The Arsenic Induced Endoplasmic Reticulum Stress Response Via The Activating Transcription Factor 6 In Human Bronchial Epithelial Beas-2b Cells

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THE ARSENIC INDUCED ENDOPLASMIC RETICULUM STRESS RESPONSE VIA
THE ACTIVATING TRANSCRIPTION FACTOR 6 IN HUMAN BRONCHIAL
EPITHELIAL BEAS-2B CELLS

by

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DISSERTATION

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Approved By:

________________________________________
Advisor Date
DEDICATION

I would like to dedicate my dissertation to my parents Mr. Amod Wadgaonkar and Mrs. Asha Wadgaonkar, and my grandparents who have always inspired me to achieve higher goals in life. A special feeling of gratitude to my husband, Mr. Akhil Sharma, my in-laws Mr. Pushpinder Kumar Sharma, and Mrs. Aruna Sharma whose words of encouragement have been pillars of support during my research work. I would also like to thank my close family and friends in the USA, Mr.&Mrs. Ambekar, Mr.&Mrs. Bhogaonkar, Mr.&Mrs. Wadgaonkar, Mr. Lingnurkar, their unwavering support, and encouragement in difficult times have been crucial for completion of my doctorate degree at Wayne State University.
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CHAPTER 1: INTRODUCTION

This chapter contains data from published work in which I was the first author. The co-authors in the publication agree to use data in this dissertation. Please note that the content in chapter one is adapted from the peer-reviewed publication “Connections between endoplasmic reticulum stress-associated unfolded protein response, mitochondria, and autophagy in arsenic-induced carcinogenesis ” published in journal Seminars in Cancer Biology (2021).

1.1 Arsenic

1.1.1 Introduction

Arsenic is one of the commonly found components in the earth's crust. It is found as a key element in many organic and inorganic compounds [1]. Organic compounds of arsenic include arsenobetaine, tetramethylarsonium salts, arsenosugars and arseno lipids. The combination of arsenic with chlorides, sulfurs, and oxygen forms inorganic arsenic compounds such as arsenic trichloride, arsenic trisulfide and arsenic trioxide. Various natural and anthropogenic activities lead to the release of arsenic into the environment. Anthropogenic sources of arsenic include drainage from mines, tailing wastes, combustion of coal and fossil fuels, and untreated industrial wastes. Arsenic is also used in commercial processes like pharmaceuticals, agricultural pesticides and herbicides, chemical and optoelectronics industries, glassmaking, and paints, leading to its exposure to humans. Various natural phenomena like volcanoes, weathering of arsenic-bearing rocks, and geothermal activities release arsenic into the environment. As per the Centers for Disease Control & Prevention (CDC) data, anthropogenic activities result in nearly three times higher arsenic release in the environment than natural activities [2, 3].
Exposure to elevated levels of arsenic causes a series of acute and chronic health problems. Acute arsenic toxicity leads to nausea, vomiting, diarrhea, acute tubular necrosis, convulsions, and organ failure. At the same time, chronic arsenic toxicity gives rise to cancers of the lungs, skin, liver, kidney, bladder, and prostate. Diabetes mellitus, nervous system disorders, cardiovascular diseases, and gastrointestinal disturbances are some of the other non-cancer disorders that develop due to chronic arsenic toxicity [4-6]. Humans are primarily exposed to arsenic via polluted drinking water, food, and air. Of these sources, arsenic exposure via contaminated drinking water is a significant health hazard in the world. To protect human health, arsenic use was prohibited and minimized in various industrial and agricultural applications. The new permissible limit of arsenic in drinking water as per World Health Organization (WHO), the US Environment Protection Agency (EPA), and EU Pharmacopoeias is 10ppb (10ug/L) [5, 7]. However, despite the regulations in place, in many countries such as the USA, China, Chile, Argentina, Mexico, India, Bangladesh, and Mongolia, an estimated 94-220 million people are exposed to high levels of arsenic in drinking water (>10ug/L) [4].

1.1.2 Arsenic-induced lung cancers

Cancer is a disease where there is an uncontrolled division of cells. The cells undergo malignant transformation and invade the surrounding tissues or reach distant organs, known as metastasis. Metastasis is the primary reason for cancer-related deaths. The inability of the body to repair the genetic damage or control the malignant transformation of cells leads to cancer. Cancer is caused by a combination of external carcinogens (physical, chemical, biological) and epigenetic and genetic changes in our body [8]. Risk factors for cancer include exposure to carcinogens like arsenic, lead and cadmium, smoking, age, sex, diet and lifestyle habits, occupational hazards, stress, viral infections, and medical conditions.
Lung cancer can be broadly categorized as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). NSCLC forms 80% of the total cancer cases, whereas SCLC accounts for 10-15% of the total cases. Smoking is a significant risk factor for most lung cancers except adenocarcinoma, a type of NSCLC. NSCLC can be further categorized based on the tissue histology as adenocarcinoma (ADC), squamous cell carcinoma (SqCC) and large cell lung carcinoma (LCLC) [9]. SCLC, an aggressive form of lung cancer, is more strongly associated with smoking. It has a neuroendocrine origin and is often seen together with NSCLC. Risk factors include exposure to tobacco smoke, halogenated ethers, arsenic, asbestos, radon, and polyaromatic hydrocarbons. According to the World Health Organization, lung cancer is the second-most common cancer (2020) after breast cancer but has the overall highest death rate. Lung cancer has the highest mortality rate of all cancers. The five-year survival rate of men and women diagnosed with lung and bronchus cancer is a dismal 21.7%, compared to a significantly higher survival rate of 90.3% for breast cancer (2011-2017). Lung cancer cases are often diagnosed at advanced stages where cure becomes difficult (https://seer.cancer.gov/statfacts/html/lungb.html). Hence, it is critical to investigate biomarkers and new anti-cancer therapeutics for the early diagnosis and treatment of lung cancer.

The lungs are a major target for arsenic toxicity. A clear correlation has been established between arsenic exposure and the development of lung cancers either from drinking contaminated water or inhalation of polluted air. When breathed in, the air containing arsenic dust leads to most arsenic particles settling in the lining of the lungs. Lung cancer development via the inhalation mode is highly associated with workers involved in mining and smelting activities. Several case-control and cohort studies conducted in Taiwan, Japan, Chile showed a positive correlation between arsenic in drinking water and development of lung cancers. The potential explanation for
arsenic induced lung cancers via drinking water is the excretion of dimethyl arsine gas from the lungs. Dimethyl arsine gas (III) is produced in vivo via reduction of dimethyl-arsenic acid (V)- a metabolite of arsenic. Dimethyl arsine can react with molecular oxygen forming superoxide anion and (CH$_3$)$_2$As$^+$ radical. High partial pressures of oxygen are found in the lungs. Arsenic may induce cancer in the lungs due to the high partial pressures of oxygen and the excretion of dimethyl arsine gas in the lungs which generates oxidative stress [10-12]. Exposure to arsenic-induced free radicals can cause DNA damage.

1.1.3 Chemistry

Arsenic is found chiefly as organic and inorganic compounds in the surroundings. It is rarely found as a pure metalloid in the environment [1]. Inorganic arsenic can be found as pentavalent arsenate (As$^{5+}$) or trivalent arsenite (As$^{3+}$) combined with elements like chlorine, sulfur, sodium, and oxygen, whereas organic arsenicals are compounds of arsenic with carbon and hydrogen. Both forms of arsenic are excreted in urine after metabolism. However, inorganic arsenic is expelled from the body after several days, although some metabolites may remain in the body for several months. The organic forms of arsenic are removed rapidly from the body within several days. Hence, in general, the inorganic forms are more toxic than the organic forms of arsenic [4, 10].

Arsenic is a metalloid, placed in Group 15 in the periodic table and a nitrogen family member. It has atomic number 33 with the electronic configuration as follows: 1s$^2$ 2s$^2$ 2p$^6$ 3s$^2$ 3p$^6$ 3d$^{10}$ 4s$^2$ 4p$^6$. The fourth shell is incompletely filled (4p orbital), which enables arsenic to readily participate in covalent bond formation. It displays four common redox states -3, 0, +3 and +5 [4, 11]. Since arsenic has a greater electronegativity (compared to nitrogen), it is more inclined to maintain an oxidative status (As$^{5+}$ and As$^{3+}$). Also, arsenate (As$^{5+}$) and arsenite (As$^{3+}$) are the most
prominent species of inorganic arsenic found in drinking water. Arsenic is known to form many biomolecules with methyl groups due to stable bond formation. Metabolism of inorganic arsenic to methylarsonic acid (MMA) and dimethylarsinic acid (DMA) in the human body after ingestion are some examples of biomolecules formed with methyl groups [11, 12].

1.1.4 Pharmacokinetics

Arsenic is a well-known carcinogen and toxicant. It is not required for any physiological processes of the body. Arsenic enters the body due to its ability to mimic essential nutrients and minerals like glycerol, glucose and phosphate and share their uptake systems and transporters [13]. The two most common oxidation states of inorganic arsenic, arsenate (As\(^{5+}\)), utilize the phosphate transporters, whereas arsenite (As\(^{3+}\)) enters the cells through aquaglyceroporins via simple diffusion. Arsenite (As\(^{3+}\)) has a higher cellular uptake and is more toxic than arsenate (As\(^{5+}\)) [4, 14]. Arsenic salts are easily absorbed in the body (via ingestion, inhalation or dermal absorption) and distributed to various organs such as the GI tract and liver, lungs, bladder, heart, spleen, and kidney. Also, they accumulate in the skin tissues [12, 14]. Post-absorption, arsenic undergoes metabolism primarily in the liver, although some in-vitro studies show that arsenic biotransformation via methylation also occurs in the cytosol of the lungs, kidneys, and testes. Biomethylation is the prime pathway by which inorganic arsenic is metabolized in almost all species [15]. A series of oxidative/reductive and methylation reactions occur, utilizing S-adenosyl methionine (SAM) as the methyl donor and reduced Glutathione (GSH) as the electron donor. These reactions are catalyzed by arsenite methyltransferases (AS3MT) and glutathione-S-transferase Omega1 (GSTO1) [4, 14]. A schematic diagram describes the cellular uptake of arsenate, its mechanism of action and excretion from the cells in Figure 1 [4].
Figure 1: Arsenic pharmacokinetics and its proposed carcinogenic mechanisms of action

Arsenate ($\text{As}^{5+}$) enters the cell via phosphate transporters, and arsenite ($\text{As}^{3+}$) through aquaglyceroporins (not shown in the figure). The arsenate is reduced to arsenite using two molecules of glutathione (GSH) by GSH reductase or by directly by arsenate reductase. The arsenite is mono or demethylated using S-adenosylmethionine (SAM) as a methyl donor in the presence of arsenic methyl transferases (AS3MT) to monomethyl arsenic acid or dimethyl arsinic acid. The methylated arsenic species are excreted from the body in urine. The inorganic arsenite and its methylated species, through oxidative stress, cause DNA, protein, and lipid damage-causing carcinogenic mutations, mitochondrial dysfunction, activation of signaling pathways related to cell proliferation, apoptosis and survival, impaired DNA repair and altered immune mechanisms. Arsenic biotransformation leads to SAM depletion causing epigenetic changes (hypomethylation of oncogenes or hypermethylation of tumor suppressors) [4]
1.1.5 Mechanisms of arsenic-induced carcinogenesis

Various epidemiological studies have linked long-term arsenic exposure to cancers of the lung, skin, liver, bladder and kidneys [1]. The International Agency for Research on Cancer (IARC) has classified arsenic and its compounds as a group I human carcinogens due to its carcinogenic ability, exposure potential and ubiquitous nature. Arsenic has been considered since 2011, the number 1 toxic substance by the United States Agency for Toxic Substances and Disease Registry (ATSDR), ahead of mercury, lead, and polychlorinated biphenyls [15]. There is sufficient evidence currently available to prove that arsenic is a human carcinogen. Various factors such as dose, exposure levels, genetics, medical condition, smoking history, occupational hazards, and metabolizing ability of individuals affect the arsenic-induced carcinogenic process. The proposed mechanisms of arsenic carcinogenesis are generation of oxidative stress, modified protein structure and functions (arsenite binding to sulfhydryl moieties in proteins), inhibition of DNA repair, activation of stress and other intracellular signaling pathways, cytotoxicity and regenerative proliferation, endoplasmic reticulum stress responses, disruption of mitochondrial dynamics and autophagic processes. Inorganic arsenic does not directly cause mutations or form DNA adducts, and it is a non-genotoxic carcinogen [16][4, 17].

Inorganic arsenic is known to induce the generation of excessive reactive oxygen species (ROS), which impacts the cellular signaling pathways and biomolecules. Arsenite (As$^{3+}$), the trivalent form of inorganic arsenic, is the primary inducer of ROS in the cells. The electrons produced during arsenic metabolism (arsenate reduction to arsenite) generate hydrogen peroxide (H$_2$O$_2$). Hydrogen peroxide by Fenton reaction produces hydroxyl radical, which is very reactive and causes macromolecule oxidation. Arsenic also produces ROS by activating the membrane-bound NADPH oxidase to produce superoxide radicals [1]. Mitochondrion, the powerhouse of the
cells, is an essential organelle of physiological ROS production. Arsenic and its methylated species induce ROS in the mitochondria through complex I and complex II of the electron transport chain (ETC) [18]. This, in turn, impacts mitochondrial dynamics and its physiological functions. Generation of oxidative stress due to ROS or reactive nitrogen species (RNS) production activates the intracellular stress signaling pathways such as JNK/AP-1, MAPK kinases, NF/κβ and Wnt/beta-catenin pathways. These signaling pathways regulate different processes such as cell proliferation, apoptosis, differentiation, and immune responses [17]. Previous studies by our group showed arsenic-induced EZH2 phosphorylation in human bronchial epithelial BEAS-2B cells and human lung cancer A549 cells via the JNK-Stat3-Akt pathway. JNK-Stat3-Akt is an intracellular pathway activated under stress conditions. Oxidative stress (ROS) was involved in the EZH2 phosphorylation as treatment with the antioxidant N-acetyl-L-cysteine (NAC) abrogated arsenic-induced EZH2 phosphorylation and inhibited the JNK, Stat3 and AKT activation in BEAS-2B and A549 cells. Enhancer of zeste homolog 2 (EZH2) is a component of the polycomb-repressive complexes (PRC2) that catalyzes the trimethylation of histone protein H3K27, leading to modification of expression of tumor suppressors and oncogenes. EZH2 is commonly found to be overexpressed in cancers [19, 20].

Excessive ROS generated by inorganic arsenite (As^{3+}) also influences the DNA-repair systems, as shown in some studies. A study by our group showed that arsenic-induced mdig, a mineral dust induced gene, in BEAS-2B and A549 cells. Proteomic studies further revealed that the mdig – non-homologous end joining repair complex (NHEJ) interaction weakened the double-strand repair function of the NHEJ complex [1]. Arsenic is also shown to inhibit base excision repair (BER), nucleotide excision repair (NER), and mismatch repair leading to genotoxicity [17]. The chemical form of the inorganic arsenic, namely arsenate (As^{5+}) and arsenite (As^{3+}), has
divergent carcinogenic effects in humans. Arsenate (As\(^{5+}\)), the pentavalent form of inorganic arsenic, is believed to be a chemical analog of phosphate. It can compete with phosphate for the phosphate ion transporters and supplant phosphate. This is especially of concern for molecules made up of phosphate, such as the energy molecule produced in the mitochondrion, the adenosine triphosphate (ATP). Trivalent inorganic arsenicals like arsenite (As\(^{3+}\)) are known to bind to thiol groups of proteins. The arsenite (As\(^{3+}\)) binding to proteins (e.g., glutathione, GSH) changes their conformation and affects their functions. Hence, it is essential to understand the chemistry and pharmacokinetics of arsenic to elucidate its carcinogenic mechanisms [15].

Genetics has traditionally been the focal point in oncology, from initiation of cancer to metastasis. However, with discoveries, such as the altered DNA methylation mark found in cancer in 1983, epigenetics is considered an equally important subject in oncology for developing anticancer therapeutics [21, 22]. Epigenetics can be defined as the heritable changes in phenotype without changes in DNA sequence. It includes the study of DNA methylation, histone tail modifications, and the non-coding RNAs’ effect on gene expression with respect to environmental changes [1]. DNA methylation at the CpG islands of gene promoter regions in dinucleotides plays a crucial role in gene expression. DNA hypomethylation will promote transcriptional activation, whereas hypermethylation will cause transcriptional repression [22]. It is known that methylation is an essential step of arsenic biotransformation where S-adenosyl methionine (SAM) is utilized as a methyl group donor by the arsenic methyltransferases (AS3MT). The arsenic biomethylation may have effects on the DNA and histone methylation status. Changes in DNA methylation were followed by altered gene expression in some in vitro and in vivo studies. These changes were linked to the expression of oncogenes and tumor suppressors, metastasis, and malignant transformation of cells [17, 23].
The histone tail modifications such as methylation, acetylation, phosphorylation, sumoylation, biotinylation and ubiquitylation also affect gene expression. There will be transcriptional activation or repression depending upon the histone tail modification and the associated amino acid of the histone protein. Non-coding RNAs, initially termed "clutter," are now important in regulating gene expression. For example, the microRNAs (miRNA), a non-coding RNA, binds to messenger RNA (mRNA) and inhibit translation. The miRNAs can act as either tumor suppressors or oncomirs. Previous studies from our group showed that arsenic-induced miR-190, a miRNA that reduces the expression of PHLPP, is a negative regulator of Akt. PHLPP is a protein phosphatase that may act as a tumor suppressor in cancers. Another study showed that arsenic also increases the expression of miR-21, a well-known oncomir [1]. Apart from epigenetics, metabolism, and other intracellular signaling pathways discussed above, the endoplasmic reticulum stress (ER stress) activated unfolded protein response (UPR) plays a significant role in arsenic-induced carcinogenesis. We will discuss in the coming sections the role of endoplasmic reticulum stress-activated unfolded protein response and mitochondrion in arsenic-induced carcinogenesis and the generation of cancer stem cells.

1.2 ER stress activated UPR and the protein quality control systems

The endoplasmic reticulum (ER) is one of the most prominent cellular organelles made up of cisternae, a series of membrane-enclosed flattened sacs and interlinked tubules encircling the ER luminal space [24]. There are two types of ER viz., rough and smooth ER. The rough ER is named so because of ribosomes, the protein synthesis units attached to its outer surface. Its membrane is continuous with the outer membrane of the nucleus. The smooth ER, on the other hand, does not possess ribosomes and is mainly concerned with the storage of lipids and calcium.
ions and the detoxification of xenobiotics. The focus of our study will be the rough ER, henceforth referred to as the ER.

Maintaining proteostasis is an essential function of the ER. Post the synthesis of proteins by the ribosome-messenger RNA (mRNA) machinery, proteins are properly folded and undergo structural maturation and post-translational modifications in the ER before being transported to the various destinations in the cells. Various quality control pathways such as the ubiquitin-proteosome system (UPS), which is a component of the ER-associated protein degradation (ERAD), the ER-phagy receptors, Golgi-phagy and plasma membrane phagy all play an important role in protein homeostasis [25]. The ER protein-folding machinery is less efficient in maintaining protein fidelity compared to the DNA error-repair systems and hence more vulnerable to physiological and pathological insults [24]. Various stimuli like oxidative stress, nutritional deficiency, viral infections, disturbances in calcium homeostasis, hypoxia, oncogene activation, increased protein folding, and a load of mutated proteins that cannot be properly folded cause ER stress. To overcome ER stress and restore normal cellular functions, the unfolded protein response (UPR) is activated. The UPR signaling pathway resolves ER stress in three ways: (1) Promotes transcriptional activation and synthesis of the ER chaperones such as GRP78 and GRP94 to increase protein folding, (2) selective mRNA translational inhibition to reduce folding load, (3) endoplasmic reticulum associated protein degradation (ERAD) to degrade the improperly folded proteins [4, 26-28]. The UPR also aims to initiate other downstream responses such as the inflammatory and antioxidant responses, autophagy, apoptosis, ER biogenesis and amino acid metabolism. Depending upon the extent and severity of the ER stress, the UPR pathway can activate the proadaptive or proapoptotic response [29].
The UPR is an evolutionarily conserved pathway first discovered in yeast. Three ER transmembrane proteins, IRE1α, PERK and ATF6α, are activated in response to ER stress. These sensors, under non-stress conditions, are bound to the BiP/GRP78 protein, which maintains them in a dormant state. However, with an overload of proteins or misfolded proteins in the ER lumen, GRP78 detaches from the sensors to assist in the protein folding process [30]. This causes activation of inositol requiring enzyme 1 alpha (IRE1α), protein-kinase R (PKR)-like ER kinase (PERK) and activating transcription factor 6 alpha (ATF6α) proteins which stimulate their respective prosurvival downstream pathways. PERK activation leads to the phosphorylation of eukaryotic translation initiation factor alpha (eIF2α), causing inhibition of mRNA translation, thus reducing protein folding load. At the same time, PERK authorizes the selective mRNA translation via the PERK-ATF4 pathway of genes related to autophagy, antioxidant response, amino acid metabolism and ER protein folding [4, 30]. IRE1α performs a dual function of both kinase and endoribonuclease under ER stress conditions. IRE1α on activation oligomerizes and autophosphorylates itself to induce its endoribonuclease activity. It excises a 26-nucleotide intron from the X-box binding protein 1 (Xbp1), leading to its activation. The spliced XBP1 increases the expression of genes related to ER protein folding and ERAD. Other pathways activated by IRE1α include the regulated IRE1-dependent decay (RIDD), which degrades specific mRNAs and miRNAs and the IRE1α-TRAF signaling pathway connected with the stress, inflammation, and autophagy pathways [30]. The ATF6 protein is a transcription factor having a basic leucine zipper domain. Under ER stress conditions, ATF6 is cleaved and transported to the Golgi body. The serine site one (S1P) and serine site two proteases (S2P) at the Golgi bodies cut the ATF6 full-length protein ATF6p90 to release the active cleaved form ATF6p50. This cleaved form of ATF6 now travels to the nucleus to induce the expression of genes associated with protein folding,
ERAD, protein secretion and ER/Golgi biogenesis [30, 31]. If these protein sensors cannot overcome ER stress via its primary prosurvival signaling pathways, apoptotic pathways are activated, leading to cell death.

PERK and IRE1α are highly investigated proteins in the UPR-cell death pathways of the three ER stress sensors. PERK-eIF2α pathway under unresolved ER stress settings activates C/EBP homologous protein (CHOP) and Growth Arrest and DNA Damage-Inducible Protein (GADD34) to promote apoptosis. The IRE1α is known to induce apoptosis via caspase-2 and caspase-8 dependent pathways. Other IRE1α pathways that induce cell death include the ASK1-JNK pathway and RIDD related mRNA degradation. IRE1α activates the apoptotic-signaling kinase 1 (ASK1) upon ER stress, stimulating Jun-N-terminal kinase (JNK) and p38 MAPK to promote apoptosis. JNK phosphorylates anti-apoptotic proteins Bim and BCl2, leading to its activation and inhibition to further apoptosis. Stress kinase p38MAPK phosphorylates the transcription factor (CHOP) to induce apoptosis. The IRE1α-mediated RIDD degradation of miRNA and mRNA has also been suggested to have a proapoptotic role [30, 32]. However, it needs further investigation. The proapoptotic role of ATF6α via the induction of CHOP or through XBP1 genes has also been suggested. Besides the UPR, the release of Ca^{2+} ions from the inositol 1,4,5-triphosphate receptor (IP_{3}R) of the ER contributes to ROS release from the mitochondrion and activation of the BAK/BAX apoptosome [30]. The ER stress-activated UPR signaling pathways have been described in Figure 2 [4].
Figure 2: ER stress-activated unfolded protein response

Under non-stress conditions, chaperone BiP/Grp78 is attached to three ER sensors, viz., the protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), inositol requiring kinase 1α (IRE1α) and activating transcription factor 6 (ATF6) and maintains them in a dormant state. Accumulation of unfolded proteins in the ER lumen leads to ER stress, which activates the unfolded protein response (UPR). During ER stress, BiP binds to the hydrophobic exposed regions of misfolded/unfolded proteins activating the ER stress sensors PERK, IRE1α and ATF6. These sensors primarily activate downstream UPR genes involved in protein folding, ER-associated protein degradation (ERAD), lipid biosynthesis, and autophagy. However, prolonged ER stress leads to the activation of pro-death UPR signaling pathways [4].

Autophagy, like the UPR, is an evolutionarily conserved process and one of the cells' central protein quality control systems besides the ubiquitin-proteasome systems (UPS). It engulfs cellular debris and improperly folded proteins to restore homeostasis. Autophagy has a prosurvival mechanism under normal physiological conditions. However, this proadaptive function becomes
pernicious when cancer cells utilize autophagy for survival [33]. Thus, it is believed that autophagy has a context-dependent role in cancer. [4] Autophagy can have a tumor-suppressive role by recycling/removing damaged and mutated organelles and proteins and lowering chromosome instability. On the other hand, autophagy can promote tumor development by helping cancer cells survive in nutrient-poor conditions and resisting chemotherapy and other anticancer treatments [33]. Autophagy is maintained constitutively at basal levels under non-stress conditions to sustain homeostasis. Stress signals such as starvation, hypoxia, and ER stress induce autophagy higher than its constitutive activation [4]. Autophagy is a highly regulated process that occurs in many steps, namely the formation of the autophagosome, the fusion of the autophagosome with the lysosome, and the degradation of the engulfed cargo by enzymes like hydrolases. The protein unc-51-like kinase (ULK1) dissociates from the mammalian target of rapamycin complex 1 (mTORC1) under autophagy inducing conditions such as starvation and ER stress. It leads to the activation of ULK1 and its autophosphorylation. The activation of AMP protein kinase (AMPK) is also essential to start the autophagy process. ULK1 then phosphorylates mAtg13 and FIP200. The activated ULK1-Atg13-FIP200-Atg101 complex assists in the formation of the isolated membrane/phagophore. ULK1 also phosphorylates Beclin1 to facilitate autophagy nucleation. Beclin1 forms a part of the PI3K complex along with AMBRA, VPS34 and p150, which directs the nucleation step in autophagy. The phagophore digests the cellular debris/cargo at this stage, followed by the phagophore’s elongation. The elongation step of autophagy is implemented by the ubiquitin-conjugating systems Atg5-Atg12-Atg16 L and LC3II-PE conjugates. Thus, the three-step initiation, nucleation, and elongation of phagophore encapsulating the cellular debris lead to autophagosome formation. The autophagosome fuses with lysosomes leading to the formation of autophagolysosomal and degradation by the lysosomal enzymes [4, 34]. The ubiquitin-
proteosome system (UPS) is another important pathway of protein degradation where approximately 80% of total proteins are degraded and sometimes is used interchangeably as ERAD. Several studies now link the UPS to ER stress especially the E3 ligases and deubiquitinases (DUBs). Out of the three ER stress protein sensors, IRE1α has been highly investigated and linked to the UPS [35].

Autophagy and apoptosis are functionally distinct cellular mechanisms, but both can be activated in response to ER stress. The threshold of stress to induce apoptosis can be increased by autophagy as it recycles and degrades damaged organelles within cells and cells within tissues. Also, dead cells or products released during apoptosis can trigger autophagy. There is interesting crosstalk between autophagy and apoptosis in the same cells or between cells, and it is known to play a role in pathological conditions like cancer and aging [36].

1.3 UPR, metabolism and autophagy in cancer development

Various mechanisms of arsenic-carcinogenesis have been extensively explored, from the generation of oxidative stress that damages the biomolecules like DNA, lipids, and proteins to the activation of intracellular stress signaling pathways like NF-κβ and JNK. Since arsenic is known to induce oxidative stress and other types of stresses, it is imperative to investigate the ER stress activated UPR pathway and its role in arsenic carcinogenesis and the generation of cancer stem-like cells [1, 4]. It is well-known that the UPR signaling pathway is highly active in many cancers such as the breast, prostate, colon, and lung. Many studies show that UPR is involved in almost all the stages of cancer development, from oncogenesis to metastasis [37]. Loss of tumor suppressor genes or activation of oncogenes leads to the malignant transformation of cells. Higher rates of cellular proliferation require higher protein folding capacity. The tumor also faces a harsh internal environment such as low oxygen levels, nutrient deprivation, and acidosis. Utilizing the pro-
survival pathways of UPR becomes essential to sustain the needs of the increased cell proliferation and to overcome the external and internal stresses. Increasing protein folding capacity, angiogenesis, and metabolic reprogramming are some of the proposed UPR pathway mechanisms promoting tumor progression [37-39].

Angiogenesis is essential in maintaining a continuous supply of oxygen and nutrients to the cancerous cells. VEGF-A is one of the well-known angiogenic factors activated either by the IRE1α-XBP1 or the PERK-ATF4 pathways [37]. Very few studies have described a direct link between UPR pathway stimulated angiogenesis factors and arsenic-induced cancers. However, a study by He et al. demonstrated that chronic arsenite (As\textsuperscript{3+}) exposure-induced ROS via DNMT1-methylation inhibited miR199a (microRNA) expression, which resulted in HIF1α and COX2 activation. Two-way regulation between HIF1α and COX2 promoted angiogenesis and tumor growth through positive feedback in human bronchial epithelial cells [40]. Another study by Bi et al. in human bronchial epithelial BEAS-2B cells established that short-term arsenite (As\textsuperscript{3+}) treatment induced PERK, Grp78 and HIF1α via Nrf2 activation. UPR-related angiogenesis was not the primary area of study in this report; however, it would be interesting to explore the crosstalk between UPR and angiogenic factor VEGF-A since another study using the same cell line suggested that arsenite (As\textsuperscript{3+}) induced HIF1α which promoted angiogenesis [41]. The IRE1α-XBP1 pathway has also been implicated in HIF1α-induced VEGFA production and angiogenesis. Further studies should investigate a possible connection between UPR and angiogenesis in arsenic-induced cancers.

A multifaceted process, metastasis requires cancer cells to undergo epithelial-to-mesenchymal transition (EMT) to enter the blood circulation and metastasize to distant sites in the
body. It is the leading cause of fatality in cancers. A study by Wu et al. had shown that human bronchial epithelial BEAS-2B cells treated with a sub-lethal dose of sodium arsenite ($\text{As}^{3+}$) (0.25-2.5 $\mu$M) lead to the malignant transformation of cells via the activation of the MEK/ERK1/2 pathway. It also induced EMT [39, 42]. UPR also seemed to play a role in tumor-associated inflammation and immune responses. In mouse macrophage cell line RAW 264.7, arsenic trioxide (ATO) disrupted the macrophage function via PERK-ATF4 signaling. This disruption was alleviated by using antioxidant N-acetyl cysteine (NAC) or chemical chaperone PBA treatment, suggesting the role of ROS and UPR. Also, the chronic exposure to sodium arsenite As$^{3+}$ (10ppb and 100ppb) resulted in significant alterations in the expression of genes related to the innate immune response in mouse lungs [4].

The ER regulates lipid homeostasis, glucose, and amino acid metabolism. Cancer cells must rewire their metabolism to sustain the rapid cell proliferation rates, satisfy the increased energy demand, generate metabolic precursors for anabolism and maintain the redox balance in the cells [43]. In most cancers, a commonly found metabolic shift is from mitochondrial oxidative phosphorylation to glycolysis in the presence of oxygen called the “Warburg effect.” A study by Li et al. found that long-term (6 months) arsenite ($\text{As}^{3+}$) treatment at an environmentally relevant concentration (0.125-0.25 $\mu$M As$^{3+}$) in BEAS-2-B cells induced malignant transformation of cells. Some of the transformed cells showed cancer-stem-like (CSCs) features. The transcriptomic studies in this arsenic-induced CSCs revealed a significant downregulation in genes associated with mitochondrial oxidative phosphorylation and TCA cycle, ER stress and autophagy. It may be possible that reduced ROS generation in the CSCs, due to downregulation of mitochondrial oxidative phosphorylation genes is important to maintain the cell stemness, as increased ROS levels promote cell differentiation [44]. On the other hand, upregulation of glycolysis genes was
observed in the CSCs compared to the control or non-CSCs. Such a change leads to the accumulation of glycolytic intermediates or metabolites in the pentose phosphate pathway for biosynthesis of NADPH and nucleotides, one-carbon metabolism of the serine-glycine pathway for S-adenosylmethionine (SAM) generation and hexosamine pathway for post-translational modification of key proteins [4, 39]. Glycolysis and other metabolic pathways like the serine-glycine, pentose phosphate and hexosamine pathway are essential in maintaining the cancer cell stemness. SAM is an important methyl donor in DNA and histone methylation. In mouse and human embryonic stem cells (ESC), SAM is required for self-renewal and maintenance of ESCs as SAM depletion leads to differentiation. The hexosamine synthesis is pivotal for glycosylation and activation of the key transcription factors Oct4 and Sox2 for the self-renewal of the ESCs and CSCs [45].

Uridine diphosphate N-acetyl glucosamine (O-GlcNAc) is an essential post-translational modification that is upregulated in cancers. Its formation is catalyzed by OGlcNAc transferase (OGT). The Hexosamine biosynthetic pathway (HBP) generates O-GlcNAc and plays crucial roles in cancer-associated processes such as cell division and signaling, metabolism and cytoskeletal regulation [46]. In recent studies, ER stress-induced UPR has been reported to be an upstream activator of HBP. XBPI and ATF4 knockdown was demonstrated to hamper glucose starvation-activated O-GlcNAcylation suggesting that O-GlcNAc post-translation modification (PTM) was modulated by UPR and may assist metabolic reprogramming of cancer cells [37]. These studies demonstrate that there may be a relationship between the UPR and metabolism in arsenic-induced carcinogenesis and cancer stem cell maintenance.

Autophagy is known to have an oncogenic or tumor suppressor role in cancers. Some studies suggest that autophagy has an anticancer role in early cancer stages by reducing
oncogenic/mutated proteins via degradation, maintaining organelle integrity and protein quality, and suppressing inflammation. Autophagy can also help cancer cells survive by providing nutrition during stress conditions and protection against oxidative stress via the p62/Nrf2 antioxidant response pathway and other immunosuppressive functions [4, 33, 47]. Thus, a context-dependent role has been suggested for autophagy in cancer. It is known that autophagy can be induced by all three UPR signaling pathways. Fewer studies, however, have focused on the direct connections between ER stress and autophagy in arsenic-induced cancers and the generation of cancer stem cells [4]. We discuss in the next section the connections between the ER stress activated UPR, mitochondrial dysfunction and autophagy in arsenic-induced carcinogenesis.

1.4 Connections between UPR, mitochondria and autophagy in arsenic-induced malignant transformation

Arsenic is known to induce the malignant transformation of cells by the generation of ROS that damages macromolecules, activation of intracellular signaling pathways, causes epigenetic and genetic alterations, mitochondrial dysfunction and altered immune/inflammatory responses. The ER, mitochondria and autophagy are closely related and collaborate to respond to stress signals in the cells. During stress conditions such as nutrient deficiency, autophagy, and the ER, stress-induced UPR is essential in recycling improperly folded proteins and organelles which are used for energy production. Similarly, ER and autophagy play an essential role in maintaining mitochondrial function and dynamics [4, 39]. The ER and mitochondrion are interlinked via specialized subcellular compartments called mitochondrial-associated ER membranes (MAMs), which are shaped by ER subdomains collocated to mitochondria. MAMs are physical and biochemical contact sites between ER and mitochondrion that play an important role in Ca^{2+} exchange, mitochondrial dynamics, ER stress, lipid metabolism and mitophagy (mitochondrial
autophagy) [48]. Thus, MAMs which connect ER and mitochondrion play an essential role in major signaling pathways such as the ER stress response-UPR and autophagy that affect cellular functions [4]. Apart from being connected to the mitochondrion physically via MAMs, the ER stress activated UPR directly activates the autophagy pathway under stress conditions. Autophagy is a catabolic process that recycles damaged organelles and misfolded proteins using the lysosomal machinery. It also protects against oxidative stress by clearing damaged mitochondria and peroxisomes, which are important sources of ROS generation. Defects in autophagy have been associated with mitochondrial dysfunction, genomic instability, and increased cancer risk.

The UPR pathway proteins, ATF6α, IRE1α and PERK, are involved in the activation of the autophagy pathway. In the PERK branch, activation of the transcription factor ATF4 leads to the upregulation of the autophagy genes LC3 and Atg12. The transcription factor CHOP activated via the PERK- eIF2α-ATF4 or the transcription factor ATF6α can transcriptionally upregulate Atg5 and p62 levels. In the ATF6α UPR pathway, cleaved ATF6α transcription factor induces DAPK1 kinase expression, which further, via Atg9 activation, affects the autophagy process. ATF6α activates TSC2 via the Rheb-mTOR pathway, which inhibits mTORC1, the negative regulator of the ULK1-mATG13-FIP200-ATG101 complex to induce autophagy [4]. Thus, all three ER stress mediated UPR pathways are involved in autophagy induction.

The UPR, hypoxia, mitochondrial function and autophagy process are all part of the global ER stress response (ERS) pathway. A pathological insult or imbalance in one of the components of this system impairs the other leading to an overall disturbance in cell homeostasis [49, 50]. The intracellular contacts between ER stress mediated UPR, mitochondria and autophagy have been illustrated in Figure 3.
The mitochondrion and ER are physically connected through ER subdomains, outer mitochondrial membrane, and other proteins (Mfn1/2) to form the mitochondria associated membranes (MAMs). The MAMs play an important role in ER stress response-UPR, autophagy, calcium exchange, lipid transport and inflammation. Arsenite metabolism or via activation of NADPH oxidase generates reactive oxygen species (ROS) that causes mitochondrial dysfunction that disturbs the protein folding in the ER causing ER stress. The ER activates the unfolded protein response (UPR) to restore the ER homeostasis [4].

Several studies have also associated arsenic with the induction of ER stress, mitochondrial dysfunction, and autophagy. In human lung adenocarcinoma A549 cells, arsenic trioxide (ATO)-induced ER stress was observed by an increase in the protein expression of GRP78, CHOP and caspase-12. Treatment of A549 cells with NAC, a ROS scavenger, reduced ER stress suggesting
that ATO induces ER stress via ROS generation. Mitochondrial dysfunction was also observed in the ATO-treated cells [51]. Low doses of sodium arsenite (<1μM) upregulated ROS levels triggering ER stress in human lung epithelial cells BEAS-2B and skin keratinocytes. ER stress-activated the UPR, as observed by an increase in BiP and phosphorylated PERK levels. Accordingly, low dose arsenite treatment increased ROS levels, activating ER stress related UPR and masked p53 function, leading to malignant transformation of cells [52]. In mouse testis Leydig tumor cell lines, arsenic exposure (0-9 μM, 24 hrs) up-regulated autophagy markers LC3, Atg7, Beclin1 and Vps34 expressions and significant accumulation of autophagosomes was observed. The autophagosome formation was downregulated on silencing of Beclin1, and inhibition of Vps34/PI3K and mTOR. To summarize, the studies showed that exposure to arsenic induced autophagosomes formation via regulation of the Beclin1-Vps34/PI3K complex and mTOR pathway. The blockage of autophagosomes degradation maybe due to impaired function of lysosomes [53]. In summary, these studies suggested that arsenic activated the ER stress response-the UPR via ROS generation, in different human cell lines. Also, mitochondrial dysfunction and induction of autophagy was observed in some of the studies.

1.5 Project Overview

1.5.1 Background and Rationale

It is known that environmental arsenic exposure results in the development of lung cancer. The proposed mechanisms of arsenic carcinogenicity are oxidative stress, epigenetic alterations, metabolic reprogramming, ER stress and other stress responses that lead to the malignant transformation of cells. However, how these arsenic-induced stress responses lead to the generation of cancer stem cells (CSCs) is largely unknown. CSCs are a subset of stem cells that can self-renew and differentiate into cancer cells that form new tumors. They may develop from
normal stem cells due to inhibition of differentiation or from normal cells or terminally differentiated cancer cells due to dedifferentiation [33, 53]. It has been proposed that CSCs contribute to cancer heterogeneity, metastasis, sustained growth, recurrence, and chemotherapy resistance [11]. Targeting the CSCs can be a promising approach to preventing cancer recurrence and drug resistance.

We have previously shown that long-term (6 months) arsenic treatment in human bronchial epithelial BEAS-2B cells with an environmentally relevant concentration (0.125-0.25 μM As\(^{3+}\)) leads to the malignant transformation of the cells and some of these transformed cells show CSCs features. An increased expression of stemness transcription factors such as Sox2, Oct4, Klf4, Myc, etc., was observed in the arsenic-induced CSCs. Other characteristics seen in the transformed cells were asymmetric division and formation of tumor spheres in the tumorsphere formation medium. On the other hand, the control cells cultured without arsenic (As\(^{3+}\)) for six months could not survive in the tumorsphere medium for more than 4-7 days. By subcutaneous (s.c.) inoculation of 10,000 control cells and the arsenic (As\(^{3+}\))-induced CSCs into athymic nude mice, respectively, only CSCs, but not the control cells, could form fast-growing tumors [57]. To summarize, the long-term arsenic As\(^{3+}\) treatment-induced malignant transformation of the BEAS-2B cells and some of these transformed cells showed cancer stem-like cell features. The arsenic-induced CSCs on s.c. inoculation in nude mice were able to form tumors.

Transcriptomics and global metabolomics analysis in the arsenic-induced CSCs revealed a significant downregulation in the mitochondria oxidative phosphorylation and tricarboxylic acid (TCA) cycle genes. A key mitochondrial protein, mitochondrial transcription factor A (TFAM) and mitochondrial DNA (mt. DNA) were highly depleted in the As\(^{3+}\) -induced CSCs compared to non-CSCs. However, there was an increase in the glycolysis genes expression and glycolytic
intermediates in the As\textsuperscript{3+}-induced CSCs compared to the non-CSCs. This metabolic switch from mitochondrial oxidative phosphorylation to glycolysis has been observed in embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adult stem cells (ASC), and CSCs. Several reports suggested that this metabolic switch was prior to the expression of stemness transcription factor. However, how metabolic reprogramming contributes to CSCs development is not fully understood [39].

It is known that the ER stress activated UPR, mitochondria and autophagy work in conjunction to maintain cell homeostasis under physiological and stress conditions. The endoplasmic reticulum and autophagy also regulate mitochondrial fission, fusion, and turnover under stress conditions. Impairment and/or pathological insult to either the ER, mitochondria or autophagy affects the other cellular component and further the ability of cells to cope with stress conditions. Based on the mitochondrial functional and metabolic impairments in the As\textsuperscript{3+}-induced CSCs, we proposed the ER stress response and autophagy abnormalities in the CSCs. Like our propositions, gene ontology study of the transcriptomics data revealed significant downregulation of genes related to ER stress and autophagy in the As\textsuperscript{3+}-induced CSCs compared to non-CSCs. Importantly, most of the genes securing ER to the mitochondrion, such as Mfn2, BECN1 (Beclin1), ATG14 L, PIK3C3 (Vps34), DNM1L (Drp1), etc., were inhibited in the CSCs. These deficiencies suggested abnormalities in mitochondrial fission and fusion. There was a significant loss of expression of the genes involved in the ER-regulated initiation, separation, and maturation of the autophagosomes, including WIPI2, ATG5, ATG16L, ATG9, RB1CC1 (FIP200), VMP1, etc. in the CSCs. The CSCs also exhibited a decreased expression of the genes contributing to mitophagy or interaction between autophagosome and mitochondria, such as BNIP3, p62, LC3, BNIP3L (Nix), PINK, etc. [39].
Considering the importance of the UPR pathway in the regulation of stemness and differentiation, it is being studied for affecting the differentiation status of tumor cells and the regulation/formation of the CSCs. The ER stress activated UPR regulates important functions such as tissue development, stem cell maintenance and differentiation, apart from the upkeep of protein homeostasis. UPR activity was found to be upregulated in many cancers such as the breast, gastric, colon, ovary, and brain. A study in breast cancer cell line MDA-MB-231 demonstrated that the pharmacological inhibition of all three UPR pathways, PERK, IRE1α and ATF6, could reduce cellular proliferation. The specific inhibition of PERK and ATF6 reduced mammospheres (mammary epithelial stem cells aggregates) formation, and studies revealed that silencing of PERK and ATF6, but not IRE1, strongly reduced SOX2 levels (stemness transcription factor), suggesting their importance in breast CSC maintenance [54]. Another in vitro study in triple-negative breast cancer (TNBC) cells found that XBP1 (transcription factor activated by the IRE1α pathway) stimulated stemness involving the cooperation of Hypoxia-inducible factor 1α (HIF1α) [55]. In contrast, studies in the glioblastoma stem cells (GSCs) revealed that ER stress reduced cell stemness and tumor-forming potential partly through Sox2 inhibition [56]. It is possible that the ER stress-activated UPR pathways PERK, IRE1α, and ATF6 can play pro- or anti-stemness function in the cancer stem cells depending on the tissue type, stressor duration, and the specific type of UPR stressor that is activated.

We have very limited knowledge regarding the potential role of ER stress, autophagy, and morphological/functional changes of the mitochondria during the generation of As\textsuperscript{3+} induced CSCs. Therefore, there is a need to better understand the mechanistic insights into the carcinogenicity of short-term As\textsuperscript{3+} exposure and identify key metabolic or UPR pathway targets which have therapeutic potential for eliminating the CSCs. Based on our preliminary findings, we
hypothesize that altered stress responses and metabolic shift induced by As\(^{3+}\) regulate the gene transcription required for the formation and maintenance of the CSCs. We will use high throughput techniques like ChIP-seq and RNA-seq to test this hypothesis to uncover the mechanistic links between ER stress-activated UPR and mitochondrial dysfunction.

### 1.5.2 Specific aims

**Specific aim 1:** To investigate the short-term effects of As\(^{3+}\) on the ER stress-associated unfolded protein response (UPR) and metabolism in human bronchial epithelial BEAS-2B cells.

**Specific aim 2a:** To investigate the regulation of the As\(^{3+}\)-induced ER stress sensor proteins in BEAS-2B cells.

**Specific aim 2b:** To determine the prognostic value of ER stress sensor proteins (ATF6 and ATF6B) in human lung cancer patients.
CHAPTER 2: IMPACT OF ARSENIC TREATMENT ON THE ER STRESS-ACTIVATED UPR AND MITOCHONDRIAL METABOLISM IN HUMAN BRONCHIAL EPITHELIAL BEAS-2B CELLS

This chapter contains material from published work in which I was the first author/co-author. The co-authors agree to the use of the published data in this dissertation. Please note that the content in chapter two is adapted from the peer-reviewed publication “Arsenic Activates the ER Stress-Associated Unfolded Protein Response via the Activating Transcription Factor 6 in Human Bronchial Epithelial Cells” published in journal Biomedicines (2022).

2.1. Introduction

Arsenic (As\textsuperscript{3+}) is a well-known carcinogen that elicits its effects via the generation of free radicals, reaction with thiol containing-molecules such as glutathione (GSH) and affecting other biological functions. One of the targets of arsenic (As\textsuperscript{3+}) toxicity is the endoplasmic reticulum (ER) [26, 57]. The ER is the center of protein homeostasis in the cell. Proteins synthesized by the ribosome machinery enter the ER, where the chaperones correctly fold them prior to transport to their respective destinations in the cell. Recent evidence has indicated that As\textsuperscript{3+} may induce ER stress by undermining the equilibrium between a load of proteins to be folded and the chaperone-mediated ability to fold them. Disturbances in the ER redox environment, calcium homeostasis and mitochondrial dysfunction may be the possible causes of the As\textsuperscript{3+}-induced ER stress. [39, 58-60]. The induction of ER stress triggers the unfolded protein response (UPR), a pro-adaptive response, via its three ER stress sensor proteins PERK, IRE1\textalpha and ATF6 to restore ER homeostasis. The UPR pathway is known to be involved in various stages of cancers, from tumor progression to chemoresistance [37].
Our previous findings have shown that long-term $\text{As}^{3+}$ treatment induced the malignant transformation of the BEAS-2B cells, and some of the transformed BEAS-2B cells exhibited cancer stem like cells (CSCs) features. The CSCs were also able to form tumors in vivo compared to non-CSCs cells. The transcriptomic data from the $\text{As}^{3+}$-induced CSCs displayed a substantial downregulation of the mitochondrial oxidative phosphorylation, ER stress and autophagy genes. On the other hand, the glycolysis was significantly upregulated, indicating a metabolic reprogramming in the $\text{As}^{3+}$- induced CSCs [39]. As discussed previously, the increased glycolysis in the CSCs leads to the accumulation of glycolytic intermediates and their subsidiary metabolites in the pentose phosphate pathway (PPP), one-carbon metabolism of the serine and glycine pathway for S-adenosylmethionine (SAM) generation and hexosamine synthesis pathways for post-translational modifications of the key proteins. Thus, metabolic reprogramming in the $\text{As}^{3+}$-induced CSCs may prove beneficial in maintaining the cell stemness. We have very limited knowledge of the short-term effect of the ER stress response, mitochondrial metabolism on the arsenic ($\text{As}^{3+}$)-carcinogenesis and the generation of the CSCs. Hence, to broaden our understanding of the subject, the human bronchial epithelial BEAS-2B cells were treated with $\text{As}^{3+}$ for a short period (6-8 hours) using various doses (0.25, 0.5, 1, 2, 4μM) that mimic the environmental exposure.

2.2 Results

2.2.1 Long-term arsenic treatment induces malignant transformation of BEAS-2B cells

Arsenic is a well-known human carcinogen that causes several cancers [1]. Previous studies from our lab have shown that long-term $\text{As}^{3+}$ treatment (6 months) in human bronchial epithelial BEAS-2B cells with an environmentally relevant concentration (0.125-0.25 μM $\text{As}^{3+}$) resulted in the transformation of cells [39, 61]. Some of these transformed cells showed cancer stem like
features (CSCs) such as asymmetric division and forming tumorsphere in tumor formation media. The control cells (no arsenic treatment for six months), on the other hand, were unable to survive for more than 4-7 days in the same media. The arsenic-induced CSCs also showed higher expression of stemness factors such as Sox2, Oct4, Klf4, Nanog, c-myc, TCF4, PBX1, and EBF3 compared to BEAS-2B cells. Transcriptomic studies were conducted to analyze the metabolic profile of these CSCs. Significant downregulation of the genes associated with mitochondrial oxidative phosphorylation was observed in the arsenic-induced CSCs. NDUFB5, NDUFC2, SDHB, ATP5O, ATP6V1D, UOCR10, NDUFA9, ATP5B, COX6C, COX7C, TFAM, etc. are some of the OXPHOS genes that were significantly downregulated (>10 folds decrease). On the other hand, glycolysis was significantly upregulated in the CSCs, indicating a metabolic reprogramming [39, 61].

2.2.2 Mitochondrial dysfunction and reduced ER stress and autophagy gene expression in the As$^{3+}$-induced CSCs

We had observed that in the As$^{3+}$-induced CSCs, mitochondrial oxidative phosphorylation genes (mt. OXPHOS genes) were significantly downregulated, indicating a functional impairment in the mitochondrion. To confirm this notion, Lingzhi Li. et al. evaluated morphology, mtDNA and other essential mitochondria proteins (unpublished data). It was observed that 1 μM As$^{3+}$ treatment for 12 hours in the BEAS-2B cells reduced the numbers of mitochondria and caused perinuclear clustering of the mitochondria in the cells (unpublished data, not shown). A gradual but significant reduction in the mitochondrial transcription factor A (TFAM) was observed in BEAS-2B cells treated with 1μM As$^{3+}$ for four months. (Unpublished data, not shown) [39]. In the As$^{3+}$-induced CSCs, mitochondrial DNA (mtDNA) and mitochondrial transcription factor A (TFAM) were significantly diminished compared to BEAS-2B cells (Figure 4 A-B). The changes
in mitochondrial number/morphology in \textit{As}^{3+}-treated cells and CSCs are like the documented description of undeveloped mitochondria in the induced pluripotent stem cells (iPSCs) and the embryonic stem cells (ESCs) (Unpublished data) [39, 62].

Since the ER stress response and autophagy are closely connected to the mitochondrial dynamics under physiological and stress conditions, it is imperative to investigate the ER stress and autophagy genes in the \textit{As}^{3+}-induced CSCs to observe any possible changes/abnormalities in gene expression. Interestingly reduced expression of genes related to ER stress and autophagy were observed in the \textit{As}^{3+}-induced CSCs compared to the control cells (\textit{Figure 4 C}) [4, 39].

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Mitochondrial dysfunction and decreased gene expression of the ER stress and autophagy in the arsenic-induced CSCs}
\end{figure}

\textbf{A-B.} Mitochondrial DNA (mtDNA) and mitochondrial transcription factor A (TFAM) were significantly reduced in the \textit{As}^{3+}-induced CSCs.
\textbf{C.} Most of the ER stress and autophagy gene expression was substantially reduced in the \textit{As}^{3+}-induced CSCs. Blue color depicts decrease gene expression and Red color depicts increased gene expression [39].
### 2.2.3 Effect of short-term As\(^{3+}\) treatment on the ER stress activated UPR

To observe the short-term effect of As\(^{3+}\) on the ER stress-activated UPR, BEAS-2B cells were treated with 2μM As\(^{3+}\) for 6 hours. This experiment served as a primary screen to identify the significantly upregulated and downregulated genes in the human unfolded protein response. The real-time RT2 Profiler PCR array consisted of 84 genes associated with the ER stress-activated UPR response (Qiagen, Cat. No. PAHS-089Z). The significantly upregulated (>2 folds) UPR genes were Activating transcription factor 6 (ATF6A), CCAAT/enhancer binding protein (C/EBP), beta, Heat shock 70kDa protein 1 B (HSPA1B), Mitogen-activated protein kinase 10 (MAPK10) and Ubiquitin-conjugating enzyme E2G2 (UBE2G2) (Figure 5 A). Three genes, including endoplasmic to nucleus signaling 2 (ERN2), HtrA serine peptidase 4 (HTRA4) and SREBF chaperone (SCAP), were downregulated to 1.4 to 1.8-fold in response to As\(^{3+}\) (Figure 5 B). However, the ERN2, HTRA4, and SCAP protein levels were not changed in the cells treated with As\(^{3+}\) (data not shown).

The above-upregulated genes ATF6, CEBPB, HSPA1B (Hsp70), MAPK10 (JNK3), and UBE2G2 are involved in the signaling pathways of the ER stress activated UPR. ATF6 (90kDa) is one of the three UPR sensors and a basic leucine zipper transcription factor activated under ER stress conditions. It is cleaved by serine proteases in the Golgi body to release an active form of ATF6 (50kDa). The cleaved form of ATF6 translocates to the nucleus to induce UPR genes (X-box binding protein, Grp78) through activation of ER stress element (ERSE) to resolve ER stress [63]. CEBPB is a transcription factor involved in regulating genes that play an important role in immune and inflammatory responses. It also plays a significant role in modulating adipogenesis, gluconeogenic pathway, liver regeneration and hematopoiesis. Under metabolic stress, the UPR pathway through PERK/eIF2α can stimulate CEBPB expression, promoting lipogenesis [64].
HSPA1B is a heat shock protein that, along with other members of its family, stabilizes proteins against aggregation and mediates the folding of newly translated proteins. It is also involved in the ubiquitin-proteosome pathway. MAPK10 is a member of the MAP kinase family that is activated via the IRE1α-TRAF2 UPR signaling pathway. MAPK10 act as integration points for multiple biochemical signals and thus is involved in a wide variety of cellular processes, such as proliferation, differentiation, transcription regulation and development. UBE2G2 belongs to the family of ubiquitin-conjugating enzymes, which catalyze the transfer of ubiquitin from ubiquitin-activating E1 enzymes to ubiquitin-conjugating E2 enzymes. This protein is part of the ER-associated degradation (ERAD) pathway of the UPR, which degrades misfolded or improperly folded proteins in the cytoplasm [65, 66].

**Figure 5: Short-term As^{3+} effect on the ER stress activated UPR**

A. UPR upregulated genes by As^{3+} treatment in BEAS-2B cells  
B. UPR downregulated genes by As^{3+} treatment in BEAS-2B cells
2.2.4 As\(^{3+}\) induced ATF6 and UBE2G2 are dose-dependent

To confirm the inducibility of the ER stress-activated UPR genes by As\(^{3+}\), the BEAS-2B cells were treated with 0.25, 0.5, 1, 2, 4 μM As\(^{3+}\) for 6 or 8 hours. Negative control 1 (NC1) represents a reverse transcriptase-free cDNA master mix, and negative control 2 (NC2) represents the use of nuclease-free water instead of cDNA in RT-PCR. RT-PCR analysis revealed a significant dose-dependent induction in the mRNA expression of both ATF6 and UBE2G2 (Figure 6 A & C). It is known that ATF6 protein is proteolytically cleaved during the ER stress response. The N-terminus of ATF6 has a transcriptional activation domain (TAD), whereas C-terminus has a basic, leucine-zipper (Leu-Zip) and ER transmembrane (ERTM) domains. Upon ER stress, the serine proteases in the Golgi body cleave the ERTM domains of ATF6 (378-398aa) to release the activated cleaved N-ATF6 transcription factor. A cleaved form of the ATF6 proteins indicates that the UPR has been activated [66, 67]. Indeed, we observed a cleaved form for ATF6 at 55 and 45 kDa, which are also roughly in an As\(^{3+}\)dose-dependent manner (Figure 6 D). The other two ER stress sensors, PERK and IRE1α, did not show significant gene expression changes with short-term arsenic treatment in the UPR array. For the UBE2G2 protein, although the induction was marginal, there was a notable induction by As\(^{3+}\) at concentrations between 0.25 to 2 μM (Figure 6 B) [66].
Figure 6: Dose-dependent induction of ATF6 and UBE2G2 by As\textsuperscript{3+}

A & B. Dose-dependent induction of ATF6 mRNA (A) and protein (B) by As\textsuperscript{3+} in BEAS-2B cells

C & D. Dose-dependent induction of UBE2G2 mRNA (A) and protein (B) by As\textsuperscript{3+} in BEAS-2B cells. The cells were treated with the indicated concentrations of As\textsuperscript{3+} for 6h.

2.2.5 As\textsuperscript{3+} induced ATF6B is dose-dependent

It is known that ATF6 has two isoforms encoded by two different genes. The gene for the ATF6, also named ATF6A, is located at the genomic region of chromosome 1 (chr1) q23.3, whereas the gene for ATF6B is positioned at chromosome 6 (chr6) p21.32. The ATF6 and ATF6B can form heterodimers due to the conserved DNA binding domain to bind to the ER stress response element for the transcription of UPR genes. Mice with genetic deficiency of ATF6 or ATF6B do not elicit major phenotype, whereas the dual deficiency is lethal, suggesting equal or similar importance of ATF6 and ATF6B for the normality of the cells. Since the potency of As\textsuperscript{3+} on the induction of ATF6 was observed, we also checked the effect of arsenic As\textsuperscript{3+} on ATF6B. Dose-dependent induction of ATF6B by As\textsuperscript{3+} was also detected [66]. Although not well-studied, As\textsuperscript{3+} treatment also promoted the generation of the proteolytic products of ATF6B at a molecular weight of around 70 and 55kDa (Figure 7).
Figure 7: Dose-dependent induction of ATF6B by As$^{3+}$

Dose-dependent induction of ATF6B by As$^{3+}$ in BEAS-2B cells treated with the indicated concentrations of As$^{3+}$ for 8h. The bottom panel shows the average of the ATF6B proteins of three independent experiments. One-way ANOVA was used for statistical analysis with Tukey’s post hoc test *p<0.05, **p<0.01, ***p<0.001.

2.2.6 As$^{3+}$ induces the CEBPBeta and HSPA1B in a dose-dependent manner

In addition to ATF6 and UBE2G2, a real-time RT2 profiling of the human UPR PCR array also indicated a strong induction of CEBPB, HSPA1B, and MAPK10/JNK3. To confirm our human UPR RT2 array results, we treated BEAS-2B cells with As$^{3+}$ (0.25, 0.5, 1, 2, 4 μM) for 6 hours. As$^{3+}$ was able to induce protein expression of CEBPB isoform, LAP, especially at 0.5 and
1 μM concentrations (Figure 8 B). There was a slight induction in the protein levels of CEBPB isoform LIP and HSPA1 (Figure 8 A&C). We have previously shown that As$^{3+}$ activates the JNK-STAT3 pathway during carcinogenesis [72]. CEBPB is a single exon gene at the locus of chr20 (q13.13). The CEBPB gene encodes a transcription factor that contains a basic leucine zipper (bZIP) domain. The transcription factor C/EBPβ has three isoforms: full length (FL), liver activating protein (LAP), and liver inhibitory protein (LIP). The utilization of alternative in-frame AUG codons results in multiple protein isoforms. These transcription factors can bind to the CAAT enhancer site of many gene promoters to regulate several processes such as immune and inflammatory processes, metabolism, growth, and differentiation [68]. HSPA1B is also a single exon gene at the gene locus of chr6 (p21.33). It arbitrates the folding of newly synthesized proteins and stabilizes existing proteins against aggregation. It is also involved in the ubiquitin-proteasome pathway.
Figure 8: Dose-dependent induction of CEBPB and HSPA1B by arsenic As$^3+$

A-C. Western blotting and quantification of the Western blotting data from 3 independent experiments for CEBPB isoform LIP (A), LAP (B) and HSPA1B (C). One-way ANOVA was used for statistical analysis with Tukey’s post hoc test. *: p<0.05, **: p<0.01, *** p<0.001.

2.2.7 Regulation of glycolysis and mitochondrial respiration by As$^3+$

The connection between ER stress to metabolic reprogramming has been well-established [39, 69]. To observe the changes in the metabolic profile, the BEAS-2B cells were treated with different doses of As$^3+$ for 6 hours. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a Seahorse bioanalyzer. The ECAR and OCR values are indicators of glycolysis and mitochondrial respiration, respectively. A significant dose-
dependent increase was observed in glycolysis (ECAR) and mitochondrial respiration (OCR) with As$^{3+}$ treatment in BEAS-2B cells (Figure 9 A-B). The BEAS-2B cells seem to go towards a more energetic, glycolytic pathway with short-term As$^{3+}$ treatment (Figure 9 C). However, unlike in the As$^{3+}$-induced CSCs and transformed cells, the short-term As$^{3+}$ treatment also significantly increases mitochondrial oxidative phosphorylation.

Figure 9: Real-time metabolic phenotype assay in As$^{3+}$-treated BEAS-2B cells using Seahorse bioanalyzer

A. Significant upregulation was observed in the extracellular acidification rate (ECAR), especially at 1μM and 4 μM As$^{3+}$-treated BEAS-2B cells for 6h compared to control cells. Increased ECAR (mpH/min/μg protein) is indicative of active glycolytic metabolism.

B. Significant upregulation was also observed in the oxygen consumption rate (OCR) 1μM and 4 μM As$^{3+}$ doses in BEAS-2B cells. OCR (pmol/min) represents the mitochondrial respiration in the live cells.
C. The ECAR versus OCR energy map shows that the BEAS-2B cells treated with As\(^{3+}\) shift towards a more energetic, glycolytic phenotype.

2.3 Discussion

An increased number of studies now provide evidence that arsenic exposure leads to the development of lung cancer and other diseases. Hence, it is crucial to investigate the intracellular targets affected by environmental arsenic exposure. Additionally, characterizing the signaling pathways affected by arsenic during both the onset/early stages and at the later stages of disease progression will be vital in developing treatments to mitigate arsenic-linked cancers [70]. Previous studies from our group have demonstrated that long-term (6 months) arsenic (As\(^{3+}\)) treatment in human bronchial epithelial BEAS-2B cells with an environmentally relevant concentration (0.125-0.25 \(\mu\)M As\(^{3+}\)) resulted in the malignant transformation of cells and some of the transformed cells showed cancer stem cells like features. Transcriptomics and global metabolomic analysis in the arsenic-induced CSCs revealed that genes associated with mitochondrial oxidative phosphorylation (OXPHOS), ER stress and autophagy were significantly downregulated [61, 71]. In contrast, most of the genes in the main glycolytic pathway were upregulated, such as all members of the SLC2A family, HKs, PFKs, ALDOB, GAPDHS, PGK2, PGAMs, ENOs, and CDC19. These results indicate metabolic reprogramming in the As\(^{3+}\)-induced CSCs from mitochondrial oxidative phosphorylation (OXPHOS) to glycolysis [39]. Based on observations in embryonic stem cells, adult stem cells, induced pluripotent stem cells and other cancer stem cells, we believe that the metabolic switch to glycolysis promotes the stemness properties of the As\(^{3+}\)-induced CSCs [39]. ER stress may also promote the differentiation of some types of CSCs, and a possible explanation of why lower ER stress genes have been observed in the arsenic-induced CSCs [72, 73].
The ER, mitochondrion and autophagy are closely related and work in conjunction under stress conditions. It is also well-known that the tri-components of the global ER stress response (UPR, mitochondrion, autophagy) can restore cellular homeostasis or activate apoptosis under unresolved stress conditions. However, we have limited knowledge regarding the potential role of ER stress, autophagy, and morphological/functional changes of the mitochondria during the generation of As$^{3+}$ induced CSCs. Therefore, there is a need to better understand the mechanistic insights into the carcinogenicity of short-term As$^{3+}$ exposure and identify metabolic, epigenetic or UPR pathway targets which have therapeutic potential for eliminating the CSCs. Our treatment of the BEAS-2B cells with 2 μM As$^{3+}$ for 6 hours caused a more than 2-fold upregulation of five UPR genes, including ATF6 (also known as ATF6A), CEBPB, HSPA1B, MAPK10 and UBE2G2. ATF6 is an ER-resident UPR protein and a member of the basic leucine zipper family of transcription factors. It is a 90kDa protein that is cleaved to a 50kDa protein in the Golgi body under ER stress. The cleaved form of N-ATF6 translocates to the nucleus to activate ER stress response genes [26]. Mammals express two isoforms of ATF6 proteins, the bona fide ATF6, ATF6A (670 aa), and ATF6B (703 aa). The ATF6 and ATF6B proteins have similar highly conserved b-Zip domains that allow them to bind to ER stress response element (ERSE) as homo or heterodimers. However, they have a divergent N-terminal domain and vary in their ability to induce an ER stress response. The ATF6 protein is more studied than the ATF6B protein [67]. In the present study, we found that As$^{3+}$ can induce both ATF6 and ATF6B protein levels, and both proteins showed proteolytic products in the As$^{3+}$-treated cells (Figures 6 B and 7). It has been implicated that only the cleaved forms of ATF6 and ATF6B can function as transcription factors for the transcription of the ER stress response genes in human bronchial epithelial cells, such as Grp78, and Grp94, Xbp1, and VEGF.
CCAAT enhancer-binding protein beta (CEBPB) is a single exon gene that encodes a transcription factor containing a basic leucine zipper domain. This transcription factor can function as a homodimer or heterodimer with other CEBP protein isoforms such as α, δ and γ and regulate immune and inflammatory responses. There are limited studies that describe the connection between ER stress-UPR and CEBPB and its possible role in cancer development. However, a recent study demonstrated that siRNA mediated CEBPB knockdown in RAW264.7 macrophage cells, attenuated oxidized LDL (low-density lipids) induced ER stress response genes ATF6 and ATF4, suggesting a possible link between CEBPB and ER stress sensor protein ATF6 and PERK-ATF4 signaling pathway. It would be interesting to study ATF6 and CEBPB links in As³⁺-treated BEAS-2B cells [74]. Besides the CEBPB (LAP isoform), As³⁺ was also found to induce UBE2G2 and HSPA1B proteins in BEAS-2B cells (Figure 8 B&C). There are very few studies conducted that specifically describe the roles played by UBE2G2 and HSPA1B in As³⁺-induced cancers and hence, require further investigation. The human UPR array analysis in As³⁺-treated BEAS-2B cells also showed a significant gene upregulation of MAPK10, also known as JNK3 (Figure 2). Previous studies from our group have proven that As³⁺ activates the JNK-STAT3 signaling pathway in BEAS-2B cells which is involved in the expression of mdig, a mineral dust-activated oncogene [75].

Studies by other groups have also shown that As³⁺ induces ER stress response-the UPR pathway. Arsenic trioxide (ATO) induced the UPR via the IRE1α pathway in human osteosarcoma and fibrosarcoma cells. It was also concluded that the autophagy and apoptosis observed in these cells with ATO treatment were partly mediated by IRE1α [76]. Another study demonstrated that arsenic trioxide (As2O3) induced ER stress in laryngeal squamous cell line Hep-G2. Increased expression of Grp78, CHOP, phosphorylated eIF2α and ATF4 were considered as indicators of
ER stress [77]. A recent study in BEAS-2B cells treated with arsenic trioxide (ATO) focused on the induction of Glucose-regulated protein 78 (GRP78), a chaperone and an important ER stress sensor protein. Similar to our findings, the study showed that the ATF6 protein was cleaved with ATO treatment in BEAS-2B cells along with activation of the other UPR stress sensor protein IRE1α. However, the GRP78 protein was only induced by IRE1α and not ATF6 [58]. The different ER stress sensors activated in each case may be due to the different chemical form and concentration of the As\(^{3+}\) used and the tissue type or cell line variation.

Metabolic reprogramming was observed in the As\(^{3+}\)-induced CSCs from mitochondrial oxidative phosphorylation to glycolysis. To investigate if short-term As\(^{3+}\) treatment also alters the metabolic profile, BEAS-2B cells were treated with arsenic (0.25, 0.5, 1,4 μM As\(^{3+}\)) for 6 hours. It was observed that acute As\(^{3+}\) treatment significantly increased glycolysis and mitochondrial respiration in BEAS-2B cells, especially at concentrations of 1 and 4μM As\(^{3+}\) concentrations (Figure 9 A&B). Unlike what we observed in the CSCs, mitochondrial oxidative phosphorylation was also upregulated with short term As\(^{3+}\) treatment. Such a difference in mitochondrial metabolism between short- and long-term As\(^{3+}\) treatment is very likely due to the accumulative damage of the functional proteins in mitochondria during long-term As\(^{3+}\) exposure. This finding also agrees with the report by Zhao et al., who showed that As\(^{3+}\) exposure in BEAS-2B cells induced an increased rate of extracellular acidification, which was inhibited by non-metabolized glucose analog 2-deoxy-D-glucose [78].

Based on the findings in this chapter, we can conclude that short-term As\(^{3+}\) treatment induces ER stress-activated UPR in human bronchial epithelial BEAS-2B cells via the ATF6 pathway. The other UPR upregulated genes such as CEBPB, HSPA1B, MAPK10, and UBE2G2 were also induced in a dose-dependent manner in As\(^{3+}\) treated BEAS-2B cells. Their role in the
As$^{3+}$ induced CSC generation requires further investigation. Glycolysis and mitochondrial oxidative phosphorylation were also induced by As$^{3+}$ treatment in BEAS-2B cells.

2.4 Materials and methods

Cell Culture

The human bronchial epithelial (normal, immortalized) cell line BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA). The BEAS-2B cells were cultured in Dulbecco’s Modified Eagle’s Medium-High Glucose (Sigma-Aldrich #D5796) with added 5% Fetal Bovine Serum, 1% L-Glutamine and 1% Penicillin/Streptomycin in a humidified incubator at 37°C and 5%CO2. The cells were starved with serum-free medium (overnight), followed by treatment of the cells with various concentrations of As$^{3+}$ (arsenic [III] chloride, Sigma-Aldrich, St. Louis, MO) for the indicated times.

Real-time PCR (Human Unfolded Protein Response Qiagen RT² Profiler PCR Array)

Total RNA was isolated from BEAS-2B cells (control/ As$^{3+}$ treated cells) using Qiagen RNeasy plus mini kit. The RNA quality was determined by agarose gel electrophoresis, and concentration was calculated using the Thermofisher nanodrop spectrophotometer. RNA was reverse transcribed to cDNA using a High-Capacity RNA-to cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. The cDNA and SYBR-Green qPCR Master mix (Qiagen Cat. no. 330529) were added to the Qiagen Human UPR array plate (QIAGEN, Cat. no. PAHS-089Z) to detect changes in gene expression in the UPR w/o As$^{3+}$ treatment.

RT-PCR
Total RNA was prepared using Qiagen RNeasy plus mini kit according to the manufacturer’s protocol. Reverse transcription was performed to convert RNA to cDNA using a High-Capacity RNA-to cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. ATF6α and UBE2G2 primers were designed using NCBI primer blast. GAPDH was used as a loading control. RT-PCR primer sequences for ATF6α (exon 16) are forward primer, 5’-GAAGCTTATGGCAGAGATGCAC-3’; and reverse primer, 5’CAGTGCTTTCAAAATAGATGGGTA-3’, UBE2G2 (exon 4) are forward primer, 5’-CATTTGTCAATTGTGGTCGACGTT-3’; and reverse primer, 5’AATACCACCATGCTTTACTGGCT-3’. Primer sequences for GAPDH are as follows: sense primer, 5’-CTGAACGGGAAGCTGGCATGGCCTTCC -3’; and anti-sense primer, 5’ CATGAGGTCCACCTGTTGCTAGCC-3’. PCR products were run on 1% agarose gels with DNA ladders. Samples without cDNA or RT templates served as negative controls.

**Western blotting**

BEAS-2B cells were seeded in a 6-well plate (3.0×10^5 cells per well) or 10 cm dish (1.0×10^6 cells per plate) and treated with various concentrations of inorganic arsenic (As^{3+}) for the indicated times. Cells were lysed by 1×RIPA lysis buffer (Cell signaling) supplemented with protease and phosphatase inhibitors cocktail (Roche, Indianapolis, IN) and 1mM PMSF. Cell lysates were homogenized by sonication, and insoluble debris was removed through centrifugation of 12,500g for 15 minutes at 4 °C. The protein concentrations were then determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). The protein samples were prepared using 4 × LDS sample buffer (Invitrogen) with dithiothreitol (final concentration of 200 mM) and were denatured by boiling at 95°C for 5 minutes before separation by SDS-PAGE gel. Separated samples were then transferred onto PVDF membrane (Invitrogen) and blocked with 5% non-fat
milk diluted in 1 X TBST for 1 hour at room temperature, followed by a quick TBST rinse. The membranes were then incubated with the indicated primary antibodies overnight at 4°C. The membranes were further washed thrice, each wash of TBST for 10 minutes. The membranes were further incubated at room temperature with the corresponding HRP-linked secondary antibodies for 1 hour, followed by a TBST wash thrice. ECL substrates (Thermo Scientific, Millipore and Westpico Plus) were used to visualize the signals. Primary antibodies against p-CEBPβ Threonine 235 (3084S), SCAP (13102s), Sox2, Klf4, C-myc, GAPDH and HRP-linked rat, mouse and rabbit IgG were purchased from Cell Signaling technology. Antibodies against ATF6α and ATF6β were purchased from Biolegend for western blot. UBE2G2 (ab235790), HSPA1B/Hsp70 (ab231637), anti-htrA4 antibody-catalytic domain (ab65915),anti-ERN2 antibody (ab135795) and CEBPβ primary antibodies (ab32358) were purchased from Abcam.

Seahorse bioanalyzer (Cell energy phenotype test)

The Seahorse XF24 Extracellular Flux Analyzer was used to obtain real-time measurements of extracellular acidification rates (ECAR) and oxygen consumption rate (OCR) in BEAS-2B cells. Cells (1.0×10^4) were plated in a 24-well Agilent Seahorse XF24 plate in DMEM media. The plate was coated with 0.1% gelatin prior to cell seeding to improve cell adhesion. The extracellular flux assay kit cartridge was hydrated with XF calibrant/well, and the hydrated cartridge was incubated overnight in the CO2-free incubator prior to use. After the cells reached 70-80% confluency, media was changed to serum-free DMEM media (overnight), and cells were treated with the indicated doses of arsenic. The media was changed to Seahorse media with appropriate supplements and arsenic (As^{3+}) 45 mins-1 hour prior to ECAR and OCR measurements.

Statistical analysis
All cell culture experiments were performed independently in triplicate at a minimum (unless otherwise indicated). Western blot images were analyzed using NIH ImageJ software. One-way Anova with 95% confidence interval followed by Tukey’s post hoc test was performed using IBM SPSS statistical software. Figures were prepared using GraphPad Prism 5 and plotted as mean values with SEM. A p-value of < 0.05 was considered statistically significant.

CHAPTER 3: REGULATION OF THE ER STRESS-INDUCED UPR GENES IN SHORT TERM ARSENIC As³⁺ TREATED BEAS-2B CELLS

This chapter contains material from published work in which I was the first author/co-author. The co-authors agree to the use of the published data in this dissertation. Please note that the content in chapter three is adapted from the peer-reviewed publication “Arsenic Activates the ER Stress-Associated Unfolded Protein Response via the Activating Transcription Factor 6 in Human Bronchial Epithelial Cells” published in journal Biomedicines (2022).

3.1 Introduction

The endoplasmic reticulum (ER) is a central organelle where protein handling, folding, and post-translation modifications occur that determine cell function and homeostasis. Although these processes are tightly regulated, multiple external and intrinsic factors such as hypoxia, nutrient deprivation, redox and calcium disturbances, and exposure to xenobiotics like arsenic can trigger ER stress [79]. Studies have shown that arsenic and its compounds cause ER stress which activates the proadaptive UPR signaling pathways in various in vitro and in vivo studies [58, 80-82]. However, finite studies specifically investigate how arsenic-induced ER stress activated UPR genes are regulated.
A recent study demonstrated that the activation of cancer-associated fibroblasts (CAF) from normal fibroblasts is a protective response to stresses (e.g., hypoxia) via the p62-Nrf2 pathway. One of the transcriptional targets of Nrf2 was ATF6 which increased the expression of ER stress response genes. NFE2 like bZIP transcription factor 2 or Nrf2, is a gene located at the locus of chromosome 2 (2q31.2) that encodes a transcription factor which is a member of a family of basic leucine zipper (bZIP) proteins. The Nrf2 transcription factor regulates genes that are implicated in injury and inflammation conditions including free radicals production, and these genes contain antioxidant response elements (ARE) in their promoters [83]. The Nrf2 transcription factor is also known to be activated by the UPR protein PERK under ER stress conditions. Some studies also identified that the regulatory ROS-independent ER stress pathway (GRP78/p-PERK/NRF2 signaling) was involved in mediating the metabolic shift (Warburg effect) and stemness of cancer-initiating cells (CICs) or the CSCs [84]. Hence, we investigated the relationship between the arsenic (As\(^{3+}\)) upregulated UPR genes ATF6, CEBPB, HSPA1B MAPK10, and UBE2G2 and the transcription factor Nrf2 in BEAS-2B cells.

3.2 Results

3.2.1 Nrf2 dependency of the As\(^{3+}\)-induced ATF6 and ATF6B

To understand how As\(^{3+}\) regulates the expression of ATF6 proteins, we retrieved our Chromatin Immunoprecipitation-sequencing (ChIP-seq) data from recent studies for transcription factors Nrf2 and HIF1α in the control BEAS-2B cells and the BEAS-2B cells treated with 1μM As\(^{3+}\) for 6h [69]. Visualization of the gene loci for both ATF6 and ATF6B in the genome browser revealed that As\(^{3+}\) induced a substantial enhanced enrichment of Nrf2 in the gene promoter of ATF6 (left panel Figure 10 A) and downstream of ATF6B (right panel of Figure 10 A). There is no significant difference in the enrichment of HIF1α in both ATF6 and ATF6B between control
and As$^{3+}$-treated cells. We previously had shown that the conserved Nrf2-binding element contains a core sequence motif TGAGTC or TGACTC [69]. Manual inspection of the Nrf2 peak region of the ATF6 gene showed that there are two less conserved Nrf2 binding motifs (indicated on the top of the ATF6 panel in Figure 10 A). For ATF6B, there are two major Nrf2 enrichment peaks downstream of the gene. The far downstream Nrf2 peak contains one conserved Nrf2 element, GACTCA, which is complement to the Nrf2 core element, TGAGTC. The proximal downstream Nrf2 peak contains two conserved Nrf2 elements (Figure 10 A, right panel). These data, thus, clearly indicated a Nrf2 dependency of the As$^{3+}$-induced ATF6 and ATF6B.

To confirm the importance of Nrf2 in As$^{3+}$-induced ATF6 and ATF6B, we also checked the mRNA expression of ATF6 and ATF6B between wild type (WT) BEAS-2B cells and the Nrf2 knockout (KO) cells that were made through CRISPR-Cas9 gene editing. Corroborating to the ChIP-seq findings, the RNA-sequencing (RNA-seq) showed a notable decrease of both ATF6 and ATF6B in the Nrf2 KO cells relative to the WT cells (Figure 10 B). Thus, we can conclude that Nrf2 plays a critical role in mediating As$^{3+}$-induced expression of both ATF6 and ATF6B [66, 69].
Figure 10: ChIP-seq analysis of Nrf2 dependency for the As$^{3+}$-induced ATF6 and ATF6B

A. ChIP-seq shows an enhanced enrichment of Nrf2 (pointed by red arrows) on the promoter region of ATF6 and downstream of ATF6B gene in the cells treated with 1 μM As$^{3+}$ for 6h. The corresponding Nrf2-binding elements for each of these Nrf2 peaks are indicated on top of the panels. Numbers above the Nrf2 motifs are genomic positions of nucleotides in the human genome hg19.

B. RNA-seq revealed reduced expression of ATF6 and ATF6B in the Nrf2 knockout BEAS-2B cells.

3.2.2. Contribution of Nrf2 to other As$^{3+}$-induced UPR genes.

In addition to ATF6 and ATF6B, a real-time RT2 profiling of the human UPR PCR array indicated a strong induction of CEBPB, HSPA1B, MAPK10, and UBE2G2 (Figure 2). Analysis of the ChIP-seq data revealed that except MAPK10, all other genes, including CEBPB, HSPA1B and UBE2G2, exhibited an increased enrichment of Nrf2 binding on these gene loci in the cellular response to As$^{3+}$ (Figure 11 A). CEBPB is a single exon gene at the locus of chr20 (q13.13). At 24.4kb upstream of the CEBPB gene, there is a conserved Nrf2 peak that contains two Nrf2-
binding elements with the core motif of TGACTC and TGACTC. In addition to Nrf2, As$^{3+}$ also induced an enhanced enrichment of HIF1$\alpha$ at the gene body of CEBPB (pointed by a green arrow in **Figure 11 A**). HSPA1B is also a single exon gene at the gene locus of chr6 (p21.33). There are two As$^{3+}$-induced Nrf2 enrichment peaks, one is at the promoter region, and another one is about 5.2kb upstream of the HSPA1B gene. A conserved Nrf2-binding element was found in the upstream peak (**Figure 11 A**). An enhanced HIF1$\alpha$ enrichment by As$^{3+}$ was noted at the promoter region of HSPA1B. As$^{3+}$ is also highly capable of elevating the binding of Nrf2 and HIF1$\alpha$ at the promoter region of the UBE2G2 gene (**Figure 11 A, right panel**). The notion that Nrf2 contributes to the As$^{3+}$-induced expression of CEBPB, HSPA1B and UBE2G2 was supported by the dose-dependent increase of these proteins in the cells treated with As$^{3+}$ (**Figure 6 D and Figure 8 A-C**)[66, 69].

![Figure 11](image)

**Figure 11: Nrf2 contributes to As$^{3+}$-induced expression of CEBPB, HSPA1B and UBE2G2**

**A.** Screenshot for the indicated genes in ChIP-seq showing As$^{3+}$-induced enhanced enrichment of Nrf2 (pointed by red arrows) and HIF1$\alpha$ (pointed by green arrows).
3.2.3 Expression status of ATF6 and ATF6B in human lung adenocarcinoma.

Considering the fact that $\text{As}^{3+}$ is a human carcinogen and $\text{As}^{3+}$ induces ATF6 and ATF6B, we next evaluated the expression of ATF6 proteins in human lung adenocarcinoma tissues that were case-matched with the adjacent normal lung tissues using human tissue microarray (TMA) panels (LC10013c, US Biomax Inc.). We scored cancer and matched non-cancerous lung tissues into negative (1), weak positive (2), medium positive (3) and strong positive (4) based on visual staining intensity (Figure 12). We observed that of the 48 patients’ samples (cancer and matched non-cancer tissue pair), the ATF6 expression was higher in non-cancer tissue of 22 cases compared to their cancer tissue, whereas 15 cases had no difference and 11 cases had higher expression of ATF6 in cancer tissue compared to their non-cancerous tissue (Figure 12 A, left panel & B, top panel). Similarly, the ATF6B expression was higher in the non-cancer tissue of 28 participants compared to their non-cancerous tissue, whereas 11 cases had no difference and 9 cases had higher expression of ATF6B in cancer tissue compared to their non-cancerous tissue (Figure 12 A, right panel & B, bottom panel). No significant differences were observed in ATF6 and ATF6B among the cancer tissues stratified by grade, stage, age, and sex (data not shown).
A. Representative immunohistochemistry panels show ATF6 and ATF6B staining in lung adenocarcinoma and the case-matched non-cancerous lung tissues of 48 patients. Staining scores range from negative (1), weak positive (2), medium positive (3), strong positive (4).

B. Quantification of the ATF6 (top panel) and ATF6B (bottom panel) staining in case-matched cancer and non-cancer tissues of 48 patients.

### 3.2.4 The Prognostic Predictions of ATF6 and ATF6B Expression in Lung cancer Patients

We have detected the expression of ATF6 and ATF6B in both normal lung tissues and lung cancer tissues. To additionally investigate whether the ATF6 genes contribute to the pathogenesis of human lung cancer, we evaluated the prognostic value of ATF6 expression on overall survival (OS) of lung cancer patients using the publicly available dataset from Kaplan–Meier Plotter [13]. We randomly selected 3 probe sets for both ATF6 and ATF6B (Figure 13). Setting the analytic option of the best perform threshold as a cutoff showed that a high level of ATF6 expression predicts a better OS in lung cancer patients as determined by three different ATF6 probes (upper panels of Figure 13 A). In contrast, the high level of ATF6B predicts a poorer OS for the patients.
(bottom panels of Figure 13 A). The differences in the prognostic values between ATF6 and ATF6B are also reflected by the different expression levels of these two ATF6 genes between normal lung tissue and tumor tissues. In both lung adenocarcinoma (AD) and lung squamous cancer (SC), the tumors expressed much higher levels of ATF6B relative to ATF6 (Figure 14 B). These data, thus, suggested possible different roles played by ATF6 and ATF6B in the development of human lung cancer, although ATF6 and ATF6B can form heterodimers for transcriptional regulation. ATF6 may more likely tend to be a tumor suppressor, whereas ATF6B appears to be more oncogenic.
Figure 13: Different prognostic values of ATF6 and ATF6B for lung cancer patients

A. Data are derived from Kaplan–Meier database containing gene expression information from 1925 lung cancer patients. Probe IDs for ATF6 and ATF6B are indicated on the top of each panel. Analytic options of “Auto select best cutoff” for overall survival (OS) were used in the graphic generation. High ATF6 expression predicts better OS, whereas high ATF6B expression predicts poor OS in human lung adenocarcinoma patients.
Figure 14: Comparison of ATF6 and ATF6B gene expression in normal lung and tumor tissues

B. ATF6 and ATF6B expression in n human lung adenocarcinoma (AD, top panels) and lung squamous cell carcinoma (SC, bottom panels) patients using the TNMplot. In AD, both ATF6 and ATF6B are significantly increased compared to normal lung tissues. In SC, only ATF6B was found to be significantly increased in the tumor tissues. The numbers in each column are the total cases included in this analysis.

3.2.5 Impact of clinicopathological parameters on the prognostic value of ATF6 and ATF6B in human lung cancer patients

We have studied the prognostic value of ATF6 and ATF6B on the overall survival (OS) in human lung cancer patients (Figure 13 A). To examine if the prognostic value of ATF6 and ATF6B is affected by various clinicopathological parameters such as lung cancer type, grade, stage,
sex, and smoking history, we next plotted the OS using the Kaplan Meier plotter [85]. Using the same analytical tools as mentioned in the previous section, we observed that high ATF6 expression corresponded to better OS (upper panels of Figure 15 A), whereas a high level of ATF6B expression corresponded to poor OS in human lung adenocarcinoma patients (bottom panels of Figure 15 A). However, in the case of squamous cell carcinoma, no clear trend was observed in the OS with high and low expression of both ATF6 and ATF6B (Figure 16 A). Cancer stages 1 and 2, smoking status and gender showed a similar trend in the OS as in the case of lung adenocarcinoma (data not shown), with high expression of ATF6 correlated to better OS and high expression of ATF6B predicted a poor OS. The OS curves for ATF6 and ATF6B could not be determined for cancer stages 3 and 4 and cancer grades due to the limited sample size.
Figure 15: Different prognostic values of ATF6 and ATF6B for lung adenocarcinoma patients

A. High ATF6 expression predicts better OS, whereas high ATF6B expression predicts poor OS in human lung adenocarcinoma patients.
3.3 Discussion

The endoplasmic reticulum (ER) is the center of protein folding in cells and a target of As\(^{3+}\)-induced toxicity besides the mitochondrion [66, 86]. We have demonstrated that short-term As\(^{3+}\) treatment induces the ER stress response via the ATF6 proteins and upregulates other UPR genes such as CEBPB, HSPA1B, MAPK10 and UBE2G2. Mutual regulation of ER stress and Nrf2 activation has been studied in C. elegans and certain neurodegenerative diseases [87]. In this report, we provided the first evidence showing that Nrf2 is also a master regulator for the...
expression of the ER stress-associated UPR in the cellular response to As$^{3+}$ through ChIP-seq and RNA-seq. On the genomic level, all of the As$^{3+}$-induced UPR genes contain conserved Nrf2-binding elements in either the promoter region or upstream of the transcription start site. Furthermore, some of them also showed significant enrichment of HIF1a binding in the promoter or gene body, such as CEBPB, HSPA1B and UBE2G2. HIF1a is a well-established oncogenic transcription factor during tumorigenesis. We had shown that the non-hypoxic induction of HIF1a by As$^{3+}$ is also Nrf2 dependent [69]. Accordingly, there is a self-amplification and forward feedback loop among ER stress, Nrf2 and HIF1a induced by As$^{3+}$, which may serve as one of the key mechanisms of As$^{3+}$-induced carcinogenesis.

Studies have demonstrated the connection between the transcription factor Nrf2 and the UPR genes ATF6, CEBPB, and MAPK10. A recent study showed that the p62-Nrf2 pathway was involved in the activation of cancer-associated fibroblasts (CAFs), promoting lung tumorigenesis. Both the HIF1a and ATF6 were the transcriptional targets of Nrf2 during the activation of the CAFs, which mediated the hypoxia and ER stress responses respectively [88]. Recent research has provided evidence suggesting the pro-tumorigenic role of Nrf2-CEBPB in non-small lung cancers. A study in lung cancer cell lines A549 and H2023 indicated that Nrf2 is activated in lung cancers and, in cooperation with CEBPB, contributes to the tumor-initiating activity and drug resistance by activating NOTCH3 and drug metabolism-related genes, respectively, in lung cancer cells [89]. In hepatocellular carcinoma (HCC) patients, low MAPK10 and NRF2 expression levels were associated with shorter survival and poorer prognosis due to a positive correlation with microvascular invasion, tumor thrombus, elevated AFP levels, and larger tumor size [90]. A context-dependent different role may be possible for Nrf2 and MAPK10 in lung cancer.
Although IHC evaluation of the ATF6 and ATF6B in lung cancer tissues vs. case-matched non-cancerous tissues is somewhat inconclusive, the overall survival analysis for the lung cancer patients unraveled an opposite prognostic value between ATF6 and ATF6B. Higher expression of ATF6 and ATF6B predicts a better and poorer OS of the lung cancer patients, respectively, indicating a possible tumor suppressor-like activity of ATF6 and an oncogenic property of the ATF6B for lung carcinogenesis. These findings also suggest that ATF6 and ATF6B may have different target genes for transcriptional regulation under physiological and carcinogenic conditions. Future studies are needed to define what sets of genes are regulated by these two transcription factors. Taken together, the data presented in this report demonstrated that As\(^{3+}\) is capable of activating the ER stress response signaling that may be actively involved in mediating the carcinogenic and/or tumorigenic processes associated with environmental As\(^{3+}\) exposure.

### 3.4 Materials and methods

#### Cell culture

The human bronchial epithelial (normal, immortalized) cell line BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA). The BEAS-2B cells were cultured in Dulbecco’s Modified Eagle’s Medium-High Glucose (Sigma-Aldrich #D5796) with added 5% Fetal Bovine Serum, 1% L-Glutamine and 1% Penicillin/Streptomycin in a humidified incubator at 37\(^\circ\) C and 5%CO\(_2\). The cells were starved with serum free medium (overnight), followed by treatment of the cells with various concentrations of As\(^{3+}\) [arsenic (III) chloride, Sigma-Aldrich, St. Louis, MO] for the indicated times.

#### Chromatin Immunoprecipitation with Global Parallel DNA Sequencing (ChIP-seq)

ChIP-seq for Nrf2 and HIF1a was performed as reported previously [91]. Briefly, BEAS-2B cells were seeded in 10 cm dishes and treated with 1 \(\mu\)M arsenic As\(^{3+}\) or without treatment for
6 h. At the end of culture, approximately 10 million control cells and the As^{3+}-treated cells were fixed using formaldehyde solution. The fixation was then stopped by adding glycine solution. The cell pellet was washed twice with 1× PBS-Igepal and snap-frozen in dry ice. The cells were then subjected to immunoprecipitation using ChIP-grade antibodies against Nrf2 and HIF1α from Active Motif (Carlsbad, CA, USA). The procedures of ChIP, preparation of input and control DNA, DNA sequencing, and data analysis were performed as what we had recently reported [69, 92]. The sequence tags were aligned to the reference genome hg19 using the Burrows–Wheeler Aligner (BWA) algorithm with default settings. The enrichment data of Nrf2 and HIF1a were visualized using University of California Santa Cruz (UCSC) genome browser. All ChIP-seq data can be accessed at [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145834](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145834) (accessed on 25 February 2020).

**Immunohistochemistry**

Lung adenocarcinoma tissue microarray slide LC10013c (lung cancer and matched adjacent normal lung tissue array) was purchased from US Biomax, Inc. (Rockville, MD, USA). It was processed for immunohistochemical staining using ATF6 and ATF6B antibodies (Novus biologicals, Littleton, CO, USA). Paraffin-embedded tissue sections were deparaffinized with xylene and hydrated in a series of alcohol gradients. To quench endogenous peroxidase activity, slides were incubated with 1.5 to 3% H2O2 in PBS for 20 min at room temperature. Heat-mediated antigen retrieval was performed by boiling tissue sections in citrate buffer with pH 6.0 for 20 min in a microwave. To block nonspecific binding of immunoglobulin, slides were incubated with a solution containing 5% goat serum, 0.2% Triton X-100 in PBS for 2 h at room temperature, followed by incubation with primary antibodies against ATF6 and ATF6B (1:50) overnight at 4 °C. Goat anti-mouse and goat anti-rabbit biotinylated secondary antibodies were subsequently
applied at 1:100 dilution and incubated for 2 h at room temperature. Slides were then incubated with ABC reagent (Vectastatin Elite ABC kit, Vector Laboratories Inc., Burlingame, CA, USA) for 45 minutes at room temperature. The chromogen was developed with diaminobenzidine (DAB). Slides were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) and mounted with Entellan® (Electron Microscopy Sciences, Hatfield, PA, USA). All incubation steps were carried out in a humidified chamber, and all washing steps were performed with 1 × PBS. Images were captured under a bright field of a Nikon Eclipse Ti-S Inverted microscope (Mager Scientific, Dexter, MI, USA).

Kaplan–Meier Survival Analysis

A Kaplan–Meier survival analysis was carried out using the Kaplan–Meier plotter (lung cancer) from mRNA database [85], which contains overall survival (OS) of 1925 lung cancer patients, using the option of the best perform threshold as a cutoff. The probe IDs are indicated on the top of each panel in the figures. Survival curves resulting in p values of <0.05 were considered statistically significantly. The gene expression of ATF6 and ATF6B in human lung cancer was determined by using TCGA platforms that contain genomic and transcriptomic data of the lung cancer patient databases. The differential expression of ATF6 and ATF6B in normal lungs and lung tumors was calculated by TNMplot [93].

Statistical Analysis

All cell culture experiments were performed independently in triplicate at a minimum (unless otherwise indicated). Wilcoxon signed-rank tests and Kruskal–Wallis H tests were used for immunohistochemistry data. Figures were prepared using GraphPad Prism 5 and plotted as mean values with SEM. A p-value of < 0.05 was considered statistically significant.
REFERENCES


ABSTRACT

THE ARSENIC INDUCED ENDOPLASMIC RETICULUM STRESS RESPONSE VIA THE ACTIVATING TRANSCRIPTION FACTOR-6 IN HUMAN BRONCHIAL EPITHELIAL BEAS-2B CELLS

by

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Arsenic is a well-known human carcinogen associated with a number of cancers, including lung cancers. We have previously shown that long-term exposure to an environmentally relevant concentration of inorganic arsenic (As$^{3+}$) leads to the malignant transformation of the BEAS-2B cells, and some of the transformed cells show cancer stem-like features (CSCs) with a significant upregulation of glycolysis and downregulation of mitochondrial oxidative phosphorylation. In the present report, we investigate the short-term effect of As$^{3+}$ on the endoplasmic reticulum (ER) stress response—the “unfolded protein response (UPR)” and metabolism in human bronchial epithelial cell line BEAS-2B cells. Treatment of the cells with inorganic As$^{3+}$ upregulated both glycolysis and mitochondrial respiration. Analysis of ER UPR signaling pathway using a real-time human UPR array revealed that As$^{3+}$ induced a significant up-regulation of some UPR genes, including ATF6, CEBPB, MAPK10, Hsp70, and UBE2G2. Additional tests confirmed that the induction of ATF6, ATF6B and UBE2G2 mRNAs and/or proteins by As$^{3+}$ is dose dependent. Chromosome immunoprecipitation and global sequencing indicated a critical role of Nrf2 in
mediating As\(^{3+}\)-induced expression of these UPR genes. In summary, our data suggest that As\(^{3+}\) is able to regulate the ER stress response, possibly through activating the ATF6 signaling.
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