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Arsenicals Produce Stable Progressive Changes in DNA Methylation Patterns that are Linked to Malignant Transformation of Immortalized Urothelial Cells

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Abstract

Aberrant DNA methylation participates in carcinogenesis and is a molecular hallmark of a tumor cell. Tumor cells generally exhibit a redistribution of DNA methylation resulting in global hypomethylation with regional hypermethylation; however, the speed in which these changes emerge has not been fully elucidated and may depend on the temporal location of the cell in the path from normal, finite lifespan to malignant transformation. We used a model of arsenical-induced malignant transformation of immortalized human urothelial cells and DNA methylation microarrays to examine the extent and temporal nature of changes in DNA methylation that occur during the transition from immortal to malignantly transformed. Our data presented herein suggest that during arsenical-induced malignant transformation, aberrant DNA methylation occurs non-randomly, progresses gradually at hundreds of gene promoters, alters expression of the associated gene, and these changes are coincident with the acquisition of malignant properties, such as anchorage independent growth and tumor formation in immunocompromised mice. The DNA methylation changes appear stable, since malignantly transformed cells removed from the transforming arsenical exhibited no reversion in DNA methylation levels, associated gene expression, or malignant phenotype. These data suggest that arsenicals act as epimutagens and directly link their ability to induce malignant transformation to their actions on the epigenome.

Keywords

DNA Methylation; Epigenetic; Arsenic; Histone acetylation; Bladder

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Introduction

Aberrant DNA methylation occurs in nearly all tumor types and likely plays a causal role in carcinogenesis (Jones and Baylin, 2007). Relative to their normal counterparts, tumor cells generally exhibit a redistribution of DNA methylation, resulting in a pattern of global hypomethylation with regional hypermethylation (Riggs and Jones, 1983; Jones and Baylin, 2007). The loss of DNA methylation during carcinogenesis occurs primarily in repetitive elements and intragenic regions, which can result in chromosome instability and an increased mutation rate (Lengauer *et al.*, 1997; Chen *et al.*, 1998; Shann *et al.*, 2008). DNA hypermethylation is typically associated with the promoter regions of genes or clusters of genes (Feinberg and Vogelstein, 1983; Goelz *et al.*, 1985; Costello *et al.*, 2000; Frigola *et al.*, 2006; Novak *et al.*, 2006; Novak *et al.*, 2008). The DNA hypermethylation of gene promoters has been mechanistically linked to the alteration of other epigenetic modifications, such as the reduced histone acetylation, and these alterations lead to transcriptional silencing (Jones *et al.*, 1998; Nan *et al.*, 1998). Additionally, the changes in DNA methylation that occur during tumorigenesis are stable and may be further propagated within an individual tumor *in vivo* as well as in cell lines derived from these tumors (Markl *et al.*, 2001). Recent studies have shown that the number of differentially methylated regions in an individual tumor is estimated to be in the hundreds or low thousands (Markl *et al.*, 2001; Jones and Baylin, 2007; Novak *et al.*, 2008; Irizarry *et al.*, 2009).

While disruption of normal DNA methylation patterns is a known contributor to cancer, the manner in which these lesions accumulate during carcinogenesis is not completely understood and may depend upon their location in the process of malignant transformation, for example such as after the acquisition of the immortal phenotype. Early in tumorigenesis, epithelial cells go through distinct proliferative barriers that must be overcome prior to reaching immortality (Brenner *et al.*, 1998; Romanov *et al.*, 2001; Stampfer and Yaswen, 2003; Garbe *et al.*, 2007). Multiple epigenetic aberrations are acquired in a precipitous fashion coincident with overcoming these barriers. Step-wise methylation changes are thus characteristic of pre-immortal stages of tumorigenesis (Novak *et al.*, 2009). On the other hand, during the malignant transformation of an immortalized, non-tumorigenic cell or the progression of an initiated tumor cell, DNA methylation may proceed in a more gradual, progressive manner (Salem *et al.*, 2000; Jiang *et al.*, 2008; Watts *et al.*, 2008).

As a model of post-immortalization epigenetic dysfunction, we used an immortalized, non-tumorigenic human urothelial cell line (UROtsa) and its malignantly transformed variants, created by independent exposures to either arsenite [As (III)] or monomethylarsonous acid [MMA (III)] (Petzoldt *et al.*, 1995; Rossi *et al.*, 2001; Sens *et al.*, 2004; Bredfeldt *et al.*, 2006). With increasing time of arsenical exposure, these cells exhibit a progressively increasing malignant phenotype. Exposure to MMA (III) for 12 weeks (URO-MS12) results in cells with an increased growth rate. Those exposed to arsenicals for 24 weeks (URO-MS24) grow in an anchorage independent fashion, and those exposed to either MMA (III) (URO-MS52) or As (III) (URO-ASSC) for 52 weeks form tumors when injected into immunocompromised mice (Sens *et al.*, 2004; Bredfeldt *et al.*, 2006). Earlier studies have demonstrated that exposure to arsenicals results in changes in DNA methylation and histone tail modifications globally as well as in select promoter regions; however, studies have yet to examine DNA methylation changes in a genome-wide and temporal manner (Zhao *et al.*, 1997; Jensen *et al.*, 2008; Zhou *et al.*, 2008; Jensen *et al.*, 2009).

In this study, we acquired temporal snapshots of DNA methylation patterns during the arsenical-induced malignant transformation of the immortalized UROtsa cells. These DNA methylation microarray experiments detected hypomethylation of repetitive elements such as satellite sequences, in support of earlier observations (Zhao *et al.*, 1997). In addition, these

experiments also revealed changes in DNA methylation at target gene promoters that progress gradually during the process of malignant transformation, and manifest as both hyper- and hypomethylated promoter regions. Aberrant DNA methylation of target promoters was confirmed using MALDI-TOF mass spectrometry (MassARRAY) and was linked to changes in the expression of the associated gene, as assessed by quantitative real time RT-PCR. Long-term removal of arsenicals did not reverse these DNA methylation changes, gene expression, or the malignant phenotype. These data indicate that epimutagens, such as arsenicals, lead to changes in DNA methylation that occur in a gradual, progressive, and stable fashion in immortalized cells, and that these epigenetic changes are coincident with the acquisition of an increasingly malignant phenotype.

Materials and Methods

Cell Culture

UROtsa, URO-ASSC, and URO-CDSC cells were a kind gift from Drs. Donald and Maryann Sens. The UROtsa cell line was created from the urothelial cells of a 12-year-old female donor and were immortalized using a SV40 large-T antigen construct (Petzoldt *et al.*, 1995; Rossi *et al.*, 2001). URO-ASSC cells were created through continuous exposure of UROtsa to 1 μ M As (I II) (Sens *et al.*, 2004). URO-MSD cell lines were created through continuous exposure of UROtsa cells to 50 nM MMA (III) as previously described (Bredfeldt *et al.*, 2006). Each of the UROtsa cell lines were cultured as previously described (Novak *et al.*, 2008). Similar to the arsenical selection, URO-CDSC cells were created by continuous exposure of UROtsa to 1 μ M cadmium for one year (Sens *et al.*, 2004).

Nucleic Acid Isolation

Total RNA was isolated using the RNeasy Mini Kit, and genomic DNA was isolated using the DNeasy Blood and Tissue Kit according to manufacturer's protocol (Qiagen, Valencia, CA) (Qiagen, Valencia, CA). The quantity and relative quality of each sample was assessed using absorbance at 260 nm using the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

MeDIP Coupled to Human Promoter Microarray Analysis

Primers for the 13,000 human promoter microarray probes were obtained from the Whitehead Institute (Odom *et al.*, 2004). Each primer pair was designed to span approximately 750 bp upstream and 250 bp downstream of the transcription start site of these genes based upon the April, 2001 build of the NCBI database (Odom *et al.*, 2004). Methylcytosine Immunoprecipitations (MeDIP) were performed as previously described (Novak *et al.*, 2008). Equal amounts of input and immunoprecipitated DNA were amplified using the BioPrime Array CGH Genomic Labeling Module (Invitrogen, Carlsbad, CA) according to modified manufacturer's protocol. Two rounds of amplification were performed, during the second of which input DNA was labeled with Cy-5 and immunoprecipitated DNA was labeled with Cy-3. Labeled DNA was quantified to ensure proper amplification and incorporation of cy-labeled dUTP using the microarray function of the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE). Resultant products were co-hybridized to the human promoter microarray as previously described (Novak *et al.*, 2008).

MassARRAY DNA methylation Analysis

Sodium bisulfite (NaBS) treated genomic DNA was prepared according to manufacturer's instructions (Zymo Research, Orange, CA). NaBS treated DNA (5 ng) was seeded into a region specific PCR reaction incorporating a T7 RNA polymerase sequence as described by the manufacturer (Sequenom, San Diego, CA). Resultant PCR product was then subjected to *in*

vitro transcription and RNase A cleavage using the MassCLEAVE T-only kit, spotted onto a Spectro CHIP array, and analyzed using the MassARRAY Compact System MALDITOF mass spectrometer (Sequenom, San Diego, CA). Each NaBS treated DNA sample was processed in two independent experiments. Data were analyzed using EpiTyper software (Sequenom, San Diego, CA) as described (Coolen *et al.*, 2007; Novak *et al.*, 2008). Primers were designed using Epidesigner software (<http://www.epidesigner.com>) to amplify bisulfite-modified DNA and analyze CpG sites contained within the probe sequence present on the human promoter microarray. Primer sequences are provided in Supplemental Table 1 and genomic locations of the regions analyzed are provided in Supplemental Figure 1.

CpG Island Microarray

Six thousand eight-hundred clones from a human CpG island library were arrayed, DNA sequence validated, and prepared for microarray as described (Cross *et al.*, 1994; Nouzova *et al.*, 2004). DNA methylation analysis using McrBC digestion coupled to CpG island microarray hybridization was performed as previously described (Cross *et al.*, 1994; Nouzova *et al.*, 2004). Briefly, genomic DNA was cut by MseI (New England Biolabs, Beverly, MA), and then a catch-linker was ligated to the MseI fragments. These fragments were then cut with a methylation-sensitive restriction enzyme, McrBC (New England Biolabs). “Mock-cut” samples were samples exposed to the same conditions and reagents of the digested samples, except no GTP was added to drive the restriction digest. Twenty nanograms of the mock-cut or uncut samples and 20 ng of McrBC-cut MseI-linked genomic DNA was amplified by PCR. The PCR products were purified with the QIAquick PCR purification kit (Qiagen). Fluorescent Cy3 or Cy5 dye was incorporated into the PCR product using the BioPrime DNA labeling system (Invitrogen, Carlsbad, CA). After the labeling, the cut and mock reactions were mixed together and again cleaned with the QIAquick PCR purification kit (Qiagen). After purification, the labeled target was lyophilized to dryness and resuspended in 10 μ l of hybridization buffer [2x SSC, 0.1% SDS, 100 ng/ μ l Cot1 DNA, 100 ng/ μ l oligo(dA)], denatured by boiling for 10 min, and added to a processed array slide. A coverslip (22 $\times$ 22 mm) was applied, and the array was placed in a hybridization chamber (GeneMachines) at 62°C for 4 to 8 h. Following hybridization, slides were washed by placing them into 50-ml conical tubes containing 2x SSC, 0.1% SDS for 5 min, 0.06x SSC, 0.1% SDS for 5 min, and 0.06x SSC for 2 min, all at room temperature. Slides were scanned for Cy3 and Cy5 fluorescence using an Axon GenePix 4000 microarray reader (Axon Instruments, Inc., Foster City, CA).

Real-Time RT-PCR

Gene expression was measured using quantitative real-time RTPCR as previously described (Jensen *et al.*, 2008). Roche Universal Probe Library information and primer sequences are provided in Supplemental Table 1.

Clonal Sodium Bisulfite Sequencing

Bisulfite sequencing was performed as previously described (Novak *et al.*, 2006) using the method described by Clark, et al (Clark *et al.*, 1994).

Data Analysis

All microarray data were processed in R programming environment (R, 2007). For normalization of all data, the Linear Models for Microarray Data (Limma) package was used (Smyth, 2005). Differentially methylated elements were identified using statistical approaches previously described (Smyth, 2004). To control for false discovery rate, a multiple testing correction was performed according to methods described by Benjamini and Hochberg (Benjamini and Hochberg, 1995). Regions were considered statistically significant if the adjusted p-value was $p < 0.05$.

Gene Ontology Testing

Data from the human promoter microarrays were analyzed based upon gene ontology (GO) terms. GO terms over-representation testing was performed using GStats package (Falcon and Gentleman, 2007). The overlapping probabilities of DMR sets were calculated using a hypergeometric test (Fury *et al.*, 2006).

Tumor Growth in SCID Mice

A severe combined immunodeficiency (SCID) mouse colony was developed at the University of Arizona using original SCID (C.B-17/IcrACCscid) obtained from Taconic (Germantown, New York). The mice were housed in microisolator cages (Allentown Caging Equipment Company, Allentown, NJ) and maintained under specific pathogen-free conditions. Sentinel mice were screened monthly for mycoplasma, mouse hepatitis virus, pinworms, and Sendai virus via ELISA. Cells (10×10^6) from each cell line (UROtsa, URO-MS52, and URO-MS52 + 6mo) were injected subcutaneously. Subcutaneous tumors were measured twice a week for tumor volume estimation (mm^3) in accordance with the formula ($a^2 \times b / 2$) where a is the smallest diameter and b is the largest diameter. All procedures were performed in accordance with approved protocols of the University of Arizona Institutional Animal Care and Use Committee.

Results

Aberrant DNA methylation of specific loci during Arsenical-Induced Malignant Transformation

Previous studies have reported the derivation of malignant cell line variants from immortalized urothelial cells (UROtsa) following exposure to different environmental toxicants. Table 1 describes characteristics of these variant cell lines, which provide unique models with which to examine the molecular mechanisms that underlie malignant transformation. To assess the DNA methylation patterns in our model of malignant transformation, we utilized two complimentary techniques coupled to two different microarray platforms. First, DNA from UROtsa, URO-ASSC, and the URO-MS cell lines was analyzed by 5-methylcytosine immunoprecipitation (MeDIP) coupled to a 13,000 element human promoter microarray (Odom *et al.*, 2004; Jensen *et al.*, 2008). These microarrays provided data about methylation changes localized within promoter regions and therefore candidate genes whose expression may be directly affected. To obtain information about methylation changes occurring in CpG rich regions apart from gene promoters as well as high copy repetitive elements, we also analyzed DNA methylation using an approach where DNA is digested with the methylation-sensitive restriction enzyme MspI, amplified, and hybridized to a 6000 element CpG island microarray (Cross *et al.*, 1994; Nouzova *et al.*, 2004).

The DNA methylation profile of gene promoters in each cell line was determined. MMA (III) and As (III) treated cell lines were compared to the parental UROtsa. After 52 weeks of exposure to MMA (III), the number of aberrantly methylated promoters increased by more than 400 (Figure 1A). We detected both hypermethylation and hypomethylation events with hypermethylation being the more prevalent event in gene promoters. The changes observed in promoter regions represented approximately 4% of all elements examined in URO-MS52, demonstrating that changes in DNA methylation are a common event during arsenical-induced

malignant transformation. For a list of the results for all promoter regions analyzed, refer to Supplemental Table 2.

We assessed how these individual promoter regions accumulated DNA methylation temporally during malignant transformation, by querying their DNA methylation state at earlier time points in the derivation of URO-MSC52. Figure 1B shows that the individual promoter regions which become hypermethylated in UROMSC52 cells began to exhibit hypermethylation early in the transformation process and these levels increased progressively through malignant transformation. A similar but opposite effect is seen in regions which become hypomethylated in URO-MSC52. Even though only five promoter (four hypermethylated, one hypomethylated) regions pass our statistical threshold in URO-MSC52, these data show changes in DNA methylation status can be detected in target promoters as early as 12 weeks after exposure to MMA (III). These data suggest that arsenic induces methylation of target promoter regions in a gradual time-dependent fashion and is linked to an increasingly malignant phenotype.

We next assessed whether the observed DNA methylation changes were targeted to specific promoter regions within the genome. URO-ASSC and URO-MSC52 cells were created independently using different arsenicals, thereby providing biological replicates of arsenical-induced malignant transformation. It was calculated that the expected number of shared aberrantly methylated promoters based on a random distribution between these two cell lines would be 6.89 - we observed 108 (Figure 1C). It is unlikely that the observed overlap between these two cell lines is due to random chance ($p < 2.2 \times 10^{-16}$), based on statistical analysis using a hypergeometric test. These data suggest that chronic exposure to arsenicals may lead to non-random alterations in the DNA methylation levels of target promoter regions within immortalized human cells.

To analyze the potential biological significance of the observed alterations in methylation in URO-MSC52, we performed a GO analysis of the genes whose promoters were differentially methylated. Promoter regions were categorized using GO terms and Supplemental Table 3 shows the groups of genes which are significantly ($p < 0.05$) enriched based upon GO classification. Hyper- and hypo-methylated elements were analyzed separately with both groups shown. Hypermethylated promoters exhibited more significantly enriched subcategories than did hypomethylated promoters. The most significant hypermethylated groups of genes when categorized based upon cellular component were those associated with the plasma membrane. Functionally, the most commonly enriched genes were those involved in receptor activity, in particular those categorized as contributing to G-protein coupled receptors. There were fewer categories which exhibited significantly enriched hypomethylated promoters. The most commonly altered were those involved in sensory perception, nucleosome assembly, and pigment metabolism. Results from this analysis provide possible cell functions and pathways that may be preferentially targeted by epigenetic control during malignant transformation.

To analyze differential DNA methylation in genomic regions located outside of gene promoter regions, UROtsa and URO-MSC52 cells were analyzed using McrBc restriction enzyme digestion coupled to CpG island microarray hybridization (Nouzova *et al.*, 2004; Watts *et al.*, 2008). Relative to the parental UROtsa cells, URO-MSC52 cells exhibited DNA hypermethylation more frequently in single copy regions close to transcription start sites or directly in genes while hypomethylation was more prevalent in repetitive elements such as satellite sequences (Figure 1D). Such a distribution of hyper- and hypo-methylated elements between gene-related single copy sequences and repetitive satellites is in agreement with the pattern commonly observed in many types of cancer, including bladder cancer (Salem *et al.*, 2000; Enokida and Nakagawa, 2008), and suggests that DNA methylation changes in our UROtsa cell model recapitulate the *in vivo* process.

MassARRAY and Sodium Bisulfite Sequencing Confirm Microarray Data

The DNA methylation status of eleven promoter regions identified in our DNA methylation microarray studies was confirmed and extended using MassARRAY or bisulfite sequencing. The DNA methylation of *KRT7*, *FAM83A*, *CYP24A1*, *CIQTNF6*, *THEM4*, *G0S2*, *NR0B1*, *GNA14*, and *EREG* was measured using MassARRAY in UROtsa, URO-MSC12, URO-MSC24, URO-MSC36, URO-MSC52, and URO-ASSC. The location of the promoter microarray probe and the region analyzed using MassARRAY is shown in Supplemental Figure 1. The DNA methylation levels of each amplicon analyzed confirmed that the exposure to arsenicals leads to gradual changes in DNA methylation and the magnitude of changes grows with prolonged exposure (Figure 2; Supplemental Figure 2A). Data obtained from the second microarray platform, the CpG island microarray, were validated using clonal sodium bisulfite sequencing. Analysis of two CpG island islands associated with the genes *THBD* and *ADAMTS5* also confirmed the methylation microarray data (Supplemental Figure 3).

To test the possibility that the changes in DNA methylation could have occurred as a result of long term *in vitro* culture, the DNA methylation status of the nine gene promoters shown in Figure 2 and Supplemental Figure 2 were analyzed in a UROtsa cell culture at time zero and then again after six months of continuous culture. Results from the MassARRAY analysis indicate DNA methylation state did not change in any of the promoter regions due solely to continuous culture (Supplemental Figure 4).

DNA Methylation is Linked to Changes in Gene Expression and Histone H3 Acetylation

We performed quantitative real time RT-PCR on parental UROtsa cells as well as URO-ASSC and each of the URO-MSC cell lines to assess the effect of DNA methylation on the transcription of the target genes. The transcript levels of *KRT7*, *FAM83A*, *CIQTNF6*, *THEM4*, *G0S2*, and *EREG*, each confirmed to be differentially methylated, were inversely correlated with DNA methylation. Expression levels of those genes that exhibited progressive hypermethylation exhibited a loss in gene expression that mirrored this pattern (Figure 3). Taken together, these data suggest that arsenical-induced aberrant DNA methylation is linked to changes in gene expression in this model of malignant transformation.

The hypermethylated promoters identified in this study were integrated with the promoter histone acetylation data obtained in our previous study (Jensen *et al.*, 2008). Normalized log2 ratios (ChIP/Input) from the human promoter microarray analysis presented herein and those published previously were plotted and correlation coefficients were calculated for each promoter region analyzed. There is a consistent inverse correlation between DNA methylation and histone H3 acetylation with negative coefficients ranging from 0.750 - 0.997 (Supplemental Figure 5). These data suggest that epigenetic remodeling of target loci in our model system involves multiple epigenetic components.

Aberrant DNA Methylation is Stable After Arsenical Removal

To assess the stability of aberrant DNA methylation in a set of target promoter regions, URO-MSC24 and URO-MSC52 cells were cultured in the absence of MMA (III) for three (URO-MSC+3 mo) and six months (URO-MSC+6 mo). DNA methylation was measured using MassARRAY technology with each sample analyzed in two independent experiments. In each of the nine promoter regions examined, DNA methylation remained elevated at levels similar to those measured in URO-MSC24 (Figure 4A, Supplemental Figure 2B) and URO-MSC52 (Figure 4B; Supplemental Figure 2C). These data indicate that the changes in DNA methylation are stable after arsenical-induced malignant transformation. Furthermore, for each of the genes analyzed, withdrawal of MMA(III) from URO-MSC24 (Figure 5A) and URO-MSC52 (Figure 5B) had no or little effect upon gene expression. These data suggest that the changes in DNA

methylation observed in the malignantly transformed cells are not dependent upon the presence of the transforming arsenical and are linked to stable changes in associated gene expression.

Epigenetic Stability is Linked to the Maintenance of a Tumorigenic Phenotype

As a marker of the malignant phenotype, we determined if cells removed from arsenical exposure retained the ability to form tumors as xenografts in immunocompromised mice. UROtsa, URO-MSC52, and URO-MSC52+6 mo cells were injected subcutaneously into SCID mice (n=4). Tumor growth was assessed by measuring the volume of the palpable tumor. URO-MSC52+6 mo cells formed tumors at a similar rate and size to its antecedent cell line URO-MSC52 (Figure 6). As previously demonstrated, passage matched UROtsa cells did not form tumors in mice while UROMSC52 cells did (Bredfeldt *et al.*, 2006). These data indicate that stable epigenetic remodeling is correlated with the maintenance of a tumorigenic phenotype.

Discussion

Epigenetic aberrations, including the redistribution of DNA methylation, play a participative role in carcinogenesis (Riggs and Jones, 1983; Jones and Baylin, 2007). The rate and pattern in which these epigenomic alterations emerge is still being elucidated and may vary depending on the location of a cell along its path from normality to malignancy. The link between arsenic exposure and cancer of the lung, skin, liver, and bladder has been well established; however, the mechanisms by which arsenicals participate in carcinogenesis are not completely understood (Chen *et al.*, 1985; Marshall *et al.*, 2007). One mechanism by which arsenicals appear to participate is through the altering the epigenetic landscape (Zhao *et al.*, 1997; Chen *et al.*, 2004; Marsit *et al.*, 2006; Xie *et al.*, 2007; Jensen *et al.*, 2008; Jensen *et al.*, 2009). Using an *in vitro* human urothelial cell line model of arsenical-induced malignant transformation, we set out to identify the gene targets of epigenetic change, the speed of the changes and their stability, and how these changes related to the emergence of malignant characteristics.

We performed DNA methylation microarrays and found that hundreds of alterations in DNA methylation occur during arsenical-induced malignant transformation. These data are consistent with previous studies demonstrating that exposure to arsenic results in the loss of global DNA methylation and the alteration of promoter region DNA methylation in a small number of regions examined (Zhao *et al.*, 1997; Chen *et al.*, 2004; Marsit *et al.*, 2006; Xie *et al.*, 2007; Jensen *et al.*, 2008). These data thereby reinforce that there is likely an epigenetic component of arsenic-associated malignancy. The data presented herein also show that arsenical-induced aberrant DNA methylation is acquired gradually during the malignant transformation of immortalized cells. The changes in DNA methylation occur non-randomly and are linked to altered transcriptional regulation of affected genes. These changes appear stable since long-term removal of the arsenical from the malignantly transformed cells did not result in reversion of promoter region DNA methylation, associated gene expression, and a malignant phenotype. These data suggest environmentally relevant species of arsenicals can act as epimutagens and may participate in human carcinogenesis by altering the epigenetic landscape of immortalized cells.

Furthermore, aberrant DNA methylation is known to act in concert with histone tail modifications, in particular histone acetylation, to regulate gene expression. For example, members of the methyl-binding domain (MBD) class of proteins bind to methylated cytosines and recruit histone deacetylase (HDAC) enzymes, resulting in the concurrence of DNA hypermethylation, histone hypoacetylation and transcriptional silencing (Jones *et al.*, 1998; Nan *et al.*, 1998). Overall, our data show gene targets of arsenical exposure show strong correlations between their DNA methylation, histone acetylation, and gene expression states, suggesting that this epigenetic control pathway may participate in arsenical-mediated malignant transformation (Supplemental Figure 5).

The DNA methylation microarray analyses presented herein uncovered hundreds of aberrantly methylated genes that may warrant future study, of which the gene *G0S2* (G0/G1 Switch Gene 2) may prove to be of particular interest. The CpG island promoter region associated with *G0S2* is an early target of epigenetic remodeling during arsenical-induced malignant transformation with significant changes detected after 12 weeks of exposure. *G0S2* exhibits promoter hypermethylation and transcriptional silencing in URO-MSC12 cells, coincident with the acquisition of hyperproliferation (Bredfeldt *et al.*, 2006). Recent studies suggest that *G0S2* likely plays a functional role in cell cycle regulation and differentiation (Zandbergen *et al.*, 2005; Kitareewan *et al.*, 2008). Consistent with these results, *G0S2* is expressed at low levels in rapidly proliferating hepatoma cell lines when compared to growth-arrested murine liver cells, indicating a potential role in carcinogenesis as well as cell cycle regulation (Zandbergen *et al.*, 2005). Taken together with its potential functional importance, hypermethylation of the *G0S2* promoter may provide an early biomarker for the detection of pre-malignant cellular changes associated with arsenic exposure.

Since epigenetic patterns can be heritably transmitted from parent to daughter cell providing a means of cellular memory (Bird, 2002), changes to the patterns and levels of these marks by an epimutagenic toxicant may compromise the epigenetic cellular memory of subsequent generations of cells. Indeed, the changes in DNA methylation after arsenic exposure appear to be stable; therefore, they may act as a long-term detectable mark of arsenic exposure. Interestingly, these aberrations are stable even in those cells that have not yet reached complete malignant transformation, such as in URO-MSC24 cells. As such, arsenical exposure may affect normal cellular physiology before the appearance of pathological lesions, and this exposure may result in epigenetic changes that exist and can be detected as earlier markers of risk. Consistent with these findings have been epidemiological studies performed in humans that note that the increase in the relative risk of developing cancer after arsenic exposure may not occur until 20-50 years after exposure (Morales *et al.*, 2000; Smith *et al.*, 2006; Marshall *et al.*, 2007; Ahlborn *et al.*, 2008). A similar theory has been proposed that suggests that the alteration of DNA methylation at specific loci within gastric mucosae is a stable lesion that results due to exposure to *heliobacter pylori* (Maekita *et al.*, 2006). Taken together, these data suggest that arsenic exposure may alter epigenetic memory prior to malignancy, and these epigenetic changes may ultimately prove useful as stable biomarkers of arsenical exposure.

These data lead us to question whether the aberrations detected in URO-ASSC and URO-MSC cells were arsenic specific or whether they would also be found in cells which were malignantly transformed using other metalloids or transition metals. Cadmium is a naturally occurring transition metal that is known to be associated with human cancer, potentially inducing carcinogenesis through epigenetic mechanisms (Takiguchi *et al.*, 2003; Waalkes, 2003). Additionally, cadmium has been used to malignantly transform UROtsa cells, producing the URO-CDSC cell line (Sens *et al.*, 2004). To address whether the changes found in our study were specific to arsenic or whether they may be common to multiple metals, the levels of the six transcripts described in Figure 3 were measured in URO-CDSC cells using real-time RT-PCR. Results indicate that four of the six (67%) genes examined showed transcriptional suppression in URO-CDSC similar to the levels seen in the arsenical associated cell lines (Supplemental Figure 6A). Each of these downregulated genes was associated with a hypermethylated gene promoter (Supplemental Figure 6B). These data suggest that the carcinogenicity of various metals may be linked by a common ability to remodel the epigenetic state of specific loci; future studies will explore this prediction in more detail.

Although changes in DNA methylation are evident during arsenical-induced malignant transformation, the mechanism by which these changes occur is not well understood. DNA methyltransferase (DNMT) enzymes, including the maintenance methyltransferase DNMT1, catalyze the methylation of cytosine residues within DNA. In order to determine whether the

changes that observed were the result of altered expression of DNMT1, we performed quantitative real-time RT-PCR to quantify its expression (Supplemental Figure 7). The results show that DNMT1 expression is not altered during arsenical-mediated malignant transformation suggesting that other mechanisms are likely responsible for the multifaceted epigenetic changes observed during arsenical exposure. Future studies will focus on delineating the mechanisms involved in the epigenetic remodeling associated with arsenical-induced malignant transformation.

In conclusion, this study links arsenical-induced malignant transformation to the changes that the arsenicals induce in DNA methylation state. The DNA methylation changes in gene promoters emerge and progress over time, are linked to altered expression of the associated gene, and appear concurrently with the acquisition of increasingly malignant properties. Importantly, target promoter DNA methylation, associated gene expression, and the malignant phenotype were stable in the absence of the arsenical. Taken together, these studies provide early insights into the dynamics of the DNA methylation changes at specific loci that occur during arsenic-driven cancer progression as well as to establish further arsenicals as epimutagens associated with arsenic-mediated carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

As (III), Arsenite; MeDIP, methylcytosine immunoprecipitation; MMA (III), monomethylarsonous acid; SCID, severe combined immunodeficiency.

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Figure 1.

DNA methylation changes during arsenical-induced malignant transformation as determined by DNA methylation microarrays. **(A)** The number of hyper- and hypomethylated elements in each of the MSC cell lines relative to parental UROtsa cells, as detected by MeDIP coupled to human gene promoter microarray analysis. **(B)** Each individual line shows the progression of DNA methylation state of individual gene promoters from parental UROtsa through MSC12, MSC24, and MSC36 to MSC52 cells. Gene promoters shown are those which exhibited a statistically significant (adjusted $p < 0.05$) change in methylation between URO-MSC52 cells and parental UROtsa. Genes that become hypermethylated (red) and hypomethylated (green) are shown. **(C)** As (III) and MMA (III) target common gene promoters during malignant transformation. A Venn diagram shows the number of gene promoters which were differentially methylated in a statistically significant fashion (adjusted $p < 0.05$) in URO-MSC52 (MSC52) and URO-ASSC cell lines relative to parental UROtsa. The total number of promoters analyzed was 10350. The p -value is the probability of the overlap being due to random chance and was calculated using a hypergeometric test. **(D)** Single copy elements show predominantly hypermethylation while repetitive elements show significant hypomethylation in URO-MSC52 cells, as determined by MspBc-coupled CpG island microarray analysis. The y -axis values show the fraction of differentially methylated elements (adjusted p -value $p < 0.05$) present in URO-MSC52 relative to UROtsa for each of five genomic categories. Single copy elements are designated TSS/gene (probe is located within 2000bp of transcription start site of associated gene) or no gene (probe is located greater than 2000bp from a transcription start site). Transcription start sites are designated as such according to the UCSC Genome Browser (<http://genome.ucsc.edu>). Ribosomal RNA elements are designated as rRNA. Repetitive elements are designated as either Alu or Satellite sequences.

Figure 2.

MassARRAY confirms the arsenical-mediated differential DNA methylation detected by gene promoter microarray analysis. DNA methylation levels in parental UROtsa cells and each of the malignantly transformed variants (MSC12, MSC24, and MSC36, MSC52, and ASSC cells) were assessed in six differentially methylated gene promoter regions using Sequenom MassARRAY. The values presented show the overall percent CpG methylation for each promoter region analyzed.

Figure 3.

Decreased gene expression is linked to arsenical-mediated aberrant DNA hypermethylation of the target gene promoter. Expression levels were determined for select genes that had verified differentially methylated promoters. Quantitative real-time RT-PCR was performed on three independent samples from parental UROtsa and each of the arsenical exposed variants. The average expression value shown on the y-axis is normalized to β -Actin expression and is relative to expression levels seen in parental UROtsa cells. Error bars show the standard deviation.

Figure 4.

Altered DNA methylation patterns are stable after long term removal of arsenicals. Sequenom MassARRAY was used to analyze the DNA methylation status of each sample. **(A)** DNA methylation levels were determined for six differentially methylated gene promoter regions in parental UROtsa, URO-MSC24 and URO-MSC24 cells grown in the absence of MMA (III) for 3 or 6 months. **(B)** DNA methylation levels were determined for six differentially methylated gene promoter regions in parental UROtsa, URO-MSC52, and URO-MSC52 cells grown in the absence of MMA (III) for 3 or 6 months. The values presented show the overall percent CpG methylation for each promoter region analyzed.

Figure 5.

Gene expression changes are stable after long-term removal of MMA (III). **(A)** Gene expression levels were determined in UROtsa, URO-MSC24, and URO-MSC24 cultured in the absence of MMA (III) for 3 or 6 months using quantitative real-time RT-PCR. **(B)** Gene expression levels were determined in UROtsa, URO-MSC52, and URO-MSC52 cultured in the absence of MMA (III) for 3 or 6 months using quantitative real-time RT-PCR. The average expression value shown on the y-axis is normalized to β -Actin expression and is relative to expression levels seen in parental UROtsa cells. Experiments were performed on three independent samples for each cell line with the average expression value shown. Error bars show the standard deviation.

Figure 6.

The tumorigenic phenotype of URO-MSC52 cells persists after long-term removal of MMA (III). UROtsa, URO-MSC52, and URO-MSC52 cells cultured in the absence of MMA (III) for 6 months (MSC52+6mo) were injected into SCID mice and allowed to grow for 76 days to assess tumorigenicity. Mean tumor burden is the average tumor volume of four independent (n=4) experiments. Error bars indicate the standard error of the mean at each time point for each cell line.

List of cell lines used in this study

Cell line name, the treatment metal, concentration (exposure), and duration of treatment for each cell line are shown. In addition, the phenotypic properties of each cell line including increased growth rate relative to UROtsa (Hyperproliferation), anchorage independent growth (AIG), and ability of each cell line to form tumors when injected subcutaneously in to immunocompromised mice is described. Reference (Ref) describes previous publications describing part or all of the information presented for a given cell line. NA=not applicable; ND=not determined.

Cell Line	Treatment	Exposure	Duration	Hyper-proliferation	AIG	Tumors in Mice	Reference
UROtsa	None	None	None	NA	NO	NO	Rossi <i>et al.</i> , 2001;
URO-MSC12	MMA (III)	50 nM	12 wk	YES	NO	NO	Bredfeldt <i>et al.</i> , 2006
URO-MSC24	MMA (III)	50 nM	24 wk	YES	YES	NO	Bredfeldt <i>et al.</i> , 2006
URO-MSC36	MMA (III)	50 nM	36 wk	YES	YES	NO	Bredfeldt <i>et al.</i> , 2006
URO-MSC52	MMA (III)	50 nM	52 wk	YES	YES	YES	Bredfeldt <i>et al.</i> , 2006
URO-MSC24 + 3mo	MMA (III)	50 nM	24 wk	ND	YES	ND	Bredfeldt <i>et al.</i> , 2006;
URO-MSC24 + 6mo	MMA (III)	50 nM	24 wk	ND	YES	ND	Jensen <i>et al.</i> , 2009
URO-MSC52 + 3mo	MMA (III)	50 nM	52 wk	ND	YES	YES	Bredfeldt <i>et al.</i> , 2006;
URO-MSC52 + 6mo	MMA (III)	50 nM	52 wk	ND	YES	YES	Jensen <i>et al.</i> , 2009
URO-ASSC	As (III)	1 μ M	52 wk	YES	YES	YES	Bredfeldt <i>et al.</i> , 2006;
URO-CDSC	Cd (II)	1 μ M	52 wk	YES	YES	YES	Jensen <i>et al.</i> , 2004
							Sens <i>et al.</i> , 2004