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 big family 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 (p53^{low}HBECs) to low levels of arsenite (NaAsO₂, 2.5 μ M) 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-p53^{low}HBECs) 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 p53^{low}HBECs 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 p53^{low}HBECs 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.

Arsenic is one of the most common environmental pollutants, and human arsenic exposure mainly occurs through contaminated drinking water. Arsenic levels in drinking water often exceed by many folds the World Health Organization's

recommendation, affecting tens of millions of people in the United States and many other countries (States *et al.*, 2009; Tapio and Grosche, 2006). Studies have shown that long-term arsenic exposure through drinking water is associated with increased risks of skin, lung, bladder, liver, and prostate cancer (Benbrahim-Tallaa and Waalkes, 2008; Celik *et al.*, 2008; Liu and Waalkes, 2008; Tapio and Grosche, 2006). Despite the recognition that arsenic is a human carcinogen, the mechanism by which it causes cancer has not been elucidated. Although the proposed mechanisms for arsenic carcinogenesis include oxidative stress, DNA repair inhibition, and genotoxic effects (Beyersmann and Hartwig, 2008; Kitchin and Conolly, 2010), accumulating evidence suggests that nongenotoxic modifications that lead to aberrant gene expression may play crucial roles in arsenic carcinogenesis (Arita and Costa, 2009; Hernandez *et al.*, 2009; Ren *et al.*, 2011).

MicroRNAs (miRNAs) are a large class of small noncoding RNAs that regulate protein-coding gene expression posttranscriptionally by interacting with messenger RNAs (mRNAs) and inducing translation suppression and mRNA degradation (Hobert, 2008). The interaction of miRNAs with mRNAs occurs through imperfect base pairing between the miRNA and mRNA 3'-untranslated region, principally involving the seed region at the 5' end of the miRNA. Consequently, a single miRNA is usually capable of negatively regulating the expression of many genes. Recent studies reveal distinct miRNA expression signatures in a variety of human tumors, with expression levels of many miRNAs being reduced in tumor cells and tissues (Calin and Croce, 2006; Gaur *et al.*, 2007). Moreover, it was also found that decreasing miRNA levels by knocking down miRNA-producing molecules in tumor cells enhances their colony formation in soft agar and tumorigenic effect in mice (Kumar *et al.*, 2007). Together, these findings suggest that systemic loss of miRNA expression in tumor cells may play important roles in tumor progression. Nevertheless, it remains to be determined whether loss of

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 μ M 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^{low}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^{low}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^{low}HBECs form colonies in soft agar or produce tumors in nude mice, which makes HBECs and p53^{low}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^{low}HBECs were cultured in chemically defined serum-free medium (K-SFM) (Invitrogen, Carlsbad, CA) supplemented with 20 μ g/ml of bovine pituitary extract (BPE) and 0.8 μ g/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^{low}HBECs were continuously exposed to Vehicle Control (deionized H₂O) or 2.5 μ M of arsenite (NaAsO₂, S225I-100, Fisher Scientific, Fair Lawn, NJ), re-

spectively. 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 μ g/ml) but no EGF at a concentration of 5×10^4 cells/ml. Normal melting point agar (5 ml of 0.6% agar in K-SFM containing 10% FBS and BPE), (20 μ g/ml) but no EGF was placed into each 60-mm tissue culture dish as the bottom agar. After solidification of the bottom agar, 4 ml of cell mixture consisting of 2 ml of cell suspension (5×10^4 cells/ml) and 2 ml of 0.8% lower melting point agar in K-SFM containing BPE (20 μ g/ml) but no EGF were poured over the bottom agar. After solidification of the upper agar, 3 ml of K-SFM containing BPE (20 μ g/ml) (no EGF and no FBS) 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 μ m) under a phase-contrast microscope after 4-week incubation.

Nude mice xenograft tumorigenesis studies. Arsenite-transformed cells stably expressing green fluorescence protein without (As-p53^{low}HBECs-GFP) or as well as miRNA-200b (As-p53^{low}HBECs-GFP-200b) (5×10^6 of each cells in 0.1 ml of 1:1 growth factor-reduced matrigel) were injected subcutaneously into the right flank of nude female mice (Nu/Nu, Charles River laboratories), five mice in each group. Animals were maintained under specific pathogen-free conditions, and animal protocols were reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee. All mice were euthanized 12 weeks after injection, and the xenograft tissues were harvested and fixed with 10% formalin solution for histology analysis and immunofluorescent staining.

MiRNA microarray and quantitative PCR analysis of miRNAs. Total RNAs from HBECs and p53^{low}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 miRBase 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 0.5 μ M of each primer in a final reaction volume of 15 μ l. The primers and PCR conditions were specific for bisulfite-modified DNA and did not amplify unmodified 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'-GTTTATGGGAGTTTATAGGGGAT-3'; miR200b reverse, 5'-AAACT-CRCCTTACAAAAACAATAC-3'; miR-200c~141 (miR-200c) forward, 5'-GGTAGTTTATGGTAGGAGGATA-3'; miR-200c reverse 5'-AAAA-CRAAAAAACTTTAAACCCCAA-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 the first 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 transduced 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 Dharmacon (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 as described above. 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 SuperScript™ II RT (Invitrogen). The resulting cDNA was used for PCR amplification using the following primers: ZEB1 forward, 5'-GCACCTGAAGAGGACCAGAG-3'; ZEB1 reverse, 5'-GTGTAAGTGCACAGGGAGCA-3'; ZEB2 forward, 5'-CGCTTGACATCACTGAAGGA-3'; ZEB2 reverse, 5'-CTTGCCACACTCTGTGCATT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-CCCTTCATTGACCTCAACTACATGG-3'; and GAPDH reverse, 5'-CATGGTGGTGAAGACGCCAG-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., Downingtown, 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 p53^{low}HBECs (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 p53^{low}HBECs and their p53 protein levels are shown in Supplementary figure 1A. Both HBECs and p53^{low}HBECs 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 p53^{low}HBECs from epithelial to spindle-like mesenchymal morphology starting from 8 weeks of exposure (17.2 ± 3.9% cells) (Fig. 1A). The majority of p53^{low}HBECs 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 p53^{low}HBECs, Western blot analysis showed that arsenite-induced expression of the mesenchymal marker vimentin in p53^{low}HBECs starting from 8 weeks of exposure (Fig. 1B). The epithelial marker E-cadherin expression was completely lost in p53^{low}HBECs 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 p53^{low}HBECs but were detected in arsenite-exposed p53^{low}HBECs starting from 8 weeks of exposure (Fig. 1C). Although the protein levels of snail, slug, and twist were readily detected in control p53^{low}HBECs, their levels were not dramatically changed during the entire arsenite exposure period (data not shown). Soft agar colony

FIG. 1. Sixteen-week exposure to low concentration of arsenite (As) causes depletion of miRNA-200, EMT, and malignant transformation of HBECs. HBECs and p53^{low}HBECs were continuously exposed to arsenite (2.5 μ M of NaAsO₂) for 16 weeks to induce cell transformation. (A) Representative images of p53^{low}HBECs exposed to vehicle control (deionized H₂O) or arsenite for 4, 8, 12, or 16 weeks. Scale bar, 100 μ m. (B and C) Time-course of arsenite exposure depletes the expression of E-cadherin and induces the expression of vimentin and ZEB1/ZEB2. E-cadherin, vimentin, and p53 levels were determined by Western blot (B). ZEB1 and ZEB2 levels were detected by RT-PCR (C). (D) Soft agar colony formation analysis for HBECs and p53^{low}HBECs exposed to control vehicle or arsenite for 16 weeks, respectively (mean $\pm$ SDs, $n = 3$). (E and G) Q-PCR analysis of miR-200 expression levels in Control- and arsenite-transformed p53^{low}HBECs (E) and a panel of human lung cancer cells (G). The miR-200 levels were expressed relative to nontransformed HBECs (mean $\pm$ SDs, $n = 3$). (F) Time-course of arsenite exposure causes depletion of miR-200b and 200c. The expression levels of miR-200b and 200c were measured by Q-PCR and expressed relative to the control cells at each time point (mean $\pm$ SDs, $n = 3$). Similar results were obtained in two additional experiments.

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^{low}HBECs exposed

to arsenite for 16 weeks (As-p53^{low}HBECs) formed colonies in soft agar (Fig. 1D). Moreover, subcutaneous inoculation of As-p53^{low}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-p53^{low} HBECs cells were generated by lentiviral transduction followed by FACS sorting as described in Materials and Methods. (A) Vector control (As-p53^{low} HBEC-GFP) and miR-200b stable expressing (As-p53^{low} HBEC-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 μ m. (B) Stably reexpressing miR-200b in As-p53^{low} HBECs cells restores E-cadherin

p53^{low}HBECs or arsenite-treated p53-intact HBECs formed tumors. Together, these results indicate that chronic arsenite exposure causes EMT and malignant transformation of p53^{low}HBECs but not of p53-intact HBECs.

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 p53^{low}HBECs, Control p53^{low}HBECs, and As-p53^{low}HBECs. 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-p53^{low}HBECs) (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 p53^{low}HBECs but were depleted in arsenite-transformed cells (As-p53^{low}HBECs) (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 p53^{low}HBECs 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 NHBECs and a panel of human lung cancer cells. It was found that the miR-200s are similarly expressed in NHBECs (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-p53^{low}HBECs). 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-p53^{low}HBECs 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-p53^{low}HBECs). 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 stably expressing As-p53^{low}HBECs were generated by lentiviral transduction, followed by FACS and named As-p53^{low}HBEC-GFP and As-p53^{low}HBEC-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-p53^{low}HBECs on plastic. However, As-p53^{low}HBEC-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-p53^{low}HBEC-GFP cells exhibited similar soft agar colony formation capability to that of As-p53^{low}HBECs (Fig. 2C), but As-p53^{low}HBEC-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-p53^{low}HBEC-GFP cells produced xenograft tumors (Fig. 2E). In sharp contrast, subcutaneous injection of As-p53^{low}HBEC-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

expression (determined by Western blot), decreases the levels of ZEB1 (determined by Western blot), and ZEB2 (measured by RT-PCR) but has no effect on vimentin levels (determined by Western blot). (C) Stably reexpressing miR-200b in As-p53^{low}HBECs completely inhibits their soft agar colony formation capability (mean $\pm$ SDs, $n = 3$). (D) Representative bright field, and bright field merged with fluorescent field images of As-p53^{low}HBEC-GFP and As-p53^{low}HBEC-GFP-200b cells cultured in soft agar for 4 weeks. At the end of 4-week culture, cells or colonies in soft agar were visualized and photographed with a fluorescence microscope. The captured bright field images were overlaid with fluorescent field images (direct GFP fluorescence). Scale bar, 100 μ m. Similar results were obtained in two additional experiments. (E–H) Stably reexpressing miR-200b inhibits xenograft tumor formation by arsenite-transformed cells in nude mice. Five million of As-p53^{low}HBEC-GFP or As-p53^{low}HBEC-GFP-200b cells were injected subcutaneously into right flank of five female nude mice as described in Materials and Methods. Twelve weeks after injection, mice were euthanized. Xenograft tissues were harvested and fixed in 10% formalin solution for histology and pathology examinations. (E and F) Representative H & E staining histology images of xenograft tumor tissues (E) or scar-like tissues (F) produced from nude mice injected with As-p53^{low}HBEC-GFP (E) or As-p53^{low}HBEC-GFP-200b cells (F), respectively. (G and H) The overlaid fluorescent images from anti-GFP immunofluorescent staining (red color) and nucleus DAPI staining (blue color) in xenograft tissues formed from As-p53^{low}HBEC-GFP (G) or As-p53^{low}HBEC-GFP-200b (H) injection, respectively. The arrow in panel (F) points to the fibrous components.

mice inoculated with As-p53^{low}HBEC-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-p53^{low}HBEC-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 p53^{low}HBECs using the same miR-200b lentiviral transduction, followed by FACS sorting. The GFP vector control and miR-200b stable expressing nontransformed cells were named p53^{low}HBEC-GFP and p53^{low}HBEC-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 p53^{low}HBEC-GFP and p53^{low}HBEC-GFP-200b cells. Similar to the previously observed effect of arsenite exposure on parental p53^{low}HBECs, 16 weeks of arsenite exposure downregulated miR-200b and 200c and caused EMT and transformation of the vector control cells (p53^{low}HBEC-GFP) (Figs. 3C–F). In sharp contrast, arsenite exposure did not cause EMT or transformation of miR-200b stable expressing cells (p53^{low}HBEC-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 p53^{low}HBEC-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-p53^{low}HBECs) were methylated because there was a shift to the right in the melt curves. Demethylation of As-p53^{low}HBECs 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 p53^{low}HBECs 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-p53^{low}HBECs) (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-p53^{low}HBECs by 4- and 6-fold, respectively. Furthermore, simultaneously knocking down both ZEB1 and ZEB2 caused a significant decrease of colony formation by As-p53^{low}HBECs 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 p53^{low}HBECs by lentiviral (pLenti7.3-ZEB1) transduction, followed by FACS sorting. The pLenti7.3 vector control and ZEB1 stable expressing cells were named p53^{low}HBEC-pLenti7.3 and p53^{low}HBEC-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 p53^{low}HBECs induced EMT as evidenced by morphology changes (more than 90% of cells

FIG. 3. Stably expressing miR-200b in parental nontransformed cells completely prevents arsenite exposure from inducing EMT and malignant transformation. Vector control and miR-200b stable expression nontransformed p53^{low}HBECs were generated by lentiviral transduction followed by FACS sorting as described in Materials and Methods. (A) Vector control (p53^{low}HBEC-GFP) and miR-200b stable expression (p53^{low}HBEC-GFP-200b) cell representative images in bright field, and bright field merged with fluorescent field. Cells were visualized and photographed with a fluorescence microscope. The captured bright field images were overlaid with fluorescent field images (direct GFP fluorescence). Scale bar, 100 μm. (B) Q-PCR analysis of miR-200b and 200c levels in Control and miR-200b stable expression p53^{low}HBECs. MiR-200b and 200c levels were expressed relative to the control cells (mean ± SDs, $n = 3$). (C–F) Stably expressing miR-200b in p53^{low}HBECs completely prevents arsenite exposure inducing EMT, cell transformation, and depletion of miR-200b and 200c. Representative images of p53^{low}HBEC-GFP and p53^{low}HBEC-GFP-200b cells exposed to vehicle control or arsenite (2.5 μM) for 16 weeks, respectively (C). Western blot analysis of E-cadherin, ZEB1, and vimentin levels and RT-PCR determination of ZEB2 level in p53^{low}HBEC-GFP and p53^{low}HBEC-GFP-200b cells exposed to vehicle control or arsenite (2.5 μM) for 16 weeks, respectively (D). Soft agar colony formation analysis for p53^{low}HBEC-GFP and p53^{low}HBEC-GFP-200b cells exposed to vehicle control or arsenite (2.5 μM) for 16 weeks, respectively (mean ± SDs, $n = 3$) (E). Q-PCR analysis of miR-200b and 200c levels in p53^{low}HBEC-GFP and p53^{low}HBEC-GFP-200b cells exposed to vehicle control or arsenite (2.5 μM) for 16 weeks, respectively (F). MiR-200b and 200c levels were expressed relative to the control cells (mean ± SDs, $n = 3$). Similar results were obtained in two additional experiments.

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, p53^{low}HBEC-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

FIG. 4. Methylation status of miR-200 promoters in control and arsenite-transformed p53^{low}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-p53^{low}HBECs) are methylated. Genomic DNA from Control- and As-p53^{low}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 $\pm$ 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-p53^{low}HBECs were treated with freshly prepared with 5-Aza-2'-deoxycytidine (5Aza) (2.5 or 5 μ M) 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 $\pm$ SDs, $n = 3$). Similar results were obtained in one additional experiment.

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-200b 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; Korpai *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. 5. Knocking down ZEB1 and ZEB2 in arsenite-transformed cells increases miR-200b/200c promoter activity and expression levels and attenuates soft agar colony formation. Effect of knocking down ZEB1, ZEB2, or both in arsenite-transformed cells (As-p53^{low}HBEcs) on the promoter activity (A) and expression levels (B) of miR-200b/200c and soft agar colony formation (C) (mean $\pm$ SDs, $n = 3$). The efficiency of ZEB1 and ZEB2 knockdown was analyzed by RT-PCR (D). ZEB1 and ZEB2 knockdown procedures were described in detail in Materials and Methods. MiR-200b/200c promoter luciferase activity and expression levels were measured using dual luciferase reporter assay and Q-PCR, respectively, and expressed relative activity/levels to the Control siRNA group. Similar results were obtained in two additional experiments.

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 finding has 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;

FIG. 6. Stably expressing ZEB1 in nontransformed cells depletes miR-200s, induces EMT, and causes cell transformation. The p53^{low}HBECs stably expressing human full-length ZEB1, and vector control cells were generated by lentiviral transduction followed by FACS sorting as described in Materials and Methods. (A) Representative images of vector control (p53^{low}HBEC-pLenti7.3) and ZEB1 stable expressing (p53^{low}HBEC-pLenti7.3-ZEB1) cells and their protein levels of ZEB1, E-cadherin, and vimentin were determined by Western blot. Scale bar, 100 μ m. (B) Q-PCR analysis of miR-200 levels in p53^{low}HBEC-pLenti7.3 and p53^{low}HBEC-pLenti7.3-ZEB1 cells. MiR-200 levels were expressed relative to the p53^{low}HBEC-pLenti7.3 cells (mean $\pm$ SDs, $n = 3$). (C) Soft agar colony formation assay for p53^{low}HBEC-pLenti7.3 and p53^{low}HBEC-pLenti7.3-ZEB1 cells (mean $\pm$ SDs, $n = 3$). Similar results were obtained in two additional experiments.

Ren *et al.*, 2011). 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

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

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