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Up-regulation of RNA m^{6}A methyltransferase like-3 expression contributes to arsenic and benzo[*a*]pyrene co-exposure-induced cancer stem cell-like property and tumorigenesis

Zhishan Wang[a,b,*,1], **Mohammad Burhan Uddin**[c,1,2], **Po-Shun Wang**[a], **Zulong Liu**[a], **David Barzideh**[a], **Chengfeng Yang**[a,b]

[a] Stony Brook Cancer Center, Stony Brook University, Stony Brook, NY, USA

[b] Department of Pathology, Renaissance School of Medicine, Stony Brook University, Stony Brook, NY, USA

[c] Department of Toxicology and Cancer Biology, University of Kentucky School of Medicine, Lexington, KY, USA

Abstract

While arsenic or BaP alone exposure can cause lung cancer, studies showed that arsenic plus BaP co-exposure displays a significantly stronger lung tumorigenic effect. However, the underlying mechanism has not been well understood. Studies showed that RNA molecules are chemically modified. The most frequently occurring RNA modification in eukaryotic messenger RNAs is the N6-methyladenosine (m^{6}A) methylation. This study aimed to determine whether arsenic plus BaP exposure alters RNA m^{6}A methylation and its role in lung tumorigenic effect of arsenic plus BaP exposure. Human bronchial epithelial cells transformed by exposure to arsenic or BaP alone, and arsenic plus BaP and mouse xenograft tumorigenesis models were used in this study. It was found that arsenic plus BaP exposure-transformed cells have significantly higher levels of RNA m^{6}A methylation than arsenic or BaP alone exposure-transformed human bronchial epithelial cells. Western blot analysis showed that arsenic plus BaP exposure greatly up-regulates the m^{6}A writer methyltransferase like-3 (METTL3) expression levels in cultured cells and mouse lung tissues. METTL3 knockdown in cells transformed by arsenic plus BaP exposure drastically

* Corresponding author at: Stony Brook Cancer Center and Department of Pathology, Stony Brook University, Stony Brook, NY, USA. zhishan.wang@stonybrook.edu (Z. Wang).

[1]These authors contribute equally to this work.

[2]Current address: Department of Pharmaceutical Sciences, North South University, Bashundhara, Dhaka 1229, Bangladesh.

Declaration of Competing Interest

The authors declare they have no actual or potential competing financial interests.

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During the preparation of this work the authors did not use any Generative AI and AI-assisted technologies.

CRediT authorship contribution statement

Zhishan Wang: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Mohammad Burhan Uddin:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Po-Shun Wang:** Data curation, Investigation, Methodology. **Zulong Liu:** Data curation, Investigation. **David Barzideh:** Investigation. **Chengfeng Yang:** Conceptualization, Writing – original draft, Writing – review & editing.

Appendix A. Supplementary data

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reduced their RNA m^6A methylation levels. Functional studies revealed that METTL3 knockdown in cells transformed by arsenic plus BaP exposure greatly reduces their anchorage-dependent and -independent growth, cancer stem cell characters and tumorigenesis. The findings from this study suggest that arsenic plus BaP co-exposure causes epitranscriptomic dysregulation, which may contribute significantly to arsenic plus BaP co-exposure-caused synergistic lung tumorigenic effect.

Keywords

Arsenic; Benzo(*a*)pyrene (BaP); Mixture exposure; RNA m^6A modification; Epitranscriptome; METTL3

1. Introduction

Both arsenic and benzo[*a*]pyrene (BaP) are Group I carcinogens classified by the International Agency for Research on Cancer (IARC) (IARC, 1983, 2012). Exposure to either arsenic or BaP can cause lung cancer. Non-occupational human exposure to arsenic mainly occurs through consuming drinking water that is contaminated by arsenic (IARC, 2004). BaP is generated when organic matters are not completely burned (IARC, 1983). Non-occupation human BaP exposure may happen through cigarette smoking or consuming well-done barbecued meat, which usually has high BaP levels (Hecht, 2003; Kazerouni et al., 2001). In addition, wildfire frequency, severity and duration are increasing around the world and wildfire smoke may contain high levels of polycyclic aromatic hydrocarbons (PAHs) including BaP, representing an emerging source of BaP exposure by humans. As both arsenic and BaP are widely present in the general environment and workplaces, human exposure to arsenic and BaP could be common. This may cause significantly worse adverse health effects compared to that caused by arsenic or BaP single exposure. Indeed, others and our recent studies reported that arsenic plus BaP exposure displays a synergistic effect in inducing cell transformation, cancer stem cell-like property and lung cancer (Pershagen et al., 1984; Wang et al., 2020; Xie et al., 2020; Yang et al., 2020). However, the mechanism of how arsenic plus BaP co-exposure synergizes in promoting lung carcinogenesis has been poorly understood.

Studies from our group and others suggest that arsenic plus BaP exposure-caused synergistic lung carcinogenic effect may be due to the enhanced genetic, epigenetic toxic effects and increased activation of oncogenic signaling pathways (Wang, 2021). Although arsenic may enhance DNA damage by inhibiting DNA repair; and metabolic activation of BaP generates highly carcinogenic BPDE-DNA adduct, the literature reports on the effect of arsenic plus BaP exposure on BPDE-DNA adduct levels are not consistent and sometimes even controversial (Wang, 2021). The inconsistent or controversial genotoxic effects of arsenic plus BaP exposure suggest that other mechanisms such as epigenetic or epitransciptomic dysregulations may have critical roles in the synergistic carcinogenic effect of arsenic plus BaP exposure. Our recent report showed that arsenic plus BaP exposure acts synergistically by epigenetically down-regulating the expression of SOCS3 (suppressor of cytokine signaling 3) to promote cancer stem cell-like property and tumorigenesis (Wang et al.,

2020). It has not been determined, however, whether arsenic plus BaP co-exposure alters epitranscriptome.

Chemical modifications occur at DNA and nuclear histone proteins. Similarly, chemical modfications also are observed at RNA molecules (Uddin et al., 2020, 2021). The demonstration of important biological functions of RNA chemical modifications resulted in the birth of “RNA epigenetics” or “Epitranscriptome” terms (Saletore et al., 2012; Roundtree and He, 2016). Like DNA methylation and nuclear histone protein chemical modifications, RNA chemical modifications are also subject to the regulations of writers, erasers, and readers. The “Writer” proteins deposit chemical modifications to RNA molecules. The “Eraser” proteins remove chemical modifications on RNA molecules. The “Reader” proteins recognize and bind to the chemically modified RNAs, mediating RNA chemical modification functional outcomes (Shi et al., 2019; Flamand et al., 2023). Among >150 types of modifications in RNAs, the N6-methyladenosine (m^6A) methylation is found the most abundant modification in eukaryotic messenger RNAs (mRNAs). The RNA m^6A modification is achieved by a writer protein complex that is composed of methyltransferase like-3 (METTL3), METTL14, WTAP (Wilms’ tumor 1-associating protein), etc. In contrast, the RNA m^6A methylation can be removed by eraser protein FTO (fat mass and obesity-associated protein) and ALKBH5 (AlkB homolog 5) (Uddin et al., 2021). Functionally, the RNA m^6A methylation regulates RNA trafficking, structure, stability, and translation thus changing gene expression (Cesaro et al., 2023; Gilbert and Nachtergaele, 2023; Liu et al., 2023). As a result, abnormal RNA m^6A modification as reflected by the dysregulated expression and function of m^6A writers, erasers or readers have been linked with cancer and other disease initiation and progression (Deng et al., 2023; Diao et al., 2023). The purpose of this study was to determine whether arsenic plus BaP exposure dysregulates RNA m^6A modification and its role in arsenic plus BaP exposure-caused cancer stem cell-like property and tumorigenesis.

2. Materials and methods

2.1. Cell culture

The process of exposing immortalized nontumorigenic human bronchial epithelial BEAS-2B cells to a vehicle control, arsenic ($NaAsO_2$, 1 μM), BaP (2.5 μM), or arsenic ($NaAsO_2$, 1 μM) plus BaP (2.5 μM) for 30 weeks to induce BEAS-2B cell malignant transformation was described and reported in our recent publications (Wang et al., 2020; Xie et al., 2020; Yang et al., 2020). The passage-matched control cells, arsenic alone exposure-transformed cells, BaP alone exposure-transformed cells and arsenic plus BaP co-exposure-transformed cells were designated as BEAS-2B-Control, BEAS-2B-As, BEAS-2B-BaP, and BEAS-2B-As+BaP, respectively. All BEAS-2B cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

2.2. METTL3 expression stable knockdown in arsenic plus BaP co-exposure-transformed cells (BEAS-2B-As+BaP)

Control shRNA non-targeting lentiviral particles (pZIP-hCMV-ZsGreen-Puro-Control shRNA), shRNA lentiviral particles specifically targeting METTL3 (pZIP-hCMV-ZsGreen-

Puro-METTL3 shRNA) were used to produce the shRNA control and METTL3 stable knockdown BEAS-2B-As+BaP cells, which are designated as BEAS-2B-As+BaP-shRNA control and BEAS-2B-As+BaP-METTL3 shRNA, respectively. The control shRNA lentiviral particles and the METTL3-specific targeting shRNA lentiviral particles were obtained from Transomic Technologies (Huntsville, Alabama). The procedures for viral particles transduction and knockdown cell selection were reported in our recent publication (Wang et al., 2022). The METTL3 stable knockdown was confirmed using Western blot analysis.

2.3. The m^{6}A RNA modification ELISA-like colorimetric assay

Cellular total RNAs were extracted using the TRIzol reagent following the manufacturer's instruction (Invitrogen, California). Total RNA m^{6}A levels were analyzed using the EpiQuik m^{6}A RNA Methylation Quantification Kit from EpiGenTEK (Farmingdale, New York) following the manufacturer's detailed instructions.

2.4. Cell proliferation and clonogenic assays

The effect of METTL3 stable knockdown on cell proliferation was analyzed by the MTT assay as described in our previous publications (Wang et al., 2014; Zhao et al., 2011). The clonogenic assay was performed according to the protocol reported in our recent publications with minor modifications (Clementino et al., 2020; Li et al., 2019). Briefly, one hundred BEAS-2B-As+BaP-shRNA control or BEAS-2B-As+BaP-METTL3 shRNA cells were seeded into 6cm dishes and cultured in DMEM supplemented with 5% FBS. The culture medium was changed every three days and cells were cultured for 12days. At the end of the experiment, cell clones were fixed with 4% paraformaldehyde and stained with 0.01% (*w*/*v*) crystal violet, photographed and counted.

2.5. Serum-free suspension culture sphere formation assay

The serum-free suspension culture sphere formation assay was performed to determine the effect of METTL3 stable knockdown on the cancer stem cell (CSC)-like property of arsenic and BaP co-exposure-transformed cells following the protocol described in our recent publications with minor modifications (Lin et al., 2021a; Wang et al., 2019). Briefly, BEAS-2B-As+BaP-shRNA control or BEAS-2B-As+BaP-METTL3 shRNA cells (2.5×10^3) were seeded into the ultra-low attachment 24-well plates (Corning, NY). The culture media were serum-free DMEM supplemented with human recombinant basic fibroblast growth factor (bFGF, 20 ng/mL), human recombinant epidermal growth factor (EGF, 20 ng/mL) (R&D, Minneapolis, MN), B27 (Invitrogen, Carlsbad, CA) and heparin (4 μg/mL, Sigma-Aldrich). Cells were cultured for 10 days at 37 °C with 5% CO_2. At the end of culture, suspension spheres >100 μm were counted and photographed under a phase-contrast cell culture microscope.

2.6. Western blot analysis

Western blot analysis was performed as previously described and repeated three times with different biological replicates (Lin et al., 2021b; Wang et al., 2018). The following primary antibodies were used: anti-METTL3, anti-WTAP, anti-FTO, and anti-ALKBH5 (Abcam,

Cambridge, Massachusetts); anti-KLF4, anti-KLF5, anti-METTL14, and anti-NANOG, and anti-Oct4 (Cell Signaling Technology, Beverly, Massachusetts); and anti-β-actin (Millipore Sigma, St Louis, Missouri). The Western blot images were obtained using an Amersham AI680 imager. The Western blot band intensities were quantitated using NIH ImageJ software.

2.7. Soft agar colony formation assay

The soft agar colony formation assay was carried out following the protocol described in our previous publications (Wang et al., 2011; Yang et al., 2005).

2.8. Immunohistochemistry (IHC) staining of METTL3 in mouse lung tissues

The lung tissues of mice exposed to BaP alone or arsenic plus BaP were from our recent study and the details of mouse arsenic and BaP exposure were reported in our recent study (Wang et al., 2020). The IHC staining of METTL3 in mouse lung tissues was performed using the VECTASTAIN Elite ABC Universal Kit following the manufacturer's instructions. Briefly, mouse lung tissues were fixed in 10% formalin solution overnight at room temperature, and dehydrated, embedded in paraffin and sectioned to make 5 μm-thick section. Next, sections were deparaffinized and hydrated through xylenes and graded alcohol series followed by antigen retrieval by autoclave heating for 15 min in citrate-based retrieval solution. The mouse lung tissue sections were incubated overnight at 4 °C with the anti-METTL3 antibody (abcam) and counter stained with hematoxylin solution. The images were captured by an Olympus microscope (Model IX83).

2.9. Nude mouse xenograft tumorigenesis assay

BEAS-2B-As+BaP-shRNA control and BEAS-2B-As+BaP-METTL3 shRNA cells were used to determine the effect of METTL3 knockdown on the tumor forming capability of arsenic and BaP co-exposure-transformed cells in nude mice. The animal study protocol was reviewed and approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC). Briefly, BEAS-2B-As+BaP-shRNA control and BEAS-2B-As+BaP-METTL3 shRNA cells (1.0×10^6 cells) were suspended in growth factor-reduced Matrigel and PBS and injected subcutaneously into the flank areas of 7-week-old female nude mice (Nu/Nu, Charles River Laboratories) as described previously (Wang et al., 2020). Five weeks after the cell injection, mice were euthanized and the xenograft tumors were harvested, weighed and photographed.

2.10. Statistical analysis

The significance of differences of treatment effects between two groups (mean ± SD) were analyzed by statistical analyses using two-tailed *t*-tests. One-way analysis of variance (ANOVA) was performed to analyze the significance of treatment effects of multiple group data sets. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic arsenic plus BaP co-exposure increases METTL3 expression

To explore whether combined chronic arsenic and BaP exposure causes the RNA m^6A modification dysregulation, we first determined the levels of the RNA m^6A main writers and erasers in chronic arsenic alone exposure-transformed cells (BEAS-2B-As), BaP alone exposure-transformed cells (BEAS-2B-BaP); arsenic and BaP co-exposure-transformed cells (BEAS-2B-As+BaP), and the passage-matched control cells (BEAS-2B-Control). As shown in Fig. 1A, no significant differences of the RNA methyltransferase METTL3 protein levels are observed among passage-matched control cells, chronic arsenic alone exposure-transformed cells and BaP alone exposure-transformed cells. However, the protein level of METTL3 in chronic arsenic and BaP co-exposure-transformed cells is drastically higher than the METTL3 level in passage-matched control cells, chronic arsenic alone exposure-transformed cells and BaP alone exposure-transformed cells. These results suggest that, although chronic arsenic or BaP alone exposure does not significantly affect METTL3 expression, chronic arsenic and BaP co-exposure acts differently and is capable of drastically increasing METLL3 expression levels. Additional Western blot analyses showed that chronic arsenic or BaP alone exposure, or arsenic plus BaP co-exposure do not significantly change the expression levels of METTL14 and WTAP, the other two important components of the m^6A writer complex (Fig. 1A). Moreover, further Western blot analyses revealed that chronic arsenic or BaP alone exposure, or arsenic plus BaP co-exposure do not significantly alter the expression levels of FTO and ALKBH5 (Fig. 1B), two known m^6A erasers. Together, these results indicate that chronic arsenic and BaP co-exposure up-regulates the RNA methyltransferase METTL3 expression but does not significantly affect the expression of other important regulators of the RNA m^6A modification in cultured human bronchial epithelial cells.

We further determined whether chronic arsenic and BaP co-exposure also up-regulates METTL3 expression level in mouse lung tissues. Our recent study showed that mice exposed to arsenic alone via drinking water for 34 weeks did not develop lung tumors and mice exposed to BaP alone developed lung tumors; however, arsenic plus BaP co-exposure synergized in inducing lung tumorigenesis evidenced by significantly increasing lung tumor multiplicity and tumor burden (Wang et al., 2020). We performed immunohistochemistry (IHC) staining to compare METTL3 protein levels in lung normal and tumor tissues of mice exposed to BaP alone and to arsenic plus BaP. Fig. 2A shows the representative H&E staining images of lung tumor and the adjacent normal lung tissues from mice exposed to BaP alone and to arsenic plus BaP (Fig. 2A). Fig. 2B shows the representative METTL3 IHC staining images of lung tumor and the adjacent normal lung tissues from mice exposed to BaP alone and to arsenic plus BaP (Fig. 2B). From these figures it was found that METTL3 positive staining in BaP alone exposure- and arsenic plus BaP co-exposure-induced mouse lung tumor tissues are stronger than their corresponding adjacent normal lung tissues (Fig. 2B). However, METTL3 positive staining in arsenic plus BaP co-exposure-induced mouse lung tumor tissue is more and stronger than that in the adjacent normal lung tissue and BaP alone exposure-induced mouse lung tumor tissue (Fig. 2B). Our quantification analysis using QuPath software showed that the average percentage of cells

with METTL3 positive staining in BaP alone exposure- and arsenic plus BaP co-exposure-induced mouse lung tumors are 22.77 ± 6.04 ($n = 3$) and 49.98 ± 10.11 ($n = 4$) ($p < 0.05$), respectively. These results indicate that arsenic and BaP co-exposure also acts synergistically in increasing METTL3 expression in mouse lung tissues.

3.2. Chronic arsenic plus BaP co-exposure increases RNA m^{6}A levels through up-regulating METTL3 expression

We next determined whether chronic arsenic and BaP co-exposure increases RNA m^{6}A modification levels. An ELISA-like colorimetric RNA m^{6}A methylation quantification kit was used to quantitatively measure total RNA m^{6}A levels. Similar to the non-significant effect of chronic arsenic alone or BaP alone exposure on the m^{6}A writer METTL3 expression, no significant differences of total RNA m^{6}A levels are observed among chronic arsenic alone exposure-transformed cells (BEAS-2B-As), BaP alone exposure-transformed cells (BEAS-2B-BaP) and the passage-matched control cells (BEAS-2B-Control) (Fig. 3A). In sharp contrast, total RNA m^{6}A levels in chronic arsenic and BaP co-exposure-transformed cells (BEAS-2B-As+BaP) are significantly higher than chronic arsenic alone exposure-transformed cells, BaP alone exposure-transformed cells and the passage-matched control cells (Fig. 3A). These results indicate that chronic arsenic and BaP co-exposure acts synergistically in increasing total RNA m^{6}A modification levels.

To demonstrate whether chronic arsenic and BaP co-exposure increases total RNA m^{6}A levels by up-regulating the RNA methyltransferase METTL3 expression, we next stably knocked down METTL3 expression in chronic arsenic and BaP co-exposure-transformed cells. An efficient knockdown of METTL3 protein level is confirmed by Western blot (Fig. 3B). It was found that METTL3 knockdown in chronic arsenic and BaP co-exposure-transformed cells significantly reduces their total RNA m^{6}A levels (Fig. 3C). These results indicate that chronic arsenic and BaP co-exposure increases total RNA m^{6}A modification levels mainly by up-regulating METTL3 expression.

3.3. METTL3 knockdown in chronic arsenic plus BaP co-exposure-transformed cells reduces their anchorage-dependent and -independent growth

We next sought to determine whether up-regulation of METTL3 expression and m^{6}A modification plays a role in maintaining malignant phenotypes of chronic arsenic and BaP co-exposure-transformed cells. Carcinogen-transformed cells and tumor cells usually display fast anchorage-dependent proliferation and anchorage-independent growth capability. The MTT assay and clonogenic assay were used to determine the effect of METTL3 knockdown on arsenic and BaP co-exposure-transformed cell anchorage-dependent growth. It was found that METTL3 knockdown cells grow slower determined by the MTT assay and form significantly less clones in the clonogenic assay than the shRNA control cells (Fig. 4A–B). The soft agar colony formation assay is commonly used to assess the anchorage-independent growth of carcinogen-transformed cells and tumor cells. It was found that METTL3 knockdown cells grow significantly less colonies in the soft agar than the shRNA control cells (Fig. 4C). Together, these results suggest that METTL3 up-regulation plays an important role in maintaining the malignant phenotypes of arsenic and BaP co-exposure-

transformed cells as evidenced by the fact that METTL3 knockdown significantly reduces their anchorage-dependent and independent growth.

3.4. METTL3 knockdown in chronic arsenic plus BaP co-exposure-transformed cells reduces their CSC-like property

Our recent study demonstrated that chronic arsenic and BaP co-exposure synergizes in inducing cancer stem cell (CSC)-like property and enhancing mouse lung tumorigenesis (Wang et al., 2020). We next determined whether METTL3 up-regulation plays a role in maintaining the CSC-like property of arsenic and BaP co-exposure-transformed cells. The serum-free suspension culture sphere formation assay was used for assessing the CSC-like property of arsenic and BaP co-exposure-transformed cells (Wang et al., 2020; Xie et al., 2020; Yang et al., 2020). As shown in Fig. 5A, arsenic plus BaP co-exposure-transformed cells expressing control shRNA are capable of growing big suspension spheres, however, METTL3 knockdown cells produce much smaller and significantly less suspension spheres. These results suggest that knocking down METTL3 expression in arsenic and BaP co-exposure-transformed cells significantly reduces their CSC-like property. This finding is supported by further Western blot analysis showing that METTL3 knockdown drastically reduces the protein levels of common CSC markers such as KLF4, KLF5, NANOG and Oct4 in arsenic and BaP co-exposure transformed cells (Fig. 5B). Together, these results suggest that METTL3 up-regulation plays an important role in maintaining the CSC-like property of arsenic and BaP co-exposure-transformed cells.

3.5. METTL3 knockdown in chronic arsenic plus BaP co-exposure-transformed cells reduces their tumorigenicity

Cancer stem cells (CSCs) or CSC-like cells are considered tumor initiating cells (Nguyen et al., 2012; Wang and Yang, 2019). Finally, we determined whether up-regulation of METTL3 expression plays a role in the tumorigenicity of arsenic and BaP co-exposure-transformed cells. The nude mouse tumorigenesis assay revealed that subcutaneous injection of 1 million arsenic plus BaP co-exposure-transformed cells expressing control shRNA produces rapidly growing xenograft tumors in all injection sites (Fig. 6A–C). This is consistent with the finding in our recent study showing that arsenic and BaP co-exposure-transformed cells display a significantly stronger tumor forming capability than arsenic alone or BaP alone exposure-transformed cells (Wang et al., 2020). In contrast, subcutaneous injection of 1 million METTL3 stable knockdown cells produces very slowly growing xenograft tumors in about 70% injection sites (Fig. 6A–C). These results along with the findings of METTL3 knockdown reducing serum-free suspension culture sphere formation and CSC marker gene expression shown in Fig. 5 strongly suggest that up-regulation of METTL3 expression plays a critical role in maintaining the strong tumorigenicity of arsenic and BaP co-exposure-transformed cells.

4. Discussion

Arsenic and BaP co-exposure could be common in general populations and certain occupational workers as both arsenic and BaP are commonly presented environmental pollutants, representing a significant environmental health concern. Ours and others'

studies showed that arsenic and BaP co-exposure causes a significantly stronger lung tumorigenic effect than arsenic or BaP exposure alone, however, the underlying mechanism has not been well understood (Pershagen et al., 1984; Wang et al., 2020; Wang, 2021). The findings from this study indicate that arsenic and BaP co-exposure causes epitranscriptomic dysregulations, revealing a previously unidentified mechanism responsible for the synergistic lung tumorigenic effect of arsenic and BaP co-exposure.

The epitranscriptome includes all forms of modifications in all types of RNA transcripts (Saletore et al., 2012; Roundtree and He, 2016). The RNA m^6A methylation is now known as the most prevalent modification in mRNAs. RNA m^6A levels are dynamically and reversibly regulated by its writers and erasers. Recent studies showed that abnormal expression and function of m^6A writers and erasers play important roles in many diseases including cancer (Zhang et al., 2021; Fang et al., 2022; Qi et al., 2023). Particularly in lung cancer, studies showed that abnormal expression of the m^6A writers and erasers are linked with lung cancer development, progression and poor prognosis (Khan and Malla, 2021; Zhang and Xu, 2022; Diao et al., 2023). However, the mechanisms of how the expressions of m^6A writers and erasers are dysregulated during lung cancer development and progression have not been well understood. Arsenic and BaP are both classified as Group I carcinogen causing lung cancer and are important lung cancer etiology factors. The finding from this study showing that arsenic and BaP co-exposure up-regulates the m^6A writer METTL3 expression provides one mechanistic insight for understanding the up-regulation of METTL3 during lung cancer development.

Currently, METTL3 is considered as one of the key components of the m^6A writer complex consisting of METTL3, METTL14, WTAP, and other proteins (Yue et al., 2015; Balacco and Soller, 2019; Uddin et al., 2021). The methyltransferases METTL3 and METTL14 form a heterodimer via the interaction of their methyltransferase domain, which serves as the catalytic core of the RNA m^6A writer complex. Although other components of the m^6A writer complex such as WTAP do not have the methyltransferase activity, they serve as supportive and regulatory subunits to help the localization of the METTL3–METTL14 heterodimer into nuclear speckles and facilitate m^6A deposition to RNA molecules (Yue et al., 2015; Huang et al., 2022). In this study, we found that arsenic and BaP co-exposure significantly increases the expression of METTL3 but does not affect the expression of other components in the m^6A writer complex and the expression of m^6A erasers. Moreover, knocking down the expression of METTL3 in arsenic and BaP co-exposure-transformed cells significantly reduced their total RNA m^6A levels. Taken together, these findings indicate that arsenic and BaP co-exposure causes epitranscriptomic dysregulation mainly through increasing the expression of METTL3.

How may METTL3 up-regulation contribute to the synergistic lung tumorigenic effect of arsenic and BaP co-exposure? Cancer stem cells (CSCs) are considered as cancer initiating cells although the origin of CSCs remains unclear (Nguyen et al., 2012; Wang and Yang, 2019). Ours and other recent studies showed that exposure to chemical carcinogens such as metal carcinogens produce CSC-likes cells and promotes tumorigenesis (Wang and Yang, 2019; Bi et al., 2020; Yang and Wang, 2022). Indeed, our recent study found that arsenic and BaP co-exposure significantly increases mouse lung tumor multiplicity and tumor burden

and mechanistic studies revealed that arsenic and BaP co-exposure produces significantly more CSC-like cells (Wang et al., 2020). In this study, it was further determined that, one potential mechanism for arsenic and BaP co-exposure producing more CSC-like cells could be the epitranscriptomic dysregulation. This is supported by the findings showing that (i) METTL3 knockdown in arsenic and BaP co-exposure-transformed cells significantly reduces their serum-free suspension culture sphere formation; (ii) METTL3 knockdown drastically reduces the expression levels of several CSC markers in arsenic and BaP co-exposure-transformed cells; and (iii) METTL3 knockdown significantly reduces the volumes and weights of mouse subcutaneous xenograft tumors produced by the injection of arsenic and BaP co-exposure-transformed cells.

While this study demonstrated an important role of METTL3 upregulation in maintaining the malignant phenotypes and CSC-like property of arsenic and BaP co-exposure-transformed human bronchial cells, further studies are needed to answer some additional important questions: (i) How does arsenic and BaP co-exposure up-regulate METTL3 expression? (ii) How does METTL3 up-regulation enhance arsenic and BaP co-exposure-induced CSC-like property? Or in other words, which genes and signaling pathways are the targets of the m^6A modification mediated by METTL3 upregulation? (iii) Does METTL3-mediated m^6A modification function through other mechanisms such as reprograming tumor microenvironment to enhance lung tumorigenic effect of arsenic and BaP co-exposure? (iv) Could total RNA m^6A modification levels or certain specific RNA m^6A level be used as a biomarker for early diagnosis of arsenic and BaP co-exposure-caused lung cancer? (v) Could METTL3 serve as a therapeutic target for treating arsenic and BaP co-exposure-caused lung cancer?

5. Conclusion

It was determined that the expression level of the RNA methyltransferase METTL3 in chronic arsenic and BaP co-exposure-transformed cells is much higher than that in arsenic or BaP alone exposure-transformed cells. Moreover, the METTL3 expression level is also drastically higher in mouse lung tumors induced by arsenic and BaP co-exposure than that in mouse lung tumors caused by BaP alone exposure. As a result, arsenic and BaP co-exposure-transformed cells have significantly higher levels of total RNA m^6A modification than the arsenic or BaP alone exposure-transformed cells. Stably knocking down METTL3 expression in arsenic and BaP co-exposure-transformed cells significantly reduces their total RNA m^6A levels. Further functional studies revealed that METTL3 knockdown significantly reduces the malignant phenotypes and CSC-like property of arsenic and BaP co-exposure-transformed cells. Based on these findings, it is concluded that chronic arsenic and BaP co-exposure is capable of causing epi-transcriptomic dysregulation mainly through up-regulating METTL3 expression, which may play an important role in the synergistic lung tumorigenic effect of arsenic and BaP co-exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

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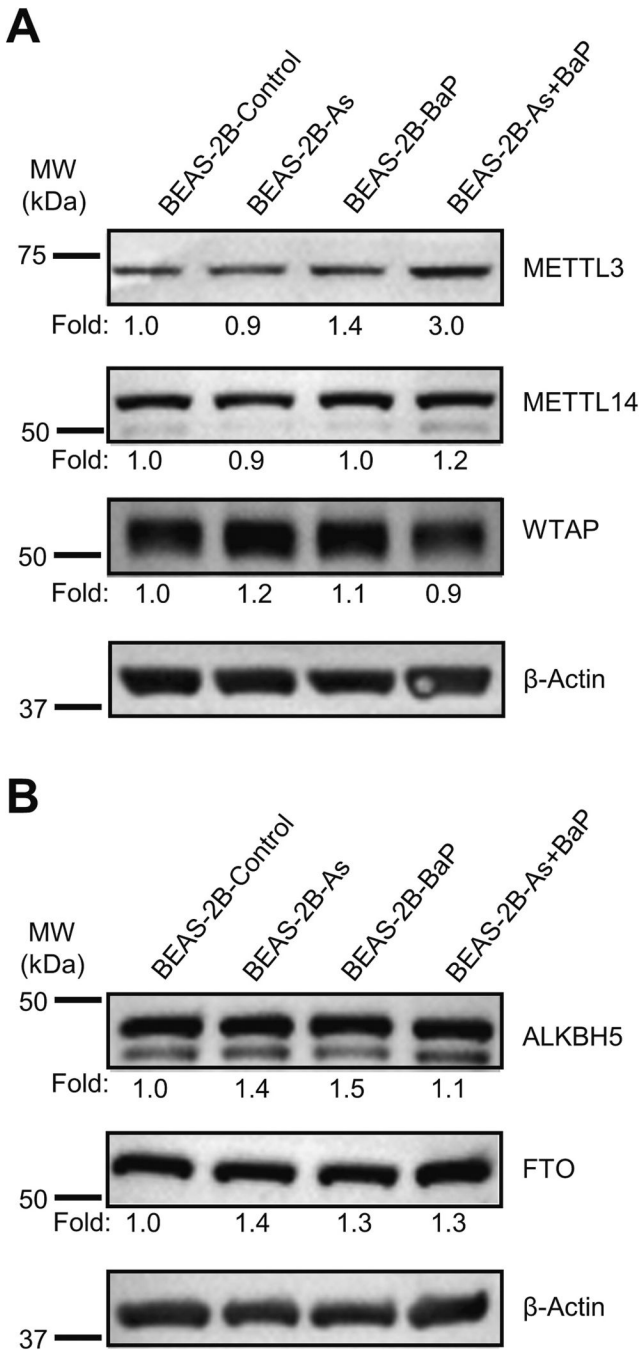

Fig. 1.

Chronic arsenic plus BaP co-exposure increases METTL3 expression level in human bronchial epithelial cells (*A, B*) Representative images of Western blot analysis of the m^6A writer complex major components METTL3, METTL14 and WTAP (*A*), and m^6A erasers FTO and ALKBH5 (*B*) in passage-matched control cell (BEAS-2B-Control), arsenic alone exposure-transformed cells (BEAS-2B-As), BaP alone exposure-transformed cells (BEAS-2B-BaP), and arsenic plus BaP co-exposure-transformed cells (BEAS-2B-As+BaP). The details of chronic arsenic alone, BaP alone exposure and arsenic plus BaP co-exposure

to induce cell transformation were described in our recent publication (Wang et al., 2020). The Western blot band intensities were quantitated using NIH ImageJ software and expressed as the ratio of a specific protein band intensity divided by the corresponding β-actin band intensity. The fold change of a specific protein level in a treatment group is expressed relative to that protein level in the control group.

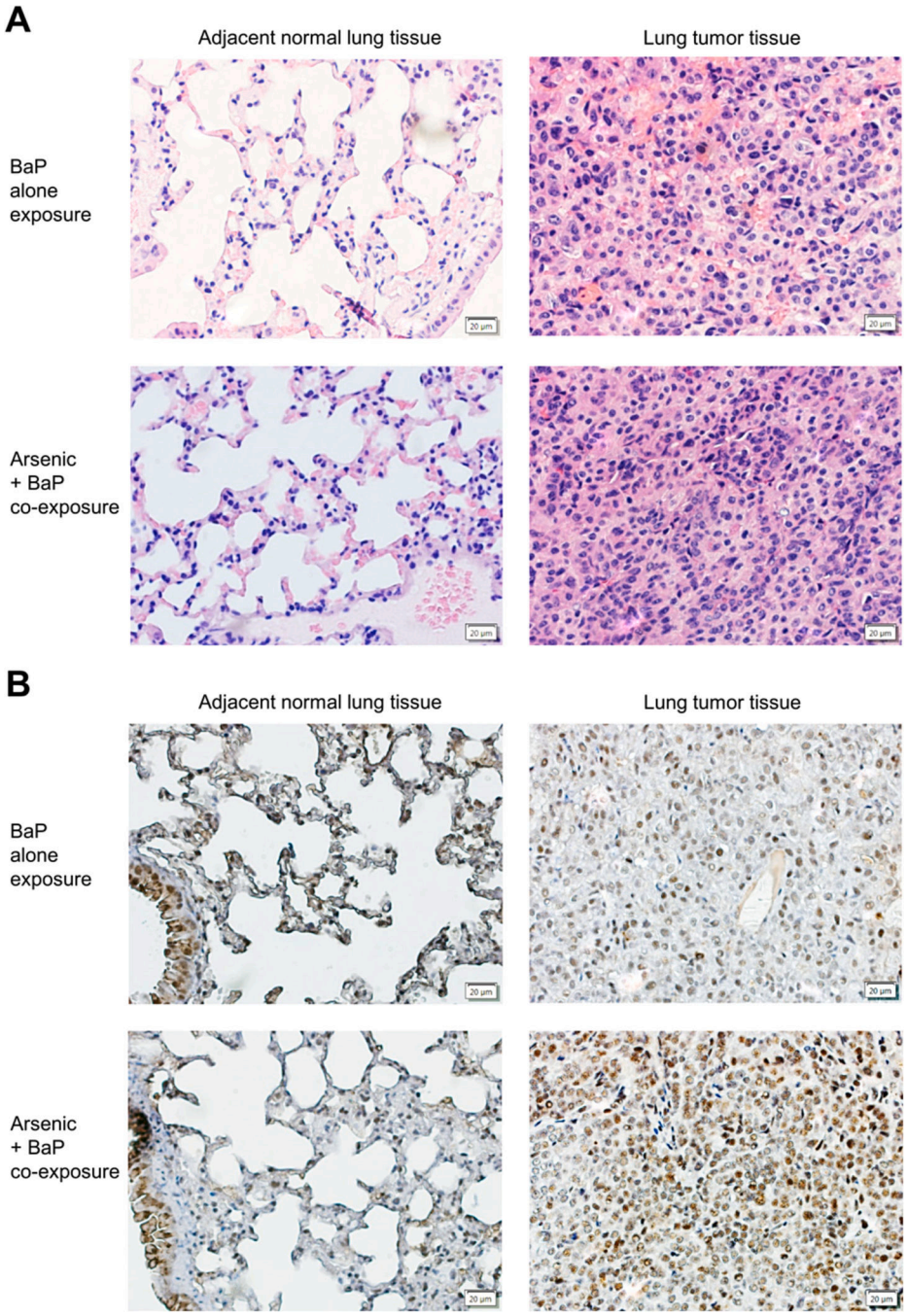

Fig. 2.

Chronic arsenic plus BaP co-exposure increases METTL3 expression level in mouse lung tumor tissues (*A*) Representative H&E staining images of lung tumor tissues and adjacent normal lung tissues of mice exposed to BaP alone or arsenic plus BaP co-exposure. (*B*) Representative METTL3 IHC staining images of lung tumor tissues and adjacent normal lung tissues of mice exposed to BaP alone or arsenic plus BaP co-exposure. The details of mouse BaP alone exposure and arsenic plus BaP co-exposure were described in our recent publication (Wang et al., 2020). Scale bar: 20 μm.

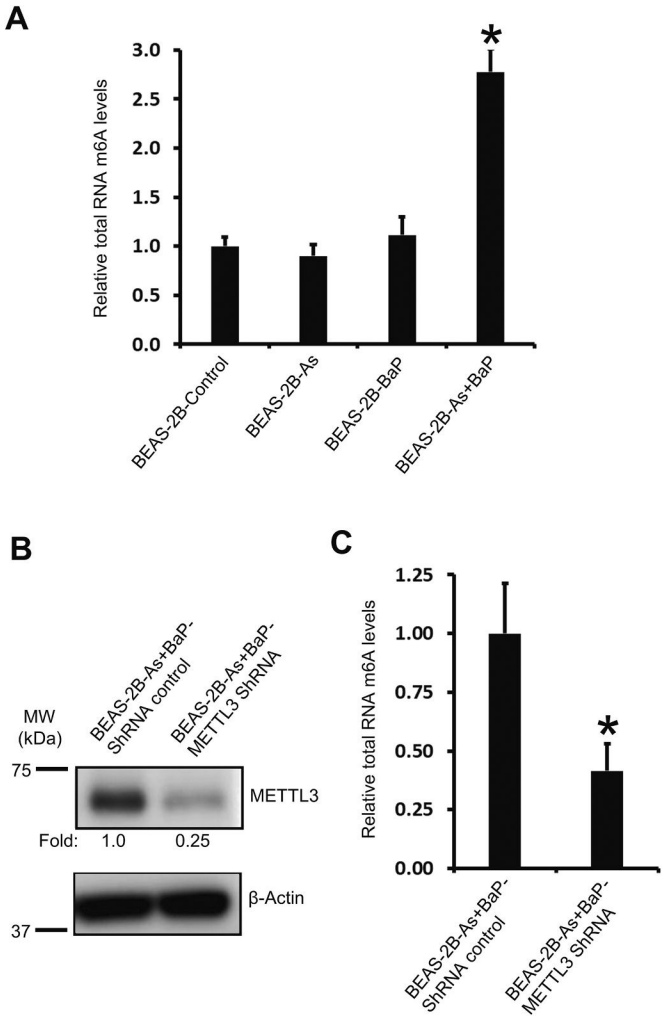

Fig. 3.

Chronic arsenic plus BaP co-exposure increases RNA m^6A levels through up-regulating METTL3 expression (*A*) Relative total RNA m^6A levels determined by using the EpiQuik m^6A RNA Methylation Quantification Kit. The total RNA m^6A levels in arsenic alone exposure-transformed cells (BEAS-2B-As), BaP alone exposure-transformed cells (BEAS-2B-BaP), and arsenic plus BaP co-exposure-transformed cells (BEAS-2B-As+BaP) are expressed relative to the passage-matched control cells (BEAS-2B-Control) (means ± SD, $n = 3$). * $p < 0.05$. (*B*) Representative images of Western blot analysis of METTL3 in arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-As+BaP-METTL3 shRNA). The Western blot band intensities were quantitated using NIH ImageJ software and expressed as the ratio of METTL3 protein band intensity divided by the corresponding β-actin band intensity. The fold change of METTL3 protein level in METTL3 shRNA group is expressed relative to METTL3 protein level in the shRNA control group. (*C*) Relative total RNA m^6A levels determined by using the EpiQuik m^6A RNA Methylation Quantification Kit. The total RNA m^6A levels in BEAS-2B-As+BaP-METTL3 shRNA cells

are expressed relative to the BEAS-2B-As+BaP-shRNA control cells (means ± SD, n = 3). * $p < 0.05$.

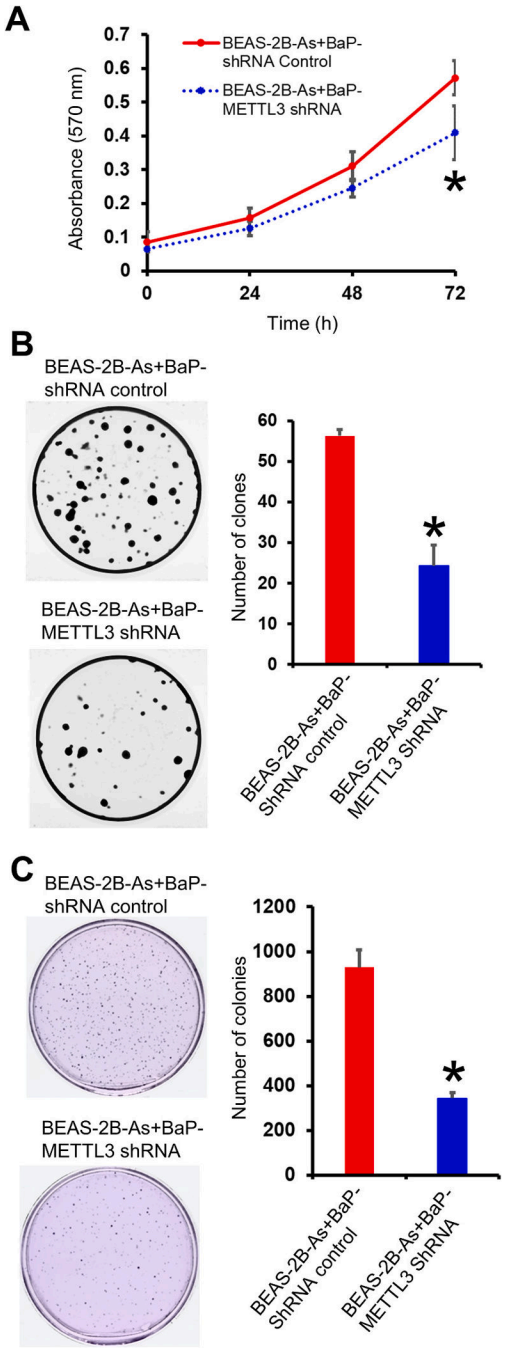

Fig. 4.

METTL3 knockdown reduces chronic arsenic plus BaP co-exposure-transformed cell anchorage-dependent and -independent growth (*A*) The MTT assay showing the effect of METTL3 stable knockdown on the proliferation of arsenic plus BaP co-exposure-transformed cells. The results are presented as means ± standard deviations ($n = 8$). * $p < 0.05$. (*B*) Representative images of clonogenic assay (left) and the quantification of clones formed by arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-

As+BaP-METTL3 shRNA) (right) (means ± SD, n = 3). * $p < 0.05$. (*C*) Representative images of soft agar colony formation assay (left) and the quantification of soft agar colonies formed by arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-As+BaP-METTL3 shRNA) (right) (means ± SD, n = 3). * $p < 0.05$.

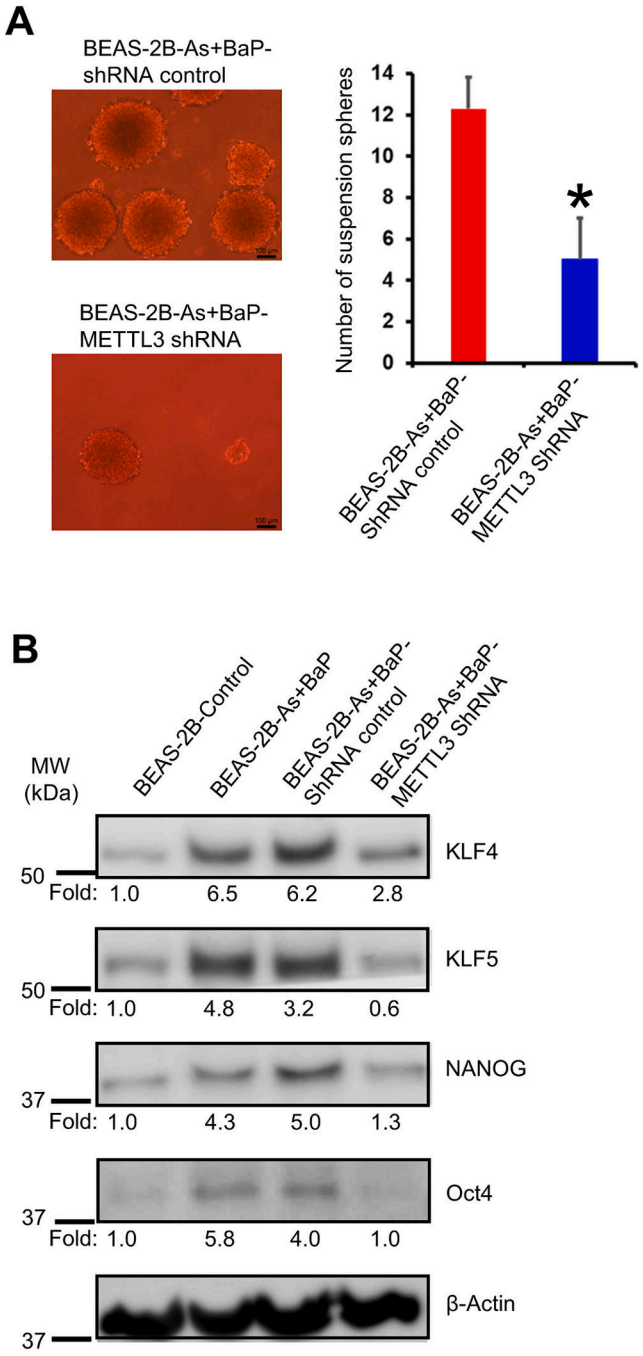

Fig. 5.

METTL3 knockdown reduces chronic arsenic plus BaP co-exposure-transformed cell CSC-like property (*A*) Representative images of serum-free suspension culture sphere formation assay (left) and the quantification of suspension spheres formed by arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-As+BaP-METTL3 shRNA) (right) (means ± SD, n = 3). * $p < 0.05$. (*B*) Representative images of Western blot analysis of cancer stem cell markers in the passage-matched control cells (BEAS-2B-Control),

arsenic plus BaP co-exposure-transformed cells (BEAS-2B-As-+ BaP), and arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-As+BaP-METTL3 shRNA). The Western blot band intensities were quantitated using NIH ImageJ software and expressed as the ratio of a specific protein band intensity divided by the corresponding β-actin band intensity. The fold change of a specific protein level in a treatment group is expressed relative to that protein level in the control group.

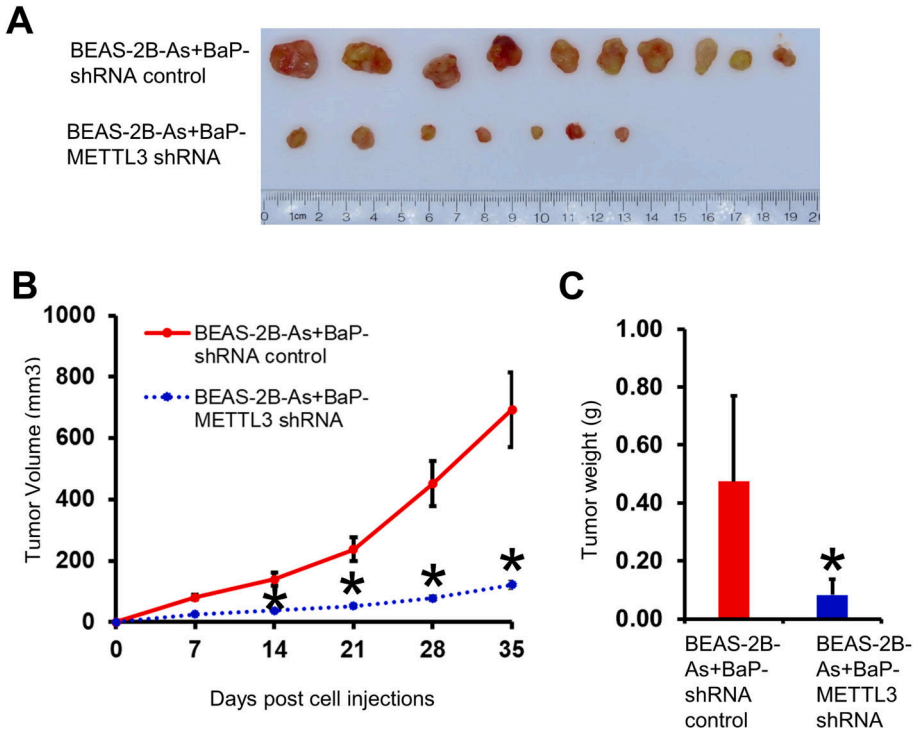

Fig. 6.

METTL3 knockdown in chronic arsenic plus BaP co-exposure-transformed cells reduces their tumorigenicity (*A*) Images of nude mouse subcutaneous xenograft tumors produced by injection of arsenic plus BaP co-exposure-transformed cells that express a control shRNA (BEAS-2B-As+BaP-shRNA control) or METTL3 specific targeting shRNA (BEAS-2B-As+BaP-METTL3 shRNA). Tumors were harvested when mice were euthanized 5 weeks after cell injections. (*B*) Nude mouse subcutaneous xenograft tumor growth curve (means ± SD, $n = 7–10$). * $p < 0.05$. (*C*) The weight of nude mice subcutaneous xenograft tumors harvested when mice were euthanized 5 weeks after cell injections (means ± SD, n = 7–10). * $p < 0.05$.