydroxymethyltransferase; THF: Tetrahydrofolate.

Table 1

Summary of studies investigating the epigenetic effects of arsenite.

Report	Model	Arsenic exposure	Treatment protocol	Methylation end point	Effect of arsenic	Assay	Ref.
<i>Cell culture studies</i>							
Mass and Wang (1997)	A549 human lung adenocarcinoma cells	Na arsenite up to 2 μ M	2 weeks	Genomic 5-meC	Hypermethylation	SssI methyl acceptance HpaII resistance/PCR	[20]
		Na arsenate up to 300 μ M	2 weeks	<i>p53</i> promoter	Hypermethylation	Bisulfate sequencing	
		DMA up to 2000 μ M	2 weeks	<i>p53</i> promoter	Hypermethylation	HpaII resistance/PCR	
				<i>p53</i> promoter	No change	HpaII resistance/PCR	
Zhao <i>et al.</i> (1997)	TRL1215 rat liver epithelial cells	Na arsenite up to 0.5 μ M	18 weeks, passaged weekly	Genomic DNA digestion by HpaII	hypomethylation	Probed by Southern blot for satellite I DNA fragments	[49]
Chen <i>et al.</i> (2001)	TRL1215 rat liver epithelial cells	Na arsenite up to 0.5 μ M	18 weeks, passaged weekly (from Zhao <i>et al.</i> [49])	c-Myc expression correlates with arsenic exposure and genomic DNA hypomethylation and reported by Zhao <i>et al.</i>		Linear regression with a calculation of a parametric correlation coefficient	[75]
Chen <i>et al.</i> (2004)	129/SvJ mice; liver	Na arsenite in drinking water at 45 ppm	48 weeks	Genomic 5-meC	Hypomethylation	SssI methyl acceptance HPLC quantification of 5-dmeC	[81]
			48 weeks	<i>ER-α</i> promoter	Hypomethylation	Bisulfate sequencing	
			48 weeks	Cyclin D1 promoter	No change	Bisulfate sequencing	
Benbrahim-Tailla <i>et al.</i> (2005)	RWPE-1 human prostate epithelial cells	5 μ M Na arsenite	Up to 30 weeks of exposure	Genomic 5-meC	Hypomethylation	Methyl acceptance	[70]
				<i>K-ras</i> promoter	No change	Bisulfate sequencing	
Cui <i>et al.</i> (2006)	Human derived cell lines: HepG2 and Huh-7 cells*	2 μ M and 10 μ M arsenite	24, 48 and 72 h	<i>p16^{INK4a}</i> promoter <i>RASSF1A</i> promoter E-cadherin promoter <i>GSTP1</i> promoter	Hypomethylation by 48 h with 2 μ M arsenite; cytotoxicity at 10 μ M	Bisulfate methyl sensitive/PCR	[71]
Reichard <i>et al.</i> (2007)	Human keratinocyte HaCaT cells	0.2 μ M Na arsenite	10 passages (relative to passage-matched controls)	Genomic 5-meC	Hypomethylation	HPLC quantification of 5-dmeC	[50]
Coppin <i>et al.</i> (2008)	RWPE-1 human prostate epithelial cells	5 μ M Na arsenite	Up to 16 weeks	Arbitrary 5-meC	Hypomethylated	HpaII resistance/arbitrary primed PCR	[51]

Report	Model	Arsenic exposure	Treatment protocol	Methylation end point	Effect of arsenic	Assay	Ref.
Animal studies							
Okaji <i>et al.</i> (2002)	C57BL/6J mice; liver	Na arsenite in drinking water at 2.6, 4.3, 9.5 or 14.6 mg/kg/day	7 days/week for 130 days plus methyl deficient diet	Genomic 5-mC Ha-ras promoter	Hypomethylation Hypomethylation [‡]	Methyl acceptance methyl-sensitive restriction digest/PCR	[104]
Xie <i>et al.</i> (2004)	Tg.AC mice; liver	Na arsenite 150 ppm Na arsenate 200 ppm MMA(V) 1500 ppm DMA(V) 1000 ppm	17 weeks of As treatment with 2 weeks of topical TPA (1.25 µg/200 ml) from week 4	Genomic 5-mC	Hypomethylation: As (III) is the most potent; MMA (V) is the least	Methyl acceptance	[86]
Waalkes <i>et al.</i> (2004)	Male C3H mice; liver (control liver compared with normal-appearing liver tissue with grossly visible tumors)	Arsenite, 85 ppm	<i>In utero</i> exposure to maternal drinking water from gestation days 8 through 18; offspring were assayed after 32 weeks of age	Genomic 5-mC Cyclin D1 promoter ER-α promoter	No change No change hypomethylation	Sssl methyl acceptance Bisulfate sequencing Bisulfate sequencing	[82]
Uthus and Davis (2005)	Fisher 344 rats; liver	Na arsenite 0.5 µg/g diet Na arsenite 50 µg/g diet	3 weeks of As diet, two i.p. injections of DMH, 8 more weeks of diet: 12 weeks total	Genomic 5-mC	Hypermethylation [§] Hypomethylation [§]	Methyl acceptance	[112]
Cui <i>et al.</i> (2006)	A/J mice; lung and lung adenocarcinomas	Arsenite 0, 1, 10 or 100 ppm	Food and water containing arsenate <i>ad libitum</i> for 18 months	<i>p16^{INK4a}</i> promoter <i>RASSF1A</i> promoter	Hypermethylation [¶]	Bisulfate methyl sensitive/PCR	[74]
Xie <i>et al.</i> (2007)	Newborn C3H pups; liver	Arsenite, 85 ppm	<i>In utero</i> exposure same as Waalkes <i>et al.</i> (2004) [82]; Pups assayed shortly after birth	Genomic 5-mC Arbitrary 5-mC	No change Hypomethylation	Sssl methyl acceptance Arbitrary 5-mC	[100]
Human studies							
Marsit <i>et al.</i> (2006)	Human; DNA methylation in bladder tumors from arsenic exposed and unexposed subjects [#]	Dicotomously stratified into exposed and unexposed on the basis of toenail arsenic level (≥0.26 µg/g)		<i>p16^{INK4a}</i> promoter <i>RASSF1A</i> promoter <i>PRSS3</i>	No change Hypermethylation Hypermethylation	Bisulfate methyl sensitive/PCR	[73]
Chanda <i>et al.</i> (2006)	Human; DNA methylation in blood from arsenic-exposed and unexposed subjects ^{**}	Up to 1000 µg/l in drinking water	Stratified by drinking water concentration	<i>p53</i> promoter <i>p16</i> promoter	Hypermethylation with some individuals having hypomethylation	<i>Hpa</i> II methyl sensitive/PCR Bisulfate methyl sensitive/PCR	[72]

Report	Model	Arsenic exposure	Treatment protocol	Methylation end point	Effect of arsenic	Assay	Ref.
Pilsner <i>et al.</i> (2007)	Human; blood leukocytes	Tube well drinking water	Prospective case-control; subjects stratified based on plasma folate levels	Genomic 5-meC	Hypermethylation in high-folate subjects ^{§,¶}	Methyl acceptance	[106]
Majumdar <i>et al.</i> (2009)	Same as Chanda <i>et al.</i> [72]	Up to 1000 µg/l in drinking water	Stratified by drinking water concentration	<i>p53</i> promoter <i>p16</i> promoter	Hypermethylation with some individuals having hypomethylation	<i>HpaII</i> methyl sensitive/PCR Bisulfate methyl sensitive/PCR	[101]
Pilsner <i>et al.</i> (2009)	Human subjects with skin lesions; blood leukocytes	Tube well drinking water	Prospective case-control; subjects matched, in part, based on drinking water As levels	Genomic 5-meC	Hypomethylation with folate insufficiency/hypomethylation with adequate folate	Methyl acceptance	[107]

* This paper also demonstrated that promoter hypermethylation coincides with the occurrence of hepatocellular carcinoma in liver tissue from 25 patients. No association was made between the occurrence of these tumors and arsenic exposure.

[‡] Hypomethylation of non-CpG dinucleotides; GpG dinucleotides remained unchanged. Hypomethylation observed with arsenite applies to the proximal CpG island only (segment 474/976 of HaRas); the distal CpG island region (962/1487 segment) remained unmethylated.

[§] The authors reported that arsenic treatment was associated with DNA hypomethylation; however, hypermethylation was observed in the 0.5 µg/g of diet group and hypomethylation was observed in the 50 µg/g group

[¶] Hypermethylation coincides with the occurrence of adenocarcinoma in lung tissue and was greater in arsenic-treated samples relative to untreated controls. Arsenic increased the rate of poorly differentiated adenocarcinomas.

The authors also drew conclusions with regards to tobacco smoke exposure.

** *The authors also investigated p53 and p16 promoter methylation in subjects with skin tumors.*

^{‡‡} Folate is positively associated with DNA methylation and did not reach statistical significance after adjustment for covariant.

5-mdC: 5-methyl-2'-deoxycytidine 5'-monophosphate; 5-meC: 5-methylcytosine; As: Arsenic; DMA: Dimethylarsenic acid; DMH: Dimethylhydrazine; MMA: Monomethylarsonic acid.