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Differential DNA methylation profile of key genes in malignant prostate epithelial cells transformed by inorganic arsenic or cadmium

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Abstract

Previous work shows altered methylation patterns in inorganic arsenic (iAs)-or cadmium (Cd)-transformed epithelial cells. Here, the methylation status near the transcriptional start site was assessed in the normal human prostate epithelial cell line (RWPE-1) that was malignantly transformed by 10 μ M Cd for 11 weeks (CTPE) or 5 μ M iAs for 29 weeks (CAsE-PE), at which time cells showed multiple markers of acquired cancer phenotype. Next generation sequencing of the transcriptome of CAsE-PE cells identified multiple dysregulated genes. Of the most highly dysregulated genes, five genes that can be relevant to the carcinogenic process (*S100P*, *HYAL1*, *NTM*, *NES*, *ALDH1A1*) were chosen for in depth analysis of the DNA methylation profile. DNA was isolated, bisulfite converted, and combined bisulfite restriction analysis was used to identify differentially methylated CpG sites, which was confirmed with bisulfite sequencing. Four of the five genes showed differential methylation in transformants relative to control cells that was inversely related to altered gene expression. Increased expression of *HYAL1* (>25-fold) and *S100P* (>40-fold) in transformants was correlated with hypomethylation near the transcription start site. Decreased expression of *NES* (>15-fold) and *NTM* (>1000-fold) in transformants was correlated with hypermethylation near the transcription start site. *ALDH1A1* expression was differentially expressed in transformed cells but was not differentially methylated relative to control. In conclusion, altered gene expression observed in Cd and iAs transformed cells may result from altered DNA methylation status.

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Keywords

Inorganic Arsenic; Cadmium; DNA methylation; Prostate cancer; Gene expression

Introduction

Cadmium (Cd) and inorganic arsenic (iAs) are known human carcinogens that occur naturally in the environment (2012b; 2012a). Human exposure to Cd in the general population occurs primarily through consumption of contaminated food, although an additional source can be cigarette smoke (2012b). Cd is a human lung carcinogen and is possibly carcinogenic in the kidney, prostate, and breast (2012b; Strumylaite *et al.*, 2014). Human exposure to environmental iAs occurs primarily by consumption of contaminated water (2012a). In humans iAs is carcinogenic in the lung, skin, and urinary bladder, and probably in the kidney, liver, and prostate (2012a).

Despite extensive research, it is unclear how either Cd or iAs cause cancer. Neither element appears to be directly mutagenic (2012b; 2012a). Several other mechanisms have been proposed for these inorganics including induction of oxidative stress, secondary genotoxic damage, and chromosomal abnormalities (Waalkes, 2003; 2012a; 2012b). Recent evidence suggests that epigenetic modifications play a role in both Cd- and iAs-induced cancer (Ren *et al.*, 2011; Rossman and Klein, 2011). Epigenetic modifications are heritable changes in gene transcription that are not caused by changes in the DNA sequence. For example altered DNA methylation, histone modification, or microRNA expression can promote oncogene expression or silence tumor suppressor gene expression thereby creating a more favorable environment for cancer (Ren *et al.*, 2011).¹

The prostate is a potential target of both Cd and iAs carcinogenesis (2012b; 2012a). The non-tumorigenic immortalized human prostate epithelial cell line RWPE-1 is a well-characterized model for exploring the mechanism of malignant transformation by carcinogenic inorganics (Bello *et al.*, 1997; Achanzar *et al.*, 2001; Webber *et al.*, 2001; Achanzar *et al.*, 2002). Long-term exposure of RWPE-1 cells to environmentally relevant levels of Cd or iAs induces the acquisition of a malignant phenotype with both physical (e.g. invasion, colony formation) and genetic (e.g. loss of tumor suppressor gene expression) characteristics of cancer cells that form aggressive carcinoma upon inoculation into nude mice (Achanzar *et al.*, 2001; Achanzar *et al.*, 2002).

The cancer phenotype induced by Cd or iAs in these cells appears independent of mutagenesis or DNA damage events (Achanzar *et al.*, 2001; Kojima *et al.*, 2009). In this regard, Cd is not redox active so it cannot directly cause oxidative DNA damage (ODD), but given at very high concentrations it could displace redox active endogenous metals from their cellular binding sites. However, ODD was not observed during Cd-induced malignant transformation of RWPE-1 cells or the human lung epithelial cell line HPL-1D (Achanzar *et al.*, 2001; Person *et al.*, 2013). In fact, oncogene over-expression appears more important

¹Abbreviations: Cadmium (Cd), inorganic arsenic (iAs), kerinocyte-serum free medium (K-SFM), combined bisulfite restriction analysis (COBRA), Bisulfite Sequencing DNA Methylation Analysis (BISMA), Quantitation Tool for Methylation Analysis (QUMA)

than oxidant stress in Cd-induced malignant transformation of liver epithelial cells (Qu *et al.*, 2005). Furthermore, an analysis of four different rodent cell lines found no evidence of direct genotoxicity for Cd (Misra *et al.*, 1998). These data indicate non-genotoxic or epigenetic mechanisms of transformation for Cd.

On the other hand, ODD with iAs exposure can occur in cells that biomethylate the metalloid (Kojima *et al.*, 2009). RWPE-1 cells, however, are not able to biomethylate iAs and show no evidence of ODD during iAs-induced malignant transformation (Kojima *et al.*, 2009). In contrast, liver cells, which will biomethylate iAs, show more rapid transformation and ODD when exposed to iAs suggesting that the ability of the target cell to methylate iAs dictates DNA damage (Kojima *et al.*, 2009). Taken together, this lends further support for the potential role of epigenetic mechanisms in the malignant transformation by both iAs and Cd in these prostate epithelial cells.

Based on emerging evidence that epigenetic factors may be of etiologic significance in both Cd and iAs induced oncogenic phenotype, we sought to study such events in the current work. We used combined bisulfite restriction analysis (COBRA) and bisulfite sequencing to evaluate changes in DNA methylation patterns of five highly dysregulated genes in human prostate epithelial (RWPE-1) cells transformed either by chronic exposure to Cd or iAs, called CTPE or CAsE-PE cells, respectively (Achanzar *et al.*, 2001; Achanzar *et al.*, 2002; Merrick *et al.*, 2013). The results were also compared to prostate epithelial cells that had previously been malignantly transformed with the direct mutagen N-Nitroso-N-methylurea (MNU) called B26 cells.

Materials & Methods

Cell Culture/ Inorganic Transformation

Cells were cultured in K-SFM (Life Technologies, Grand Island, NY) containing 50 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, and a 1× antibiotic/antimycotic solution. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and passed weekly. Non-tumorigenic human prostate epithelium cells (RWPE-1; control) (Bello *et al.*, 1997) were malignantly transformed following chronic exposure to Cd (termed CTPE) (Achanzar *et al.*, 2001) or iAs (termed CAsE-PE) (Achanzar *et al.*, 2002) as described previously. Transformed cells showed multiple molecular and cellular markers of acquired cancer cell phenotype and have the ability to form carcinoma upon inoculation into nude mice (Achanzar *et al.*, 2001; Achanzar *et al.*, 2002). For comparison, control RWPE-1 cells were transformed with the mutagenic organic carcinogen, MNU resulting in the WPE1-NB26 cell line (herein referred to as B26 cells), which also produces carcinoma after injection in nude mice (Webber *et al.*, 2001).

Gene Expression

Preliminary work in this laboratory using next generation sequencing of the transcriptome identified multiple dysregulated genes in CAsE-PE cells (presented as a meeting abstract (Merrick *et al.*, 2013)). Of the most highly dysregulated genes, five genes that were associated with stem cell function (*ALHD1A1* and *NES*), extracellular matrix (*HYAL1*), cell

adhesion (*NTM*), or aggressive cancers (*S100P*) were chosen for in depth analysis of expression and promoter region DNA methylation patterns across the four cell types evaluated in the current work.

Sub-confluent cells (1×10^6) from three flasks per cell type were collected in 1 mL Trizol (Life Technologies, Grand Island, NY) each and frozen at -80°C . Samples were warmed to room temperature, chloroform was added, and after centrifugation, the aqueous phase was removed, mixed with an equal volume of 75% ethanol, applied to an RNeasy mini-spin column (Qiagen, Valencia, CA), and purified following the manufacturer's recommendation. RNA was treated on-column with DNase I, ethanol precipitated, resuspended in nuclease free water, and quantified on a Nanodrop 2000 spectrophotometer (Wilmington, DE). RNA quality was assessed by examination of the 18S and 28S bands following separation on an ethidium bromide stained 1% agarose gel. All agarose gels were visualized on a Kodak Gel Logic 2200 Imaging System using CareStream Molecular Imagine Software version 5.0 (Rochester, NY).

Two hundred nanograms of total RNA from three separate samples per cell type was reverse transcribed using the GeneAmp RNA PCR kit (Applied Biosystems, Grand Island, NY). The 20 μL cDNA reaction containing MuLV reverse transcriptase, random hexamers, and oligo d(T)₁₆ primers and was carried out on a GeneAmp PCR 9700 system with incubation at 25°C for 10 minutes, 48°C for 60 minutes, and 95°C for 5 minutes. As a negative control (noRT), an identical reaction was set up for each sample except that no reverse transcriptase was added. cDNA was diluted 2-fold with nuclease free water prior to use in real time RT-PCR.

Real time RT-PCR was performed with SYBR green technology on a BioRad MyiQ 2 PCR machine (BioRad, Hercules, CA) using primers designed with Primer-BLAST (NCBI, Bethesda, MD) as previously described (Pelch *et al.*, 2010) and synthesized by Sigma (St. Louis, MO) (Supplementary Table 1). Real time RT-PCR reactions were performed in duplicate in 25 μL with Absolute qPCR master mix containing Rox (Thermo, Lafayette, CO) according to the manufacturer's recommendations with primers at 70-100 nM each (Supplementary Table 1). The mRNA processing gene *NONO* was found to be constitutively expressed in the cell types investigated and was used to normalize the expression of the other mRNAs (Livak and Schmittgen, 2001). Fold changes ($2^{-\text{CT}}$) relative to control RWPE-1 cells are plotted as the geometric mean $\pm 95\%$ confidence interval ($\pm 95\% \text{CI}$) in Graphpad Prism version 6.00 for Mac (La Jolla, California). Fold changes were transformed logarithmically and compared by one way analysis of variance (ANOVA) and Dunnett's post-hoc test to compare transformed cells to control (RWPE-1).

Analysis of Differential DNA Methylation Profiles by Combined Bisulfite Restriction Analysis (COBRA) and Bisulfite Sequencing

Sub-confluent cells (1×10^6) from three flasks per cell type were lifted with trypsin, pelleted, and snap frozen in liquid nitrogen in triplicate. At processing, cell pellets were warmed to room temperature and DNA was isolated on DNEasy spin columns (Qiagen). DNA was treated with RNase A, ethanol precipitated, and quantified on a Nanodrop 2000 spectrophotometer. DNA was bisulfite treated using the EZ DNA Methylation Direct Kit

(Zymo, Irvine, CA) according to the manufacturer's directions except that the samples were passed through the column four times during processing. During bisulfite treatment unmethylated cytosines were converted to uracil whereas methylated cytosines occurring in a CpG context were retained.

PCR primers specific for bisulfite converted DNA (Table 1) were designed using MethPrimer software (Li and Dahiya, 2002) based on DNA sequences obtained from BLAT utilizing build hg19 from February 2009, of the human reference sequence (GRCh37). Primers targeted methylated or unmethylated regions within 1000 bases upstream or downstream of the transcription start site. When possible, multiple assays within this region were designed for each gene.

Bisulfite converted DNA was amplified in a 25 μ L PCR reaction using 2 μ L converted DNA, 1 $\times$ ZymoTaq (Zymo), and primers at 150-500 nM each (Table 1). Targets were amplified with hot start PCR with touchdown to 55°C for annealing and a final extension at 72°C for 10 minutes. A single PCR product from each sample was confirmed on an ethidium bromide stained 1.5% agarose gel.

Differential methylation was first examined using COBRA, in which the specific conversion or conservation of cytosines results in the creation of restriction endonuclease sites whose products are then visualized on an agarose gel (Xiong and Laird, 1997). Eight microliters of PCR product were digested in 25 μ L reactions according to the manufacturer's instructions except that all digestion times were increased to four hours using restriction enzymes (Hpy99I, HpyCH4IV, BstUI, and Taq^QI) from New England Biolabs (Ipswich, MA). The entire reaction was resolved on a SYBR Safe stained (Invitrogen) 1-5% agarose gel depending on the expected fragment sizes. Universally methylated or unmethylated DNA (Zymo) was included as a positive or negative control, respectively.

Amplicons that appeared differentially methylated by COBRA were further analyzed by bisulfite sequencing (Frommer *et al.*, 1992). PCR amplicons were passed through DNA Clean and Concentrator-5 (Zymo) spin columns, eluted in nuclease free water, and quantified on a Nanodrop 2000 spectrophotometer. TopoTA cloning was performed at a 1:3 M vector:insert ratio. Plasmids were transformed into TOP10 One Shot Chemically Competent Cells (Invitrogen) and grown up on kanamycin and $\times$ -gal containing plates overnight. The following day eight colonies were amplified overnight with the TempliPhi Kit (GE Healthcare, Pittsburgh, PA) according to the manufacturer's recommendations. Amplified product was diluted 1:2 with nuclease free water and 7 μ L was sequenced with 1 μ L M13 reverse primer using Big Dye V1.1 Terminator Kit (Life Technologies). Sequence reactions were purified using a perform DTR Ultra 96-well spin plate (EdgeBio) and electrophoresed on a Perkin-Elmer ABI 3130 sequencer. Sanger sequencing reactions were carried out by the NIEHS Sequencing Lab Group.

Sequencing results were viewed and edited in CLC Main Workbench Vs 6.9 (CLC Bio, Cambridge, MA). Sequences were then uploaded into the Bisulfite Sequencing DNA Methylation Analysis (BISMA) program (Rohde *et al.*, 2010), where sequence identity, conversion rate, and gaps were evaluated and data images were generated. CpG locations are

reported relative to the transcription start site of each gene. The overall percentage of methylation across each clone was calculated and the non-parametric Kruskal-Wallis test was used to look for differences across the four cell types. Dunn's post-hoc test was then used to compare the percentage of methylation in the transformants relative to the control cells.

Gene expression following treatment with a hypomethylating agent

To determine the extent to which DNA methylation contributed to differential gene expression cells were treated with 5-aza-dC as described previously (Benbrahim-Tallaa *et al.*, 2007). After incorporation into DNA, 5-aza-dC inhibits DNA methyltransferase (DNMT) activity leading to DNA hypomethylation. Briefly, cells were plated in triplicate at 50% confluence and treated every 48 hours with 0 (DMSO) or 0.5 μ M 5-aza-dC. After six days cells were collected and processed for analysis of gene expression as described above except that data was analyzed by two way ANOVA and all multiple comparisons post-hoc test to compare 5-aza-dC treated cells to untreated cells of the same type.

Analysis of microRNA (miRNA) expression

To determine if *ALDH1A1* expression is regulated by miRNA expression, total RNA including nucleotides >18 bases in length was isolated from untreated and 5-aza-dC treated cells using the miRNeasy mini kit (Qiagen) following the manufacturer's recommendations. RNA was quantified and quality was evaluated as described above.

MicroRNAs that may regulate *ALDH1A1* were identified by searching the literature (miR-29a) (Muniyappa *et al.*, 2009) or searching within the microRNA (www.microRNA.org) and MicroCosm Targets (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) databases as outlined by Kuhn *et al.* (Kuhn *et al.*, 2008). Only microRNAs identified by both databases were evaluated. TaqMan microRNA real time RT-PCR assays (Applied Biosystems) were performed on 5-aza-dC treated or untreated samples for the seven miRNAs obtained by this method (Supplementary Table 2). Briefly, 10 ng RNA was used for each 15 μ L reverse transcription reaction that contained target specific primers. Subsequently, 1.33 μ L of reverse transcribed target were used in each 20 μ L real time RT-PCR reaction that contained target specific primers and probe. The miRNA *RNU6B* was found to be constitutively expressed in the cell types investigated and was used to normalize the expression of the other miRNAs as described above.

Results

Differential gene expression in inorganic transformants

Our preliminary work using next generation sequencing of the transcriptome in CAsE-PE cells identified multiple dysregulated genes (Merrick *et al.*, 2013). We selected five highly expression-altered genes that were associated with stem cell function (*ALDH1A1* and *NES*), extracellular matrix (*HYAL1*), cell adhesion (*NTM*), and aggressive cancers (*S100P*) for in depth analysis in the current work. Relative expression of these five genes was assessed by real time RT-PCR. Three genes were found to be upregulated and two genes downregulated in the Cd and iAs transformants (Table 2). The magnitude of upregulation of *HYAL1* and

S100P was similar in both the Cd and iAs transformants. Expression of *HYAL1* was increased 49- or 25-fold and expression of *S100P* increased 50- or 41-fold in Cd or iAs transformed cells, respectively. Expression of both *NTM* and *NES* was decreased in the metal transformants. Expression of *NTM* was decreased >1000-fold in Cd and iAs transformants. For example, *NTM* was detected at a cycle threshold (Ct) value of 27 in controls, but was completely undetected in Cd or iAs transformants. Expression of *NES* was decreased 5.2 or 7.4-fold in Cd or iAs transformants, respectively. Expression of *ALDH1A1* was upregulated 10-fold in iAs transformants and downregulated 6.1-fold in Cd transformants. With the exception of *ALDH1A1*, expression of these genes in the cells transformed with the directly mutagenic organic carcinogen MNU (B26 cells) was more similar to what was observed in the controls (Table 2). That is, the magnitude of effect in the B26 cells was smaller than in the inorganic transformants.

Differential methylation profiles detected by COBRA and bisulfite sequencing

DNA was isolated, bisulfite converted, and COBRA was used to identify differentially methylated CpG sites within 1000 bases of the transcription start site of selected genes. When possible, two or more amplicons were evaluated in order to increase coverage of the CpG sites near the transcription start site. In COBRA, restriction digestion sites are only maintained after bisulfite conversion when a CpG site is methylated, thus allowing for the detection of differential methylation patterns. Representative agarose gels indicating differential methylation by COBRA are shown in Figures 1B, 2B, 3B, and 4B. COBRA also detected sites that are not differentially methylated, as in Figures 3B (amplicon #1 digested with Hpy99I, amplicon #2 digested with Taq^qI, and amplicon #3 digested with HpyCH4IV), and 4B (amplicon #1 digested with Hpy99I or BstUI and amplicon #2 digested with HpyCH4IV).

Bisulfite sequencing was performed on amplicons that were identified as differentially methylated by COBRA. Bisulfite sequencing confirmed the methylation status of all CpGs interrogated by both methods, provided information on sites that could not be interrogated by COBRA, and indicated that *S100P*, *HYAL1*, *NTM*, and *NES* were differentially methylated in Cd and iAs transformants when compared to controls (Table 3). *S100P* and *HYAL1* were hypomethylated whereas *NTM* and *NES* were hypermethylated in the inorganic transformants. In general, the methylation status in B26 cells was quite similar to controls for the genes examined.

Methylation analyses revealed that *S100P* was hypomethylated in Cd and iAs transformants at CpG sites +132 and +149 (relative to the transcription start site) (Figure 1C). Likewise, *HYAL1* was hypomethylated in inorganic transformants at CpG sites -324, -291, -281, -181, -72, -60, and -7 (Figure 2C).

NTM was generally unmethylated in controls and B26 cells (Table 3), but there was increased methylation present in both of the inorganic transformants, specifically at CpG sites -383, -355, -305, and -273 (Figure 3C). Whereas some CpG sites were only hypermethylated in the Cd transformants (-380, -341, +541, +545, +548, +550, and +559), other sites were only hypermethylated in the iAs transformants (-378, -353, -317, -260, +515, and +517). Bisulfite sequencing also revealed an interesting pattern of methylation

within CTPE cells in which there appears to be at least three unique populations of cells: those with methylation at all CpG sites from +433 to +659, those with methylation only at CpG sites from +541 to +559, and those that are unmethylated at CpG sites from +433 to +659.

NES was hypermethylated at +827 and +878, in the Cd transformants (CTPE) (Figure 4C). Because there was no indication of differential methylation by COBRA, *NES* amplicon #1 was not sequenced.

ALDH1A1 was largely unmethylated across all cell types (Figure 5C, Table 3). COBRA suggested increased methylation in inorganic transformants at CpGs at +78 and/or +201. Bisulfite sequencing suggested partial methylation at the CpG at +201 in the Cd and iAs transformants.

Effect of treatment with hypomethylating agent

As a means of confirming that altered gene expression was a result of differential DNA methylation, each of the various cell lines were treated with 5-aza-dC to inhibit DNMT activity thereby resulting in DNA hypomethylation. Cells were treated with 5-aza-dC for six days at which time gene expression was again evaluated.

For the two up-regulated genes, *S100P* and *HYAL1*, treatment with 5-aza-dC had an expected minimal effect on the expression in the inorganic transformants. Treatment with 5-aza-dC did, however, result in a marked increase in *S100P* and *HYAL1* gene expression in the control and B26 cells (Figures 1D and 2D). For example, expression of *S100P* was increased 8.6- and 11-fold and expression of *HYAL1* was increased 14.9- and 22-fold in the 5-aza-dC treated control and B26 cells, respectively, relative to the untreated RWPE-1 control cells.

The opposite effect was observed for the hypermethylated gene *NES* and the unmethylated gene *ALDH1A1*. The expression of *NES* and *ALDH1A1* was increased in all cell types following 5-aza-dC treatment (Figures 4D and 5D). Expression of *NTM* was relatively unchanged in control and B26 cells following 5-aza-dC treatment, and remained undetectable in the CTPE and CAsE-PE cells (Figure 3D).

Expression analysis of miRNAs targeting ALDH1A1

The expression of miRNAs that potentially target *ALDH1A1* was evaluated in cells that were untreated or treated with 5-aza-dC. There were no cell type specific changes in expression of miR-21, -23a, -23b, -29a, -132, -221 or -222. Treatment of cells with 5-aza-dC had no effect on the expression of the miRNAs assessed (Supplemental Figure 1).

Discussion

The present work indicates the altered gene expression observed with acquired malignant phenotype in Cd and iAs transformation of human prostate epithelial cells can arise from epigenetic factors, and specifically in this case, altered DNA methylation status. Here, five genes that were highly dysregulated in preliminary next generation sequencing of the

transcriptome of CAsE-PE cells (Merrick *et al.*, 2013) underwent in-depth DNA methylation profile analysis. The genes of interest were linked to stem cell function/dysfunction, extracellular matrix, cell adhesion, or aggressive cancers and can be relevant to cellular transformation (Ekici *et al.*, 2004; Liu *et al.*, 2004; Kasper, 2008; Ma and Allan, 2011; Jiang *et al.*, 2012). Altered DNA methylation near the transcriptional start site appeared critical in regulating four of the five genes in the inorganic transformants. When the parental line is transformed with the direct acting alkylating carcinogen MNU there is little evidence of altered DNA methylation. Thus, although all three transformed cell lines produce xenograft carcinomas in mice, the mechanism by which they acquire a malignant phenotype appears quite distinct. This is consistent with prior work showing little evidence of ODD during Cd- or iAs-induced transformation of these prostate epithelial cells or other cell types (Qu *et al.*, 2005; Kojima *et al.*, 2009; Person *et al.*, 2013). Given the large differences in exposure time needed to achieve transformation by MNU, Cd, or iAs (four hours, eight weeks, or 29 weeks, respectively) it is not surprising that the mechanism driving transformation with each agent is unique (Achanzar *et al.*, 2001; Webber *et al.*, 2001; Achanzar *et al.*, 2002)). In the case of all five genes interrogated, exposure to Cd or iAs altered the gene expression in such a way that would favor carcinogenesis. Yet it is unclear if the changes in promoter region DNA methylation and expression of the genes observed in the current work are a cause or consequence of transformation. In this regard, a time course study would be of great value, comparing acquisition of cellular and molecular cancer cell characteristics with iAs and Cd and epigenetic changes.

The genes studied in the present work all have potential relevance to carcinogenesis. For instance, *S100P* is over-expressed in many malignancies including prostate carcinomas and is linked with particularly aggressive tumors (Jiang *et al.*, 2012) increased cell growth, and anchorage independent growth of prostate cancer cells in soft agar (Basu *et al.*, 2008). Proteins in the *S100* family are often regulated by DNA methylation (Lesniak, 2011). Indeed, *S100P* is hypomethylated in human prostatic cancers and gene expression is increased in a non-cancerous prostate cell line by 5-aza-dC treatment (Wang *et al.*, 2007). In our study *S100P* expression was highly increased in both Cd and iAs transformants and the gene was hypomethylated upstream of the transcription start site. Furthermore, 5-aza-dC treatment increased *S100P* expression in all cells except the Cd transformant, which already had a very high level of expression. This suggests that *S100P* is regulated by DNA methylation in the inorganic transformed cells, which would increase the anchorage independent cell growth and tumor forming potential in these cells.

The *HYAL1* gene encodes an enzyme that degrades hyaluronan, a component of the extracellular matrix. *HYAL1* expression is considered an angiogenic prognostic marker for prostate cancer (Ekici *et al.*, 2004). *HYAL1* expression was greatly increased in the inorganic transformants and was hypomethylated upstream of the transcriptional start site. *HYAL1* expression was increased in all cell types after 5-aza-dC treatment, but most notably in the control and B26 cell lines that were highly methylated in the promoter region. The observed hypomethylation occurs in the region that is important in the transcriptional regulation of *HYAL1* in prostate cancer cells. These CpGs occur in a region of overlapping SP1/Egr-1 transcription factor binding sites and near NF κ B and AP-2-binding sites (Lokeshwar *et al.*,

2008). The methylation status of these key CpGs regulates which transcription factors bind in prostate cells that differentially express *HYAL1*, suggesting a mechanism by which differential methylation of *HYAL1* results in altered gene expression. Increased *HYAL1* in the inorganic transformants likely promotes growth and invasion by remodeling the extracellular environment.

Our preliminary genome wide expression and methylation analyses in control and iAs transformed cells suggested that two poorly expressed genes, *NTM* and *NES*, were hypermethylated in CAsE-PE cells (Merrick *et al.*, 2013). *NTM* is a cell surface adhesion molecule and a member of the IgLON immunoglobulin superfamily (Liu *et al.*, 2004). Little is known about *NTM* expression in normal or cancerous prostate tissue. In our work, *NTM* expression was detected at modest levels in control cells but was undetectable in inorganic transformants. Since *NTM* acts in cell adhesion this could suggest that the iAs and Cd transformed cells may have lost contact inhibition, which would be consistent with their greater capacity for invasion (Achanzar *et al.*, 2001; Achanzar *et al.*, 2002; Liuet *et al.*, 2004). *NTM* was largely unmethylated in the control and B26 cells and hypermethylated in the inorganic transformants, however, treatment with 5-aza-dC did not consistently activate *NTM* expression in the inorganic transformants. An interesting pattern of methylation was, however, detected in the Cd transformed cells raising the possibility that there may be multiple subsets of differentially methylated cells. Specific epigenomic and growth characteristics of such putative subpopulations within Cd transformants should be explored.

NES is a marker of a wide range of multi-lineage progenitor cells (Wiese *et al.*, 2004), is detected in various tumor types including prostate cancers, and is often associated with a poor prognosis (Kasper, 2008). Transcriptional regulation of *NES* in adults is not well understood (Wiese *et al.*, 2004), but seems to be linked to disease progression in prostate cancer cells (Kleeberger *et al.*, 2007). In this work, decreased *NES* expression in the inorganic transformants was linked to gene hypermethylation. Indeed, preliminary genome-wide analysis in our laboratory shows a 27-fold increase in methylation near the promoter region of *NES* in the iAs transformed cells (Merrick *et al.*, 2013). *NES* expression was increased by 5-aza-dC treatment in all cell types, suggesting DNA methylation regulates *NES* expression. However other controlling factors may also be at play, since in a mouse neuronal cell line histone acetylation, rather than DNA methylation, is required to regulate *NES* expression (Han *et al.*, 2009). Whereas initial reports indicated that *NES* is involved in prostate cancer cell migration, invasion, and metastasis (Kleeberger *et al.*, 2007), more recent work demonstrates an inhibitory role for *NES* in prostate cancer cell migration and invasion (Hyder *et al.*, 2014). In the work by Hyder *et al.*, (2014), decreased *NES* expression regulates phosphorylated focal adhesion kinase activity, integrin localization on the cell membrane, and extracellular matrix proteolysis resulting in larger cells that spread out upon the tissue culture growth surface and have increased invasive properties. Interestingly, the iAs transformed cells in our study often show a similar phenotype, with increased size, more extensive spreading, and increased invasion. The downregulation of *NES* in Cd and iAs transformants may, therefore, reflect a mechanism more similar to that identified by Hyder *et al.*

ALDH1A1 is a marker of normal and cancer stem cells (Ma and Allan, 2011). Despite being differentially expressed in the transformed cell lines, there were no differences in the methylation pattern of *ALDH1A1* between the inorganic transformants and control cells. However, 5-aza-dC treatment increased *ALDH1A1* expression in all four cell types, implicating methylation changes as relevant to expression and suggesting several possibilities. One is that the bisulfite sequencing failed to cover the key regulatory element. However, the closest CpG island is over 1.5 million bases upstream of the transcriptional start site, thus making direct regulation by promoter region DNA methylation less likely. More likely is the possibility that *ALDH1A1* expression is regulated by an upstream signaling factor that is methylation sensitive. To explore this, expression of seven microRNAs predicted to target *ALDH1A1* was assessed after 5-aza-dC treatment but none appeared to regulate *ALDH1A1* expression.

An additional possibility is that the increased *ALDH1A1* expression might be related to an increased number of cancer stem cells in the iAs transformants but not in the Cd and MNU transformants as previously reported (Tokar *et al.*, 2010; Tokar *et al.*, 2011). This may also explain why *ALDH1A1* was differentially expressed, being upregulated in the CAsE-PE but downregulated in the CTPE and B26.. This, however, does not explain the further increase in *ALDH1A1* expression following 5-aza-dC treatment and thus, the mechanism by which *ALDH1A1* expression is increased in response to iAs or 5-aza-dC remains to be elucidated. The differential expression of *ALDH1A1* amongst the transformants is likely further evidence that even between Cd and iAs different mechanisms are used to achieve malignant transformation, which is reflected in the length of time each chemical requires to induce transformation.

Cd and iAs are well known human carcinogens yet the carcinogenic mechanism is poorly understood. The RWPE-1-based isogenic cell model is useful for studying the epigenetic mechanisms associated with inorganic transformation of prostate cells since RWPE-1 cells have a relatively normal global DNA methylation profile (Severson *et al.*, 2012). In this model, Cd transformants have increased global DNA methylation content, increased DNMT activity, and DNMT3b transcript expression (Benbrahim-Tallaa *et al.*, 2007). In contrast, iAs transformants have decreased global DNA methylation and DNMT activity (Benbrahim-Tallaa *et al.*, 2005). This global hypomethylation is often attributed to depletion of S-adenosyl-methionine (SAM), the methyl-donor during both conversion of cytosine to 5-methylcytosine and biomethylation of iAs. Chronic exposure to iAs can deplete SAM reducing the availability for DNA methylation (Reichard and Puga, 2010). Although RWPE-1 cells do not biomethylate iAs they do, however, have depleted levels of SAM due to adaptive mechanisms that shuttle more homocysteine to the transsulfuration pathway in the presence of chronic iAs for iAs efflux (Coppin *et al.*, 2008; Kojima *et al.*, 2009). Furthermore, although global hypomethylation occurs with chronic iAs exposure, there are also significant amounts of gene specific hypermethylation (Severson *et al.*, 2012).

In summary, we studied altered DNA methylation near the transcriptional start site as a basis for the differential expression of five of the most highly dysregulated genes in inorganic transformed prostate cell lines. In this analysis the inorganic transformants appear more similar to each other while the B26 cells appear more similar to the control in both

expression and DNA methylation status. This suggests that the mechanism of transformation by these inorganics is distinct from the organic alkylating carcinogen MNU. Finally, while the genes examined in this work are associated with the malignant process, it is unclear if the changes in methylation and expression are causes or consequences of oncogenic transformation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- Cd and iAs are known human carcinogens, yet neither appears directly mutagenic
- Prior data suggest epigenetic modification plays a role in Cd or iAs induced cancer
- Altered methylation of four misregulated genes was found in Cd or iAs transformants
- The resulting altered gene expression may be relevant to cellular transformation

Figure 1.

Differential methylation pattern of *S100P* in transformed cell lines. A) Genomic locus showing location of CpGs (red lines) relative to the transcription start site (arrow). Genomic DNA was bisulfite converted and PCR amplified (gray bars). B) Representative agarose gels from COBRA analysis. PCR amplified bisulfite converted DNA was enzymatically digested with BstUI, Hpy99I, HpyCH4IV, or Taq^QI as indicated. Triplicate samples of each cell type and universally methylated (+ Con) or unmethylated (– Con) DNA were separated on each gel. C) Bisulfite sequencing results. Each box represents a CpG site where red is a methylated CpG site and blue is an unmethylated CpG site. White boxes represent locations where a base call could not be accurately made. Each row is a different clone that was sequenced. Summary data for each cell type is also displayed. D) Relative gene expression in untreated cells (open bars) or cells treated with 0.5 μ M 5-aza-dC (gray bars). Data is geometric mean of the fold changes + 95% confidence interval. Data were analyzed by two way ANOVA and all multiple comparisons post-hoc analysis. * p <0.05 for comparisons of treated and untreated cells of the same type and † p <0.05 for comparison of untreated transformants to untreated controls.

Figure 2. Differential methylation pattern of *HYAL1* in transformed cell lines. See Figure 1 legend for description of panels A-D.

Figure 3. Differential methylation pattern of *NTM* in transformed cell lines. See Figure 1 legend for description of panels A-D. ND indicates not detected.

Figure 4. Differential methylation pattern of *NES* in transformed cell lines. See Figure 1 legend for description of panels A-D.

Figure 5. Methylation pattern of *ALDH1A1* in transformed cell lines. See Figure 1 legend for description of panels A-D.

Table 1

COBRA/bisulfite sequencing primers

	Primer #	Primer Sequence	Genomic Location	Amplicon Size (bp)	[Primer] nM	CpG sites
<i>ALDH1A1</i>	1	F: TGGTTTTFAGTGGTTAGAGTAGTTGT R: ACACCTTAACCTTATTTATTCCTTTTT	chr9(-): 75,567,973-75,568,256	284	200	6
	1	F: TTTTAAAAAGTGTAGGATTATAGG R: TACTCAAAAAATATCTTTATCCAC	chr3(-): 50,341,463-50,341,302	162	500	4
<i>HYAL1</i>	2	F: GGGTTTATTAATGTTAGATGTAGTTA R: CTACACCAAAAACTCCTAAAAA	chr3(-): 50,341,353-50,340,980	374	150	7
<i>NES</i>	1	F: GTTTGATTATTGAGGATGGATAGA R: TCTAAAAAATTAAACCTAAATCCC	chr1(-): 156,647,064-156,647,284	221	300	18
	2	F: ATTAGGTTTTTGAGAAATGGTTTTT R: AAACACAACTCCAACTAAAC	chr1(-): 156,646,139-156,646,387	250	300	7
<i>NTM</i>	1	F: AAATGGTTAAATTTTGTGTTGG R: TATCATAACAACAACCTCCATCCCTA	chr11(+): 131,780,281-131,780,480	200	200	24
	2	F: AGGAGGGAGTTTTTTTTTGGT R: AAACAAAATACCCACAAACCCC	chr11(+): 131,781,124-131,781,399	276	200	27
	3	F: TTTTGTGTTTGGAAAGTGT R: TACAACCCCAAAAAACCAAC	chr11(+): 131,781,398-131,781,668	272	200	14
<i>S100P</i>	1	F: ATGAGGATGTTATTGTGGTTTAGTG R: CACCTTCCTCCTAAAAACTAACAAA	chr4(+): 6,695,348-6,695,642	295	150	5
	2	F: AGGAAGGTGGGTTTGAAATTTAGTAT R: CCTAATAACTCCTTCTCCATCAACA	chr4(+): 6,695,634-6,695,787	154	150	6

Table 2

Relative gene expression in transformed cells.

Gene Name	CTPE	CAsE-PE	B26
<i>ALDH1A1</i>	-6.1 [*] (-15, -2.4)	10 [*] (5.4, 22)	-20 [*] (-117, -2.8)
<i>HYAL1</i>	49 [*] (47, 51)	25 [*] (24, 27)	1.1 (-1.1, 1.2)
<i>NES</i>	-5.2 [*] (-8.3, -3.2)	-7.4 [*] (-14, -3.9)	1.7 [*] (1.0, 3.0)
<i>NTM</i>	>-1000 ^{*a}	>-1000 ^{*a}	-23 [*] (-36, -15)
<i>S100P</i>	49.5 [*] (43, 57)	41.0 [*] (36, 47)	-3.6 [*] (-5.4, -2.4)

Fold change relative to control RWPE-1 cell equal to 1.0.

^{*} $p < 0.05$ by one-way ANOVA of log transformed fold change values followed by comparison to RWPE-1 control cells by Dunnett's multiple comparisons post-hoc test. (95% CI)

^a *NTM* detected at a cycle threshold of 27 in control RWPE-1 cells and undetected in CTPE or CAsE-PE cells.

Table 3

Percentage (%) of methylated CpGs across the entire amplicon.

Gene name	Amplicon	RWPE-1	CTPE	CAsE-PE	B26
<i>S100P</i>	Amplicon #1	42.0	25.7	22.5	42.2
<i>S100P</i>	Amplicon #2	71.7	43.9	29.2 *	90.0
<i>HYAL1</i>	Amplicon #1	100	71.9 *	71.9 *	97.2
<i>HYAL1</i>	Amplicon #2	92.9	15.4 *	7.6 *	97.1
<i>NTM</i>	Amplicon #1	1.4	19.0 *	25.5 *	2.4
<i>NTM</i>	Amplicon #2	0.4	29.4 *	5.7	0.6
<i>NES</i>	Amplicon #2	68.6	88.6	75.4	84.1 *
<i>ALDH1A1</i>	Amplicon #1	3.3	11.3	7.4	5.0

The percentage of methylated CpGs in each clone were compared by Kruskal-Wallis test

* $p < 0.05$ relative to RWPE-1 by Dunn's post-hoc analysis.