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Role of miR-31 and SATB2 in arsenic-induced malignant BEAS-2B cell transformation

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Abstract

Arsenic is a naturally occurring and highly potent metalloid known to elicit serious public health concerns. Today, approximately 200 million people around the globe are exposed to arsenic-contaminated drinking water at levels greater than the World Health Organization's recommended limit of 10 parts per billion. As a class I human carcinogen, arsenic exposure is known to elicit various cancers, including lung, skin, liver, and kidney. Current evidence suggests that arsenic is capable of inducing both genotoxic and cytotoxic injury, as well as activating epigenetic pathways to induce carcinogenesis. Our study identifies a novel pathway that is implicated in arsenic-induced carcinogenesis. Arsenic down-regulated miRNA-31 and the release of this inhibition caused overexpression of special AT-rich sequence-binding protein 2 (SATB2). Arsenic is known to disrupt miRNA expression, and here we report for the first time that arsenic is capable of inhibiting miR-31 expression. As a direct downstream target of miR-31, SATB2 is a prominent transcription factor and nuclear matrix binding protein implicated in many types of human diseases including lung cancer. Results from this study show that arsenic induces the overexpressing SATB2 by inhibiting miR-31 expression, which blocks the translation of SATB2 mRNA, since levels of SATB2 mRNA remain the same but protein levels decrease. Overexpression of SATB2 induces malignant transformation of human bronchial epithelial (BEAS-2B) cells indicating the importance of the expression of miR-31 in preventing carcinogenesis by suppressing SATB2 protein levels.

Keywords

Carcinogenesis; metals; human bronchial epithelial cells; non-coding RNAs

Introduction

As a ubiquitous metalloid highly carcinogenic in its inorganic form, arsenic occurs naturally in the Earth's crust. In addition to corrosion from rocks and mineral ores, anthropogenic sources of arsenic including pesticides, combustion of fossil fuel, as well as chemical and industrial wastes from mining, all contribute to the exacerbation of world wide arsenic contamination. As a potent human carcinogen, the World Health Organization's maximum recommended limit for arsenic in drinking water is set at 10 parts per billion (ppb).

However, alarmingly high levels of arsenic that often exceed these levels in drinking water have been detected in various parts of the world including, Bangladesh, Pakistan, the French Mediterranean coast, China, Mexico, United States, etc. (Brinkel et al., 2009). Even though most people are exposed via contaminated drinking water, inhalation of toxic dusts in occupational settings such as nickel-refineries and ingestion of food containing arsenic are also prominent exposure routes (Axelson et al., 1978; Carpenter and Jiang, 2013; Enterline et al., 1987).

Today, approximately 200 million people around the globe are exposed to potentially toxic levels of arsenic. Arsenic is an IARC Class I human carcinogen, chronic arsenic exposure has been implicated in serious human cancers including cancers of the lung, skin, kidney, urinary bladder and liver (Bjorklund et al., 2017). Extended research efforts have been carried out to elucidate the mechanisms responsible for arsenic-induced carcinogenesis. Current evidence suggests that arsenic is capable of causing cancer through both genotoxic and cytotoxic, as well as epigenetic pathways (Huang et al., 2004). Here we report that arsenic induces malignant transformation in human bronchial epithelial (BEAS-2B) cells by inhibiting miRNA-31 expression, which subsequently leads to the overexpression of special AT-rich sequence-binding protein 2 (SATB2) to levels that can induce malignant transformation of BEAS2B cells.

SATB2 resides in the gene poor region, 2q32-q33, and was first isolated from human fetal brain cDNA library as a part of the Human Unidentified Gene-Encoded (HUGE) protein database project (Kikuno et al., 2002; Fitzpatric et al., 2003). SATB2 is a transcription factor critically important for the biological development of various tissues such as osteoblast, as well as differentiation of neurons and stem cells (FitzPatric et al. 2003; Dobрева et al. 2006; Britanova et al. 2006; Alcamo et al. 2008; Britanova et al. 2008; Savarese et al. 2009; Zhao et al. 2014; Zarate and Fish, 2017). In addition, SATB2 is also considered a nuclear matrix attachment region (MAR) binding protein (FitzPatric et al., 2003). Nuclear matrix attachment regions contain numerous AT sequences that can non-specifically bind to the minor groove of the target DNA. Through its structural recognition, MARs can regulate gene expression and higher-order chromatin states by having multiple binding sites that target more than one gene (Bode et al. 1992; Strick and Laemmli 1995; Laemmli and Tjian 1996; Strick et al. 2000; Patani et al. 2009). As a prominent MAR-binding protein, the ability for SATB2 to bind to AT-rich DNA sequences allows for its interaction with specific metastasis-associated proteins as well as histone acetylases and deacetylases, which can in turn influence epigenetic signals and overall genome stability (Gyorgy et al. 2008; Zhang et al. 2011). The levels of SATB2 expression vary by tissue type. In fact, an extensive immunohistochemistry-based screening of 65 different types of human cells showed that the

highest expression of SATB2 was found in the glandular cells of the gastrointestinal tract and neuronal cells of the cerebral cortex and hippocampus (Magnusson et al. 2011; Zhao et al. 2014, Ordonez 2014). While expression levels are more conserved in the epididymis and lymphoid cells, most other types of cells such as those of the lung, breast, stomach, thyroid, etc. showed no SATB2 expression. Interestingly, while SATB2 is not expressed in BEAS-2B cells, its mRNA and protein expression will significantly increase following arsenic exposure. The critical roles of SATB2 in multiple tissue development, differentiation, and genomic stability prompted an investigation into its potential role in carcinogenesis.

Recent studies have linked the possible role of SATB2 in heavy metal-induced cell transformation (Clancy et al., 2012; Wu et al., 2016). In fact, microarray analysis suggests that SATB2 is the only gene found up regulated in all 4 heavy metal-induced (arsenic, nickel, chromium, and vanadium) BEAS-2B transformed cells (Clancy et al., 2012). However, existing studies show that SATB2 can exert both oncogenic and tumor suppressive functions, depending on the type of tumor tissue or cell line (Wang et al., 2009; Magnusson et al., 2011; Yang et al., 2014; Jiang et al. 2015; Mansour et al. 2015; Fukuhara et al. 2016; Wu L et al., 2016; Ma et al., 2017; Tian et al., 2017; Yu et al., 2017). Overall, the finding that SATB2 expression is induced by multiple heavy metals as well as its contrasting functions in different cancer tissues and cell lines lead to an examination of its role in arsenic-induced BEAS-2B cell transformation.

In the past two decades, mounting epigenetic studies have focused on non-coding RNAs (ncRNAs). With only 2% of the transcribed genome being actively translated into proteins from transcribed mRNA, researchers are finding that small ncRNAs such as endogenous small interfering RNAs (endo-siRNAs), PIWI-interacting RNAs (piRNAs), and microRNAs (miRNAs) play important roles in gene regulation and overall genome stability (Khanduja et al., 2016). In this paper, we specifically focus on the role of miRNAs in arsenic-induced carcinogenesis. Endogenous miRNAs are approximately 22 nucleotides long, single-stranded, and were first discovered in *Caenorhabditis elegans* (Lee et al., 1993; Vrijens et al., 2015). In eukaryotes, miRNAs are responsible for many physiological processes including cell growth, differentiation, and stress responses (VanRooji et al., 2007; Xiao et al., 2007). MicroRNA is a type of regulatory RNA that silences gene expression through post-transcriptional regulation. Specifically, miRNAs function by base pairing with the target mRNA noncoding sequences at the 5' end, and subsequently promote the degradation of the targeted mRNA (Ambros 2004). In consideration of their biological and epigenetic importance, disruptions in miRNA expression have been implicated in various diseases including cardiovascular disorders and cancer (Calin et al., 2005). Several studies have reported that arsenic exposure causes changes in miRNA expression (Marsit et al., 2006; Sturchio et al., 2014; Ren et al., 2016; Beezhold et al., 2017). Examples of arsenic-deregulated miRNAs include miR-29, -183, -192, etc. (Ren et al., 2016; Beezhold et al., 2017). Here we report for the first time that arsenic is able to trigger the down-regulation of miR-31, a known negative regulator of SATB2 (Aprelikova et al., 2010; Deng et al., 2013; Brocato and Costa, 2015). Using human bronchial epithelial cells, our study provides a novel mechanism that illustrates the functional roles of miR-31 in suppressing SATB2 expression and thereby preventing cell transformation.

Materials and methods

Cell culture

Immortalized human bronchial epithelial (BEAS-2B) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BEAS-2B cells were adapted to serum growth immediately after their purchase and have been carefully maintained. The cells were recently tested through Genetica DNA Laboratories and proved to be 100% authentic against a reference BEAS-2B cell line (Burlington, NC). Cells are cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 1% penicillin/streptomycin (GIBCO, Grand Island, NY) and 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA). BEAS2B cells were treated with 2 uM sodium meta-arsenite (NaAsO₂) for 6 weeks and transformed colonies were selected by their growth in soft agar. Doses of acute arsenic treatment range from 0 to 10 uM for 48-h. All cells were cultured in a 37°C incubator containing 5% CO₂. BEAS2B cells were authenticated by Genetica DNA Laboratories (Burlington, NC) on July 22, 2015. Cells were matched 100% to 15 short tandem repeat (STR) loci and amelogenin to the reference profile of BEAS-2B (ATCC CRL-9609).

Stable transfection of plasmid into cells

Normal BEAS2-B cells and arsenic transformed-BEAS2-B clones were cultured in DMEM supplemented with 10% FBS and 1% penicillin. SATB2 shRNA constructs (TG301833C) and scramble control shRNA plasmid (TR30013) were purchased from OriGene (Rockville, MD) (Cartularo et al., 2016). The miR-31 overexpression (HmiR0138-MR04-10) and scramble negative control plasmids (CmiR0001-MR04) were purchased from GeneCopoeia (Rockville, MD, USA).

Plasmids were purified using a Qiagen QIAprep Spin Midiprep kit prior to transfection. Knockdown transfections were performed using Lipofectamine® LTX Reagent with PLUS reagent (Invitrogen) following the manufacturer's protocol. Briefly, 150,000 cells were seeded into 6-well dishes 24 hours prior to transfection. The following day, 1 ug of purified plasmid was transfected into each well using 10 µL of Lipofectamine LTX and 2.5 µL of PLUS reagent per transfection. 24 hours post-transfection, the media was removed and replaced with fresh DMEM. After three days, 0.5 µg/ml of puromycin selection agent was added to the transfected cells. The cells were grown under selection for three weeks and harvested for western blot and qPCR analysis to test for knockdown efficiency.

Western blotting

Cells were lysed with boiling buffer (1% SDS, 10 mM Tris (pH 7.4), 1 mM sodium orthovanadate) and 50 µg of whole cell lysate were separated by 12% SDS-PAGE and transferred to a PVDF membrane. After blocking in 5% skim milk in TBST for 1 h at room temperature, the membrane was incubated with SATB2 mouse monoclonal antibody (Abcam, Cambridge, MA., ab51502, 1:100) overnight at 4 °C, and then probed with HRP labeled goat anti mouse secondary antibody (1:2000) for 1 h at room temperature before the visualization by the chemiluminescence. Quantification of immunodetected proteins was performed using Image J software.

Quantitative Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen) and subsequently synthesized into single-stranded cDNA using ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs) in accordance with manufacturer's instructions. Quantitative real time PCR analysis was performed using Power SYBR Green PCR Master Mix (Qiagen) on the ABI PRISM 7900HT system. All experiments were performed in triplicates. Relative gene expression levels were normalized to β -actin expression. The SATB2 primers used are as follows: forward, 5'-TCTCCCCAAACACACCATCA-3'/ reverse, 5'-GCAGCTCCTCGTCCTTATATTCA-3'. Beta-actin primers used are as follows: forward, 5'-ACCAGTTCGCCATGGATGAC-3'/ reverse, 5'-TGCCGGAGCCGTTGTC-3'.

Anchorage independent growth assay

Cells were rinsed with PBS to remove the metal from the media then seeded in low gelling temperature Agarose Type VII (Sigma Aldrich, St. Louis, MO). 5000 cells were seeded in triplicate in 6-well plates in a top layer of 0.35% agarose onto a bottom layer of 0.5% agarose. Cells were allowed to grow for four weeks until individual colonies were large enough to select from the agar. Colonies were picked from each treatment and control group. These colonies were grown out into monolayers for four weeks. After monolayer growth, cells were collected in Trizol for RNA and sequencing (RNA-seq) or quantitative real-time PCR (qRT-PCR). A second set of plates was stained with INT/BCIP solution (Roche Diagnostics, Indianapolis, IN) for visualization and quantification of colony growth in agar, according to the manufacturer's protocol.

Transwell Cell Invasion assay

For cell invasion analysis, Biocoat Matrigel® Invasion Chambers (Corning, Corning, NY) were incubated with DMEM containing 0.1% FBS at 37°C for 3-h. BEAS-2B cells were trypsinized, centrifuged, and washed to remove excess FBS. 5×10^4 cells were resuspended with 400 μ L of 0.1% DMEM. The cells containing 0.1% DMEM were seeded in wells containing 10% DMEM and grown for 24-h in a 37°C incubator containing 5% CO₂. The migrated cells were fixed with 3.5% formaldehyde for 5 minutes at room temperature and subsequently incubated with methanol for 20 minutes. The wells were then incubated with 5% Giemsa solution overnight at room temperature and washed with water the next day for observation under the microscope.

Wound healing assay

Cells (5×10^5) were plated into 6-well plates with 2×2 mm grids (Nunc) on the bottom. Upon reaching confluency, a single scratch was made across the monolayer using a 200 μ L pipette tip held perpendicular to the plate bottom. The plate was then gently washed with medium to remove detached cells, and fresh DMEM media containing 10% of FBS were added back into the plates. Images were acquired at 0 and 24 h after the scratch using a Nikon digital DS-Fi1-U3 camera unit controlled by NIS-Elements F3.2 software on an Nikon Eclipse TS100 microscope (Nikon Instruments Inc., Tempe, AZ).

Statistical analysis

Image J image processing software (National Institute of Health) was used to quantify western blotting gel intensities. All statistical significance was calculated and assessed using unpaired, 2 tailed t-test, where * indicates $p < 0.05$ and ** indicates $P < 0.01$.

Results

Arsenic exposure promotes anchorage-independent growth, migration, and overexpression of SATB2

Exposure to arsenic has been shown to induce malignant transformation, and a number of studies have demonstrated its ability to transform human bronchial epithelial cells (Stueckle et al., 2012). While the amount of time and concentration required for malignant transformation may differ by cell type (Chen and Costa, 2017), here we report that 6 weeks of 2 μ M arsenic exposure was sufficient to induce anchorage-independent growth in BEAS-2B cells (Fig 1A). Of note, two micromolar of arsenic is equivalent to 150 ppb, which are similar to levels found in the environment (Kile et al., 2014; Brocato et al., 2015;). As shown in Fig 1B, arsenic-transformed BEAS-2B cells showed significantly more colony growth in soft agar compared to the control group. In addition to anchorage-independent growth, we also examined the rate of cell migration using As-transformed cells selected from soft agar colonies. Scratch tests were implemented to compare the difference in migration abilities between As-transformed and normal BEAS-2B cells. As expected, after the first 24-h period, As-transformed cells exhibited significantly faster wound-healing capability than the control cells (Fig 1C/D). In light of previous studies illustrating up regulated SATB2 expression following exposure to heavy metals (Clancy et al., 2012; Wu et al., 2016), the mRNA and protein levels of SATB2 were measured following various doses and time periods of arsenic treatment. Using quantitative real-time PCR (qPCR), significant increases in SATB2 mRNA levels were detected in arsenic-transformed cells (Fig 1E). In addition, arsenic-transformed clones also displayed markedly increased SATB2 protein levels compared with control cells (Fig 1F). Furthermore, as shown in Fig 1G, western blotting analysis of BEAS-2B cells exposed to 1.25, 2.5, 5, or 10 μ M of arsenic for 48-h displayed a dose-dependent increase in SATB2 protein levels. We next examined the changes in SATB2 level after exposure to 2 μ M of arsenic for various time intervals. The increase in SATB2 protein level was directly correlated with exposure time (Fig 1H). Collectively, these results demonstrate that SATB2 protein level increases with higher doses and length of time of arsenic exposure. Given that SATB2 is not expressed in normal BEAS-2B cells, arsenic-induced elevation suggested a potential role of SATB2 in arsenic-induced malignant cell transformation.

SATB2 knockdown in As-transformed BEAS-2B cells rescues anchorage-independent growth, cell migration, and invasion

Mounting experimental studies have found a correlation between increased SATB2 expression and various types of human cancers (Magnusson et al., 2011). Notably, several studies have indicated SATB2 up regulation in lung cancer (Wu et al., 2016a). To experimentally address our hypothesis, we transfected shRNA constructs into arsenic-transformed BEAS-2B cells to stably knockdown SATB2 expression (arsenic-

SATB2shRNA). Quantitative real-time PCR results confirm a striking reduction in mRNA levels in SATB2 knockdown cells (Fig 2A). In addition, the shRNA construct showed a significant reduction in SATB2 protein level compared with the empty vector cells (Fig 2B).

These results confirmed the efficient knockdown of SATB2 in arsenic-transformed cells. In order to examine the effect of reduced SATB2 expression on anchorage-independent growth, arsenic-transformed cells were transfected with either an empty vector or SATB2 shRNA and cells were tested for growth in soft agar. As indicated by the number of colonies in Fig 2C/D, knocking down SATB2 significantly reduced anchorage-independent growth in arsenic-transformed cells while transfection of the vector alone had no effect. In addition, scratch tests indicated that arsenic-transformed-SATB2shRNA cells displayed attenuated wound healing ability in comparison with cells transfected with the empty vector (Fig 2E/F). Invasion abilities of arsenic-transformed cells after SATB2 knockdown or cells transfected with empty vector alone were also tested. Figures G/H indicated that SATB2 shRNA displayed a remarkably reduced invasion capacity compared to empty vector transfected arsenic-transformed cells. Collectively, our results showed that knocking down SATB2 eliminated a number of hallmarks of cancer including anchorage-independent growth, cell migration and invasion abilities.

SATB2 knockdown prevents As-induced transformation in BEAS-2B cells

In line with the finding that SATB2 reduction eliminated the transformation properties of arsenic-transformed cells, we investigated whether knocking down of SATB2 in normal BEAS-2B cells prior to arsenic exposure would prevent arsenic-induced cell transformation. Of note, under normal conditions, SATB2 is only weakly expressed in the lung, but not detectable in BEAS-2B cells (Seong et al., 2015; Wu et al., 2016). We knocked down SATB2 in BEAS-2B cells that do not express this gene, using stable shRNA transfection to prevent its expected increase in expression after exposure to arsenic. SATB2 was stably knocked down in BEAS-2B cells using shRNA (BEAS-2B/SATB2 shRNA) and cells were treated with 2 μ M of arsenic for 6 consecutive weeks. As illustrated in Fig 3A, after 6 weeks of arsenic exposure, the empty vector BEAS-2B cells demonstrated significantly higher levels of SATB2 mRNA than the knockdown cells. With regard to protein levels, SATB2 knockdown cells showed no sign of elevation in SATB2 protein after arsenic exposure, while empty vector cells demonstrated a striking increase in SATB2 protein levels (Fig 3B). These findings confirm that knocking down SATB2 in normal BEAS-2B cells prevented its mRNA and protein levels from rising after arsenic exposure.

We next investigated the effect of arsenic on cell transformation in the absence of SATB2 expression. As indicated in Fig 3C/D, after exposure to arsenic, BEAS-2B cells with knockdown of SATB2 demonstrated significantly less colony growth compared to cells transfected with the empty vector. These results are consistent with Fig 2 D/E and suggest that the presence of SATB2 was essential for arsenic-induced anchorage-independent growth. With regard to the scratch test shown in Fig3 E/F, 24 hours after the initial wound induction, arsenic-transformed BEAS-2B cells healed at a significantly faster rate than arsenic-treated SATB2 knockdown cells. Collectively, our data show that arsenic-induced anchorage-independent growth and cell migration were attenuated when SATB2 expression

was lowered, which further suggest that SATB2 was essential for the hallmarks of arsenic-induced cell transformation.

miR-31 is inhibited by As exposure and functions as an upstream negative regulator of SATB2

Non-coding mi-RNAs participate in attenuating gene expression by base pairing with complementary mRNA sequences and thereby reducing mRNA stability and preventing their translation. In order to investigate the role of miRNAs in arsenic-carcinogenesis, we searched for possible upstream regulators of SATB2. Of the currently available 17 studies, 5 have suggested that miR-31 was an upstream negative regulator of SATB2 (Aprelikova et al., 2010; Deng et al., 2013; Yang et al., 2014; Ge et al., 2015; Zhen et al., 2017). Furthermore, of the 7 known miR-31 targets, SATB2 was by far the most frequently identified (Aprelikova et al., 2010; Deng et al., 2013; Yang et al., 2014; Ge et al., 2015; Luo et al., 2016; Zhen et al., 2017). Physically, miR-31 was capable of binding to two sites in the SATB2 3'UTR region; Fig 4A demonstrates the sequences that bind with miR-31 (miRmap: <http://mirmap.ezlab.org/>). Furthermore, in an effort to investigate the specific biological functions of miR-31, we utilized two databases, miRmap and miRSystem, to examine the potential target genes of miR-31 and their biological functions. Fig 4B provides a graphical representation of the top 20 biological functions regulated by miR-31, including cell migration, locomotion, motility, and positive regulation of gene expression, all of which were important characteristics which correlated with SATB2 expression (Fig 4C). These data suggest that miR-31 may play a role in As-induced cell transformation by attenuating SATB2 expression.

Although several studies have illustrated the ability of arsenic to interfere with various miRNA expression, including miR-29, -183, -192, etc. (Ren et al., 2016; Beezhold et al., 2017), there were no studies that have investigated the correlation between arsenic exposure and miR-31 expression. As indicated in Fig 4D/E, our study showed that both acute and chronic arsenic exposure that induced arsenic-transformed BEAS2B cells, have been shown to significantly reduce miR-31 levels. As a negative regulator of SATB2, loss of miR-31 expression would presumably lead to higher levels of SATB2, which has been observed in arsenic-exposed BEAS-2B cells. Collectively, these results suggest that as an upstream regulator of SATB2, miR-31 may play an important role in arsenic-carcinogenesis.

Overexpression of miR-31 rescues As-induced anchorage-independent growth, cell migration, and invasion.

MiR-31 was overexpressed in arsenic-transformed cells by stable transfection (Fig 5A). Since miR-31 is a negative regulator of SATB2, overexpression of miR-31 should presumably decrease SATB2 expression. As indicated in Fig 5B, overexpressing of miR-31 in arsenic-transformed cells caused a reduction in SATB2 protein level as compared with empty vector. However, as shown in Fig 5C, SATB2 mRNA levels remain unchanged. Similar findings were reported in osteogenic differentiation of bone mesenchymal stem cells (Deng et al., 2013). These results suggest that miR-31 affected SATB2 translation without changing the levels of its mRNA (Fig 5D). After verifying miR-31 overexpression in arsenic-transformed cells, the level of cell transformation was assessed by growth in soft

agar (Fig 5E). There was significantly less colony growth in arsenic-transformed cells overexpressing miR-31 compared with empty vector cells. This result suggests that miR-31 may play an important role in arsenic-induced anchorage-independent growth. In addition, scratch tests were employed to examine the rate of cell migration in the first 24-h period. Figure 5F/G showed that in comparison to the arsenic-transformed cells harboring an empty vector, miR-31 overexpressing cells demonstrated delayed wound-healing abilities. Furthermore, arsenic-transformed cells overexpressing miR-31 exhibited a significant loss in cell invasiveness as compared with empty vector cells (Fig 5 H/I). Collectively these findings showed that overexpression of miR-31 effectively reduced arsenic-induced anchorage-independent growth, cell migration, and invasion. As a negative regulator of SATB2, arsenic-induced down-regulation of miR-31 and the subsequent overexpression of SATB2 may be a potential mechanism of arsenic carcinogenesis.

Discussion

Our study focuses on a potential pathway involving miR-31 and SATB2 in arsenic-induced BEAS-2B cell transformation. We have established that both SATB2 mRNA and protein levels increased following either acute or chronic arsenic exposure (Fig 6). Secondly, we showed that knocking down SATB2 in arsenic-transformed BEAS-2B cells significantly reduced hallmarks of carcinogenesis including anchorage-independent growth, greater cell migration, and invasion capabilities. These data suggest a direct functional relationship between SATB2 expression and arsenic-induced cell transformation. In addition, knocking down SATB2 prior to arsenic treatment (2uM for 6 weeks) prevented arsenic-induced anchorage-independent growth as well as increased cell migration abilities. MiR-31 is an upstream negative regulator of SATB2, and the expression of this miRNA was attenuated by arsenic treatment. Overexpression of miR-31 in arsenic-transformed BEAS-2B cells significantly reduced anchorage-independent growth, cell migration, and invasion abilities, apparently through inhibition of SATB2 mRNA translation because SATB2 mRNA levels were unchanged. Together, these results suggest that arsenic-induced miR-31 inhibition and subsequent SATB2 overexpression may be a potential new mechanism for arsenic-induced BEAS-2B cell transformation.

This is the first study to describe a role of miR-31 and SATB2 in arsenic-induced cell transformation. As a potent class I human carcinogen, mounting scientific research efforts have been conducted to uncover the mechanisms and pathways by which arsenic exerts its carcinogenicity. In addition to the commonly studied epigenetic alterations such as DNA methylation and histone post-translational modifications, miRNA-mediated silencing also plays an important role in arsenic-induced carcinogenesis (Sturchio et al., 2014). Defects in the proper regulation of epigenetic marks can lead to inappropriate activation or suppression of critical oncogenes or tumor suppressor genes, which can lead to cancer development (Jones et al., 2002; Egger et al., 2004). The expression of SATB2 is often found disrupted in various types of cancers including lung, breast, colon, and ovarian cancers. However, the precise role of SATB2 in carcinogenesis remains unclear due to its varied apparent functions in different types of tissues from which cancers can arise. For example, evidence suggests that SATB2 is overexpressed and acts as an oncogene in lung, colorectal, Merkel cell, and hepatocellular carcinoma (Magnusson et al., 2011; Fukuhara et al. 2016; Yu et al., 2017). In

addition, elevated mRNA levels of SATB2 is linked to poor breast cancer grade (Patani et al. 2009; Yu et al. 2017). On the other hand, SATB2 has also been shown to function as a tumor suppressor gene in lung, gastric, as well as colorectal cancers (Wang et al., 2009; Yang et al., 2014; Mansour et al. 2015; Wu L et al., 2016; Ma et al., 2017; Tian et al., 2017). The opposite roles of SATB2 are cell-specific, and further evidence is still needed to better understand its role in cancer development.

In the past two decades, interest in non-coding RNAs has increased substantially. MicroRNAs are a class of ncRNAs that suppress gene expression by inducing degradation of target mRNAs. Several studies have indicated that arsenic exposure can interrupt miRNA expression, but the present study is the first to demonstrate that arsenic can induce the down-regulation of miR-31. Specifically, miR-31 has been shown to play an important role in many types of cancers including lung, colon, breast, etc. (Stepicheva and Song, 2016). Through database searches such miRSys, miRMap, and Toppgene, miR-31 emerged as a direct upstream negative regulator of SATB2. Specifically, miR-31 was found to promote SATB2 mRNA degradation by binding to two different sites in its 3' UTR region. The direct physical and functional interaction between miR-31 and SATB2 indicated a possible role in regulating SATB2 expression. However, like SATB2, miR-31 has also been shown to display both oncogenic and tumor suppressive functions, depending on the type of cancer (Stepicheva and Song, 2016). Here SATB2 is described as a potential tumor suppressor, and when miR-31 was significantly reduced in arsenic-transformed cells, this led to the overexpression of SATB2. In order to confirm the functional effect of miR-31 in arsenic-induced cell transformation, we stably transfected arsenic-transformed BEAS-2B cells to overexpress miR-31. Overexpression of miR-31 in arsenic-transformed cells reduced SATB2 protein and suppressed hallmarks of transformation including anchorage-independent growth, cell migration, and invasion. The data further confirm miR-31 as a negative regulator of SATB2 as well as its direct role in arsenic-induced cell transformation. However, it is important to note that unlike protein levels, SATB2 mRNA levels remained unchanged in miR-31 overexpressed cells, as illustrated in Fig 7. This may be explained by the nature of miRNAs as post-transcriptional regulators. Rather than mRNA degradation, miRNAs are predominantly known to induce translational repression (Bartel, 2004; Carrington et al., 2003; Lewis et al., 2005). Although recent microarray analyses were able to show that some miRNAs may be capable reducing transcript levels, it remains unknown whether miRNA-induced gene expression is directly through mRNA degradation or if the phenomenon is due to off-target effects of miRNA-like activity (Lim et al., 2005). In addition, online database searches using miRSys and miRMap as well as gene list analyses using Toppgene, suggested that both miR-31 and SATB2 were directly involved in cell migration, motility, and gene expression regulation, all of which may be point to ways that these processes induce cell transformation.

Arsenic exposure reduces miR-31 expression, which slows the degradation of SATB2 mRNA and expression. As a possible tumor promoter, down regulation of miR-31 and overexpression of SATB2 were directly linked to the hallmarks of cancer including anchorage-independent growth, cell invasion, and accelerated cell migration in arsenic-transformed cells. However, further research is still needed to understand how arsenic induces the down-regulation of miR-31. Some evidence suggests that expression of Runt-

related transcription factor 2 (RUNX2) was also regulated by arsenic, and that this gene participates in a regulatory loop with SATB2 and miR-31 to induce osteogenic differentiation (Hu et al., 2012; Deng et al., 2013), but no studies are currently available in human bronchial epithelial cell models. Given that SATB2 overexpression is induced by other heavy metals such as nickel and chromium, which are also carcinogenic, the pathways reported here may be a common phenomenon for heavy metal-induced lung carcinogenesis.

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Abbreviations

BEAS-2B	Human bronchial epithelial cells
Endo-siRNAs	Endogenous small interfering RNA
HUGE	Human unidentified gene-encoded
MAR	Matrix attachment region
MiRNA	Micro RNA
NaAsO₂	Sodium meta-arsenite
NcRNA	Non-coding RNA
PiRNA	PIWI-interacting RNA
Ppb	Parts per billion
qPCR	Quantitative real-time PCR
SATB2	Special AT-rich sequence-binding protein 2

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Fig 1. Anchorage-independent growth in soft agar and SATB2 expression following six-week treatment with arsenic.

A and B: Image and number of colonies in soft agar after 6 weeks' treatment of 2 μ M arsenic. C and D: Image and data analysis of scratch test of arsenic transformed cells. E: mRNA level of SATB2 in arsenic-transformed clones. F: SATB2 protein overexpression in arsenic-transformed clones. G: Protein expression of SATB2 after 48h treatment of 1.25, 2.5, 5, 10 μ M arsenic. H: Protein expression of SATB2 after 1, 2, 4, and 6 weeks of 2 μ M arsenic treatment. ** $p < 0.01$, compared with the control group.

Fig 2. Small hairpin RNA knockdown of SATB2 in arsenic-transformed BEAS-2B cells.

A and B: Reduced SATB2 mRNA and protein expression after shRNA stable transfection of shRNA for SATB2 in arsenic-transformed BEAS-2B cells. C and D: Image and number of colonies in soft agar growth after SATB2 stable knockdown in arsenic-transformed BEAS-2B cells. E and F: Image and data analysis of scratch test of SATB2 stable knockdown in arsenic-transformed BEAS-2B cells. G and H: Image of cell invasion assay in arsenic-transformed BEAS-2B-SATB2 stable knockdown cells. **: $p < 0.01$, compared with arsenic-transformed BEAS-2B shRNA vs Vector Control group.

Fig 3. Inhibition of arsenic-induced hallmarks of cancer in BEAS2B cells transfected with shRNA to SATB2.

A and B: SATB2 mRNA and protein expression in stable SATB2 knockdown BEAS-2B cells with or without six weeks arsenic treatment. C and D: Image and number of colonies in soft agar in stable SATB2 knockdown BEAS-2B cells after six weeks of arsenic treatment. E and F: Scratch test images and data analysis of stable SATB2 knockdown BEAS-2B cells after six weeks arsenic treatment. **: $p < 0.01$, compared with normal BEAS-2B shControl + As group.

Fig 4. Upstream miR-31 inhibition of SATB2 following arsenic exposure.

A: Schematic illustration of the predicated miR-31-binding sites (S1 and S2) in SATB2 3'-UTR. B and C: Functional overlap graph. D: The relative levels of Mir31 mRNA in normal and arsenic-transformed BEAS-2B cells, **: $p < 0.01$, compared with Normal-BEAS-2B group. E: Levels of Mir31 mRNA after 48h treatment of 0, 2.5, 10 uM arsenic, **: $p < 0.01$, compared with Control group.

Fig 5. Overexpression of miR-31 inhibits arsenic-induced cancer hallmarks

A. Levels of miR31 mRNA after stable overexpression of miR-31 in arsenic-transformed BEAS-2B cells, **: $p < 0.01$, compared with arsenic-transformed BEAS-2B cells overexpressing empty vector control group. B: SATB2 protein expression after stable overexpression of miR-31 in arsenic-transformed BEAS-2B cells compared to a control vector. C: Levels of SATB2 mRNA after stable overexpression of miR-31 in arsenic-transformed BEAS-2B cells compared to a control vector. D and E: Image and number of colonies in soft agar of stable overexpression of miR-31 in arsenic-transformed BEAS-2B cells compared to a vector control. F and G: Scratch test images and data analysis of stable overexpression of miR-31 in arsenic-transformed BEAS-2B cells compared to vector control. H and I: Image and cell count of cell invasion assay after stable overexpression of miR-31 in arsenic-transformed cells compared with a vector control: *: $p < 0.05$, **: $p < 0.01$, compared with arsenic-BEAS2B Mir31 overexpression vs Control group.

Fig 6.
Model demonstrating the effect of acute and chronic arsenic exposure on SATB2 and miR-31

Fig 7.
Model demonstrating the potential role miR-31 in SATB2 repression