Reversal and Prevention of Arsenic-Induced Human Bronchial Epithelial Cell Malignant Transformation by microRNA-200b

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Arsenic is a well-recognized human carcinogen, yet the mechanism by which it causes human cancer has not been elucidated. MicroRNAs (miRNAs) are a large class of small noncoding RNAs and negatively regulate the expression of a large number of protein-coding genes. We investigated the role of miRNAs in arsenic-induced human bronchial epithelial cell malignant transformation and tumor formation. We found that prolonged exposure of immortalized p53-knocked down human bronchial epithelial cells (p53lowHBECs) to low levels of arsenite (NaAsO2, 2.5 μM) caused malignant transformation that was accompanied by epithelial to mesenchymal transition (EMT) and reduction in the levels of miR-200 family members. Stably reexpressing miR-200b in arsenite-transformed cells (As-p53lowHBECs) completely reversed their transformed phenotypes, as evidenced by inhibition of colony formation in soft agar and prevention of xenograft tumor formation in nude mice. Moreover, stably expressing miR-200b alone in parental nontransformed p53lowHBECs was sufficient to completely prevent arsenite exposure from inducing EMT and malignant transformation. Further mechanistic studies showed that depletion of miR-200 in arsenite-transformed cells involved induction of the EMT-inducing transcription factors zinc-finger E-box-binding homeobox factor 1 (ZEB1) and ZEB2 and increased methylation of miR-200 promoters. Stably expressing ZEB1 alone in parental nontransformed p53lowHBECs was sufficient to deplete miR-200, induce EMT and cause cell transformation, phenocopying the oncogenic effect of 16-week arsenite exposure. These findings establish for the first time a causal role for depletion of miR-200b expression in human cell malignant transformation and tumor formation resulting from arsenic exposure.

Key Words: arsenic; miRNA-200; ZEB; cell transformation; chemical carcinogenesis.
miRNA expression in nontransformed human cells results in cell malignant transformation and tumor formation.

An increasing number of studies show that miRNAs can act either as oncogenes or as tumor suppressor genes (Shenouda and Alahari, 2009). Hence, deregulation of miRNA expression may also play important roles in the initiation of tumor formation. Interestingly, recent studies show that chemical carcinogen exposure is capable of altering miRNA expression (Chen, 2010). For example, Marsit et al. (2006) found that exposure of human lymphoblastoid cells to arsenic (2 μM of sodium arsenite) for 6 days lead to global increases in miRNA expression. Jiang et al. (2011) recently reported that 55 miRNAs were differentially expressed in anti-benzo(a)pyrene-trans-7,8-diol-9,10-epoxide (anti-BPDE)-transformed human bronchial epithelial cell (HBEC) line 16HBE, and inhibition of miR-106a in anti-BPDE-transformed cells induced apoptosis and suppressed their anchorage-independent growth (Shen et al., 2009). However, it has not been determined whether abnormally expressed miRNAs causally contribute to carcinogen-caused human cell malignant transformation and subsequent tumor formation. In this study, we investigated the effect of chronic arsenic exposure on miRNA expression in immortalized HBECs, to explore the mechanism by which arsenic exposure causes deregulation of miRNA expression, and to examine the role of abnormally expressed miRNAs in human cell malignant transformation and tumor formation resulting from arsenic exposure. We report for the first time a causal role for depletion of miR-200b expression in human cell malignant transformation and tumor formation resulting from arsenic exposure.

MATERIALS AND METHODS

Cell culture. Immortalized HBECs with intact p53 expression and function (defined as HBECs) and the HBECs with p53 expression stably knocked down (defined as p53-/-HBECs) by expressing a short hairpin RNA (shRNA) targeting p53 were generated from the parental HBECs and generously provided by Dr John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX) (Ramirez et al., 2004; Sato et al., 2006). The HBECs and p53-/-HBECs are isogenic cells, have epithelial morphology, express epithelial markers, are able to differentiate into mature airway cells in organotypic cultures and have minimal genetic changes (Ramirez, et al., 2004). Unlike other immortalized HBECs such as BEAS-2B cells that form colonies in soft agar, neither HBECs nor p53-/-HBECs form colonies in soft agar or produce tumors in nude mice, which makes HBECs and p53-/-HBECs excellent models for studying the mechanism of carcinogen- and oncogene-induced malignant transformation (Damiani et al., 2008; Liu et al., 2010; Sato et al., 2006). Both HBECs and p53-/-HBECs were cultured in chemically defined serum-free medium (K-SFM) (Invitrogen, Carlsbad, CA) supplemented with 20 μg/ml of bovine pituitary extract (BPE) and 0.8 μg/ml of epidermal growth factor (EGF). Normal finite life span human bronchial epithelial cells (NHBEs) were purchased from Lonza (Allendale, NJ). Human lung cancer cells were kindly provided by Dr Yong Lin (Lovelace Respiratory Research Institute, Albuquerque, NM).

Cell transformation by arsenite exposure. The HBECs and p53-/-HBECs were continuously exposed to Vehicle Control (deionized H2O) or 2.5 μM of arsenite (NaAsO2, S2251-100, Fisher Scientific, Fair Lawn, NJ), respectively. When reaching about 80–90% confluence, cells were subcultured. Arsenite was then added to cells after overnight attachment. These procedures were repeated every 3 or 4 days for 16 weeks. During the exposure period, cell morphology changes were monitored. Cell malignant transformation was assessed by morphology change, anchorage-independent growth (soft agar colony formation), and tumor formation in nude mice.

Anchorage-independent growth assay. The anchorage-independent growth assay was performed in 60-mm tissue culture dishes in triplicates for each treatment as described previously (Yang et al., 2005). Briefly, cells were collected by trypsinization and suspended in K-SFM containing 20% fetal bovine serum (FBS) and BPE (20 μg/ml) but no EGF at a concentration of 5 × 104 cells/ml. Normal melting point agar (5 ml of 0.6% agar in K-SFM containing 10% FBS and BPE), (20 μg/ml) but no EGF were placed over the bottom agar. After solidification of the bottom agar, 4 ml of cell mixture consisting of 2 ml of cell suspension (5 × 106 cells/ml) and 2 ml of 0.8% lower melting point agar in K-SFM containing BPE (20 μg/ml) but no EGF were added, and dishes were incubated at 37°C in a humidified 5% CO2 atmosphere. Colony formation in the agar was photographed and counted (if > 100 μm) under a phase-contrast microscope after 4-week incubation.

MIRNA microarray and quantitative PCR analysis of miRNAs. Total miRNAs from HBECs and p53-/-HBECs were extracted using QIAGEN miRNeasy mini kit and used for miRNA microarray and quantitative PCR (Q-PCR) analysis. The miRNA microarray was performed using the miBase version 12.0 containing 856 human mature miRNA probes (September 2008 release) by LC Sciences (Houston, TX). Q-PCR analysis of individual miRNAs was carried out in ABI 7500 Fast Real Time PCR System using TaqMan miRNA assays. U6 snRNA was analyzed by TaqMan PCR assay and used as internal controls for normalizing relative miRNA expression levels (Applied Biosystems, Inc., Foster City, CA).

MiR-200b and 200c promoter-luciferase reporter assay. The construction of miR-200b and 200c promoter luciferase reporter vectors was described in detail previously (Bracken et al., 2008; Wellner et al., 2009). Cells were cotransfected with a miR-200b or 200c promoter luciferase vector and a pRL-TK Renilla luciferase vector. Forty-eight hours after transfection, the luciferase activities were measured using Promega Dual Luciferase Assay (Promega, Madison, WI). The relative luciferase activity was calculated as the miR-200b or 200c promoter firefly luciferase activity divided by the Renilla luciferase activity.

DNA methylation analysis of the miR-200 loci. Genomic DNA was isolated from cells using Trizol (Invitrogen), and 500 ng was bisulfite modified using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocols. Approximately, 20 ng of bisulfite modified DNA was PCR amplified using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) containing a final concentration of 0.5μM of each primer in a final reaction volume of 15 μl. The primers and PCR conditions were specific for bisulfite-modified DNA and did not amplify unmethylated DNA. Unmodified DNA and bisulfite-modified unmethylated reference and methylated reference were included in each PCR run. The following primers were used: miR200b–200a–429 (miR-200b) forward,
5'-GTGTTAGGGTGGTGGGAT-3'; miR200b reverse, 5'-AACCTT-CRCCTTACAAAAACAATAC-3'; miR-200c-141 (miR-200c) forward, 5'-GGTGGTTATGTTGAGAGATA-3'; miR-200c reverse 5'-AAAA-CRAAAAAATTTAAAAACCCCAAA-3'. The PCR was performed using a Rotor-Gene 3000 (Corbett Life Science, Sydney, NSW, Australia) with a 95°C activation step for 15 min; 95°C for 30 s, 55°C for 60 s for 45 cycles; and a final extension step of 72°C for 4 min. The melt of the PCR product was performed from 60 to 90°C, rising in 0.5°C increments, waiting for 30 s at each step and for 5 s at each step thereafter, and acquiring fluorescence at each temperature increment. The raw fluorescence data for the melt was normalized as described previously (Smith et al., 2009).

**Generation of miR-200b stable expressing cell lines.** MiRNA precursor-expressing lentiviral particles were used for generating miR-200b stable expressing cells. Cells were transfected with GFP control (pMiRNA-GFP) or miR-200b precursor-expressing (pMiRNA-GFP-200b) lentiviral particles (System Biosciences, Mountain View, CA). Cells were subcultured 48 h after lentiviral particle transduction. Fluorescence-activated cell sorting (FACS) was performed to sort GFP positive cells after 48 h culture. The overexpression of miR-200b was confirmed by Q-PCR analysis.

**Generation of ZEB1 stable expressing cells.** Human ZEB1 full-length cDNA was obtained from Thermo Scientific (Open Biosystems, Huntsville, AL) and cloned into pLenti7.3/V5-DEST vector using Gateway cloning technology (Invitrogen) following manufacturer's instructions. Control (pLenti7.3) and ZEB1-expressing (pLenti7.3-ZEB1) lentiviral particles were packaged using 293FT cells according to published protocols (Tiscornia et al., 2006). Cells were transduced with GFP control (pLenti7.3) or ZEB1-expressing (pLenti7.3-ZEB1) lentiviral particles and subcultured 48 h after lentiviral particle transduction. FACS was performed to sort GFP positive cells. ZEB1 expression was confirmed by Western blot.

**Western blot.** Cells were lysed using tris-sodium dodecyl sulfate (SDS) as described by Yang et al. (2006) and subjected to SDS-polyacrylamide gel electrophoresis (10–30 μg of protein/lane). The following primary antibodies were used: anti-E-cadherin, anti-vimentin (Cell Signaling Technology, Beverly, MA); anti-p53, anti-ZEB1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-β-actin (Sigma, St Louis, MO).

**ZEB1/ZEB2 RNA interference.** Negative control small interfering RNA (siRNA) and ON-TARGETplus SMARTpool siRNA for ZEB1 and ZEB2 were obtained from Thermo Scientific Dharmaco (Lafayette, CO). SiRNA duplexes (100nM) were transfected into cells using Lipofectamine 2000 (Invitrogen) in serum-free medium following the manufacturer’s instructions. Forty-eight to 72 h after transfection cells were collected for Q-PCR analysis of miR-200b and 200c levels or for soft agar colony formation assay as described above. For the luciferase reporter assay, 24 h after siRNA duplex transfection cells were cotransfected with a miR-200b or 200c promoter luciferase vector and a pRL-TK Renilla luciferase vector (Promega). Successful knockdown of ZEB1 and ZEB2 was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR).

**RT-PCR.** Total RNA was prepared using Trizol according to the manufacturer’s protocol and reverse transcribed using SuperScriptTM II RT (Invitrogen). The resulting cDNA was used for PCR amplification using the following primers: ZEB1 forward, 5'-GACCATGAGGACCGACAAG-3’; ZEB1 reverse, 5'-GTTGAATCTGCAACAGGAGCA-3’; ZEB2 forward, 5'-CGGTTGACATCAGCTAAAGGA-3’; ZEB2 reverse, 5'-CTTCCAGACATCGAAGCTG-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-CCCTTAGTATCTGATTACATAG-3’; and GAPDH reverse, 5'-CATGGTGTGAGGACCGAGC-3’. ZEB1-, ZEB2-, and GAPDH-specific fragments were amplified by 30, 35, and 25 cycles of PCR, respectively, each cycle comprising 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C.

**GFP immunofluorescent staining.** Nude mice xenograft tissue sections (5 μm) were prepared and subjected to hematoxylin and eosin (H & E) and immunofluorescent staining as previously described (Zhao et al., 2010). The anti-Turbo GFP primary antibody was obtained from Axxora, LLC (San Diego, CA). The Alexa Fluor 546-labeled goat anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The stained sections were visualized with a Nikon Eclipse TE2000-U fluorescence microscope (Nikon, Inc., Melville, NY). The captured red fluorescent images (GFP positive staining) were overlaid with the blue fluorescent images (nucleus DAPI staining) using MetaMorph software (Molecular Devices Corp., Downington, PA).

**Statistical analysis.** The statistical analyses for the significance of differences in numerical data (means ± SDs) were performed using two-tailed t-tests for comparison of two data sets or one-way ANOVA for multiple data sets.

**RESULTS**

**Chronic Arsenic Exposure Causes Depletion of miRNA-200, Epithelial to Mesenchymal Transition and Malignant Transformation of Immortalized Human Bronchial Epithelial Cells**

To study the mechanism of arsenic-induced human cell malignant transformation and tumor formation, we used a pair of immortalized HBEcs: HBEcs (with normal p53 expression and function) and p53lowHBEcs (HBEcs with p53 levels stably knocked down by expression of a shRNA targeting p53) (Ramirez et al., 2004; Sato et al., 2006). The representative images of HBEcs and p53lowHBEcs and their p53 protein levels are shown in Supplementary figure 1A. Both HBEcs and p53lowHBEcs were continuously exposed to a low concentration of arsenite (NaAsO2, 2.5μM) or vehicle control (deionized H2O) for 16 weeks. Arsenite caused dramatic morphology alterations in p53lowHBEcs from epithelial to spindle-like mesenchymal morphology starting from 8 weeks of exposure (17.2 ± 3.9% cells) (Fig. 1A). The majority of p53lowHBEcs obtained the spindle-like mesenchymal morphology by 16 weeks of arsenite exposure (90.2 ± 4.5% cells). In contrast, similar arsenite exposure did not change the morphology of p53-intact HBEcs (Supplementary fig. 1B). Consistent with the mesenchymal morphology of arsenite-treated p53lowHBEcs, Western blot analysis showed that arsenite-induced expression of the mesenchymal marker vimentin in p53lowHBEcs starting from 8 weeks of exposure (Fig. 1B). The epithelial marker E-cadherin expression was completely lost in p53lowHBEcs exposed to arsenite for 12 weeks, indicating the occurrence of epithelial to mesenchymal transition (EMT). We then next examined the expression levels of EMT-inducing transcription factors including zinc-finger E-box-binding homeobox factor 1 (ZEB1), ZEB2, snail, slug, and twist (Peinado et al., 2007). ZEB1 and ZEB2 expression were not detectable by RT-PCR in control p53lowHBEcs but were detected in arsenite-exposed p53lowHBEcs starting from 8 weeks of exposure (Fig. 1C). Although the protein levels of snail, slug, and twist were readily detected in control p53lowHBEcs, their levels were not dramatically changed during the entire arsenite exposure period (data not shown). Soft agar colony
formation was used for initial assessment of whether 16-week arsenite exposure caused cell malignant transformation. Consistent with significant cellular morphology changes resulting from arsenite exposure, only p53<sup>low</sup>HBECs exposed to arsenite for 16 weeks (As-p53<sup>low</sup>HBECs) formed colonies in soft agar (Fig. 1D). Moreover, subcutaneous inoculation of As-p53<sup>low</sup>HBECs formed xenograft tumors in all five nude mice injected (Fig. 2E), whereas 0/5 mice injected with control
FIG. 2. Stably reexpressing miR-200b in arsenite-transformed cells completely reverses their transformed phenotype. Vector control and miR-200b stable expressing As-p53lowHBECs cells were generated by lentiviral transduction followed by FACS sorting as described in Materials and Methods. (A) Vector control (As-p53lowHBEC-GFP) and miR-200b stable expressing (As-p53lowHBEC-GFP-200b) cell representative images in bright field and bright field merged with fluorescent field. Cells were visualized and photographed with a Nikon Eclipse TE2000-U fluorescence microscope. The captured bright field images were overlaid with fluorescent field images (direct GFP fluorescence). Scale bar, 100 μm. (B) Stably reexpressing miR-200b in As-p53lowHBECs cells restores E-cadherin.
To investigate whether 16 weeks of arsenite exposure altered the miRNA expression profile, we first performed a miRNA expression microarray to detect differentially expressed miRNAs among parental HBECs, Control-HBECs, As-HBECs, parental p53lowHBECs, Control p53lowHBECs, and As-p53lowHBECs. Among 856 human miRNAs screened in the microarray, only three miRNAs (miR-200b, 200c, and 205) were downregulated more than twofold and one miRNA (miRNA-605) was upregulated more than twofold in arsenite-transformed cells (As-p53lowHBECs) (Supplementary table 1). The mir-200 family consists of five members in two clusters: mir-200b–200a–429 and mir-200c–141. Q-PCR analysis revealed that all five members of the miR-200 family were expressed in control p53lowHBECs but were depleted in arsenite-transformed cells (As-p53lowHBECs) (Fig. 1E and Supplementary table 2). Further Q-PCR analysis showed that 16-week arsenite exposure had no dramatic effect on the levels of miR-205 and 605. We then focused on the miR-200s in this study. Time-course studies revealed that arsenite dramatically decreased the levels of miR-200b and 200c, representative members from two clusters of the miR-200 family, in p53lowHBECs starting from 4 weeks and 8 weeks of exposure, respectively (Fig. 1F). To explore the potential clinical relevance of these findings, we determined the expression levels of the miR-200s in NHBEs and a panel of human lung cancer cells. It was found that the miR-200s are similarly expressed in NHBEs (Supplementary fig. 2) but are drastically depleted in 5/6 human lung cancer cell lines examined compared with immortalized HBECs (Fig. 1G). This suggests that loss of miR-200s expression may be a common occurrence in lung cell oncogenesis.

**Stably Reexpressing miR-200b in Arsenite-Transformed Cells Completely Reverses Their Transformed Phenotypes**

To determine whether loss of miR-200 expression plays a role in arsenite-induced cell transformation, we transiently reexpressed miR-200b, 200c, or both, by transfection of synthesized miR-200b and 200c precursors into arsenite-transformed cells (As-p53lowHBECs). miR-200b, 200c, or both restored the epithelial-like morphology and the expression of E-cadherin, although the expression of vimentin was not changed (Supplementary figs. 3A and 3B). Moreover, transiently reexpressing miR-200b, 200c, or both in As-p53lowHBECs all resulted in similar but significant reduction of colony formation in soft agar (Supplementary fig. 3C).

In order to determine whether loss of miR-200b or 200c expression plays a role in tumor formation by arsenite-transformed cells, we stably reexpressed miR-200b in arsenite-transformed cells (As-p53lowHBECs). We chose to stably reexpress miR-200b rather than miR-200c because although they have identical seed regions and largely the same predicted targets, miR-200b is downregulated by arsenite more rapidly than is miR-200c (Fig. 1E). The GFP lentiviral vector control and miR-200b stable expressing As-p53lowHBECs were generated by lentiviral transduction, followed by FACS and named As-p53lowHBEC-GFP and As-p53lowHBEC-GFP-200b, respectively. Representative images are shown in Figure 2A. Transduction with miR-200b-expressing lentiviral particles usually increased miR-200b levels by about 30–50-fold as determined by Q-PCR. Stably reexpressing miR-200b had no significant effect on viability and monolayer growth of As-p53lowHBECs on plastic. However, As-p53lowHBEC-GFP-200b cells restored E-cadherin expression, regained epithelial morphology, and had dramatically decreased expression of ZEB1 and ZEB2, although the level of vimentin was not significantly changed (Figs. 2A and 2B). As-p53lowHBEC-GFP cells exhibited similar soft agar colony formation capability to that of As-p53lowHBECs (Fig. 2C), but As-p53lowHBEC-GFP-200b cells completely lost their ability of forming colonies in soft agar, although they remained alive in soft agar as indicated by their ongoing expression of GFP (Figs. 2C and 2D). Moreover, all five nude mice injected subcutaneously with As-p53lowHBEC-GFP cells produced xenograft tumors (Fig. 2E). In sharp contrast, subcutaneous injection of As-p53lowHBEC-GFP-200b cells only formed scar-like tissues mainly consisting of fibrous components in 4 of 5 injected nude mice (Fig. 2F). This conclusion was further supported by extensive anti-GFP positive staining in xenograft tumors from.
mice inoculated with As-p53lowHBEC-GFP cells (Fig. 2G), but GFP positive staining was only detected in monolayer cells of glandular-like structures surrounded by fibrous components in xenograft tissues from mice injected with As-p53lowHBEC-GFP-200b cells (Fig. 2H). Together, these results indicate that stably reexpressing miR-200b alone in arsenite-transformed cells completely reverses their transformed phenotype, suggesting that loss of miR-200b plays a crucial role in arsenite-induced human cell malignant transformation and subsequent tumor formation.

Stably Expressing miR-200b in Parental Nontransformed Cells Completely Prevents Arsenite Exposure from Inducing EMT and Malignant Transformation

To further determine the role of miR-200b in arsenite-induced human cell malignant transformation, we next examined whether forcing expression of miR-200b in non-transformed cells could prevent cell transformation by arsenite exposure. We stably expressed miR-200b in parental non-transformed p53lowHBECs using the same miR-200b lentiviral transduction, followed by FACS sorting. The GFP vector control and miR-200b stable expressing nontransformed cells were named p53lowHBEC-GFP and p53lowHBEC-GFP-200b, respectively, and representative images are shown in Figure 3A. Q-PCR analysis confirmed the overexpression of miR-200b (about 30-fold increase), but the level of miR-200c was not changed in miR-200b stable expressing cells (Fig. 3B).

Continuous 16 weeks of arsenite exposure was then carried out in p53lowHBEC-GFP and p53lowHBEC-GFP-200b cells. Similar to the previously observed effect of arsenite exposure on parental p53lowHBECs, 16 weeks of arsenite exposure downregulated miR-200b and 200c and caused EMT and transformation of the vector control cells (p53lowHBEC-GFP) (Figs. 3C–F). In sharp contrast, arsenite exposure did not cause EMT or transformation of miR-200b stable expressing cells (p53lowHBEC-GFP-200b), which maintained their epithelial morphology, expressing high level of E-cadherin but no vimentin, ZEB1 or ZEB2 (Figs. 3C–E). Moreover, arsenite exposure did not change the expression levels of miR-200c in the p53lowHBEC-GFP-200b cells (Fig. 3F). These results indicate that forcing expression of miR-200b alone is sufficient to completely prevent arsenite-induced EMT and cell transformation.

Arsenite Exposure Depletes miR-200 Expression Through Induction of ZEB1 and ZEB2 and Methylation of miR-200 Promoters

We next investigated the mechanism by which arsenite exposure depletes miR-200 expression. Because arsenic is known to silence gene expression by causing DNA methylation (Arita and Costa, 2009; Hernandez et al., 2009), we first examined whether arsenite exposure caused methylation of miR-200 promoter regions. The methylation status of miR-200b–200a–429 and miR-200c–141 cluster promoters was determined using melt curve analysis, a rapid and cost-effective method to quantitate DNA methylation (Smith et al., 2009). Unlike the methylation-specific PCR assay, melt curve analysis of DNA methylation is quantitative and does not generate false positives (Smith et al., 2009). As shown in Figures 4A and 4B, both miR-200b–200a–429 and miR-200c–141 cluster promoters in arsenite-transformed cells (As-p53lowHBECs) were methylated because there was a shift to the right in the melt curves. Demethylation of As-p53lowHBECs with 5-aza-2'-deoxycytidine (5Aza) increased the expression of miR-200b and 200c by 1.6- and 1.3-fold, respectively (Fig. 4C). Similar demethylation induced the expression of E-cadherin as determined by Western blot in MDA-MB-231 cells. It was recently reported that ZEB1 and ZEB2 are not only targets of miR-200 but also repress the expression of the miR-200 genes, resulting in a double-negative feedback loop (Bracken et al., 2008; Burk et al., 2008). Because p53lowHBECs do not express ZEB1 and ZEB2, but arsenite greatly induced the expression of ZEB1 and ZEB2 starting from 8 weeks of exposure (Fig. 1C), we examined whether induction of ZEB1 and/or ZEB2 contributes significantly to the down-regulation of miR-200b and 200c by arsenite exposure. Knocking down the expression of ZEB1 or ZEB2 individually using ON-TARGETplus SMARTpool siRNA for ZEB1 or ZEB2 only slightly increased the expression of miR-200 promoter-luciferase reporter genes and the levels of miR-200b and 200c in arsenite-transformed cells (As-p53lowHBECs) (Figs. 5A and 5B). However, simultaneous knocking down both ZEB1 and ZEB2 together increased the promoter activity and expression levels of miR-200b and 200c in As-p53lowHBECs by 4- and 6-fold, respectively. Furthermore, simultaneously knocking down both ZEB1 and ZEB2 caused a significant decrease of colony formation by As-p53lowHBECs in soft agar (Fig. 5C). About 70–80% knockdown of ZEB1 and ZEB2 mRNA level was normally achieved (Fig. 5D). These results indicate that induction of ZEB1 and ZEB2 expression also plays a critical role in arsenite-caused downregulation of miR-200b and 200c and subsequent cell transformation.

Stably Expressing ZEB1 in Nontransformed Cells Depletes miR-200, Induces EMT, and Causes Cell Transformation

The role of ZEB1 expression in cell transformation was then further examined. We generated ZEB1 stable expressing p53lowHBECs by lentiviral (pLenti7.3-ZEB1) transduction, followed by FACS sorting. The pLenti7.3 vector control and ZEB1 stable expressing cells were named p53lowHBEC-pLenti7.3 and p53lowHBEC-pLenti7.3-ZEB1, respectively, and representative images are shown in Figure 6A. ZEB1 expression was confirmed by Western blot analysis (Fig. 6A). Stable expression of ZEB1 in p53lowHBECs induced EMT as evidenced by morphology changes (more than 90% of cells...
adopted a spindle-like morphology after sorting), complete loss of E-cadherin, and induction of vimentin expression (Fig. 6A). Q-PCR analysis revealed that ZEB1 expression caused similar depletion of miR-200 family to that following 16-week arsenite exposure (Fig. 6B). Furthermore, p53lowHBEC-pLenti7.3-ZEB1 cells showed similar anchorage-independent growth to that of arsenite-transformed cells (Fig. 6C). These results indicate that forcing expression of ZEB1 alone is sufficient to
deplete miR-200, cause EMT and transformation of p53-low-HBECs, phenocopying the effect of 16-week arsenite exposure. Consistent with our previous observations that arsenite exposure did not cause transformation of p53-intact HBECs, expression of ZEB1 in p53-intact HBECs (HBEC-pLenti-7.3-ZEB1) did not cause cell transformation, as HBEC-pLenti-7.3-ZEB1 cells did not form colonies in soft agar (data not shown).

DISCUSSION

The notion that miRNAs may play a role in tumors was first supported by studies showing that certain miRNAs are critically involved in the regulation of cellular differentiation, proliferation, and apoptosis (Miska, 2005). Although substantial evidence exists in support of a tumor suppressive effect for miRNAs, this concept is established largely based on the findings from studies using tumor cells and tissues. Our results reveal a causal role for loss of miR-200 expression in arsenic-caused malignant transformation of immortalized HBECs and subsequent tumor formation. These findings provide additional novel evidence indicating that individual miRNAs can function as tumor suppressors by suppressing carcinogen-caused human cell malignant transformation and tumor formation.

EMT refers to a program during normal embryonic development featuring a loss of epithelial properties, such as cell adhesion and expression of the epithelial marker E-cadherin and an acquisition of mesenchymal properties such as increased cell motility and expression of the mesenchymal marker vimentin (Lee et al., 2006). EMT is now widely viewed as an important step in tumor invasion and metastasis (Thiery et al., 2009). However, EMT has not been regarded to be involved in normal cell malignant transformation—the initiation of tumorigenesis. It was recently reported that loss of expression of miR-200 family members in tumor cells is associated with a mesenchymal morphology, and reexpressing miR-200s in tumor cells causes mesenchymal to epithelial transition (Gregory et al., 2008; Korpal et al., 2008). We found that chronic arsenite exposure can trigger the expression of the EMT-inducing transcription factors ZEB1 and ZEB2 and cause depletion of miR-200s, resulting in EMT and malignant transformation. Forcing expression of ZEB1 alone in non-transformed cells is sufficient to phenocopy the effect of 16 weeks of arsenite exposure, depleting miR-200s and inducing EMT and malignant transformation. Furthermore, stably expressing miR-200b is capable of abolishing ZEB1 expression, completely reversing and preventing EMT and cell malignant transformation resulting from arsenic exposure. Together, these results suggest that ZEB1 is not only the major factor that causes depletion of miR-200 but also the important miR-200b target that mediates EMT and normal cell malignant transformation resulting from arsenic exposure. It was recently reported that EMT generates cells with properties of stem cells (Mani et al., 2008) and ZEB1, which

![FIG. 4. Methylation status of miR-200 promoters in control and arsenite-transformed p53low-HBECs and effect of demethylation treatment on miR-200b and 200c levels in arsenite-transformed cells. (A and B) The promoters of miR-200b–200a–429 and miR-200c–141 clusters in arsenite-transformed cells (As-p53low-HBECs) are methylated. Genomic DNA from Control- and As-p53low-HBECs was isolated and bisulfite modified for measuring the methylation status of miR-200 promoters using melt curve analysis. The raw fluorescence data for the melt are normalized as described in Materials and Methods and presented as mean ± SEMs (n = 3). U ref, Bisulfite modified unmethylated DNA reference; M ref, Bisulfite modified methylated DNA reference. (C) Demethylation treatment partially increases the levels of miR-200b and 200c in arsenite-transformed cells. As-p53low-HBECs were treated with freshly prepared with 5-Aza-2'-deoxycytidine (5Aza) (2.5 or 5 μM) or vehicle control (dimethyl sulfoxide) for 72 h and then collected for total RNA extraction. MiR-200b and 200c levels were measured by Q-PCR and expressed relative to the vehicle control treatment (mean ± SDs, n = 3). Similar results were obtained in one additional experiment.](https://academic.oup.com/toxsci/article/121/1/110/1639247)
induces EMT, also promotes tumorigenicity of human pancreatic cancer cells (Wellner et al., 2009). It is thus highly likely that in addition to promoting tumor invasion and metastasis, ZEB1-mediated EMT may also be critically involved in the initiation of tumorigenesis by promoting cell malignant transformation.

It is interesting to note that arsenite exposure induces ZEB1 and ZEB2, depletes miR-200s, and causes EMT and cell malignant transformation only in p53-low HBECs but not in p53-intact HBECs. A recent study showed that arsenic acts specifically on p53-compromised human lung cancer cells and immortalized lung epithelial cells to induce centrosomal abnormalities and soft agar colony formation (Liao et al., 2007). Another recent study reported that expression of a mutant p53 was required to expand an EGF receptor-overexpressing esophageal cellular subpopulation capable of undergoing ZEB-mediated EMT (Ohashi et al., 2010). The findings from this and other recent studies suggest that loss of p53 function is essential for the expansion of epithelial cells that obtain mesenchymal characteristics through induction of ZEB expression. Perhaps induction of ZEB expression in epithelial cells with normal levels of p53 causes its activation, which in turn results in cell cycle arrest and/or apoptosis, thus limiting EMT and protecting against ZEB-induced transformation. Further studies are warranted to determine how p53 regulates ZEB-mediated EMT.

How does arsenic exposure cause depletion of miR-200? Although abnormal miRNA expression is common in human tumors, the detailed underlying mechanisms of miRNA deregulation are still largely unknown. It has been recently reported that increased DNA methylation plays important roles in silencing the expression of the miR-200 family and miR-205 (Vrba et al., 2010; Wiklund et al., 2011), and decreased DNA methylation is associated with overexpression of miR-200a and miR-200b (Li et al., 2010). We found that arsenic exposure depletes the miR-200s through induction of ZEB1 and ZEB2 and DNA methylation. Although the mechanism of ZEB1 and ZEB2 induction by arsenic exposure remains to be determined, our findings have important implications in metal carcinogenesis. It is well accepted that arsenic is not a typical carcinogen and probably exerts its carcinogenic effect mainly through altering cell signaling and gene expression (Hernandez et al., 2009;
It is thus likely that a similar mechanism of depleting the expression of miR-200s may also be exerted by other nontypical metal carcinogens such as nickel and cadmium. Further studies to determine the mechanism by which arsenite exposure induces the expression of ZEB1 and ZEB2 are warranted.

These findings potentially have important clinical implications. Our results and other recent findings (Ceppi et al., 2010; Gibbons et al., 2009) reveal that loss of miR-200b and 200c expression is common in human lung cancer cells. Because miR-200b and miR-200c are highly expressed in normal HBECs and overexpressing miR-200b showed no toxic effect on normal HBEC viability and proliferation, our finding that reexpression of miR-200b completely reverses arsenite-induced cell transformation, and tumor formation provides a good rationale to explore the therapeutic effect of miR-200b for lung tumors caused by arsenic exposure and probably by other exposures. Indeed, it was recently reported that forcing expression of the miR-200b cluster in lung tumor cells derived from mice that develop metastatic lung adenocarcinoma owing to expression of mutant K-ras and p53 abrogated the capacity of these tumor cells to undergo EMT, invade, and metastasize (Gibbons et al., 2009). Our findings establish a causal role for loss of miR-200b expression in arsenic-caused human lung cell malignant transformation and tumor formation, suggesting that miR-200b may be a useful therapeutic target for lung cancer resulting from arsenic exposure.

Supplementary data are available online at http://toxsci.oxfordjournals.org/

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