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Dysregulation of DNA Methylation Induced by Past Arsenic Treatment Causes Persistent Genomic Instability in Mammalian Cells

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Abstract

The mechanisms by which arsenic-induced genomic instability is initiated and maintained are poorly understood. To investigate potential epigenetic mechanisms, in this study we evaluated global DNA methylation levels in V79 cells and human HaCaT keratinocytes at several time points during expanded growth of cell cultures following removal of arsenite exposures. We have found altered genomic methylation patterns that persisted up to 40 cell generations in HaCaT cells after the treatments were withdrawn. Moreover, mRNA expression levels were evaluated by RT-PCR for *DNMT1*, *DNMT3A*, *DNMT3B*, *HMLH1*, and *HMSH2* genes, demonstrating that the down regulation of *DNMT3A* and *DNMT3B* genes, but not *DNMT1*, occurred in an arsenic dose-dependent manner, and persisted for many cell generations following removal of the arsenite, offering a plausible mechanism of persistently genotoxic arsenic action. Analyses of promoter methylation status of the DNA mismatch repair genes *HMLH1* and *HMSH2* show that *HMSH2*, but not *HMLH1*, was epigenetically regulated by promoter hypermethylation changes following arsenic treatment. The results reported here demonstrate that arsenic exposure promptly induces genome-wide global DNA hypomethylation, and some specific gene promoter methylation changes, that persist for many cell generations following withdrawal of arsenite, supporting the hypothesis that the cells undergo epigenetic reprogramming at both the gene and genome level that is durable over many cell generations in the absence of further arsenic treatment. These DNA methylation changes, in concert with other known epigenome alterations, are likely contributing to

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AUTHOR CONTRIBUTIONS

Maurizio Mauro: Planning and practical realization of 50% of the analyses described in this manuscript. Writing of 50% of the manuscript;

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long-lasting arsenic-induced genomic instability that manifests in several ways, including aberrant chromosomal effects.

Keywords

genomic instability; arsenic; DNA methylation

INTRODUCTION

Inorganic arsenic is considered a carcinogen for humans associated with cancers of the skin, bladder, lung, kidney and liver [Rossman and Klein 2011; Humans, 2012] and epidemiological studies have associated arsenic exposure with other adverse health effects including but not limited to skin lesions, cardiovascular disease, neuropathies, and immunotoxic effects [Reichard et al., 2007; Sidhu et al., 2015]. Human exposure to environmental arsenic occurs primarily via ingestion of arsenic-laden drinking water [Argos et al., 2014] or via food derived from arsenic-contaminated aquatic or agricultural food chain sources [World Health, 2011]. Important insights into the mechanisms that promote arsenic carcinogenesis have been provided by investigations on cultured cells submitted to acute or chronic exposures to arsenic compounds. The effects observed are generally consistent with a weakly mutagenic mode of action and are suggestive of indirect and interacting mechanisms including, among others, aneuploidy, cytogenetic aberrations and epigenetic alterations [Klein et al., 2007; Ren et al., 2011; Rossman and Klein, 2011; Roy et al., 2015]. Genome instability is characterized by aneuploidy, chromosomal rearrangements, gene mutations/mutator phenotypes, repeat sequence instability, DNA damage response and cell cycle checkpoint perturbations, which are all regarded among the many forces driving or contributing to carcinogenesis [Fabarius et al., 2008; Yao and Dai 2014]. Maintenance of genomic instability has also been associated with epigenetic changes of gene promoter methylation, histone modifications and dysregulation of transposable sequences such as LINE-1 repeats, analyses of the latter often considered as surrogate for estimating global DNA methylation status [Watanabe and Maekawa, 2010; Kitkumthorn and Mutirangura, 2011]. Genomic hypomethylation has been associated with genomic instability in cancer [Jones and Baylin, 2002]. Arsenic exposure can alter LINE-1 methylation, although there are reports of both hypomethylation and increased methylation, and the persistence of effects have been questioned [Intarasunanont et al., 2012; Lambrou et al., 2012; Pilsner et al., 2012]. Thus, although much is known, the mechanisms by which arsenic-induced genomic instability is initiated and maintained, even in the absence of continuing arsenic exposures, are not completely clear.

In mammals and other vertebrates, methylation at the C5 position of cytosine, primarily in CpG dinucleotides, is carried out by DNA methyltransferases (DNMTs), a family of enzymes that catalyzes the transfer of methyl groups using S-adenosylmethionine (SAM) as the methyl donor. It has been demonstrated that acute arsenite treatment (25 μ M for 24 h) of human HaCaT keratinocyte cells caused upwards of 30% depletion of SAM, and at a lower arsenite dose range (0.5–5.0 μ M for 72 h) in the same study expression of *DNMT1* and *DNMT3A* was decreased; both effects likely contributing to the genome-wide DNA

hypomethylation observed when the HaCaT cells were chronically exposed to even lower doses of arsenite [Reichard et al., 2007]. Patterns of DNA methylation in cancer cells are known to differ from normal cells, since cancer cells generally exhibit reduction of global DNA methylation, as monitored, for example, by hypomethylation of LINE-1 sequences [Nusgen et al., 2015] but become hypermethylated at numerous gene promoters [Ruike et al., 2010]. Hypomethylation of genomic DNA has been associated with decondensation of chromatin into recombination permissive conformations, which can lead to the activation of transposition of repetitive elements, such as LINE-1, thus facilitating genomic instability [Yegnasubramanian et al., 2008]. Hypermethylation in distinct, often tissue-specific, subsets of gene promoter-associated CpG islands and shores can silence tumor suppressor, DNA repair, cell cycle modulating and many other cancer- and disease-associated genes, under many different conditions of acute and/or sustained stress environment [Jones and Baylin, 2002; Karpinets and Foy, 2005; Kroeger et al., 2008] perhaps leading to the establishment of cells with a methylator phenotype [Feinberg and Tycko, 2004; Morgan and Sowa, 2005; Hughes et al., 2013].

Previously, we reported that V79 Chinese hamster cells underwent early genetic instability when exposed to 10 μ M arsenite for 24 hr, and we demonstrated that the descendants of the surviving cells continued to be genetically unstable, showing ongoing gross aneuploidy and structural chromosome changes linked to DNA hypomethylation that persisted for about two months (up to 120 cell generations) of sub-culturing in arsenite-free medium [Sciandrello et al., 2004; Sciandrello et al., 2011]. This prolonged duration of genomic instability observed in the absence of continuous arsenite exposure suggested underlying epigenetic perturbations, the temporality (persistence) of which required further investigation.

Here we report the findings of follow up studies on the depletion, persistence and recovery of global DNA methylation status in the arsenic-exposed V79 cells, and on global and gene specific DNA methylation in human HaCaT keratinocyte cells at much lower sub-micromolar doses of arsenite (0.1 and 0.5 μ M). Together, the results demonstrate that arsenic exposure promptly induces genome-wide DNA hypomethylation in both V79 and human cells, which recovers gradually to pre-exposure levels by 40 or more cell generations after the arsenite treatment was removed. Analyses of promoter methylation status for some DNA repair genes (*HMLH1* and *HMSH2*) show that the mismatch repair gene *HMSH2* gene, but not *HMLH1*, was epigenetically regulated by promoter hypermethylation changes following the brief arsenic treatment in HaCaT cells, however, this hyper-methylation occurred in a delayed manner that was not evident until after 10 cell generations post-exposure and persisted through at least 40 cell generations. These findings support the hypothesis that arsenite-exposed cells undergo epigenetic reprogramming both at the gene and genome level, some of which continues for a substantial period of time in the absence of further arsenite treatment.

MATERIALS AND METHODS

Cells and Culture Conditions

For these studies we used the V79 cell line, Chinese hamster embryonic lung fibroblasts [Sciandrello et al., 1996], and the HaCaT cell line, human keratinocytes. Both of these cell lines were routinely cultured as previously described [Mauro et al., 2013].

V79 cells, which have a doubling time of 12–14 hr, (~2 cell generations per day) were exposed to 10 μ M Sodium Meta-Arsenite (SMA) (Sigma-Aldrich, Milan, Italy) for 24 hr. At the end of the brief SMA treatment, shake off-recovered cells, were maintained in culture for about 2 months (up to 120 cell generations) without any further SMA treatment. Aliquots of this cell population, called ASO (Arsenic Shake-Off), were frozen at the 6th, 50th, and 90th cell generation. For convenience, these collected populations were named ASO-A, ASO-B, and ASO-C, respectively.

HaCaT (human keratinocyte) cells, which have a doubling time of 24–25 hr (1 cell generation per day), were treated with 0.1 and 0.5 μ M SMA for 48 hr. At the end of the treatments, cells were left to grow exponentially for up to 40 cell generations without any further SMA treatment; these cell populations were named H0.1 and H0.5, respectively. Aliquots of the H0.1 and H0.5 HaCaT cell cultures were culled at 10, 20, and 40 cell generation time points for aneuploidy studies. DNA and RNA were harvested at the same time points to study LINE-1 methylation, gene expression and comparative gene promoter methylation levels. Note that both V79 and HaCaT cells were only exposed to SMA for a brief duration equivalent to 2 cell generations (24 hr for V79 and 48 hr for HaCaT).

Cytogenetic Analysis in V79 And HaCaT Cells

Cytogenetic analysis was performed on untreated V79 cells, on ASO-A, -B, -C SMA treated V79 cell populations, and in HaCaT cells treated with SMA (H0.1 – H0.5 populations). For mitotic cell recovery, approximately 10^6 cells were seeded into 75 cm² flasks, and harvested following mitotic arrest (colcemid 0.1 μ g/mL; 2 hr), then processed for metaphase fixation and microscopic observation as previously described [Catanzaro et al., 2012]. The slides were air dried, stained with 2.5% Giemsa in distilled water, and, then, observed under a microscope to evaluate the number of chromosomes and any chromosomal perturbations in each metaphase. Metaphase chromosome slides from V79 and ASO cell populations were used to visualize 5MeC staining along the chromosomes (see methods below). Chromosome slides of HaCaT cells were used for analysis of aneuploidy over 40 cell generations after removal of SMA. Since HaCat cells are hypotetraploid keratinocytes with a range of 72–88 chromosomes [Boukamp et al., 1988], we defined aneuploidy as cells with chromosome numbers outside the indicated range. For the aneuploidy studies, 60–80 metaphases were counted at each dose and time point.

Immunolocalization of Chromosomal 5-Methylcytosine in V79 Cells

Immunolocalization of 5-Methylcytosine (5MeC), by specific antibody (Megabase Research Products, Nebraska, USA) binding along the length of metaphase chromosomes, was performed according to the protocol of Bensaada [Bensaada et al., 1998] as modified in our

lab [Sciandrello et al., 2004]. This analysis was carried out for V79 cells with and without SMA treatment, as well as for the ASO-A, -B, and -C cell populations collected at 6, 50, and 90 cell generations after the removal of SMA to study the persistence of effects. The slides were observed under a Nikon fluorescence photomicroscope equipped with a HBO 100 W mercury lamp and a suitable filter. Photomicrographs were obtained with Genikon (Nikon) software and processed using Adobe Photoshop 6.0 software. This analysis was performed twice.

Methylation-Sensitive Arbitrarily Primed Polymerase Chain Reaction (MeSAP-PCR) in V79 Cells—Genomic DNA was extracted from untreated V79 cells and ASO-A, -B and -C cell populations using “Fast DNA Releaser” kits (Celbio-Euroclone, Italy), quantified by Nanodrop 1000 (Thermo Scientific, Italy) and was used as template in Methylation Sensitive Arbitrarily Primed Polymerase Chain Reaction (MeSAP-PCR) assays, as previously described by us [Naselli et al., 2014]. The amplified DNA was separated by a polyacrylamide gel (6%) electrophoresis, under nondenaturing conditions, transferred by blotting and revealed by chemiluminescence. The obtained lumigraphs were analyzed by densitometric scanning (Sigmagel software, Jandel Scientific, USA) that allowed us to obtain a graph from each sample corresponding to the intensity and weight of the individual DNA bands. The “S” and “D” scans were then overlapped with the aim of comparing any differences between them. All profiles were calculated by reference to the scanning densitometry of the internal molecular weight markers used. This analysis was performed at least twice.

LINE-1 DNA Methylation Analyses in HaCaT Cells

For analysis of methylation at the global genomic level in human HaCAT cells at 10, 20 and 40 cell generations after SMA treatment, LINE-1 pyrosequencing [Yang et al., 2004] was carried out as an acceptable surrogate of HPLC measurements of global DNA methylation [Lisanti et al., 2013]. Simultaneous extraction of genomic DNA and RNA from control and SMA treated HaCaT cells was carried out using AllPrep DNA/RNA Mini Kits (Qiagen, USA) according to the manufacturer’s protocol. 2 µg of control or treated DNA per sample was sodium bisulfite modified using EpiTect Bisulfite Kits (Qiagen, USA) following the instructions included with the kit. After the treatment with bisulfite, the methylation level was calculated from the ratio of T (indicating presence of 5MeC) to C at identified CpG sites within the sequence interrogated. Specifically, 150 ng of bisulfite-treated DNA was first amplified for 45 cycles at 50°C annealing temperature using the following LINE-1 primers:

Forward: 5-TTTTGAGTTAGGTGTGGGATATA-3’

Reverse: Biotin-5’-AAAATCAAAAATTCCCTTTC-3’

A biotin-conjugated primer was then used for subsequent purification of the PCR products with sepharose beads (Streptavidin Sepharose High Performance-Amersham Biosciences, Uppsala, Sweden). The purified PCR products were then washed in sterile distilled water and denatured in 0.2 mol/L NaOH using the Pyrosequencing Vacuum Prep Tool (Biotage, Uppsala, Sweden). Subsequently, 0.3 µmol/L of the primer sequence 5’-AGTTAGGTGTGGGATATAGT-3’ was linked to the single-stranded DNA and pyrosequencing was performed using the PSQ-H96 Pyrosequencing System (Biotage,

Uppsala, Sweden). This analysis was performed in triplicate and repeated independently at least twice.

Gene Promoter Methylation in HaCaT Cells

For analysis of methylation at the gene promoter level, methylation specific-PCR (MSP) [Herman et al., 1996] was used to examine the methylation of the promoters of the mismatch DNA repair genes *HMLH1* and *HMSH2*. Bisulfite-treated DNA (100 ng) was separately amplified using two specific primers that selectively recognize methylated sequences (M) or unmethylated (U) regions of the gene promoters. The PCR reactions (30 cycles) were carried out in a mixture containing: PCR buffer [(16.6 mmol/L ammonium sulfate (pH 8.8), 67 mmol/L Tris (pH 8.8), 6.7 mmol/L MgCl₂, 10 mmol/L 2-mercaptoethanol), dNTP (1.25 mmol/L each), Taq polymerase (1.25 U HotStarTaq DNA Polymerase Qiagen)] and primers (30 pmol). The sequences of the primers used and the annealing temperatures are shown in Table I. The amplified products were loaded onto agarose gels (2%) and then photographed under UV. This analysis has been carried out at least twice per gene.

RT-PCR—The expression of *DNMT1*, *DNMT3A*, *DNMT3B*, *hMLH1*, and *hMSH2* mRNA levels were evaluated by Reverse Transcriptase-PCR (RT-PCR) using the OneStep RT-PCR kit (Qiagen-USA) following the instructions of the manufacturer. Amplification (35 cycles) was performed with 100 ng of total RNA and β -*ACTIN* expression was monitored for quantitative internal control. The sequences of the primers used and the annealing temperatures are shown in Table II.

The amplified products were loaded onto agarose gels (2%) and then photographed under UV. This analysis has been carried out at least twice.

RESULTS

Chromosomal Methylation Detected by 5-Methylcytosine Antibody

To evaluate the methylation dynamics that occur in V79 cells after SMA treatment and during the expanded growth in arsenic free-medium, we first performed immunofluorescence with anti-5-MeC antibody on V79 and ASO cell populations at 0, 6, 50, and 90 cell generations after As exposure was discontinued. As shown in Figure 1A, metaphases from untreated V79 cells were homogeneously stained by brilliant fluorescence along the lengths of all the chromosome arms. As expected, chromosomes from V79 cells were uniformly dull immediately after treatment with SMA (Fig. 1B) (generation 0), whereas just a few discrete fluorescent signals on the chromosome arms of ASO-A cells were observed (Fig. 1C) by 6 cell generations after the removal of SMA. Interestingly, chromosomes of the ASO-B population after 50 cell generations displayed a recovery of chromosomal methylation as evidenced by increased level of discrete fluorescent grains against a background of lighter fluorescence along the overall chromosome lengths (Fig. 1D). Finally, chromosomes from ASO-C (90 cell generations, about 45 days, after SMA removal) showed a rather homogeneous distribution of methylation signals (Fig. 1E), which appears to concur with the methylation levels of untreated cells (Fig. 1A). These results show that arsenite induced genome wide hypomethylation in V79 cells within a very short

time after SMA exposure, and provides visual evidence that the global genomic demethylation seen shortly after treatment is followed by an increasing renewal of DNA methylation over the next 90 cell generations (about 45 days).

Genomic DNA Methylation Detected by MeSAP-PCR

In order to gain further insight into the methylation dynamics, and to validate the data of 5-MeC immunolocalization studies, we estimated the global DNA methylation status using MeSAP-PCR on untreated V79 and ASO-A, -B, and -C cell populations selected at 6, 50, and 90 generations during the expanded growth in the absence of SMA. Prior to arbitrarily primed PCR amplification, the DNA was first digested with a frequently cutting restriction enzyme (RsaI), and then further digested using the methylation-sensitive restriction endonuclease (MSRE) HpaII, that cleaves the C—C bond site only when those cytosines in RsaI-generated fragments are not methylated. It is worthwhile to note that this semi-quantitative technique allows us to reveal dissimilarities of methylation levels between different genomes. This is evident either by differences in the observed band patterns, specifically, the appearance/disappearance of bands, or by increase/decrease of band intensity in the DNA fingerprinting of the PCR products obtained from the single-(S) versus double-digested (D) DNA. Thus, differences between the “S” and “D” scans corresponds to regions of hypomethylation of the genome, whereas similarity of the scan patterns indicates regions of methylated DNA, as we have previously reported [Naselli et al., 2014].

The DNA fingerprint scans of the amplification products of double-digested DNA (RsaI and HpaII; D; blue scans) were compared with those of single-digested DNA (RsaI alone; S; red scans) (Fig. 2) for untreated and SMA treated V79 cells. In untreated V79 cells the “S” and “D” scans showed three major variations for the presence/absence of a band (peak) or difference in peak height (Fig. 2A) in the PCR amplified DNA. In contrast, 11–13 major variations for presence/absence of bands, or higher/lower peaks are noted in ASO-A and ASO-B cells at 6 and 50 cell generations after removal of the SMA treatment (Figs. 2B and 2C). Simply stated, these findings demonstrated that a greater number of hypomethylated CpG sequences persisted in the DNA of the ASO-A, and -B cell populations up to 6 and 50 cell generations after the SMA exposure was withdrawn. In the ASO-C cells, by 90 cell generations (~45 days) following SMA removal, evidence of slightly reduced hypomethylation (regain of some methylation) can be seen by fewer ($n=9$) major band and peak differences (Fig. 2D) compared to the hypomethylated ASO-A and ASO-B cells (Figs. 2B and 2C). Overall, in V79 cells, the MeSAP data mirrors the trends of the 5MeC immunolocalization results, with decreased genome methylation observed at early times after SMA treatment, persistence of hypomethylation for a substantial duration, and eventual regaining of methylation with longer regrowth of the cells in the absence of continued SMA exposure.

Cytogenetic Findings in SMA-Treated HaCaT Cells

In order to verify if the progressive and the long-term aneuploidy that we previously observed in SMA treated V79 cells [Sciandrello et al., 2011] was also observed in human cells, cytogenetic analyses were performed on HaCaT human keratinocytes exposed to 0.1 and 0.5 μ M SMA for 48 h (two cell cycles), then allowed to grow in arsenic-free medium

and tested for up to 40 cell generations (42 days). As shown in Figure 3, the SMA treatments induced immediate increases of aneuploid HaCaT cells to 8.6 and 10.4% aneuploidy, respectively, vs an average of 2.8% aneuploidy among untreated HaCaT cells that remained constantly low over 40 cell generations. Thereafter, the frequency of aneuploidy among SMA treated cells sharply decreased to the value of the untreated cells after 2 cell generations of subculturing in SMA-free medium; however, we subsequently observed an increasing frequency of aneuploid cells from 10 to 40 cell generations after the removal of SMA. Thus, the aneuploidy trend seen for the H0.1 and H0.5 HaCaT cells was comparable to that which was previously reported in the ASO V79 cells [Sciandrello et al., 2011], where the percentage of aneuploid ASO cells first declined to the same level as in untreated cells but gradually increased over the time even in the absence of continued SMA treatment. In contrast, in the HaCaT cells we did not find the persistent gross chromosome aberrations (data not shown) that we previously observed in the ASO V79 cells [Sciandrello et al., 2011], although we have previously reported preliminary findings on persistent changes in mitotic-regulatory BubR1 gene expression that could contribute to aneuploidy in these exposed HaCaT cells [Mauro et al., 2008], and future studies on centromeric repeats, or other mitotic control or centrosomal genes may be interesting to investigate.

HaCaT Genomic DNA Methylation Detected by LINE-1 Pyrosequencing

We used a bisulfite/pyrosequencing assay to measure DNA methylation in LINE-1 repetitive elements as a surrogate marker for global DNA methylation in untreated and SMA treated HaCaT cells collected at several time points during expanded growth following SMA withdrawal. As shown in Figure 4, we found a measureable decrease in the methylation of LINE-1 in HaCaT cells immediately after treatment with both 0.1 and 0.5 μ M SMA vs. an average of 50.2% methylation in untreated control cells. There was no dose dependent difference in the methylation levels of cells exposed to 0.1 μ M or 0.5 μ M SMA (38.4% and 40.1%). Thereafter, LINE-1 methylation levels of the SMA-treated cells increased back to the level of LINE-1 methylation of untreated HaCaT cell by ~13 cell generations of subculturing in SMA-free medium. Surprisingly, the LINE-1 methylation levels of the H0.1 and H0.5 cells at 20 cell generations after SMA withdrawal rebounded to higher than the original level in untreated HaCaT cells (68.4% and 70.4%, respectively vs. 50.6% for control cells) and remained consistently higher for up to 40 cell generations compared to untreated control cells (68.7% and 67.3%, respectively vs 49.2% of control cells). These findings suggest that the H0.1 and H0.5 SMA-treated cells may continue to undergo epigenetic reprogramming of the genome even after 20 and 40 cell generations of subculturing in SMA-free medium.

DNMT1, DNMT3A, DNMT3B Gene Expression Evaluated by RT-PCR

Since the methylation of cytosine residues in CpG dinucleotides is performed by DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, we evaluated the mRNA levels of the genes of these enzymes in untreated HaCaT cells and in H0.1 and H0.5 HaCaT cell populations at same generational time points observed in the LINE-1 experiments during expanded cell growth in SMA-free medium. Interestingly, arsenic induced a strong dose-dependent decrease of both *DNMT3A* and *DNMT3B* mRNA levels at the end of the SMA treatments that persisted for up to 10 cell generations (~ 10 days) post-withdrawal in both

H0.1 and H0.5 cell populations as seen in representative gels (Figs. 5A and 5D) and in duplicate analyses (Figs. 5B and 5E). Thereafter, recovery approaching the expression levels of these genes in untreated HaCaT cells was observed by 20 cell generations (~20 days, 3 weeks) after the end of SMA exposure, and the levels continued to increase slowly thereafter up to 40 cell generations (~40 days, 6 weeks). During this same 40 generation time period, expression of *DNMT3A* and *DNMT3B* remained stable in untreated HaCaT cells (See Figs. 5C and 5F). Thus, arsenic seems to be able to induce global genome hypomethylation via inhibition of the de novo *DNMT3A/3B* expression that persists for up to 10 cell generations after removal of SMA but returned to normal levels by 20–40 generations post exposure. In contrast, the very low 48 hr doses of SMA used here did not affect *DNMT1* mRNA production, neither immediately following treatment, nor up to 40 cell generations after SMA removal (Fig. 6A).

hMLH1 and hMSH2 Gene Methylation and Expression

Since gene-specific DNA methylation alterations are among many potential mechanisms by which arsenic can lead to carcinogenesis, we evaluated gene promoter methylation of the *hMLH1* and *hMSH2* genes involved in DNA mismatch repair (MMR) that plays a key role in maintaining genomic stability and has been associated with a mutator phenotype in some cell types. MS-PCR methylation analyses of these gene promoters at 0, 10, 20, and 40 cell generations following SMA treatment of HaCAT cells are shown in Figures 6 and 7. As seen, untreated HaCaT cells showed strongly unmethylated (U) promoters for both the *hMLH1* and *hMSH2* genes. We found virtually no methylation changes in the *hMLH1* promoter over 40 cell generations after removal of SMA in both H0.1 and H0.5 cells (Fig. 6B). Likewise, no change in *hMLH1* mRNA expression in H0.1 and H0.5 cells was found over the same time course (Fig. 6C). Interestingly, a different scenario took place in the *hMSH2* promoter; in fact, both H0.1 and H0.5 treated cells showed unmethylated (U) bands at 0 and 10 cell generations after treatment, but by 20 cell generations there was a notable shift to primarily methylated (M) bands. By 40 cell generations both unmethylated (U) and methylated (M) signals are clearly visible indicating either the presence of methylation in one of two alleles of the *HMSH2* gene, or more likely suggesting mixed subpopulations of cells some with methylated or unmethylated *hMSH2* promoters (Figs. 7A and 7B), which would be interesting to distinguish in future studies. Importantly, there was no evidence of *hMSH2* promoter methylation changes during 40 generations of growth of untreated HaCAT cells (Fig. 7C). Reduction in *hMSH2* mRNA expression concurrent with increasing promoter methylation was found both in H0.1 and H0.5 cells at 20 cell generations after SMA exposure (Figs. 7D and 7E), whereas generally stable gene expression was observed in untreated cells over the same 40 cell generation growth period (Fig. 7F). The continued reduction of *hMSH2* mRNA expression was markedly evident even at 40 cell generations in H0.1 and H0.5 cells, even though the loss of promoter methylation (recovery of the unmethylated [U] band) is already well underway by that time. This may indicate that the temporality of promoter methylation and gene expression may not track in the same time frame, since the gene expression still seems to be declining at 40 cell generations whereas promoter methylation status is already recovering.

DISCUSSION

Over many years we have extensively studied the effects of arsenic as an inducer of cytogenetic and genomic instability and associated epigenetic reprogramming [Sciandrello et al., 2002; Sciandrello et al., 2004; Catanzaro et al., 2010; Sciandrello et al., 2011]. Whereas a conventional hypothesis of carcinogenesis postulated that the accumulation of numerous somatic mutations, or a hypermutable phenotype, were drivers of cancer development, they did not offer full explanation for the observations that weakly or non-mutagenic agents, arsenite for example, also induced cancers. Studies attempting to correlate certain mutations with specific cancers have shown that cells within a tumor are usually not genetically and phenotypically homogeneous, but to the contrary they frequently show cell to cell variations in chromosomal abnormalities and aneuploidy [Sciandrello et al., 2004]. Therefore, genomic instability is a plausible driving force for the onset and development of a cancer, and genomic instability can be characterized in addition to aneuploidy/chromosomal rearrangements by gene mutations/mutator phenotypes, repeat sequence instability, DNA damage response and cell cycle checkpoint perturbations, and more recently demonstrated epigenetic perturbations of genome organization and changes in regulation of gene expression by DNA methylation and histone modifications. In fact, pre-neoplastic cells are likely to be characterized by genomic instability that allows temporal accumulation of gene mutations and epigenetic alterations which may not become profound until cumulative and persistent changes in cell phenotypes give rise to selective advantages that favor the cancer potentiating or perpetuating microenvironment [Schneider and Kulesz-Martin 2004; Karpinets and Foy, 2005].

To study the effects of a brief exposure to arsenic (SMA) on genomic instability and duration of effects versus recovery after treatment, here we have examined cytogenetic instability (aneuploidy) in HaCaT cells compared to our previously published work in V79 cells [Sciandrello et al., 2011]; we examined global genome methylation status in both V79 cells (by MeSAP) and in HaCaT cells (by LINE-1 methylation), and we delved into gene expression and gene promoter methylation studies of the DNA methylation machinery and mismatch repair genes in HaCaT cells. By all indicators, the predominant unifying finding was persistent genome instability that tracked temporally with reduced chromosomal methylation which recovered over time, reduced genome methylation (MeSAP and LINE-1) which recovered over time, reduced DNMT3A and 3B expression which recovered over time, and increased gene specific promoter methylation (*hMSH2*) with coordinated gene expression reduction that tracked with the exact same temporal timing as the remethylation of LINE-1 and the *DNMT3A/3B* expression recovery.

The results presented here confirm epigenetic outcomes contributing to the onset of genomic instability following SMA exposures, and show that even brief exposure of cells to a moderate environmental stress induces the advent of a cytogenetic mutator phenotype with concurrent epigenetic changes, some of which persisted well after the environmental exposure was withdrawn. Visualization of chromosomal DNA methylation of V79 and ASO-A, -B, -C cells using 5meC antibody staining revealed that arsenite induced initially rapid hypomethylation along the chromosomes, followed by a gradual recovery of what appears to be “full” methylation by 90 cell generations after the exposure ended (Fig. 1). These

findings of demethylation and remethylation over time were confirmed in these same exposed populations of V79 cells using MeSAP-PCR (Fig. 2), in which densitometric analysis of single- versus double- digested DNA produced fingerprinting scans that were consistent with genomic hypomethylation immediately after SMA exposure followed by reduced genomic hypomethylation by 90 cells generations (~45 days) post SMA removal. In subsequent studies with human HaCaT skin keratinocyte cells treated for two cell cycles (48 hr) with submicromolar SMA doses then followed for up to 40 cell generations (40 days) without SMA exposure, we observed quantifiable changes in aneuploidy (Fig. 3) and genomic methylation (e.g. LINE-1) (Fig. 4), both indicators of persistent genomic instability that mimicked the V79 trends. Although MeSAP and LINE-1 methylation assessments are both reported as surrogates for “measuring” global levels of genomic methylation, and the same trends were observed for remethylation in both assays here, the LINE-1 studies here more fully demonstrated the genomic methylation recovery compared to the MeSAP results. This may be due in part to the knowledge that these assays likely measure different subsets of repetitive genomic sequences, MeSAP may only target about 8% of genomic CpGs whereas LINE-1 regions contains about 17% of genomic CpGs [Yang et al., 2004; Naselli et al., 2014; Yaish et al., 2014; Agarwal et al., 2015] and further, it is also possible that there are global differences in the genome organization of repetitive sequences in Chinese hamster V79 cells compared to those in the human derived HaCaT cells.

There is accumulating literature on arsenic-induced LINE-1 methylation changes as indicators of global genomic methylation status, many of them from human epidemiological studies on arsenic-exposed populations around the world; however, there are reports of both hypomethylation and increased LINE-1 methylation, and persistence of effects have been questioned [Intarasunanont et al., 2012; Lambrou et al., 2012; Pilsner et al., 2012]. An important consideration in many of these studies is whether such effects can persist from prenatal exposure into adult life, and if so, whether their persistence can have any impacts on subsequent health outcomes in adolescents or adults [Farzan et al., 2013; Nye et al., 2014] or even on subsequent generations [Whitelaw and Whitelaw, 2008]. In our study here, LINE-1 pyrosequencing analysis showed that the percentage of methylated cytosines immediately after the arsenic treatment was lower than in untreated HaCaT cells, and although slightly increasing by the 10th cell generation after removal of SMA still remained lower than the control cells (Fig. 4). Surprisingly, however, by the 20th cell generation post-exposure, the methylation levels of LINE-1 in the SMA-treated HaCaT cells increased, surpassing the methylation level of the parental HaCaT cells, and remained consistently high up to 40 cell generations. This long duration of temporal tracking provides evidence that rapid arsenic-induced LINE-1 hypomethylation, is followed by subsequent “re-methylation” by ~13 cell generations (~2 weeks) after removal of the metalloid. Finally, it is interesting to underline that an analogous result, obtained via immunolocalization of chromosomal 5-methylcytosine, was found also in V79 cells, where the initial hypomethylation seen immediately after the arsenic treatment was followed by a recovery of methylation up to the levels of V79 untreated cells. The mechanisms of this recovery of DNA methylation are not well known, although in one scenario it was proposed that DNA methylation inhibitors induce partial DNA hypomethylation such that residual localized methylation sites could function to reseed the regional remethylation [Issa, 2005]. Indeed, similar to our

demonstration here of LINE-1 remethylation levels exceeding the original levels, some of the early work on 5-azacytidine (5-azaC) induced DNA demethylation also demonstrated a reacquisition of methylation levels that surpassed the original levels upon withdrawal of 5-azaC [Bender et al., 1999]. Although arsenic was the focus of the work reported here, there is also evidence that other environmental exposures, for example ionizing radiation, can similarly perturb genome stability, imposing so-called bystander effects characterized by a growing awareness of altered global genome methylation and LINE-1 sequence methylation coordinated with evidence of delayed effects and persistence of epigenome modulation [Goetz et al., 2011; Baulch et al., 2014; Luzhna et al., 2015].

Clearly, it is not new information that changes in gene expression attributed to epigenetic processes can occur even as the result of brief environmental exposures to arsenic compounds. Indeed, many studies, of candidate genes or gene arrays have demonstrated in vivo and in vitro that arsenite exposures can modulate gene expression of cancer and other disease-relevant genes [Reichard et al., 2007; Reichard and Puga, 2010; Clancy et al., 2012; Paul and Giri, 2015; Pelch et al., 2015]. What is important here is new knowledge of the duration of persistence (durability), and/or reversal, of a changing epigenetic landscape such that some epigenetic manifestations continue or even arise well after the arsenite exposure is withdrawn, and in some cases resolve to the original epigenetic state, although sometimes not until after a considerably long recovery duration. During the process of carcinogenesis, lengthy periods of epigenetic and/or cytogenetic genomic instability, even if not “forever permanent” can be perceived to afford cells with necessary opportunities to rearrange their genomes until some selective growth advantage arrangement emerges.

Since the early work of Mass and Wang [Mass and Wang, 1997], it is well known that arsenic can alter the methylation of CpG islands of many genes, resulting in various increased and decreased changes in gene expression [Rossman and Klein, 2011; Roy et al., 2015]. Among the vast list of arsenic-altered cell cycle, DNA repair, signal transduction, metabolism, antioxidant and stress response genes, the mismatch DNA repair genes *hMLH1* [Treas et al., 2013] and *hMSH2* [Zhang et al., 2007] have been shown to be hyper- and hypomethylated, respectively, either by chronic arsenite exposure in vitro, or by environmental arsenic (e.g. food and air) in vivo. Thus, these two genes *hMLH1* and *hMSH2* were examined here for arsenite-induced methylation changes, both immediately at the end of treatment, and after 10, 20, and 40 cell generations of growth in the absence of SMA. The results obtained show that arsenite did not induce appreciable changes in the methylation levels of the *HMLH1* promoter, or its gene expression, for up to 40 cell generations. This is in contrast to some long term continuous exposure scenarios which do show *HMLH1* hypermethylation by arsenite [Treas et al., 2013], however, our studies differ considerably from that scenario in that the SMA exposure we used was for a brief 48 h followed by persistent, and in fact increasing, genome instability after withdrawal of the exposure. The epigenetic scenario was quite different with regard to the *hMSH2* gene, which in our studies exhibited a delayed but complete shift from unmethylated promoter status to methylated promoter status after 20 cell generations, with the simultaneous decrease in expression levels compared to untreated HaCaT cells. Whether or not this effect on gene expression was reversible at later time (beyond 40 cell generations) was not yet investigated, although future

studies of *hMSH2* methylation persistence at later time points would be important to follow up on.

Maintenance or deregulation of DNA methylation patterns is often associated with aberrant expression of DNMTs. Here, we observed that the short term SMA treatment did not alter *DNMT1* expression, while it clearly inhibited the expression of *DNMT3A* and *DNMT3B* immediately following the end of the treatment and continuing for up to at least 10 cell generations in a dose dependent fashion. Subsequently, levels of *DNMT3A* and *DNMT3B* mRNA expression clearly shifted upward by 20 cell generations and thereafter slowly continued increasing until at least the 40th cell generation time point. Together, these results support the premise that arsenite induces delayed epigenetic effects and that DNA hypomethylation is mediated, at least in part, by altered expression of *DNMT3A* and *DNMT3B*, in addition to the widely known depletion of SAM [Reichard et al., 2007]. Our findings with DNMT1 following short-term low-dose exposure to SMA are in contrast to other studies [Reichard and Puga, 2010] in which continuous low dose of arsenite exposure for as long as 10 generations can indeed reduce *DNMT1* mRNA levels. We did not study long-term continuous exposures with SMA here. Moreover, it is worthwhile to mention that *DNMT3A* and *-3B* can also intervene in the maintenance of DNA methylation, as well as in de novo methylation [Jair et al., 2006]. All these observations recall what has previously been reported for cells exposed to ionizing radiations that induce persistent genomic instability and DNA hypomethylation, both in cells directly exposed to X-rays and in their progeny [Pogribny et al., 2005; Loree et al., 2006].

Further epigenetic insights into the genomic instability that may be induced by arsenic compounds go far beyond classic DNA methylation studies. Some recent insights are derived from studies showing epigenetic effects involving histone modifications in vitro, and in vivo in exposed human populations, that can mediate gene expression changes whose temporality can be examined to help further unravel the delayed and persistent, as well as the recently reported sex-specific effects of arsenic [Arita et al., 2012; Chervona and Costa, 2012; Chervona et al., 2012]. Also, the novel recent discovery that arsenic exposure can deplete stem loop binding protein (SLBP) by enhancing its degradation, and by concurrent epigenetic silencing of the SLBP promoter [Brocato et al., 2014; Brocato et al., 2015; Brocato and Costa, 2015] leading to dysregulation of typical canonical histone H3.1 expression, has broad implications that can be directly related to chromatin organization, mitotic regulation, and thus genomic instability following exposure to arsenic. It will be interesting to investigate the temporality of SLBP activity and downstream histone H3.1 and H3.3 variant activities on genomic instability in the presence of, and/or following withdrawal of arsenic treatment.

In conclusion, we show here that epigenetic reprogramming, involving both global and gene specific DNA methylation changes, occurs after short term acute arsenite treatment, and that some delayed epigenetic effects even in the absence of continued arsenic exposure have bearing on persistent genomic instability. These findings add interesting perspectives to our knowledge of the mechanisms by which arsenic can invoke adverse health effects, including cancers.

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Fig. 1.

Immunolocalization of 5MeC antibody on metaphase chromosomes of: (A) untreated V79 cells; (B) V79 cells immediately after SMA treatment (generation 0); (C) SMA exposed V79 ASO-A cells (after 6 cell generations); (D) SMA exposed V79 ASO-B cells (after 50 cell generations); and (E) SMA exposed V79 ASO-C cells (after 90 cell generations).

Fig. 2.
Examples of MeSAP fingerprinting and relative densitometric profiles for untreated V79 cells (Panel **A**) and the three SMA exposed ASO cell populations (Panels **B–D**) analyzed at 6, 50, and 90 cell generations after removal of SMA. (S (red): single-digested DNA; D (blue): double-digested DNA).

Fig. 3.

Trends of chromosome number variation from the modal # 72–88 per cell in SMA treated versus untreated HaCaT cells. Curves show the mean value of two independent cytogenetic analyses. Error bars indicate standard deviation from the average of the two independent experiments.

Fig. 4. Trends of LINE-1 genomic DNA methylation in SMA treated and untreated HaCaT cells. Curves show the mean value of two independent pyrosequencing analyses. Error bars represent the standard deviation from these independent experiments.

Fig. 5.

RT-PCR analysis of mRNA expression of DNMT3A and 3B following SMA exposure: (A) representative example of DNMT3A mRNA levels in untreated (Ct) as well as SMA treated HaCaT cell populations (H0.1–0.5); (B) average of two densitometric analyses of *DNMT3A* expression compared to *Actin* in SMA treated cells (H0.1–0.5) normalized to untreated HaCaT cells (Ct); (C) *DNMT3A* mRNA expression in untreated HaCat cells up to 40 cell generations; (D) representative example of *DNMT3B* mRNA levels in untreated (Ct) as well as SMA treated HaCaT cell populations (H0.1–0.5); (E) average of two densitometric analyses of *DNMT3B* expression compared to *Actin* in SMA treated cells (H0.1–0.5) normalized to untreated HaCaT cells (Ct); (F) representative example of *DNMT3B* mRNA expression in untreated HaCat cells up to 40 cell generations. Error bars in Panels B and E indicate standard deviation of two independent experiments. Statistical significance was determined by unpaired Student's *t*-tests. ****P*<0.001.

Fig. 6. RT-PCR analysis of mRNA expression and promoter methylation levels in genes that were unchanged by the SMA exposures in HaCaT cells: **(A)** *DNMT1A* mRNA expression in untreated and SMA treated HaCaT cells (H0.1–0.5) up to 40 cell generations after removal of SMA; **(B)** MSP analysis of Unmethylated (U) and Methylated (M) promoter sequences of the *hMLH1* gene in untreated HaCaT (Ct) and in SMA treated cells (H0.1–0.5) up to 40 cell generations; **(C)** Representative example of mRNA expression of *hMLH1* in untreated as well as SMA treated HaCaT cells (H0.1–0.5).

Fig. 7. RT-PCR analysis of mRNA expression and gene promoter methylation levels of hMSH2. (A) MSP analysis of Unmethylated (U) and Methylated (M) promoter sequences in the *hMSH2* gene of untreated HaCaT (Ct) as well as in SMA treated cells (H0.1–0.5) up to 40 cell generations after removal of SMA; (B) average of two densitometric analyses of U and M MSP products in SMA treated cells (H0.1–0.5) normalized to untreated HaCaT cells (Ct); (C) U and M MSP products of promoter analysis of *hMSH2* for 40 cell generations in untreated HaCaT cells; (D) representative samples of mRNA expression of *hMSH2* in untreated (Ct) as well as SMA treatment-derived HaCaT clones (H0.1–0.5); (E) average of two densitometric analyses of *hMSH2* expression compared to *Actin* in SMA treated cells (H0.1–0.5) normalized to untreated HaCaT cells (Ct); (F) representative example of *hMSH2* mRNA expression in untreated HaCat cells up to 40 cell generations. Error bars in Panels B and E indicate standard deviation of two independent experiments. Statistical significance was determined by unpaired Student's *t*-tests. **P*<0.05; ***P*<0.01; ****P*<0.001.

TABLE I

Nucleotide Sequences and Annealing Temperatures of the Primers Used in the MSP

Gene	Forward primer	Reverse primer	Annealing Temp. (°C)
<i>hMLH1</i> (M)	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	60
<i>hMLH1</i> (U)	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	60
<i>hMSH2</i> (M)	TCGTGGTCGGACGTCGTTT	CAACGTCTCCTTCGACTACACCG	60
<i>hMSH2</i> (U)	GGTTGTTGTGGTTGGATGTTGTTT	CAACTACAACATCTCCTTCAACTACACCA	60

M, methylated; U, unmethylated.

TABLE II

Nucleotide Sequences and Annealing Temperatures of the Primers Used in RT-PCR

Gene	Forward primer	Reverse primer	Annealing Temp. (°C)
<i>DNMT1</i>	ACCGCTTCTACTTCCTCGAGGCCTA	GTTGCAGTCCTCGTGAACACTGTGG	56
<i>DNMT3A</i>	CACACAGAAGCATATCCAGGAGTG	AGTGGACTGGGAAACCAAATACCC	58
<i>DNMT3B</i>	AATGTGAATCCAGTCAGGAAAGGC	ACTGGATTAACTCCAGGAACCGT	55
<i>hMLH1</i>	GTGCTGGCAATCAAGGGACCC	CACGGTTGAGGCATTGGGTAG	65
<i>hMSH2</i>	GTCGGCTTCGTGCGCTTCTTT	TCTCTGGCCATCAACTGCGGA	65
<i>β-ACTIN</i>	ACACTGTGCCCATCTACGAGG	AGGGGCCGGACTCGTCATACT	60